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DNA damage and cytotoxicity induced on common carp by pollutants in water from an urban reservoir. Madín reservoir, a case study

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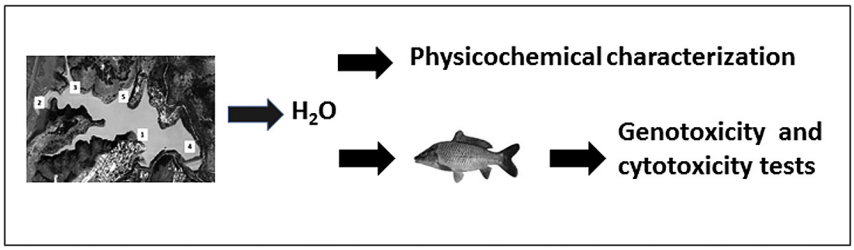
HIGHLIGHTS

- The Madín Dam is polluted by cytotoxic and genotoxic compounds, like heavy metals and pharmaceutical products.
- *Cyprinus carpio* is at risk in the Madín reservoir.
- Cytotoxicity and genotoxicity tests are a good tool to assess the risk of pollutants in water bodies.

GRAPHICAL ABSTRACT



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ABSTRACT

Madín Reservoir provides a substantial amount of drinking water to two municipalities close to Mexico City metropolitan area. However, it receives untreated wastewater discharges from domestic sources in the towns of Nuevo Madín and others, as well as diverse pollutants which are hauled by the Río Tlalnepantla from its upper reaches, so that the xenobiotics in the reservoir are highly diverse in terms of type and quantity. Previous studies showed that MR is contaminated with xenobiotics such as Al, Hg and Fe, as well as NSAIDs, at concentrations exceeding the limits established for aquatic life protection. These pollutants have been shown to induce oxidative stress on *Cyprinus carpio* and may therefore also damage the genetic material of exposed organisms, eliciting cytotoxicity as well. The present study aimed to determine the genotoxicity and cytotoxicity induced on blood, liver and gill of *C. carpio* by the pollutants present in MR water. Specimens were exposed to water from five sampling sites and the following biomarkers were evaluated: DNA damage by comet assay, frequency of micronuclei, apoptosis by TUNEL assay and caspase-3 activity. Significant increases relative to the control group ($P < 0.05$) were found with all biomarkers in all tissues evaluated, with the level of damage differing between sampling sites. In conclusion, pollutants present in MR water are genotoxic and cytotoxic to *C. carpio*, and this sentinel

Abbreviations: ANOVA, analysis of variance; BOD, biochemical oxygen demand; DCF, diclofenac; FBS, fetal bovine serum; IBP, ibuprofen; MNi, micronuclei; MR, Madín Reservoir; NPX, naproxen; NSAID, nonsteroidal anti-inflammatory drug; PAH, polynuclear aromatic hydrocarbon; PBS, phosphate buffered saline; pNA, *p*-nitroaniline; RNS, reactive nitrogen species; ROS, reactive oxygen species; SEM, standard error of the mean; SS, sampling site; TUNEL, TdT-mediated dUTP nick end labeling.

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species, coupled with the biomarkers evaluated herein, is a reliable tool for assessing the health risk to wildlife posed by exposure to pollutants in freshwater bodies.

1. Introduction

Freshwater bodies around the world have been used as reservoirs for a large number of chemical stressors of anthropogenic origin such as heavy metals, pesticides and emerging pollutants among others, which, together with changes in the physicochemical properties of the water and sediments, habitat alteration and invasive/exotic species introduction increase the toxicological risk to hydrobionts (Colin et al., 2016; Holmstrup et al., 2010). This problem is particularly important in reservoirs located near large urban areas with high populations since, in developing countries, these areas discharge their waste directly into water bodies due to the lack of adequate treatment plants, as is the case of Madín Reservoir (MR) in Mexico.

MR was constructed on the Río Tlalnepantla in the State of Mexico at the three-way meeting point of the municipalities of Naucalpan de Juárez, Tlalnepantla de Baz and Atizapán de Zaragoza, within the Mexico City metropolitan area, for the purpose of controlling river flows and as a reservoir of drinking water. In addition to its being a source of drinking water, it is also used for recreational activities such as kayaking, sailing, and fishing of common carp. There are several urban communities located in its surroundings, such as Viejo Madín, Nuevo Madín and Zona Esmeralda, which discharge all or part of their waste into MR. Previous studies conducted in 2010 and 2013 showed that MR contains, among other pollutants, heavy metals and nonsteroidal anti-inflammatory drugs (NSAIDs) at concentrations exceeding permissible limits for aquatic life protection (Galar-Martínez et al., 2010; González-González et al., 2014), with the following having been detected at diverse sampling sites: Fe (1.51–5.10 mg L^{-1}), Hg (<0.001 mg L^{-1}), Al (6.04–24.45 mg L^{-1}), diclofenac (DCF, 0.20–0.31 μg L^{1}) ibuprofen (IBP, 3.61–4.51 μg L^{-1}) and naproxen (NPX, 0.18 μg L^{-1}). Nevertheless, the presence of xenobiotics in an aquatic ecosystem is not by itself indicative of the possible induction of deleterious effects. To ascertain this, the existence of a correlation between exposure levels and signs of early damage needs to be determined with the use of biomarkers (Colin et al., 2016; Van der Oost et al., 2003).

Diverse studies have shown that both heavy metals and NSAIDs induce increases in reactive oxygen and nitrogen species (ROS and RNS) production, which in the aquatic environment lead to the generation of oxidative stress that puts the hydrobionts at risk (Rimblas, 2004; Ward et al., 2010; Lushchak, 2011; Wang and Wang, 2012; Antunes et al., 2013; Oliveira et al., 2015). In this sense, several studies carried out by our research team, using oxidative stress biomarkers such as lipid peroxidation, protein carbonyl content and the activity of antioxidant enzymes, have shown that the pollutants present in MR, including the above-mentioned metals and pharmaceuticals, increase ROS production and elicit and/or contribute to oxidative stress induction (Galar-Martínez et al., 2010; González-González et al., 2014; Morachis-Valdez et al., 2015).

