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Determination of metals and pharmaceutical compounds released in hospital wastewater from Toluca, Mexico, and evaluation of their toxic impact☆

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ABSTRACT

Due to the activities inherent to medical care units, the hospital effluent released contains diverse contaminants such as tensoactives, disinfectants, metals, pharmaceutical products and chemical reagents, which are potentially toxic to the environment since they receive no treatment or are not effectively removed by such treatment before entering the drain. They are incorporated into municipal wastewater, eventually entering water bodies where they can have harmful effects on organisms and can result in ecological damage. To determine the toxicological risk induced by this type of effluents, eight metals and 11 pharmaceuticals were quantified, in effluent from a hospital. Developmental effects, teratogenesis and oxidative stress induction were evaluated in two bioindicator species: *Xenopus laevis* and *Lithobates catesbeianus*. FETAX (frog embryo teratogenesis assay–*Xenopus*) was used to obtain the median lethal concentration (LC_{50}), effective concentration inducing 50% malformation (EC_{50}), teratogenic index (TI), minimum concentration to inhibit growth (MCIG), and the types of malformation induced. Twenty oocytes in midblastula transition were exposed to six concentrations of effluent (0.1, 0.3, 0.5, 0.7, 0.9, 1%) and negative and positive (6-aminonicotinamide) controls. After 96 h of exposure, diverse biomarkers of oxidative damage were evaluated: hydroperoxide content, lipid peroxidation, protein carbonyl content, and the antioxidant enzymes superoxide dismutase and catalase. TI was 3.8 in *X. laevis* and 4.0 in *L. catesbeianus*, both exceed the value in the FETAX protocol (1.2), indicating that this effluent is teratogenic to both species. Growth inhibition was induced as well as diverse malformation including microcephaly, cardiac and facial edema, eye malformations, and notochord, tail, fin and gut damage. Significant differences relative to the control group were observed in both species with all biomarkers. This hospital effluent contains contaminants which represents a toxic risk, since these substances are teratogenic to the bioindicators used. The mechanism of damage induction may be associated with oxidative stress.

Abbreviations: FETAX, frog embryo teratogenesis assay – *Xenopus*; LC_{50}, median lethal concentration; EC_{50}, effective concentration inducing 50% malformation; MCIG, minimum concentration to inhibit growth; TI, teratogenic index; HPC, hydroperoxide content; LPX, lipid peroxidation; PCC, protein carbonyl content; SOD, superoxide dismutase; CAT, catalase; GR, glutathione reductase; FR, free radical; ROS, reactive oxygen species; CYP, cytochrome P-450.

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1. Introduction

Emerging contaminants are compounds of diverse origin and chemical nature, whose presence and potential consequences in the environment escape notice until they produce toxic effects on the latter and/or alter the health of living organisms (Barceló and López de Alda, 2008). These compounds include personal hygene, medical and pharmaceutical, industrial and household products. The main sources of emission of these substances into the environment are domestic, municipal, industrial and hospital effluents as well as agricultural and livestock activities, and the inadequate disposal of medications. These contaminants are found in ground, surface and even potable water. Furthermore, little is known about the concentrations at which they are present, their impact, and the treatment they should undergo for removal from the environment. And, while transformation/removal rates are high, they are offset by the continuous introduction of these compounds into the environment due to their high rates of production and consumption, as a result of which they need not be persistent to be present in the environment and induce deleterious effects (Daughton, 2004).

Hospital effluent characteristics vary depending on the infrastructure of the hospital unit and its capacity, the services offered, number of beds, number of inpatients and outpatients, geographic location, and season of the year in which it is evaluated, among others (Al Aukidy et al., 2012). A major concern involving hospital effluents is that they contain waste from everyday hospital activities and may have contaminant concentrations 4 to 150-fold higher than the ones detected in municipal effluent, which makes them an important source of emission (Verlicchi et al., 2010). The types of contaminants found in hospital effluent include pharmaceutical products, chemical residues, radioelements, disinfectants and heavy metals, among others (Emmanuel et al., 2005). Since this type of effluent has usually not undergone adequate prior treatment for removal before it is released, it is mixed with other effluents from households and industry as well as municipal wastewaters (Frédéric and Yves, 2014), subsequently reaching water treatment plants where conventional methods of removal cannot eliminate it completely so that its contaminants enter water bodies and come in contact with organisms living there. It is worthwhile noting that these contaminants are persistent in the environment and can bioaccumulate. They can also generate interactions, potentiating effects and creating synergies, so that they are able to induce adverse effects on aquatic organisms, all of which makes their study relevant (Le Corre et al., 2012).

While long-term assessments have unfortunately not been carried out on aquatic organisms, acute toxicity studies have shown the toxic potential of hospital effluents in different world regions. A study in India revealed that this type of effluent induced 33–47% growth inhibition and 11–100% mortality in *Heterocypris incongruens* (Mubedi et al., 2013). A toxicity assay conducted in Mexico exposed *Cyprinus carpio* to hospital effluent, unleashing a process of oxidative stress in which the gills, brain and liver were the organs most affected (Neri-Cruz et al., 2015). The latter exposure also induced cyto/genotoxicity (Olvera-Néstor et al., 2016). Other research teams have confirmed that exposure of hospital effluent to solar radiation potentiates synergistic pharmacological interactions, with hazardous effects on organisms such as *C. carpio* and *Pseudorasbora parva* (Li and Lin, 2015).

The diverse damage that hospital effluents can induce on organisms includes, most importantly, teratogenesis or dysmorphogenesis, which is defined as a morphologic, biochemical or functional change induced during gestation and detected in the course of the latter, at birth or subsequently (Pérez-Landeiro et al., 2002). Malformations are structural defects that may compromise body systems and organs; they can be mild and even go unnoticed, or may be severe and compromise the life of the organism. Chemical substances and physical or biological agents are teratogenic if they induce permanent abnormalities in cell structure or function, or prevent growth and prompt embryo death. Diverse mechanisms give rise to malformations and no one specific mechanism has been dilucidated for this type of effects. However, oxidative stress has been shown to have a relevant role in the development of malformations. Free radical (FR) production and maintenance of redox homeostasis are essential for the physiological health of organisms. Reactive oxygen species (ROS) formation is induced by internal and external agents, including phagocytes, enzymes such as cytochrome P450 (CYP) monooxygenases, radiation and exogenous chemicals. Also, ROS production is reduced or reversed by diverse enzymes, termed antioxidant, such as superoxide dismutase (SOD), catalase (CAT) and glutathione reductase (GR) (Kovacic and Jacintho, 2001). Endogenous ROS act as secondary messengers in signal transduction (Hansen and Harris, 2013) and are believed important in the transport of ions, host immune defense, transcription and cell apoptosis (Dennery, 2007). However, ROS may also be harmful as a result of their capacity to bind covalently or irreversibly to cellular macromolecules, inducing damage to cell components and tissues, cell death, apoptosis and necrosis (Valavanidis et al., 2006).

