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Alterations to embryonic development and teratogenic effects induced by a hospital effluent on *Cyprinus carpio* oocytes

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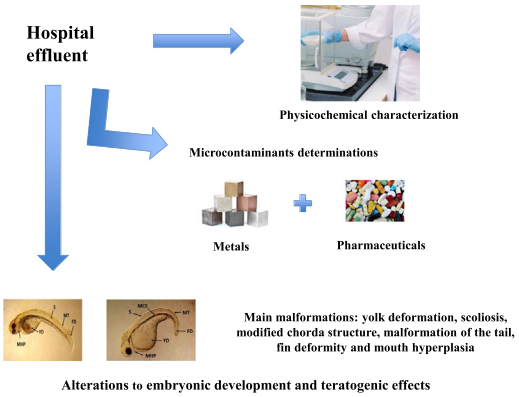
HIGHLIGHTS

- Alterations embryos and teratogenic effects in *C. carpio* after exposure to hospital effluent were evaluated.
- The hospital effluent evaluated contained mainly metals and drug.
- Proportions of 3–6% of the hospital effluent generated embryolethality in *C. carpio*.
- Yolk deformation, scoliosis, modified chorda structure, tail malformation, fin deformity and mouth hyperplasia were main teratogenic effects.
- Hospital effluent under study is capable of inducing embryotoxicity and teratogenicity.

GRAPHICAL ABSTRACT



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ABSTRACT

Hospital functioning generates a great quantity of contaminants, among which organic materials, heavy metals, and diverse pharmaceuticals are noteworthy that can affect organisms if they are not properly removed from the effluents. The hospital effluent evaluated in the present study came from IMSS (Instituto Mexicano del Seguro Social) Clinic 221 in downtown Toluca, State of Mexico, a secondary care facility. The contaminants identified in hospitals have been associated with deleterious effects on aquatic organisms; however, it is necessary to continue with more studies in order to be able to regulate the production of said contaminants which are generally dumped into the city sewage system. The present study had the purpose of evaluating the alterations to embryonic development and teratogenic effects on oocytes *Cyprinus carpio* after exposure to different proportions of hospital effluent. For said purpose, the physicochemical properties of the effluent were determined. Concentrations of the main microcontaminants were also determined. An embryolethality study out and the determination of the main alterations to embryonic development and teratogenic effects produced, due to exposure of *C. carpio* at different proportions of the effluent, were carried out. The results showed that the physicochemical properties were within the values permitted by Mexican regulation; however, the presence of contaminants such as NaClO,

Abbreviations: CAT, catalase; FET, Fish Embryo Acute Toxicity; LOD, limit of detection; LOQ, limit of quantification; NSAIDs, non-steroidal anti-inflammatory drugs; OS, oxidative stress; ROS, reactive oxygen species; SOD, superoxide dismutase; WWTP, wastewater treatment plants.

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metals, anti-biotics, anti-diabetics, non-steroidal anti-inflammatory drugs, hormones and beta-blockers, was detected. Lethal concentration 50 was 5.65% and the effective concentration for malformations was 3.85%, with a teratogenic index of 1.46. The main teratogenic alterations were yolk deformation, scoliosis, modified chorda structure, tail malformation, fin deformity and mouth hyperplasia. A high rate of hatching delay was observed. The results suggest that the hospital effluent under study is capable of inducing embryotoxicity and teratogenicity in oocytes of *C. carpio*.