The involvement of ROS in cell death has been well documented, particularly in the various stages of the apoptotic process that have now been clearly established, such as induction of mitochondrial permeability transition, release of mitochondrial death amplifying factors, intracellular caspase activation, and damage to DNA (Forman and Torres, 2001; Le Bras et al., 2005). Therefore, the pollutants present in MR can potentially induce DNA damage, reduce DNA repair, and increase susceptibility to apoptosis, factors that can all lead to cytotoxicity and mutagenic and carcinogenic events (Baršiene et al., 2013; García-Medina et al., 2013). Biomarkers of genotoxicity and cytotoxicity are useful indicators of the impact of pollutants in aquatic ecosystems since this type of damage affects the fecundity, health and life cycle of the organisms involved (Theodorakis et al., 2000).

The common carp (*Cyprinus carpio*) is a cosmopolitan species living in diverse water bodies around the world, and MR is no exception. In Mexico, in addition to its ecological value, since it is present in 80% of water bodies, it is also economically relevant as a source of food for the human population. In 2012 alone, 7 metric tons of this species, with a commercial value of 6 million US dollars, were grown and consumed in the country (RNPA, 2013; Nava-Álvarez et al., 2014). Like other fishes, *C. carpio* can bioconcentrate and bioaccumulate the pollutants present in water and sediments, which makes it a reliable sentinel organism. It has been used as a bioindicator in toxicity assays and risk assessment due to its sensitivity and easy maintenance under laboratory conditions (Fent et al., 2006). The present study aimed to determine the genotoxicity and cytotoxicity induced on blood, liver and gill of *C. carpio* by pollutants present in water from the MD.

2. Materials and methods

2.1. Study area

MR is located on the Río Tlalnepantla at 19°31′37″ N and 99°15′33″ W. Its dam is flanked by the towns of Nuevo and Viejo Madín, within the municipalities of Naucalpan de Juárez, Atizapán de Zaragoza and Tlalnepantla de Baz (State of Mexico) (Fig. 1), and was built to control river flows and supply drinking water to these municipalities. The impounded lake is also used for recreational activities such as kayaking, sailing, and fishing of common carp. Several urban communities are located in its vicinity, including Nuevo Madín, Viejo Madín, Zona Esmeralda and Lomas Verdes section VI, some of which discharge their waste directly into the reservoir.

2.2. Collection of water samples from the reservoir

Water samples were collected during the wet season (September 2015) using the procedure in the official Mexican norm on wastewater sampling (NMX-AA-003-1980). Sampling was done at surface level with a bottle fitted with an automatic sealing mechanism, at five sampling sites corresponding to: (1) discharge from the town of Nuevo Madín, (2) entry point of the Río Tlalnepantla tributary, (3) side branch of the reservoir, (4) curtain of the dam, and (5) discharge from the town of Viejo Madín (Fig. 1). Samples were placed in plastic bottles previously rinsed with 3% nitric acid, and transported to the laboratory for later analysis and use in toxicity assays. These five sampling sites represent the most relevant entry points of pollutants: domestic wastewater discharges from adjacent towns (1 and 5), influx of the main tributary (2), curtain of the dam (4) and a comparatively uncontaminated site, since there are no adjacent sources of pollutants (3). Sixty liters

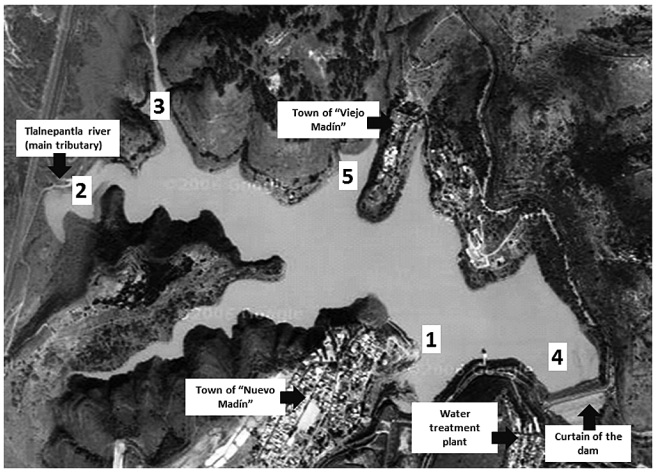

Fig. 1. Madín Reservoir. The location of sampling sites is shown: (1) discharge from the town of "Nuevo Madín", (2) entry point of the Río Tlalnepantla tributary, (3) side branch, (4) curtain of the dam, and (5) discharge from the town of "Viejo Madín". Nearby sources of pollution are indicated.

were taken from each sampling site (punctual sample collected in 3 containers) and each sample was physicochemically characterized and was used for bioassays. A control group was set up for each exposure time using dechlorinated tap water instead of MR water.

2.3. Physicochemical characterization

Water samples were physicochemically characterized as to temperature, dissolved oxygen, conductivity, pH, chloride, fluoride, hardness, ammoniacal nitrogen, nitrite, biochemical oxygen demand (BOD) and turbidity, using a HACH portable laboratory and considering the official Mexican norm on the maximum permissible limits for wastewater discharges entering national waters and resources (NOM-001-ECOL-1996). The results are expressed as values corresponding to point assessments.

2.4. Quantification of metals

The metals Al, Fe and Hg were quantified by atomic absorption spectroscopy using the method proposed in the pertinent official Mexican norm (NMX-AA-051-SCFI-2001). Concentrated nitric acid (2 mL) was added to 0.5 mL of sample (water). After digestion for 1 h in an autoclave at 120 °C and 15-lb pressure, samples were filtered and diluted with deionized water, then read on a Varian AA1475 atomic absorption spectrophotometer. Results were interpolated on an Fe, Hg or Al type curve, an Fe, Hg, or Al atomic absorption standard solution (1 mg mL-1). The percentage of recovery for all metals ranged between 97 and 100% (100% for Fe, 97% for Hg, and 97% for Al). The absorption wavelength, detection limit (DL), and quantification limit (QL) were 248.3 nm, 0.0019 ± 0.0003, and 0.007 ± 0.001 mg L-1 for Fe, 254.7 nm, 0.0025 ± 0.0004, and 0.008 ± 0.001 mg L-1 for Hg, and 309.3 nm, 0.0016 ± 0.0008, and 0.005 ± 0.003 mg L-1 for Al, respectively. Metals were quantified in their total forms. Results were expressed as mg L-1.

2.5. Quantification of NSAIDs

DCF, IBP and NPX concentrations were determined using an Agilent 1290 Infinity high performance liquid chromatography (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. NSAIDs were identified and quantified using a 6430 Triple Quadrupole mass spectrometer (Beckman Coulter, Fullerton CA) fitted with electrochemical spray ionization (ESI). The ESI positive mode was used throughout. Electrospray voltage operated at 4000 V as the unit collected data in the negative ion mode. Mass spectrometer optimization was carried out by direct infusion of a 10 μg mL^{-1} standard solution of DCF, IBP and NPX, thereafter the ionization and precursor ion modes were selected.