There are wide variations in the sensitivity to contaminants of diverse wildlife groups. Hall and Swineford, 1980 suggest that amphibians are prone to be exposed to noxious levels of chemical compounds due to the higher concentrations of these substances in irrigation canals, ponds and swamps as compared to larger water bodies. Mexico ranks fourth at world level in amphibian diversity, with 361 species (Flores-Villela and Censeco-Márquez, 2004). Amphibian exploitation in the global market comprises various sectors of the economy (Lips et al., 2001). On the other hand, frog embryos and larvae are considered excellent indicators of water quality due to their sensitivity to chemical compounds and easy maintenance (Greenhouse, 1976). Frogs are also very valuable as indicator species that integrate changes in terrestrial as well as aquatic habitats (Beiswenger, 1988). The American bullfrog *Lithobates catesbeianus* is endemic to the southern United States (US), although it is now regarded as native to Mexico. Its hindlimbs enjoy wide acceptance in the US and Europe where they are considered high-end gourmet (SEDAGRO, 2006) In southwest Mexico, consumption of this frog by the human population is low, being eaten occasionally and for medicinal purposes, while in Central and North Mexico it is more common as an alternative food complement (100 g provide 34% of the protein requirement in children and 28% in adolescents) (Calderon-Mandujano and Bahena-Basave, 2005).

The municipality of Toluca is located in the western part of the State of Mexico. It has a surface area of 429.25 sq km and a population of 873 536. There are roughly 111 medical units within the municipality, including primary, secondary and tertiary care hospitals as well as health care centers (Fig. 1). Its water resources comprise five springs, 101 wells supplying urban and rural areas, 24 intermittent streams, 43 small dams, two small lakes and two aqueducts. It is traversed by water currents flowing down from the Nevado de Toluca volcano, the main one being the Río Verdiguel which crosses the city of Toluca before flowing into the Río Lerma (Instituto de Información e Investigación Geográfica Estadística y Catastral del Estado de México (IIIGECEM), 2016; Instituto Nacional de Estadistica y Geografía, 2015; Secretaría de Desarrollo Urbano y Metropolitano (SDUM), 2014). The majority of these water bodies are contaminated by discharges of wastewater released without prior treatment (Plan Municipal de Desarrollo Urbano de Toluca, 2014); because in spite of there are laws that mark the maximum permissible concentrations of some metals in

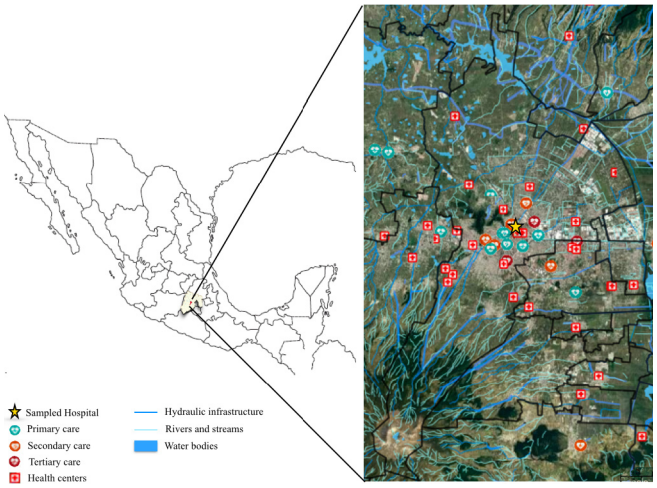

Fig. 1. Location map of the hospital sampled in Toluca, State of Mexico (Instituto de Información e Investigación Geográfica and Estadística y Catastral, 2017).

wastewater, there is no regulation on emerging contaminants present in hospital effluents or on the previous treatment that should be carried out to eliminate most of these, because of this the aim of the present study was to characterize a hospital effluent from State of Mexico (the state with the largest population in Mexico and with 1504 medical units) and evaluate its toxic potential in two amphibian species: *Xenopus laevis* and *L. catesbeianus*, a species with nutritional value due to its protein content, by determining teratogenic index and oxidative stress biomarkers.

2. Materials and methods

2.1. Test specimens

2.1.1. Xenopus laevis

Specimens were obtained from a biotechnology and aquaculture center in the state of Querétaro. The criteria used to select specimens were: males 7.5–10 cm long and 2 years old, females 10–12.5 cm long and 3 years old. Females can be identified by their larger size and the presence of protruding cloacal labia. Frogs were acclimated in natural water and the following parameters were determined monthly: pH (6.5–9), total organic carbon (<10 mg/L), alkalinity and hardness by determination of $CaCO_3$ (16–400 mg/L). Specimens were maintained in a light-protected room under a 12 h/12 h light/dark photoperiod. Males and females were placed separately in 60-L fish tanks filled to 80% capacity, with opaque sides, at 21 ± 3 °C. They were fed three times a week *ad libitum* with *Chrisotoma* sp. (0.5 ± 0.3 cm in length) or commercial pet food.

2.1.2. Lithobates catesbeianus

Specimens were acquired from an aquaculture center in Calimaya, State of Mexico. The criteria used for selection were: males 15 cm long and at least 2 years old (they differ from females in having larger tympani); females 12 cm long and 3 years old. Frogs were acclimated in 120-L fish tanks having a dry area, an area with natural water, and aquatic vegetation, simulating natural conditions, with a 12 h/12 h light/dark photoperiod, 70–80% humidity and at 23 ± 2 °C. They were fed commercial food pellets daily.

2.2. Hospital effluent

2.2.1. Sampling

The hospital effluent sampled in the present study (Fig. 1) came from IMSS (Instituto Mexicano del Seguro Social) Clinic 221 in downtown Toluca, State of Mexico, a secondary care facility with 99 beds, 34 incubators, 20 bassinets and seven specialty consulting rooms, which offers ultrasonography, X-ray, mammography and ecosonography services, and daily attends 180 specialty consultations, 21 surgeries, 30 births, and roughly 550 clinical analyses and 140 radiodiagnostic studies. Hospital effluent samples were collected during peak hospital hours (7:00–11:00 a.m.). The procedure established by the official Mexican norm for wastewater sampling (NMX-AA-003-1980) was followed. It should be noted that this hospital does not have a wastewater treatment plant (WTP) and its effluents are discharged directly into the municipal sewer system, which subsequently reaches a WTP based on activated sludge and aerobic microorganisms (Reciclagua, 2017).

2.2.2. Physicochemical characterization

The physicochemical characterization of the hospital effluent (temperature, dissolved oxygen, conductivity, pH, chloride, fluoride and hardness) was conducted using the methodology in the official Mexican norms NOM-001-SEMARNAT-1996 (establishing the maximum permissible limits of contaminants in wastewater discharged into national waters and resources) and NOM-002-SEMARNAT-1996 (setting the maximum permissible limits of contaminants in wastewater discharged into urban or municipal sewer systems).

2.2.3. Quantification of pharmaceuticals

In order to carry out the quantification in the hospital effluent, the pharmaceuticals present in their schedule of basic medications were taken into account and those that represented the greatest consumption according to the hospital's activities were selected, of these, 6 main groups were selected: beta-blockers and antidiabetics, (first and second cause of death respectively in the State of Mexico (INEGI, 2017)), beta-lactams (best-selling pharmaceuticals in Mexico (SE, 2015)), hormones and NSAIDs (highly consumed pharmaceuticals and of which two are considered to be from the watch list of the European Union: 17-β estradiol and diclofenac (OJEU, 2015)).

The methodology described by López-Serna et al. (2012) was carried out, the chromatographic separation was performed using an Acquity UHPLC system and a reversed-phase BEH C18 column (2.1 mm × 100 mm, 1.7 μm particle size), both from Waters Corporation.