MR water samples were filtered (Millipore mesh 42) and subdivided into separate glass vials and refrigerated at 4 °C prior to determination of test concentrations. Results were expressed as time-weighted average concentrations of DCF, IBP and NPX. A liquid/liquid extraction with 5 mL (1 + 1) (v/v) hexane/ethyl acetate was done to extract DCF, IBP and NPX from 1-mL water samples. The extraction was repeated, and organic layers were combined and evaporated to dryness. The method DL (MDL) and method QL (MQL) were defined and determined as the minimum detectable amount of DCF, IBP, and NPX with a signal-to-noise ratio of 3:1 and 10:1, respectively, from SS waters-spiked extract. These data (MDL and MQL) were 30 and 84 ng L-1 for DCF, 31 and 86 ng L-1 for IBP, and 26 and 72 ng L-1 for NPX. Instrumental DLs (IDLs) were determined by direct injection of decreasing amounts of the standard mixture. The IDLs were 27 pg/injected for DCF, 22 pg/injected for IBP, and 26 pg/injected for NPX (González-González et al., 2014).

2.6. Specimen procurement and maintenance

Common carp (*Cyprinus carpio*) 20 ± 0.4 cm in length and 110 ± 8 g in weight were obtained from the carp culturing facility in Tiacaque (State of Mexico), transported to the laboratory, and acclimated for two weeks. During acclimation, specimens were maintained in 80-L glass tanks equipped with filtration systems, with constant aeration and a 12:12 h light:dark photoperiod, and were fed Pedregal Silver Corp MR every third day. Tank water had the following physicochemical characteristics: dissolved oxygen 6.4 ± 0.5 mg L^{-1}, ammonia concentration 0.32 ± 0.2 mg L^{-1}, nitrate 0.26 ± 0.001 mg L^{-1}, temperature 20 ± 2 °C, oxygen saturation 90–100% and pH 7.5–8.0.

2.7. Experimental design

Toxicity assays were carried out in 20-L aquariums, one each per sampling site and exposure time. In each aquarium were placed 15 L of MR water and six carp. A control group was set up for each exposure time using dechlorinated tap water instead of MR water. Testing conditions were static without renewal and were similar to those for acclimation, except that carp were not fed during the assay. Exposure times were 12, 24, 48, 72 and 96 h. At the end of the exposure time, fish were lightly anesthetized by immersion for 30 s in 2% lidocaine, and a blood sample was obtained by puncture of the caudal vessel with a previously heparinized hypodermic syringe. Subsequently, specimens were euthanized by immersion in an ice bath, the liver and gill were removed. The samples were divided into two fractions; in the case or liver and gills the former was macerated by gently pressing in a glass mortar until the tissue was completely disintegrated and the debris was removed by decantation in order to separate the cell suspension; While the second fraction was homogenized by a tissue homogenizer at medium speed for 2 min, centrifuged at 16,000×g for 15 min at 4° C, and finally the supernatant that was used for the evaluation of caspase activity was removed. In the case of blood, the sample was first diluted 1:15 with phosphate buffer solution (PBS, 0.138 M NaCl, 0.0027 M KCl) to obtain the cell suspension; and in order to

obtain the supernatant for the determination of caspase activity, posterior to the addition of the PBS, the sample was frozen to break the cells and centrifuged at 16,000×g for 15 min at 4° C. The experiment was performed in triplicate.

2.8. Evaluation of cytotoxic and genotoxic damage

2.8.1. Comet assay

On a frosted slide were placed 25 μL of the cell suspension (diluted blood or macerated organ) and 75 μL of low melting point agarose (1%). Slides were refrigerated at 4 °C for 30 min and placed in lysis solution [2.5 M sodium chloride, 10 M EDTA, 10 mM Trizma, 10% DMSO, 1% Triton, pH 10] for 1 h. They were next placed in the electrophoresis chamber with an alkaline solution (10 N sodium hydroxide and 200 mM disodium EDTA), allowed to rest for 20 min, and electrophoresis was performed at 300 mA and 200 V for 20 min. At the end of this process, the samples were rinsed three times with a neutralizing solution (0.4 M Tris-base, pH 7.5) and allowed to dry at room temperature. The DNA was stained with 30 μL ethidium bromide (2 μg mL^{-1}), 100 measurements were obtained per treatment, and the T/N index – the ratio between comet tail length (T) and diameter of the nucleus (N) – was calculated. Measurement was made using an epifluorescence microscope equipped with digital camera (Motic, mod.BA410) and an optical filter of 450–490 nm wavelength (Tice et al., 2000). The program Image-Pro Plus v5.0 (Media Cybernetics) was used to analyze images and determine DNA damage.

2.8.2. Micronucleus test

Smears of the cell suspension (diluted blood or macerated organ) were fixed in pure ethanol for 3 min, stained with a 10% solution of Giemsa in phosphate buffered saline (PBS) (pH 6.7) for 10 min, and rinsed in tap water. A total of 2000 cells were quantified per treatment in an optical microscope (Motic BA210, immersion lens) and frequency of micronuclei (MN) was expressed as the number of nucleated cells in 2000 cells. To determine MN presence, the following criteria were used: small nuclei not attached to the main nucleus, stain color and intensity similar to those of the main nucleus, and diameter 1/5 to 1/20 of main nucleus diameter (Bolognesi et al., 2006).

2.8.3. Determination of caspase-3 activity

To determine caspase-3 activity, a Promega CaspACE™ colorimetric assay kit was used whose substrate (*N*-acetyl-Asp-Glu-Val-Asp *p*-nitroanilide) binds to the enzyme, releasing the chromophore *p*-nitroaniline (pNA). Determination of pNA was carried out with a spectrophotometer at 405 nm in order to quantify enzymatic activity, which was expressed as micromolars of released pNA per hour per milligram protein relative to the control group. A reaction blank was prepared using 32-μL caspase buffer (312.5 mM HEPES, pH 7.5; 31.25% sucrose; 0.3125% CHAPS (3-[(3-cholamido-propyl) dimethylammonio]-1-propane-sulfonate), 2 μL DMSO, 10 μL dithiothreitol (DTT, 100 mM) and 54 μL deionized water. The following were used for the control group and five sampling sites: 32 μL caspase buffer, 2 μL DMSO, 10 μL DTT (100 mM), 20 μL of the supernatant, and 54 μL deionized water; for the positive control (hepatocytes treated with 1 μg Cd^{+2} mL^{-1} fetal bovine serum (FBS): 32 μL caspase buffer, 2 μL DMSO, 10 μL DTT (100 mM), 20 μL of the supernatant, and 34 μL deionized water; for inhibited apoptosis (hepatocytes treated with 1 μg Cd^{+2} mL^{-1} FBS and 20 mM Z-VAD-FMK (Promega): 32 μL caspase buffer, 2 μL DMSO, 10 μL DTT (100 mM), 20 μL of the supernatant with inhibited apoptosis, and 34 μL deionized water. Protein content as determined by the Bradford (1976) method was used to normalize the results of enzymatic activity.