The compounds analyzed in positive ionization (PI) mode were eluted using a mobile phase consisting of acetonitrile (ACN) and 0.1% aqueous formic acid. The elution started at 10% ACN; was linearly increased to 7.5 min over 6.2 min; further increased to 100% ACN over 0.35 min; and kept isocratic for 0.5 min. Total running time for the analysis was 8 min. In the negative ionization (NI) mode, compounds were eluted with ACN: MeOH (1:1, v/v) and 10 mM ammonium acetate. The elution was linearly increased from 20% ACN to 80% ACN over 1.3 min, further increased to 90% ACN over 0.7 min, then kept isocratic for 2 min. The total running time was 5 min. The flow rate in both cases (PI and NI) was 0.4 mL/min with injection volume 20 μL. Samples were maintained at 15 °C in the injection station and the column at 30 °C.

For most compounds, two selected reaction monitoring transitions between the precursor ion and the most abundant fragment ions were monitored. The limit of detection (LOD), estimated as the concentration of analyte that produces a signal-to-noise ratio (S/N) of 3, represents the mean of the LODs for each analyte. The limit of quantification (LOQ) was estimated as the concentration of analyte producing a signal-to-noise ratio (S/N) of 10.

2.2.4. Quantification of metals

29. Quantification of 8 metals was carried out (As, Cd, Cu, Cr, Hg, Ni, Pb and Zn), which were selected because their concentration in water is regulated by Mexican laws (NOM-001-SEMARNAT-1996 and NOM-002-SEMARNAT-1996). Metals were quantified using the method proposed by Eaton et al. (1995). To 0.5 mL of effluent were added 2 mL concentrated nitric acid. The samples were placed in an autoclave at 120 °C and 15-lb pressure for 1 h. They were then filtered, diluted with deionized water and read on a Varian AA1475 atomic absorption spectrophotometer (Melbourne, Australia). Results were obtained by interpolation on a type curve with the standard atomic absorption solution for each metal (1 mg/L).

2.3. Evaluation of teratogenesis (FETAX assay)

The study was carried out in accordance with procedures in the standard guide of the American Society for Testing Materials (2012).

2.3.1. Induction of ovulation

2.3.1.1. Xenopus laevis. Male-female pairs were placed in 40-L aquariums fitted with a plastic or nylon mesh suspended roughly 3 cm over the bottom, into which oocytes could be laid. Aquarium sides were opaque and water temperature was maintained at 20 ± 2 °C, with a 12 h/12 h light/dark photoperiod. To induce amplexus, on the eve of the assay 700 IU of the hormone human chorionic gonadotropin (HCG, CHORAGON®, Ferring) dissolved in a sterile 0.9% NaCl solution were subcutaneously administered in females, and 300 IU in males, in the dorsal lymph sac, using 1-mL hypodermic syringes fitted with long, 26-gauge needles.

2.3.1.2. Lithobates catesbeianus. Induction of ovulation in *L. catesbeianus* was made using the methodology proposed by Afonso (2004). Males and females were injected 1 mL buserelin acetate (Conceptal®, Intervet-Mexico) intraperitoneally and then placed in 40-L fish tanks filled to 50% capacity with FETAX medium (neutral pH and temperature 24 ± 2 °C). After 10 h, a second dose was injected and the FETAX medium was replaced. Amplexus ensued, and 24 h after the first dose was administered, oocytes were obtained by lateral compression at oviduct height in the anteroposterior direction and were placed in 4-L polyethylene containers. Semen was collected from the males with the help of a 1-mL Pasteur pipette which was inserted in the male cloaca, then slowly extracted. The semen was diluted in FETAX medium, placed over the oocytes and gently mixed for about 3 min, followed by incubation at 23 ± 2 °C until mid-blastula transition (MBT) was reached.

2.3.2. Selection of oocytes

The following morning, fish tanks were checked for oviposition. Oocytes were extracted from the tank and terrarium using the nylon mesh, and with the help of sterile Pasteur pipettes were placed in separate containers for examination under a Zeiss Stemi 305 stereoscopic microscope to select those in MBT (stage 8).

2.3.3. Preparation of FETAX medium

FETAX medium was prepared by dissolving 625 mg NaCl, 96 mg $NaHCO_3$, 30 mg KCl, 15 mg $CaCl_2$, 60 mg $CaSO_4$ ● $2H_2O$ and 75 mg $MgSO_4$ in 1 L distilled water. All reagents were purchased from Sigma-Aldrich (St Louis). The final pH of the solution was 7.6–7.9.

2.3.4. Preparation of effluent concentrations and positive control

The six concentrations (0.1, 0.3, 0.5, 0.7, 0.9 and 1.0%) of hospital effluent tested on exposed groups were diluted in FETAX medium. The negative control was exposed to FETAX medium alone. The entire procedure was performed under a laminar flow hood. The stock solution was placed in amber glass jars; the minimum volume of each final concentration was 32 mL FETAX medium.

2.3.5. Treatment and seeding of oocytes

Under the laminar flow hood, 8 mL of each concentration of the hospital effluent were placed in previously labelled 50-mm Petri dishes. One plate was filled with 8 mL FETAX medium without hospital effluent for use as the negative control. Dissecting forceps (Moria) were used to pick up oocytes showing a regular spherical shape and homogeneous cell division, examining each under the microscope. A total of 20 oocytes in MBT were placed in each plate containing the different test concentrations for exposed groups and the control group, at ambient temperature. They were kept in the incubator inside lidded Petri dishes at 21 ± 2 °C in the dark for 96 h.

2.3.6. Culture monitoring

The FETAX medium of exposed groups and the negative control was replaced daily under the laminar flow hood. To this end, 8 mL of each test concentration prepared were placed in new, previously labelled, sterile 50-mm Petri dishes which were maintained for 1 h 30 min at ambient temperature to ensure that the medium was at ambient temperature (20 °C) before it was added to the oocytes. At 24 h, live larvae were transferred to new Petri dishes, using the microscope and dissecting forceps to separate them. At 48 and 72 h, live larvae were transferred with the help of a sterile 2-mL Pasteur pipette. A daily record was kept of observations on the number of dead larvae and precipitates (if any) in each culture, and these were removed from the plates.

2.3.7. Examination of larvae

At 96 h, larvae were checked for swimming, if not swimming, this was noted in a developmental parameter sheet used to record malformations present in larvae. Precipitates (if any) were also recorded, as well as the number of dead larvae listed in the observations sheet. Larvae were euthanized by placing them in a Petri dish containing a 0.06% MS-222 solution (lethal dose). Each larva was then measured straight from head to tail end using Zen Blue Zeiss software, noting the value in the developmental parameter sheet. Next, each larva was viewed under the microscope fitted with a Zeiss Axiocam 5s camera, to identify developmental malformations with the help of the *Atlas of Abnormalities* (Bantle et al., 1991). After examination, larvae were disposed of in accordance with institutional standards for the elimination of biological samples.

2.4. Evaluation of oxidative stress

FETAX medium was prepared and test specimens were exposed to the stated concentrations in triplicate, using the procedure described in sections 2.3.3 to 2.3.4. At 96 h, the larvae in each plate were weighed and mechanically homogenized in a 1:4 ratio (w/v) with cold (4 °C) phosphate buffer solution (pH 7.2), then centrifuged at 2500 rpm for 15 min. All determinations were made on the supernatant.