2.8.4. TUNEL assay

Manufacturer instructions provided with the ApopTag Fluorescein S7110 kit (Chemicon, Temecula CA) were followed. Samples (100 μL) of the cell suspension (diluted blood or macerated organ) diluted 1:15 in preservative solution were centrifuged at 800 rpm and 4 °C for 5 min, and the cell pellet was resuspended in 50 μL of the mounting solution; 1 μL of the cells was placed on a slide with poly-L-lysine, dried at 60 °C for 5 min, fixed in cold acetone for 10 min, and hydrated in successive changes (from 100% to 50%) of ethanol and water. The cells were then treated with proteinase K (20 μg mL^{-1}) for 10 min, followed by rinsing in PBS (0.138 M NaCl, 0.0027 M KCl) pH 7.4, addition of 60 μL equilibrium buffer and incubation with 65 μL TdT enzyme for 60 min at 37 °C. The cells were rinsed in PBS prior to addition of anti-FITC conjugate and maintained at room temperature for 30 min to identify apoptotic cells, then rinsed again in PBS, stained with propidium iodide (1.5 μg mL^{-1}) and examined in the epifluorescence microscope (Motic, mod.BA410) with digital camera. A negative control sample consisting of cells treated as described above but without addition of TdT, and a positive control sample of cells loaded with DNase I (1 μg mL^{-1}) were also included in the assay. A total of 100 cells per fish were examined, and the rate of apoptosis was expressed as the percentage of TUNEL-positive cells in 100 cells.

2.9. Statistical analysis

The results of caspase activity and TUNEL passed the test of normality and homogeneity of variances and were statistically analyzed by two-way analysis of variance (ANOVA), while for the micronuclei frequency and comet assay a non-parametric ANOVA test was performed. In both cases the differences between means were compared using the Student-Newman-Keuls multiple comparisons test. The level of significance was set at $P < 0.05$. Pearson's correlation analysis was performed to find potential correlations between biomarkers of genotoxicity and cytotoxicity with metals and NSAIDs concentrations. Sigmaplot v12.3 (Systat Software, Richmond, CA) was used.

3. Results and discussion

3.1. Physicochemical characterization, and quantification of metals and NSAIDs

Physicochemical characterization results are listed in Table 1. According to the official Mexican norms on the maximum permissible limits for wastewater discharges entering national waters and resources (NOM-001-ECOL-1996) and water for human use and consumption (NOM-127-SSA1-1994), the parameters pH, ammoniacal nitrogen, fluoride and turbidity fall outside permissible limits. It is worth noting that pH was slightly acidic at all sampling sites. The transport and distribution of metals in water depends on the metal species involved as well as on environmental characteristics, being more soluble at pH values < 6.5 (Coz et al., 2004), and their capacity to stay adsorbed to suspended solids and/or sediments also decreasing, due to competition for negatively charged binding sites (Soto-Varela et al., 2014). In our study, pH values in at least three sampling sites had this characteristic, so that Al and Fe as well as other metals in MR water are probably present in soluble form and therefore bioavailable to hydrobionts, in this case the common carp. This physicochemical characteristic may also affect the bioavailability and consequently the toxicity of ionizable xenobiotics such as NSAIDs. In evaluating the acute toxicity of DCF, IBP and NPX on *Daphnia magna* at different pH values, Boström and Berglund (2015) found that toxicity increased as pH values decreased. A similar effect cannot therefore be ruled

Table 1
Physicochemical characteristics of MD water (surface level). * Values outside the limits established by Mexican regulations (NOM-001-ECOL-1996; NOM-127-SSA1-1994), which establishes the maximum permissible limits in the discharge of waste water into national waters and goods, and water for human use. Values correspond to point assessments.

Physicochemical characteristic	SS1	SS2	SS3	SS4	SS5
Temperature (ºC)	21.7	21	23	19.9	19
Dissolved oxygen (mg L^{-1})	6.5	7.8	6.9	7.6	6.3
Conductivity (μs cm^{-1})	193.6	183.6	201.6	182.7	221.3
pH	6.3*	6.8	6.3*	6.5	6.2*
Chlorides (mg L^{-1})	201	156	173	214	142
Fluorides (mg L^{-1})	6.3*	6.1*	5.3*	4.7*	4.8*
Hardness (mg L^{-1})	175.6	195.8	207.8	197.6	201.6
Ammoniacal nitrogen (mg L^{-1}, NH_3-N)	0.375*	0.065	0.160*	0.120*	0.155*
Nitrites (mg L^{-1}, NO_2-N)	0.038	0.022	0.018	0.022	0.022
BOD 5 (mg L^{-1}, O_2)	4.7	2.20	0.20	0.30	1.30
Turbidity (UFA)	235*	89*	85.5*	91*	172*

out in the different MR sampling sites in which pH was <6.5 (SS1, SS3 and SS5).

As regards the quantification of metals and NSAIDs (Table 2), it is evident that Fe and Al are present at all sampling sites at concentrations exceeding the maximum permissible limits for aquatic life protection (i.e. Fe = 0.05, Al = 0.2 mg L^{-1}; DOF, 1989), while DCF, IBP and NPX concentrations range from 0.1 to 5.12 ng L^{-1}. Similar concentrations of Fe were found by Soto-Varela et al. (2014) in a stream draining headwaters in NW Spain, particularly after storm events. It is worth stressing that the sampling in MR was done during the wet season and the highest Fe value occurred at SS1, which corresponds to a wastewater discharge point. However, the Al concentrations at all MR sampling sites (6.04–24.44 mg L^{-1}) exceed the values reported for other water bodies, such as those evaluated by Soto-Varela et al. (2014) in NW Spain and Kumar et al. (2015) in SE Brazil, who found levels of 1.57–1.66 and 0.005–0.147 mg L^{-1} respectively. In this sense, it is worth mentioning that the drinking water purification plant located in the vicinity of SS1 uses aluminum sulfate in the purification process and cannot therefore be ruled out as a possible source of this metal.