2.4.1. Determination of hydroperoxide content (HPC)

Determination was made according to the method of Jiang et al. (1992). To 100 μL of sample [previously deproteinized with 10% trichloroacetic acid (TCA)] was added 900 μL of 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]. This mixture was incubated for 60 min at ambient temperature, and absorbance was determined at 560 nm against a blank containing reaction mixture only. Results were interpolated on a type curve and were expressed

as nM cumene hydroperoxide (CHP)/mg protein.

2.4.2. Determination of lipid peroxidation (LPX)

Determination was made by the Buege and Aust (1978) method. To 500 μL of supernatant was added 1 mL Tris-HCl buffer solution (150 mM) pH 7.4. The resulting sample was incubated at 37 °C for 30 min, then supplemented with 2 mL of 0.38% thiobarbituric acid (TBA) (Fluka, Sigma-Aldrich, Toluca) in 15% TCA, prior to incubating at 37 °C for 45 min. The sample was then centrifuged at 12,500 rpm and −4 °C for 15 min and absorbance was determined at 535 nm. Results were expressed as mM malondialdehyde (MDA)/mg protein, using the molar extinction coefficient (MEC) of 1.56×10^5/M/cm.

2.4.3. Determination of protein carbonyl content (PCC)

PCC was determined using the procedure of Levine et al. (1994), with modifications. To 100 μL of supernatant were added 150 μL of 10 mM N-dimethylformamide in 2 M HCl (Sigma), and the sample was incubated at ambient temperature for 1 h in the dark. Next, 500 μL of 20% TCA were added and the sample was allowed to rest for 15 min at 4 °C, then centrifuged at 16,000 x g for 5 min. The bud was rinsed three times with ethanol:ethyl acetate (1:1), dissolved in 150 μL guanidine (6M) at pH 2.3, and incubated at 37 °C for 30 min. Absorbance was read at 366 nm and results were expressed as nmol reactive carbonyls (C=O) formed/mg protein, based on their MEC of 21,000 M/cm.

2.4.4. Determination of superoxide dismutase (SOD) activity

SOD activity was determined by the method of Boehringer (1987). Samples were delipidized by supplementing 100 μL of serum with 30 μL chloroform and 50 μL methanol. The mixture was shaken for 1 min and centrifuged for 15 min at 6000 rpm, and the supernatant was retained.

In a quartz cuvette, 50 μL distilled water were mixed with 1.4 mL Tris-HCl buffer solution (50 mM, pH 8.20) and 25 μL EDTA solution (1 mM), subsequently supplementing with 25 μL of a pyrogallol solution (0.124 mM). Absorbance was read at 0, 10 and 60 s, at 420 nm. The assay was performed in triplicate and the difference was taken as the optical density (OD) of the blank (ΔOD_{blank}), representing the difference in ODs for the non-inhibited reaction.

For each sample, 50 μL of supernatant were mixed in a quartz cuvette with 1.4 mL of Tris-HCl buffer solution and 25 μL of EDTA solution. Next, 25 μL pyrogallol was added and absorbance was read at 410 nm, at 0, 10 and 60 s. Results were derived by the following formula:

$$| [(\text{Mean } \Delta OD_{sample} \text{ x } 100)/(\text{Mean } \Delta OD_{blank})]\text{-}100| \text{x } 0.6$$

Where:

ΔOD_{sample} = difference in optical densities in the sample
ΔOD_{blank} = difference in optical densities in the blank

2.4.5. Determination of catalase (CAT) activity

CAT activity was determined by the Radi et al. (1991) method. To 20 μL of supernatant was added 1 mL isolation buffer solution [0.3 M sucrose (Vetec, Sigma-Aldrich, St. Louis), 1 mM EDTA, 5 mM HEPES, 5 mM KH_2PO_4 (Vetec)] plus 0.2 mL of 20 mM H_2O_2 solution (Vetec).

Absorbance was read at 240 nm, at 0 and 60 sec, and CAT activity per minute was estimated using the MEC of H_2O_2 (0.093 mM/cm).

2.4.6. Determination of total protein content

Determination was made in accordance with the Bradford (1976) method. A total of 25 μL of supernatant were mixed with 75 μL deionized water and 2.5 mL Bradford reagent. The mixture was homogenized in a Vortex for 1 min and left in the dark for 5 min. Absorbance was read at 595 nm and results were interpolated on a bovine serum albumin type curve.

2.5. Data analysis

In the FETAX assay, acute toxicity was assessed by determining the median lethal concentration (LC_{50}) and the effective concentration inducing 50% malformation (EC_{50}), using probit analysis. Teratogenic index (TI) was obtained through the following equation: $TI = (LC_{50})/(EC_{50})$. To determine the minimum concentration to inhibit growth (MCIG), head-to-tail measurements at the various test concentrations were compared by one-way analysis of variance (ANOVA), with P set at < 0.05.

Oxidative stress biomarkers were analyzed by parametric ANOVA and Fisher's multiple comparison test with $P<0.05$, using the StatPlus program.

3. Results

3.1. Hospital effluent characterization

Table 1 lists the concentrations of the eleven pharmaceuticals detected in the hospital effluent, all of which are included in the schedule of basic medications of the IMSS.

Table 2 shows the concentrations of eight metals and some physicochemical properties evaluated in the hospital effluent, as well as the maximum permissible limits in the official Mexican norms for wastewater discharges into national waters and resources (NOM-001-SEMARNAT-1996) or municipal and urban sewage systems (NOM-002-SEMARNAT-1996).

4. FETAX assay

4.1. LC_{50} and EC_{50}

Table 3 lists the mortality data obtained for *X. laevis* and *L. catesbeianus* exposed to the hospital effluent. The LC_{50} values estimated were 0.508% (95% CI: 0.484–0.729) and 1.431% (95% CI: 0.9758–3.1678) respectively. Percent mortality for each species at each test concentration is also shown. On the other hand, it is also shown the data on malformation, which were used to estimate the EC_{50}. These values were 0.132% (CI: 0.101–0.351) in *X. laevis* and 0.351% (CI: 0.214–0.444) in *L. catesbeianus*.

Table 1
Pharmaceutical concentrations detected in hospital effluent.

Pharmaceutical class	Compound	Concentration (μg L^{-1})	LOD	LOQ
Antidiabetics	Glibenclamide	1.92 ± 0.02	13.54	45.13
	Metformin	1.31 ± 0.02	0.54	2.51
β-blockers	Atenolol	0.20 ± 1E-4	0.04	0.12
	Metoprolol	2.02 ± 0.30	0.12	0.39
β-lactams	Penicillin G	3.77 ± 0.03	0.12	0.40
	Penicillin V	0.42 ± 0.01	0.22	0.74
Hormones	17β-estradiol	0.08 ± 1E-3	0.01	0.03
NSAIDs	Diclofenac	0.59 ± 0.30	0.02	0.08
	Ibuprofen	0.62 ± 0.40	0.01	0.05
	Naproxen	1.79 ± 0.20	0.75	5.82
	Acetaminophen	2.66 ± 0.02	0.38	1.56

NSAIDs = nonsteroidal anti-inflammatory drugs; LOD = limit of detection; LOQ = limit of quantification.

Table 2
Metal concentrations and physicochemical properties evaluated in hospital effluent.

Physicochemical characteristics and metals	NOM-001-SEMARNAT-1996	NOM-002-SEMARNAT-1996	Hospital effluent
Temperature (°C)	40	40	14.7
Dissolved oxygen (mg/L)	NI	NI	10.1
Conductivity (μS/cm)	NI	NI	137.2
pH	6.5–8.5	6–9	6.1
Chloride (mg/L)	Maximum 250	NI	199
Flouoride (mg/L)	0–15	NI	4.3
Hardness (mg/L)	Maximum 500	NI	223.3
Ammonia (mg/L)	NI	NI	0.79
Total suspended solids (mg/L)	60	40–60	35
Total P (mg/L)	10	10	7.9
Total N (mg/L)	25	NI	20
Biochemical oxygen demand (mg/L)	60	40–60	39
NaClO (mg/L)	NI	NI	1.0
As (mg/L)	0.2	0.75	0.014 ± 0.001
Cd (mg/L)	0.2	0.75	0.039 ± 0.001
Cu (mg/L)	6.0	15	0.202 ± 0.003
Cr (mg/L)	1.0	0.75	0.051 ± 0.001
Hg (mg/L)	0.01	0.015	0.021 ± 0.001
Ni (mg/L)	4	6	0.170 ± 0.002
Pb (mg/L)	0.4	1.5	0.123 ± 0.001
Zn (mg/L)	20	9	0.205 ± 0.001

NI = Not indicated.

4.2. Type of malformations

Fig. 2 shows the malformations observed with greater frequency, the most important ones being: microcephaly, cardiac edema, facial edema and eye malformations in *X. laevis* (A), and microcephaly and damage to the notochord, tail, fin and gut in *L. catesbeianus* (B). Fig. 3shows representative images of these malformations.

4.3. MCIG

Head-to-tail length measurements are shown in Fig. 4. After 96 h of exposure, significant differences with respect to the control group were observed with all test concentrations in *X. laevis*, while in *L. catesbeianus* significant differences were observed only from the second lowest test concentration up. Thus, the statistical analysis yielded MCIG values of 0.1% and 0.3% respectively for these species.

4.4. Determination of TI

TI was determined by calculating the LC_{50} to EC_{50} ratio. American Society for Testing Materials (2012) guide states that TI values > 1.5 represent a risk. The values obtained in the present study were 3.8 for *X. laevis* and 4.0 for *L. catesbeianus*.

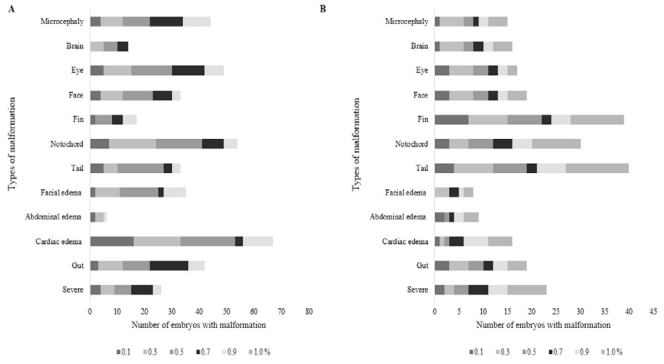

Fig. 2. Main malformations induced by exposure to hospital effluent for 96 h at the following concentrations: 0.1, 0.3, 0.5, 0.7, 0.9 and 1.0%, on (A) *Xenopus laevis* and (B) *Lithobates catesbeianus*.

5. Oxidative stress

5.1. HPC

HPC results are shown in Fig. 5. In *X. laevis*, significant differences with respect to the control group were observed with all

Table 3
Mortality and malformation data in embryos of *Xenopus laevis* and *Lithobates catesbeianus* exposed to hospital effluent.

Concentration (%)	Number of embryos exposed	*Xenopus laevis*				*Lithobates catesbeianus*			
		Number of dead embryos	% Mortality	Number of malformed embryos	% Malformation	Number of dead embryos	% Mortality	Number of malformed embryos	% Malformation
0	60	0	0	0	0	0	0	0	0
0.1	60	9	15	29	48.3	8	13.3	24	40.0
0.3	60	13	21.6	38	63.3	12	20	29	48.3
0.5	60	28	46.6	41	68.3	15	25	35	58.3
0.7	60	31	51.6	46	76.6	18	30	39	65
0.9	60	38	63.3	51	85	24	40	45	75
1	60	42	70	55	91.6	33	55	51	85
Parameter		**LC_{50}**	**0.508%** (CI = 0.484 −0.729)	**EC_{50}**	**0.132%** (CI = 0.101 −0.351)	**LC_{50}**	**1.431%** (CI = 0.976 −3.168)	**EC_{50}**	**0.351%** (CI = 0.214 −0.444)

LC_{50} = median lethal concentration; EC_{50} = effective concentration inducing 50% malformation; CI = confidence interval.

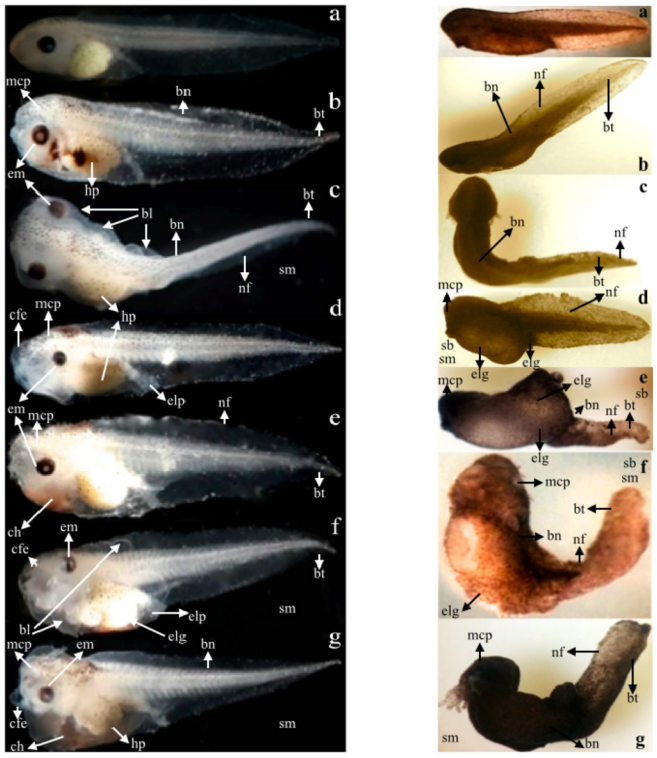

Fig. 3. Representative malformations in embryos of *Xenopus laevis* (left) and *Lithobates catesbeianus* (right) exposed to hospital effluent for 96 h at the following concentrations: (a) control, (b) 0.1%, (c) 0.3%, (c) 0.5%, (e) 0.7%, (f) 0.9%, (g) 1.0%. Abbreviations: ae: abdominal edema, he: heart edema, bl: blisters, cfe: craniofacial edema, mcp: microcephaly, bn: bent notochord, bt: bent tail, nf: narrow fin, em: eye malformation, hp: hyperpigmentation, elp: enlarged proctodeum, elg: enlarged gut, ch: cardiac hemorrhage, sm: severe malformation, sb: stunted body.