As regards NSAID concentrations, Félix-Cañedo et al. (2013) reported 15–45 ng IBP L^{-1}, 52–186 ng NPX L^{-1} and 28–32 ng DCF L^{-1} in Mexico City surface water, while Ferreira da Silva et al. (2011) found 4.91–73.2 ng IBP L^{-1}, 29.3–87.4 ng NPX L^{-1} and 4.08–148 ng DCF L^{-1} in surface water from the Río Ebro basin in Spain.

3.2. Evaluation of toxicity

Genotoxicity and cytotoxicity results include the data from sites SS2 to SS5 only, since all specimens exposed to water from SS1 died within 3 h of exposure. A subsequent lethal toxicity assay showed that a 1:20 dilution (SS1 water:dechlorinated tap water) was required to prevent specimen mortality. In previous sampling seasons (Galar-Martínez et al., 2010; González-González et al., 2014), SS1 did not produce these results and it was possible to evaluate oxidative stress biomarkers in common carp. Therefore, a different type of more dangerous contaminant may now be present at SS1 or contaminant concentrations have increased.

3.2.1. Comet assay

Comet assay results are shown in Fig. 2; T/N index values increased significantly with respect to the control group ($P < 0.05$) in liver of carp exposed to water from certain of the sampling sites: (**12 h:** SS3 15.60%, SS4 17.46%, SS5 24.32%; **24 h:** SS2 15.16%, SS3 28.11%, SS4 25.06%, SS5 16.66%; **48 h:** SS2 7.15%, SS3 13.33%, SS4 21.59%, SS5 47.44%; **72 h:** SS2 8.96%, SS3 16.37%, SS4 21.22%, SS5 18.21%; **96 h:** SS2 8.43%, SS3 16.83%, SS4 19.39%, SS5 24.87%), this damage being highest (47.77%) at 48 h in SS5, which corresponds to discharge from the town of Viejo Madín. Similar results were found in blood, but only at 12 h and to a lesser degree (SS2 11.19%, SS3 11.19%, SS4 12.42%, SS5 11.60%), while in gill the increase observed was not significant relative to the control group. Significant differences were also found between sampling sites in terms of liver and blood T/N index values, probably due to the type and concentration of the pollutants present.

The comet assay detects DNA double and single-strand breaks, adduct formation and DNA-DNA as well as DNA-protein crosslinks (Ali et al., 2008), these events being signs of premutagenic lesions. Due to its speed and sensitivity, the comet assay coupled with MN frequency has been used by diverse authors as a biomarker of damage in hydrobionts exposed to multistressors in water bodies (Frenzilli et al., 2008; Rajaguru et al., 2003; Sunjog et al., 2016). Thus, Osman et al. (2012) found increases in DNA damage in peripheral blood erythrocytes of Nile tilapia and African catfish as a result of exposure to phenol, Pb, Cd, Cr and Hg in highly contaminated areas of the Nile River. Similar results were reported by Klobucar et al. (2010) in erythrocytes of *C. carpio* exposed to water samples from a Croatian river containing polynuclear aromatic hydrocarbons (PAHs), other hydrocarbons and heavy metals (Cu, Zn, Ni, Pb, Cd, Co, Hg), while Sunjog et al. (2016) found that metal concentrations – of Al and Fe, among others – in three rivers in Serbia were correlated with DNA damage determined by comet assay in blood, liver and gill of European chub, with gill being the most affected tissue.

Diverse studies have shown that heavy metals (Al, Fe, Hg) as well as NSAIDs (IBP, NPX, DCF) and other pollutants present in MR induce increased ROS and RNS production, eliciting oxidative stress (Galar-Martínez et al., 2010; Antunes et al., 2013; González-González et al., 2014; Oliveira et al., 2015), and may consequently favor the oxidation of purine and pyrimidine bases and induce diverse DNA damage including base and sugar damage, DNA-DNA and protein-DNA crosslinks, double and single-strand breaks and abasic site formation (Bolognesi and Cirillo, 2014). One example of this is the fact that Al acts as a pro-oxidant agent by promoting the efflux of Fe from the cytosol, thus triggering the Fenton and Haber

Table 2
Metal and NSAID concentrations in MD water. Values are the mean of three replicates. SS = sampling station, ND = not detected, Fe = iron, Al = aluminum, Hg = mercury, DCF = diclofenac, IBP = ibuprofen, NPX = naproxen. * Values outside the limits established by Mexican regulations (DOF, 1989), which establishes the maximum permissible limits for the protection of aquatic life in freshwater bodies (only metals are included).

Pollutant	SS1	SS2	SS3	SS4	SS5
Fe (mg L^{-1})	4.28 ± 0.01*	1.67 ± 0.01 *	1.45 ± 0.02 *	1.42 ± 0.02*	1.58 ± 0.01*
Hg (mg L^{-1})	<0.001	<0.001	<0.001	<0.001	<0.001
Al (mg L^{-1})	24.44 ± 0.03*	6.04 ± 0.01 *	6.69 ± 0.01*	6.33 ± 0.01 *	6.35 ± 0.06 *
DFC (ng L^{-1})	0.29 ± 0.01	0.46 ± 0.02	0.31 ± 0.01	0.37 ± 0.03	0.31 ± 0.01
IBP (ng L^{-1})	4.13 ± 0.03	3.78 ± 0.03	4.02 ± 0.02	4.76 ± 0.02	5.12 ± 0.0
NPX (ng L^{-1})	0.23 ± 0.02	ND	0.10 ± 0.01	ND	0.32 ± 0.01