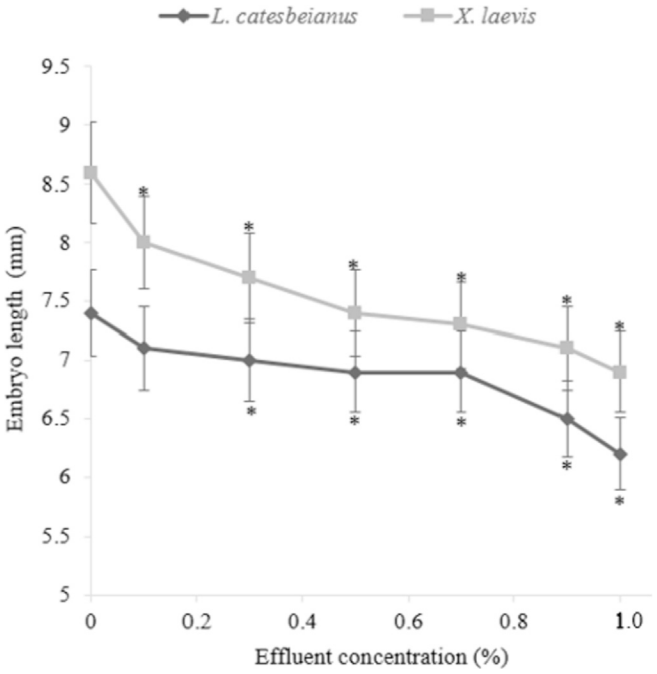

Fig. 4. Head-to-tail measurements in larvae of *Xenopus laevis* and *Lithobates catesbeianus* exposed to six different concentrations of hospital effluent for 96 h. *Significant differences relative to the control group (One-way ANOVA and Fisher's, *P>0.05*).

concentrations of the hospital effluent. Similarly, HPC increases occurred in *L. catesbeianus*, which were also statistically significant.

5.2. LPX

LPX results are shown in Fig. 5. Significant increases relative to the control group were found with all test concentrations, the most evident of these being with the 0.1 and 0.5% concentrations in *X. laevis*. In L. *catesbeianus* significant increases were noted with all concentrations as well as a concentration-dependent behavior.

5.3. Protein carbonyl content

Fig. 5 shows the results of PCC determination. In *X. laevis*, significant increases relative to the control group were found at all six test concentrations. In the case of *L. catesbeianus*, significant increases were also observed at all concentrations as well as a concentration-dependent behavior.

5.4. SOD activity

Fig. 6 shows the results of SOD activity. Larvae of *X. laevis* evidenced significant increases with the 0.1, 0.3 and 0.5% concentrations, followed by significant decreases at the 0.7, 0.9 and 1% concentrations, while in *L. catesbeianus* significant increases occurred with each increase in test concentration.

5.5. CAT activity

Fig. 6 shows the results of determination of CAT activity. In *X. laevis*, significant increases relative to the control group occurred at all test concentrations, while in *L. catesbeianus*, the same behavior was observed but showed a notable increment of this activity.

6. Discussion

The relevance of using bioassays in the assessment of the toxic potential of hospital effluents lies in the impact these effluents usually have on the health of diverse, mainly aquatic organisms, as well as the repercussions these effects may eventually have on human beings.

As can be seen in Table 3, the hospital effluent analyzed in the present study induced LC_{50} values of 0.508% and 1.431% in *X. laevis* and *L. catesbeianus* respectively. A previous study on effluent from this same hospital, using *C. carpio*, reported an LC_{50} of 5.5% (Neri-Cruz et al., 2015), showing that this effluent is toxic to aquatic organisms and its toxicity increases when the organisms exposed are at earlier life stages. In the present study, EC_{50} values of 0.132% in *X. laevis* and 0.351% in *L. catesbeianus* were obtained. Furthermore, diverse types of malformation were observed in embryos of both species (Figs. 2 and 3), evidencing a concentration-dependent behavior, i.e. they were more severe as the test concentration increased, and this was also true of the degree of growth inhibition (Fig. 4). TI values of 3.8 and 4.0 were obtained for *X. laevis* and *L. catesbeianus* respectively. These values may evidence the fact that *L. catesbeianus* is at higher risk of malformation than *X. laevis* during exposure to this particular effluent and that the latter is teratogenic to both species. In other studies evaluating hospital effluent toxicity, the effects noted include higher mortality rates, oxidative stress induction in the gills, brain and liver of *C. carpio* (Li and Lin, 2015), increased apoptotic action, cyto- and genotoxicity in correlation with increases in caspase-3 activity (Olvera-Néstor et al., 2016), behavioral changes in *Clarias gariepinus*, and higher frequency of micronuclei and necrotic erythrocytes (Alimba et al.,