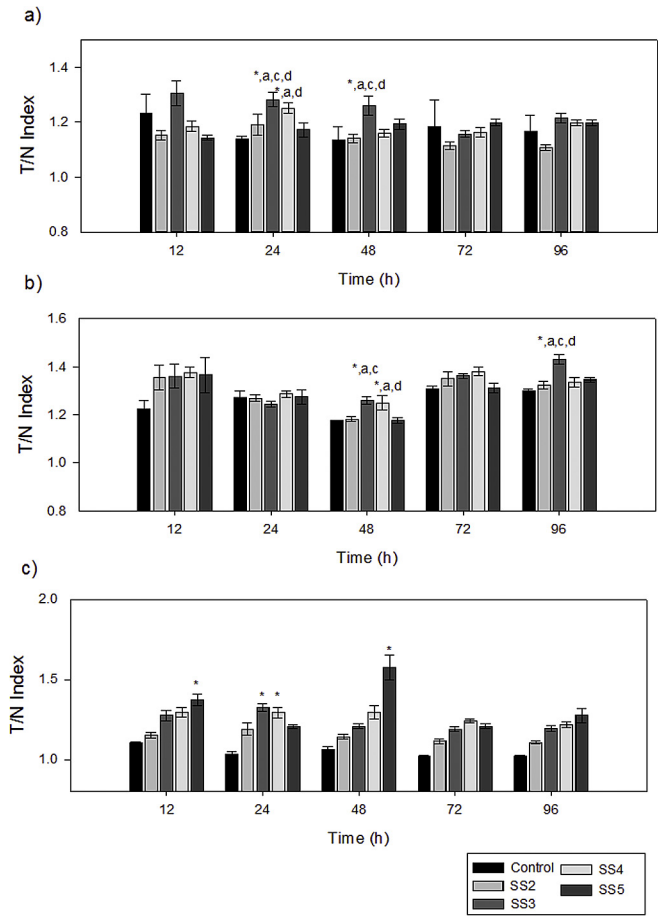

Fig. 2. Determination by comet assay of DNA damage in (a) liver, (b) blood and (c) gill of *Cyprinus carpio* exposed to water from four different sampling sites in Madín Reservoir for 12, 24, 48, 72 and 96 h. Values are the mean of three replicates ± SEM. Significantly different from: *control group, [a] SS2, [b] SS3, [c] SS4, [d] SS5. ($P < 0.05$, non-parametric ANOVA and Student-Newman-Keuls multiple comparisons test). Results from SS1 are not shown because they resulted lethal to test subjects.

Weiss reaction, which in turn induces production of hydroxyl radicals and superoxide (García-Medina et al., 2010; Razo-Estrada et al., 2013; Ramírez-Duarte et al., 2017). On the other hand, during the CYP2C9-mediated biotransformation of some NSAIDs, there is formation of hydroxyl radicals and superoxide as well as of hydroxylated metabolites that are much more toxic than the original xenobiotic (Blanco et al., 2005; Islas-Flores et al., 2013, 2014).

It is important to stress the differences in toxic response behavior found between the organs evaluated in the present study (liver > blood > gill). Lee and Steinert (2003) state that the variability in DNA damage between evaluated organs can be explained by the presence of varying numbers of alkali-labile sites, cell types with different background levels of DNA, single-strand breaks due to variations in the process of excision repair, metabolic activity and other factors such as cell-type heterogeneity, stage of the cell cycle, and the rate of cell renewal (Sunjog et al., 2014). Since liver is the major organ of biotransformation, the metabolites that are formed, which at any given moment may be more toxic than the original xenobiotic, can quickly reach concentrations high enough to elicit major damage on this organ. On the other hand, in our study, DNA damage in blood was evident only during early exposure times, thereafter decreasing and remaining constant and unchanged relative to the control group. This decrease may be due to induction of DNA repair mechanisms (García-Medina et al., 2010).

3.2.2. Micronucleus test

Micronuclei are small, round extranuclear bodies formed during the condensation of acentric chromosome fragments or whole chromosomes excluded from the cell nucleus at the end of cell division. Formation of these structures is indicative of chromosomal damage due to inefficient and/or incorrect DNA repair while an increase in MN frequency may result from environmental stress and is probably related to contaminant concentrations in a particular location (Kligerman, 1982).

As can be seen in Fig. 3, exposure of *C. carpio* to water from all MR sampling sites yielded significant increases ($P < 0.05$) in MN frequency in all organs evaluated at all exposure times, this damage being most evident in blood (**24 h:** SS2 1700%, SS3 4600%, SS4 4400%, SS5 3100%; **48h:** SS2 4300%, SS3 4400%, SS4 3900%, SS5 5000%; **72h:** SS2 4200%, SS3 5500%, SS4 5100%, SS5 4200%; **96h:** SS2 2600%, SS3 3800%, SS4 3500%, SS5 6600%) particularly in specimens exposed to SS5 water (discharge from Viejo Madín). Significant differences were also found between certain sampling sites with all organs evaluated, probably due to the type and concentration of the pollutants present. In Italy, Russo et al. (2004) noted a similar response in erythrocytes of the fish *Gambusia holbrooki* exposed chronically to water from the Sarno River contaminated with heavy metals, pesticides and detergents, while Obiakor et al. (2014) found that two fish species (*Synodontis clarias* and

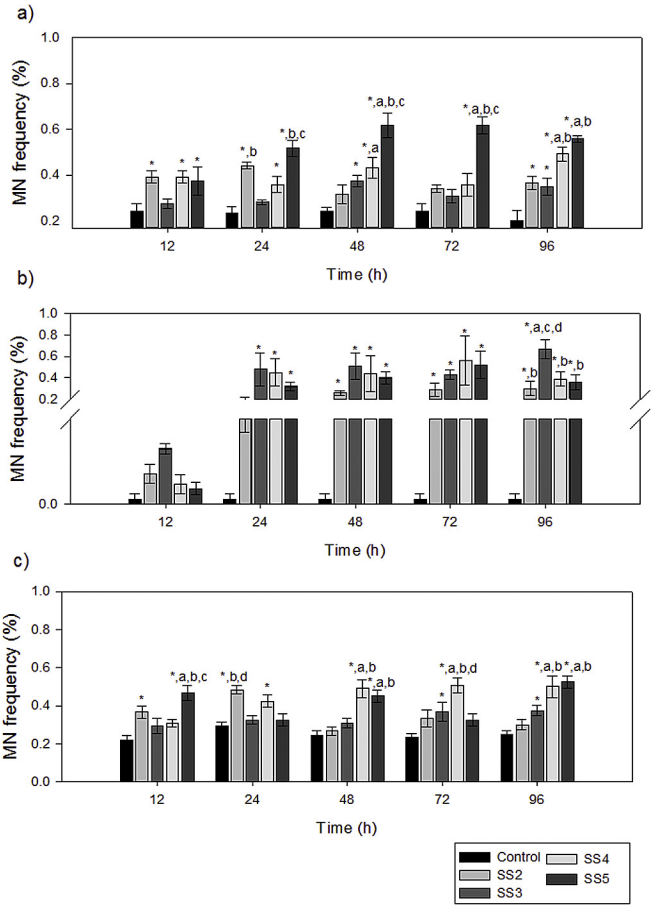

Fig. 3. Frequency of micronuclei (MN) in (a) liver, (b) blood and (c) gill of *Cyprinus carpio* exposed to water from four different sampling sites in Madín Reservoir for 12, 24, 48, 72 and 96 h. Values are the mean of three replicates ± SEM. Significantly different from: *control group, [a] SS2, [b] SS3, [c] SS4, [d] SS5 ($P < 0.05$, non-parametric ANOVA and Student-Newman-Keuls multiple comparisons test). Results from SS1 are not shown because they resulted lethal to test subjects.