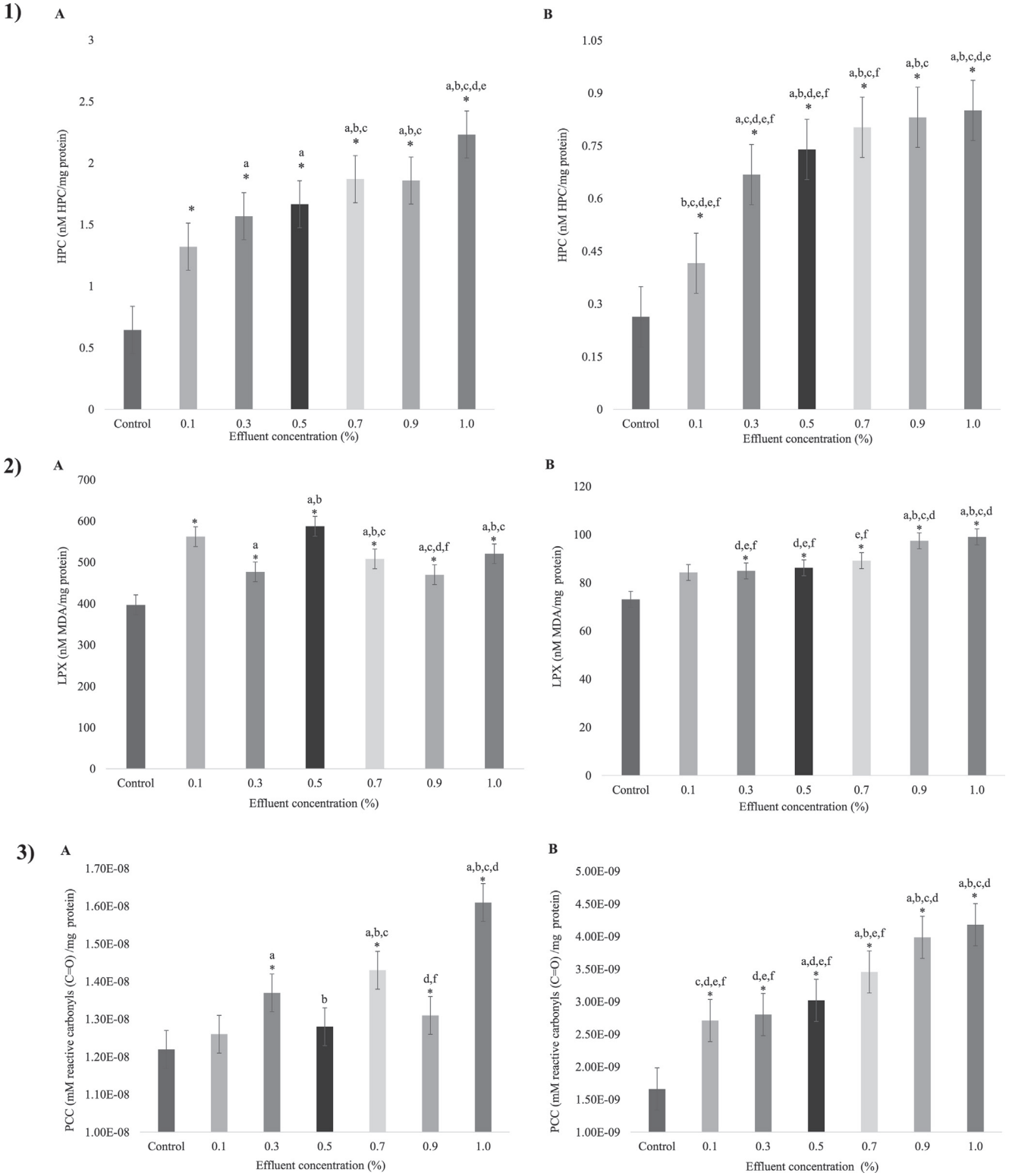

Fig. 5. Oxidative damage evaluated through biomarkers of: **(1)** Hydroperoxide content (HPC), **(2)** Lipid peroxidation (LPX) and **(3)** Protein carbonyl content (PCC) in larvae of (A) *Xenopus laevis* and (B) *Lithobates catesbeianus* exposed to six different concentrations of hospital effluent for 96 h. CHP = cumene hydroperoxide. Significant differences relative to: (*) control, (a) 0.1%, (b) 0.3%, (c) 0.5%, (d) 0.7%, (e) 0.9%, (f) 1.0%. (One-way ANOVA and Fisher's, $P<0.05$).

2017). Hospital effluent can also induce protein damage in diverse tissues including the gills and digestive gland of *Corbicula fluminea*, eliciting damage in the short term and increasing the abundance of cell and metabolic structural proteins (Bebianno et al., 2016). However, it should be recalled that the FETAX assay focuses on toxic effects induced during organogenesis, when organisms are more sensitive and contaminants are more likely to induce anomalies, and during which, occurrence of a harmful effect affects organ and

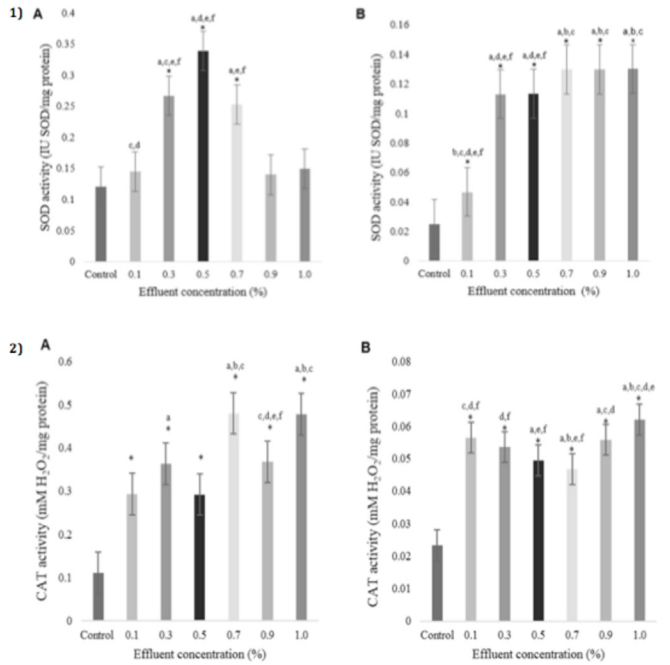

Fig. 6. Antioxidant defense evaluated through the activity of the enzymes: **(1)** Superoxide dismutase (SOD) and **(2)** Catalase (CAT) in larvae of (A) *Xenopus laevis* and (B) *Lithobates catesbeianus* exposed to six different concentrations of hospital effluent for 96 h. Significant differences relative to: (*) control, (a) 0.1%, (b) 0.3%, (c) 0.5%, (d) 0.7%, (e) 0.9%, (f) 1.0%. (One-way ANOVA and Fisher's, $P < 0.05$).

tissue development (Vallance, 1996). Results of the present study may be due to the complex mixture of pharmaceuticals (Table 1) and metals present in the effluent since, as can be seen in Table 2, the metal with the highest concentration was Hg, which exceeded the maximum permissible limits set by Mexican legislation (NOM-001-SEMARNAT-1996; NOM-002-SEMARNAT-1996). This metal is present in diverse medical devices such as thermometers and sphygmomanometers, and a certain amount is also present in dental amalgams, fluorescent lamps and light bulbs. It can enter ecosystems when inadequate disposal is made of these items (Peshin et al., 2015). In the environment, Hg is found in diverse forms which can all induce effects on different organs depending on the duration and route of exposure. It occurs as elemental mercury, which is vaporized at ambient temperature but when absorbed affects the respiratory and central nervous systems mainly (Zahir et al., 2005). It can also enter the environment and be deposited in sediments, remaining indefinitely in water bodies and entering in contact with diverse organisms which biotransform it to methylmercury, a highly toxic form that is readily uptaken by organisms and can bioaccumulate, inducing damage on the central nervous system mainly (Clarkson, 2002). Diverse reports state that Hg induces harmful effects including delayed growth and cerebral atrophy (Prati et al., 2002). It also interacts with other contaminants present in hospital effluents, such as pharmaceuticals and their metabolites, cleaning products and so on, which, when combined, may generate interactions that potentiate their toxic action. Table 2 also shows the presence of other heavy metals (As, Cd, Cu, Ni, Pb and Zn) known to induce delayed growth in early developmental stages as well as malformation of the spinal column accompanied by high mortality rates (Dong et al., 2016). Furthermore, diverse studies have evidenced the toxic potential of these metals, since they exert systemic toxic action, induce damage on diverse organs even at low doses and are carcinogenic in humans (Tchounwou et al., 2012). On the other hand, the relevance of their presence in hospital effluent lies in the fact that water bodies act as reservoirs of these contaminants, reintroducing them into the food chain through biomagnification, since if they remain for extended periods in the environment they are uptaken by aquatic organisms, or filter down into water-bearing layers, inducing damage through diverse routes (Wildi et al., 2004).