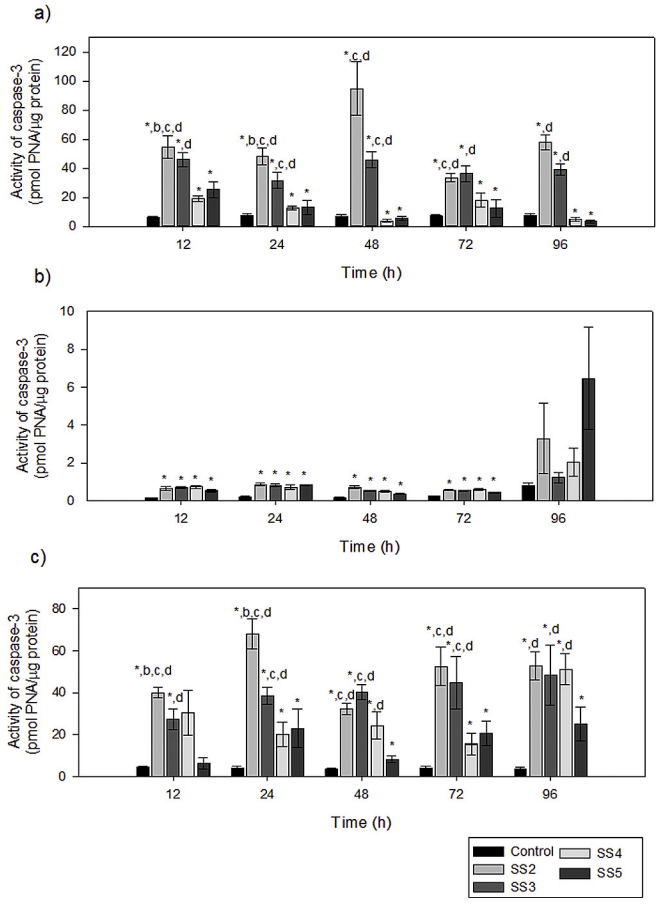

Fig. 4. Specific activity of caspase-3 in (a) liver, (b) blood and (c) gill of *Cyprinus carpio* exposed to water from four different sampling sites in Madín Reservoir for 12, 24, 48, 72 and 96 h. Values are the mean of three replicates ± SEM. Significantly different from: *control group, [a] SS2, [b] SS3, [c] SS4, [d] SS5 ($P < 0.05$, ANOVA and Student-Newman-Keuls multiple comparisons test). Results from SS1 are not shown because they resulted lethal to test subjects.

Tilapia nilotica) living in locations contaminated with heavy metals and PAHs in the Anambra River (Nigeria) show high MN induction in gill and kidney. Besides the oxidative stress and concomitant ROS and RNS increases which damage DNA and chromosomes directly, the pollutants present in MR water (i.e. the heavy metals) induce MN formation through clastogenic and aneugenic processes (Canistro et al., 2012; Russo et al., 2004).

3.2.3. Caspase-3 activity

Caspase-3 activation is the convergence of the intrinsic and extrinsic pathways of apoptosis. This enzyme is relevant in cell death and is able to cleave or degrade crucial proteins such as nuclear lamins, fodrin and the nuclear enzyme poly(ADP-ribose) polymerase (PARP), eliciting the concomitant degradation of DNA, while increases in its activity are correlated with cell death in a large variety of organisms (Abu-Qare and Abou-Donia, 2001). In the present study, caspase-3 activity (Fig. 4) increased relative to the control group ($P < 0.05$) in all tissues evaluated and at all exposure times, while important differences were also observed between sampling sites. These results show that the presence of cytotoxic pollutants interferes with the natural sequence of events of the cell cycle. It may be that, since MR pollutants induce DNA damage, the latter was detected at a restriction point, activating cell repair or cell death processes, or it could have been detected through damage to an intermediary of the cell cycle. As regards the sampling sites, SS2 and SS3 showed the largest number of differences relative to the control group and other sampling sites. This is probably due to the nature of the pollutants present at these sites since both are located near the entry point of the Río Tlalnepantla which hauls a large variety of xenobiotics along its channel. Previous studies have reported the presence of metals such as Fe and Al as well as pharmaceuticals (González-González et al., 2014) which can induce increased ROS production. On the other hand, metal concentrations have been shown to be correlated with the pathway of initiation of apoptosis, since an increase in the former induces a decrease in mitochondrial transmembrane potential, releasing cytochrome *c* which activates caspase-9, a precursor of caspase-3 (Santos et al., 2016). ROS have a major role in diverse signaling and transcription factor pathways, including the p53 suppressor factor which activates cell survival and cell death processes such as autophagia and apoptosis. These species can also induce apoptosis through disruption of calcium homeostasis, since agents such as peroxynitrite and hydroperoxide can inactivate Ca^{2+} ATPase, inducing accumulation of cytoplasmic calcium (Redza-Dutordoir and Averill-Bates, 2016). As regards SS5, where caspase-3 activity decreased in liver at 48 and 96 h, the type of death process involved may be other than apoptosis, or damage to the enzyme caspase-3 may have occurred since, being of the nature of protein, it can be affected by ROS (see Fig. 5).