On the other hand, 11 pharmaceuticals were also present in the hospital effluent (Table 1). These include relevant concentrations of NSAIDs (diclofenac, ibuprofen, naproxen, acetaminophen), a pharmaceutical class that is among the top in terms of consumption at world level (Takagi et al., 2006). Their therapeutic action involves mostly the inhibition of the enzymes cyclooxygenase-1 and 2, which are responsible for converting arachidonic acid to prostaglandins and thromboxanes which act as mediators in diverse homeostatic processes (Vane and Botting, 1998). Ibuprofen and diclofenac can delay larval eclosion by suppressing motility in early developmental stages; they may also be responsible for neurotoxic effects and behavioral changes in *Danio rerio* (Xia et al., 2017). Naproxen can affect early developmental stages including eclosion and growth. It also induces morphological damage and oxidative stress through depletion of systems coding for the enzyme glutathione peroxidase and induces increased LPX in *Cyprinus carpio* (Sehonova et al., 2017). A further group of pharmaceuticals detected in the effluent in our study were estrogens, which show highly specific genotoxic action (Liehr et al., 1986), alter the genetic material of target organs through formation of metabolite-like compounds that interact with DNA and form adducts, leading to carcinogenesis; they can also induce DNA double-strand breaks and oxidation of purines (Rajapakse et al., 2005). Estrogens are also endocrine disruptors, since they can induce changes in vitellogenin and thyrotropin levels, increasing pyruvate in the kidney and decreasing lactate dehydrogenase in *Pelophylax ridibundus* (Falfushynska et al., 2017). Some authors state that species decline and extinction may be linked to the presence of this type of compounds since they usually interfere with the reproduction process in fish (Ward and Blum, 2012). Furthermore, these pharmaceuticals are constantly released into the environment and remain in the sediment of diverse water bodies, thus being capable of inducing estrogenic activity (Hashimoto et al., 2005) since they can interfere with androgen-dependent processes such as spermatogenesis (Imai et al., 2005). A further medication found in hospital effluent in our study was metformin. It is among the most prescribed hypoglycemic agents at world level, acting in diverse ways inside the body: decreasing glucose production by the liver, increasing insulin sensitivity and reducing hepatic lipogenesis; and, while this pharmaceutical is frequently found in the environment, it has been shown not to affect embryonic development or induce severe malformations (Denno and Sadler, 1994). However, it is frequently transformed to guanylurea, a compound with higher toxic action, which has also been detected in water bodies, so that the risk these two compounds represent for diverse organisms must not be underestimated (Markiewicz et al., 2017; Trautwein et al., 2014). As regards metoprolol and atenolol, both are β-adrenergic blockers used in the treatment of diverse cardiovascular diseases, particularly hypertension, and have frequently been detected in water bodies (Kolpin et al., 2002). Acute toxicity studies on them prove that metoprolol exerts toxic action, delaying growth and inducing cardiac anomalies in early developmental stages (Van den Brandhof and Montforts, 2010). Atenolol, on the contrary, does not have significant effects on mortality or development and growth (Diniz et al., 2015; Winter et al., 2008). Fortunately, it is readily degraded as are also its transformation products (Koba et al., 2016) and it has a very low rate of bioaccumulation. Still another group of pharmaceuticals found in the present study are antibiotics, which are highly resistant to biodegradation (Kümmerer, 2001) and are

frequently found at varying concentrations in water bodies. Penicillin G is a β-lactam antibiotic used in the treatment of bacterial infections. Its mechanism of action is based on inhibition of bacterial cell wall synthesis. It has been shown to induce high (80%) mortality in *Daphnia magna* (Arslan-Alaton and Caglayan, 2006). β-lactam antibiotics interfere with renal organogenesis producing permanent renal insufficiency, since penicillin induces electrolyte imbalance. Renal development is highly sensitive to variations in potassium concentrations and a decline may lead to abnormal development (Crocker and Vernier, 1970). This effect has been evidenced in rat (Nathanson et al., 2000). In addition to the effects induced on kidneys, these antibiotics also elicit cardiac effects and diverse malformations in limb development in rat (Erić and Sabo, 2008), as well as malformation of the mandible and caudal and anal fins in embryos of *Oncorhynchus tshawytscha* whose parents were treated with antibiotics (DeCew, 1972).

On the other hand, controlled free radical (FR) production and maintenance of redox homeostasis are essential for the physiological health of organisms. Reactive oxygen species (ROS) formation is induced by internal and external agents: phagocytes, enzymes such as cytochrome P450 (CYP) monooxygenases, radiation and exogenous chemicals. Likewise, ROS production can be reduced or reversed by diverse enzymes, termed antioxidant, such as SOD, CAT and glutathione reductase (Kovacic and Jacintho, 2001). Endogenous ROS act as secondary messengers in signal transduction (Hansen and Harris, 2013) and are believed important in the transport of ions, host immune defense, transcription and cell apoptosis (Dennery, 2007). However, ROS can also be harmful due to their capacity to bind covalently or irreversibly to cellular macromolecules. Oxidative stress, an imbalance between ROS production and mechanisms of antioxidant defense in the cell or tissues, induces irreversible oxidation of DNA, proteins and lipids, which leads to inactivation of many enzymes and cell death. It can also affect genetic expression by interfering with the action of redox-sensitive transcription factors and signal transduction via thiol oxidation (Sahambi and Hales, 2006). During the prenatal period, this may result in birth defects as well as delayed growth and, in severe cases, death (Hansen, 2006). Embryonic development is especially susceptible to high levels of ROS since antioxidant defenses in the embryo are weak, particularly in the early stages of organogenesis. Oxidative stress has therefore been suggested to be involved in the pathogenesis of a wide range of birth defects including skeletal malformations and limb, neural tube and cardiovascular defects (Ryu et al., 2007; Sahambi and Hales, 2006; Yan and Hales, 2006). Based on the foregoing, the present study evaluated oxidative stress as a possible mechanism of induction of teratogenesis in organisms. Our results show concentration increases dependent on the levels of molecules evidencing oxidative damage such as hydroperoxides, lipid peroxides and protein carbonyls (Fig. 5) in larvae of two amphibian species. Similarly, increases were observed in the activity of the antioxidant enzymes SOD and CAT (Fig. 6) which are the first line of enzymatic antioxidant defense. Such results may likewise be explained by the presence of diverse contaminants that have been shown to be inductors of oxidative stress. Kanda et al. (2014) point out that Hg induces toxicity through diverse mechanisms, one of them being oxidative stress. Diverse studies show that NSAIDs are responsible for production of FRs, which alter cell membrane stability (Falfushynska et al., 2017) and increase LPX and antioxidant enzyme levels, while other studies show that their degradation products are even more toxic than the original pharmaceutical (Diniz et al., 2015). Furthermore, estradiol has proven able to induce damage on diverse organs of *Cyprinus carpio* through FR production and induction of oxidative stress (Gutiérrez-Gómez et al., 2016), in addition to inducing cyto/genotoxicity on this organism via DNA damage elicited through metabolic activation of this compound (Liehr, 2000; Orozco-Hernández et al., 2018). Yet another pharmaceutical detected in hospital effluent in the present study was glibenclamide, which owes its pharmacological action to rapid depolarization of the plasma membrane induced through changes in the flow of ions as well as calcium redistribution within the cell, in addition to stimulating the enzyme adenylate cyclase which increments cyclic adenosine monophosphate (AMP) content (Tüzün et al., 1999). Previous studies have shown that glibenclamide induces a significant decrease in CAT activity in rat liver and kidney as well as increased MDA (the final product of LPX) (Borg and Anderson, 1981). In general, it can be said that when increasing the concentration of the hospital effluent, the oxidative damage also increased, besides the activity of the antioxidant enzymes was also altered in order to counteract said damage, this activity could not be attributed to a single specific component, since it is the result of a complex mixture of pollutants and the interactions that exist between them.

7. Conclusions

Results of the present study show the presence of diverse pharmaceuticals and metals in hospital effluent and the toxic potential of the mixture of these substances on early developmental stages, eliciting oxidative stress, diverse types of malformation and notable growth inhibition in both species of amphibians. These effluents represent a risk for the environment as well as for diverse organisms, and therefore require adequate prior treatment for removal of the largest possible amount of contaminants present in them. Studies such as this one are important to reveal the toxic effects of hospital effluents on exposed organisms since, while certain pharmaceuticals do not exert a strong toxic action, products of their abiotic and/or biotic transformation are constantly present in the environment and their toxic action and persistence remain for the most part unknown. Furthermore, the mixture of diverse pharmaceutical products, metabolites, detergents, antiseptics, metals, contrast media and so on, all of which are present in hospital effluent, can generate interactions which usually potentiate the toxicity of its chemical components. Also, environmental changes may modify the physicochemical characteristics of these substances, shifting them across diverse compartments and transporting them to resources targeted for human use and consumption. Thus, studying these effluents may eventually assist in the development of actions for the rescue and preservation of the different water bodies affected.

Declaration of interest

None.

Acknowledgments

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