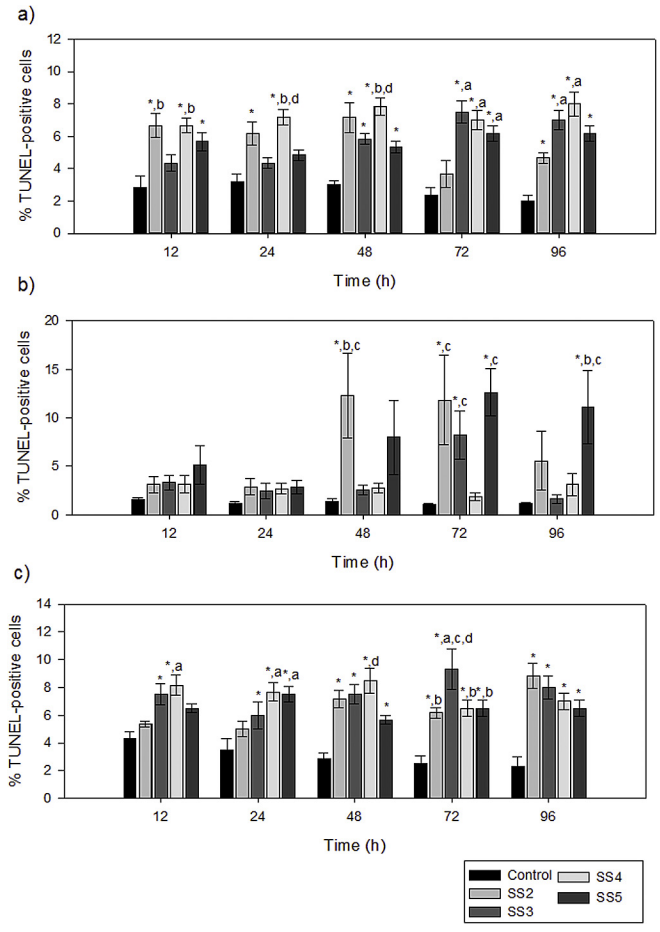

Fig. 5. Determination by TUNEL assay of the percentage of apoptotic cells in (a) liver, (b) blood and (c) gill of *Cyprinus carpio* exposed to water from four different sampling sites in Madín Reservoir for 12, 24, 48, 72 and 96 h. Values are the mean of three replicates ± SEM. Significantly different from: *control group, [a] SS2, [b] SS3, [c] SS4, [d] SS5 ($P < 0.05$, ANOVA and Student-Newman-Keuls multiple comparisons test). Results from SS1 are not shown because they resulted lethal to test subjects.

Table 3
Pearson's correlation analysis of metal and AINE's concentrations with biomarkers of genotoxicity and cytotoxicity in tissues of *C. carpio*.

Biomarkers		Al	Fe	DCF	IBP	NPX
TUNEL assay	Blood	0.2999	0.3494	0.3135	0.2949	0.2887
	Gill	**0.6471**	**0.6014**	**0.5877**	**0.6403**	0.0689
	Liver	**0.6420**	**0.6089**	**0.5447**	**0.6172**	0.1456
Micronucleus test	Blood	0.4914	0.4523	0.3595	0.4885	0.1914
	Gill	**0.5376**	**0.5338**	0.4269	**0.6138**	0.5404
	Liver	0.4867	0.4478	0.4189	**0.5302**	0.2202
Caspase-3 activity	Blood	0.1587	0.1734	0.1425	0.1819	0.1873
	Gill	0.3305	0.4129	0.4755	0.1775	−0.1819
	Liver	0.4803	**0.5103**	**0.5894**	0.3590	−0.1526
Comet assay	Blood	0.2555	0.2298	0.2212	0.2410	0.0147
	Gill	0.0755	0.0347	−0.0400	0.0817	0.0709
	Liver	**0.6303**	**0.6419**	**0.5858**	**0.6293**	0.3233

Bold indicates high correlation values.

3.2.4. TUNEL assay

To complement the information obtained by evaluating the activity of the enzyme caspase-3 which is an executor of apoptosis, the number of apoptotic cells present was determined by the TUNEL method, using the enzyme TdT as a marker. The TUNEL assay is a sensitive method for detecting apoptotic cells both in early stages and in those with morphologic changes that include apoptotic bodies. Increases in TUNEL-positive cells with respect to the control group were found in all organs evaluated, such increases being particularly evident in liver and gill at all sampling sites and at all exposure times, as follows: in liver, at **12 h**: SS2 123.08%, SS3 173.08%, SS4 188.46%, SS5 150%; **24 h:** SS2 142.86%, SS3 171.43%, SS4 219.05%, SS5 214.29%; **48 h:** SS2 252.94%, SS3 264.71%, SS4 300%, SS5 200%; **72 h:** SS2 246.67%, SS3 373.33%, SS4 260%, SS5 260%; **96 h:** SS2 378.57%, SS3 342.86%, SS4 300%, SS5 321.43%; in gill, at **12 h:** SS2 235.29%, SS3 152.94%, SS4 235.29%, SS5 200%; **24 h:** SS2 194.74%, SS3 136.84%, SS4 226.32%, SS5 152.63%; **48 h:** SS2 238.89%, SS3 194.44%, SS4 261.11%, SS5 177.78%; **72 h:** SS2 157.14%, SS3 321.43%, SS4 300%, SS5 264.29%; **96 h:** SS2 233.33%, SS3 350%, SS4 400%, SS5 308.33%.

The apoptotic elimination of cells whose function has been compromised by exposure to xenobiotics is a means for the body to minimize the deleterious effects of toxic exposure (Clearwater et al., 2002; Roberts et al., 1997; Tilly and Perez, 1997). Cytotoxicity results in the present study are consistent with those of other authors such as Nagata and Goldstein (1995), who found that high concentrations of metals such as Cu induce apoptosis and even necrosis in gill of the tropical fish *Prochilodus scrofa*, as well as Berntssen et al. (2001), who showed that Cd induces apoptotic cells in gut of *Salmo salar*. Furthermore, García-Gasca et al. (2010), using the TUNEL assay, reported increased follicular apoptosis in the mussel *Mytella strigata* exposed to pollutants in sediments from coastal ecosystems contaminated with heavy metals, PAHs and chlorinated hydrocarbons. The latter assay was likewise used by Gao et al. (2013) to evaluate DNA fragmentation in kidney cells of *C. carpio* exposed to Cd (2.5 and 10 μM Cd^{2+}).

Table 3 shows the results of the correlation between biomarkers of genotoxicity and cytotoxicity with concentrations of metals and NSAIDs. As can be seen, with the exception of the NPX, there are close correlations between the contaminants (metals and NSAIDs) in the five SS and the biomarkers evaluated, being higher in the case of the TUNEL test in the liver and blood of the exposed carp.

4. Conclusions

In conclusion, the pollutants present in Madín Reservoir water induce genotoxicity and cytotoxicity on blood, liver and gill of the common carp *Cyprinus carpio*, and this sentinel species, coupled with the biomarkers used in the present study, is a reliable tool for assessing the health risk to wildlife posed by exposure to pollutants in freshwater bodies located near large urban areas.

Conflict of interest

The authors declare they have no actual or potential competing financial interests.

Acknowledgements

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