1. Introduction

Hospitals consume a great quantity of water. On average, a hospital uses from 100 to 1200 L/person/day (Magdaleno et al., 2014; Sharma et al., 2013), generating a considerable quantity of wastewater with a great variety of contaminants that are the result of diagnostic studies, clinical analyses, research, cleaning and excretion of drugs by the patients (Magdaleno et al., 2014; Kusuma et al., 2013). Among the contaminants found, there are chemical products, heavy metals, disinfectants, sterilizers, detergents, radioactive markers and contrast agents, as well as active pharmaceutical ingredients and their metabolites (Laffite et al., 2016; Orias and Perrodin, 2013; Verlicchi et al., 2010). It is estimated that the use of pharmaceutical products will reach a worldwide level of 4500 billion doses by the year 2020 and will cost 1.4 trillion dollars (Mezzelani et al., 2018; QuintilesIMS Institute Healthcare Reports, 2016). The increasing consumption of these compounds and its inefficient elimination by wastewater treatment plants (WWTP), represent a threat to aquatic ecosystems due to its continual entry into natural environments (Novoa-Luna et al., 2016; Zenker et al., 2014). More than 300 pharmaceutical waste products and other chemical wastes have been detected in trace concentrations in hospital effluents (Fernández et al., 2014; Oliveira et al., 2017). Among the pharmaceutical products detected in Asia, Europe and North America, there are analgesic/anti-inflammatory drugs (0.02–1,36 μg L^{-1}), antibiotics (0.01–125 μg L^{-1}), psychiatric drugs (0.54–2 μg L^{-1}), anti-hypertensive drugs (0.71–2 μg L^{-1}), beta-blockers (0.42–25 μg L^{-1}), hormones (0.02–0.50 μg L^{-1}), contrast media (0.01–2,50 μg L^{-1}), anti-diabetics (0.05–0.11 μg L^{-1}), anti-viral drugs (0.01–0.60 μg L^{-1}) and anti-cancer drugs (0.004–124 μg L^{-1}) (Oliveira et al., 2017). These compounds suffer various forms of degradation when they are released onto surface waters, including biotic (biodegradation, bioaccumulation) and abiotic (oxidation, hydrolysis, photolysis) degradation, by which compounds more toxic than the original molecule can be generated (Li and Lin, 2015; Baillie and Rettie, 2011) and can lead to a negative impact on aquatic species, such as genetic lesions, organ damage, reproduction issues, changes in behavior and biochemical alterations, as well as oxidative stress (Oliveira et al., 2017). Oxidative stress (OS) is a disequilibrium between reactive oxygen species (ROS) and anti-oxidant defenses (Morachis-Valdez et al., 2015; Saucedo-Vence et al., 2015a). ROS are important for embryonic development, since they can act as cellular messengers modulating processes such as apoptosis, activation of transcription factors and cell differentiation (García-Medina et al., 2013, 2017; Nava-Álvarez et al., 2014; Razo-Estrada et al., 2013). However, if oxidative stress occurs during embryonic development, it could generate embryotoxicity and teratogenicity (Dennery, 2007), which could manifest itself through structural or functional malformations or even embryo death.

On the other hand, the toxicity of hospital effluents is not usually due to exposure to a single substance, but is the result of exposure to a mixture of several compounds (Islas-Flores et al., 2017; Neri-Cruz et al., 2015). Biomarkers are a useful tool for the detection of exposure and the effects of a compound or mixture of these, for example the trial Fish Embryo Acute Toxicity (FET). The FET test allows to determine the acute toxicity of a wide variety of chemical products which exhibit different modes of action, solubilities, volatility and hydrophobicity, in the embryonic stage of the fish (OECD, 2013). Numerous studies have been reported exploring the capacity of zebrafish assays for the assessment of the teratogenic potential through the test FET (Nishimura et al., 2016; Van den Bulck et al., 2011). However, this test has been little explored for other fish species as *C. carpio*. Fish have been used as biomarkers in order to evaluate contamination by different xenobiotics, due to the fact that they greatly interact with the water and its sediments, besides occupying different habitats and trophic levels (SanJuan-Reyes et al., 2017). *C. carpio* belongs to the *Cyprinidae* family, which is the most important group of teleost fish cultivated in the whole world for commercial ends, due to the fact that they are a food product frequently consumed by the human being (Huang et al., 2007), besides presenting various advantages as biomarker species, such as being very resistant and easy to maintain. Toluca is a city in the State of Mexico in which 111 medical facilities can be found, including primary, secondary and tertiary-care hospitals, as well as health centers, whose wastewater discharges represent a threat to water bodies (Pérez-Alvarez et al., 2018). Therefore, the present study had the objective of studying the alterations to embryonic development and the teratogenic effects in oocytes of *C. carpio* after exposure to different proportions of a hospital effluent.

2. Materials and methods

2.1. Area of study and physicochemical characterization

The hospital under study is found in the city of Toluca, State of Mexico, latitude 19.28, longitude −99.64 (Fig. 1). It is a secondary-care hospital, which tends to a million and a half people, has 100 beds, 34 incubators, 20 cribs and 4 gynecology and obstetrics doctor's offices, an internal medicine doctor's office and an epidemiology department. It also has 2 X-ray and mastography areas, three operating rooms and a clinical analysis laboratory. This hospital tends to 185–200 patients daily, carries out 30 surgical interventions, assists in 35 births and carries out 625 clinical analysis, 150 diagnostic studies and 85 echosonographies.

The samples were obtained in March of 2018 in 5 occasions (Monday through Friday), during the hours when there was the most hospital activity (11:00–14:00 h). The samples were taken from the water that flows directly from the city sewage system. This procedure was carried out according to that stipulated in norm NMX-AA-003-1980, "Wastewaters" (1980). Sampling was carried out using dark-colored 20-liter containers, which were previously washed with 30% nitric acid and perfectly rinsed with deionized water. The samples were transported to the laboratory and stored at 4 °C until their posterior analysis.

A portion of water was used in order to carry out the physicochemical characterization, considering the *Standard Methods for the Examination of Water and Wastewater* (2017), established by the American Public Health Association/American Water Works Association/Water Environment Federation and Mexican norm NOM-001-SEMARNAT-1996. Another portion was taken in order to carry out the chemical characterization of the effluent (metal and emerging contaminants analyses) and the third portion was employed in order to carry out the embryotoxicity and teratogenicity studies.

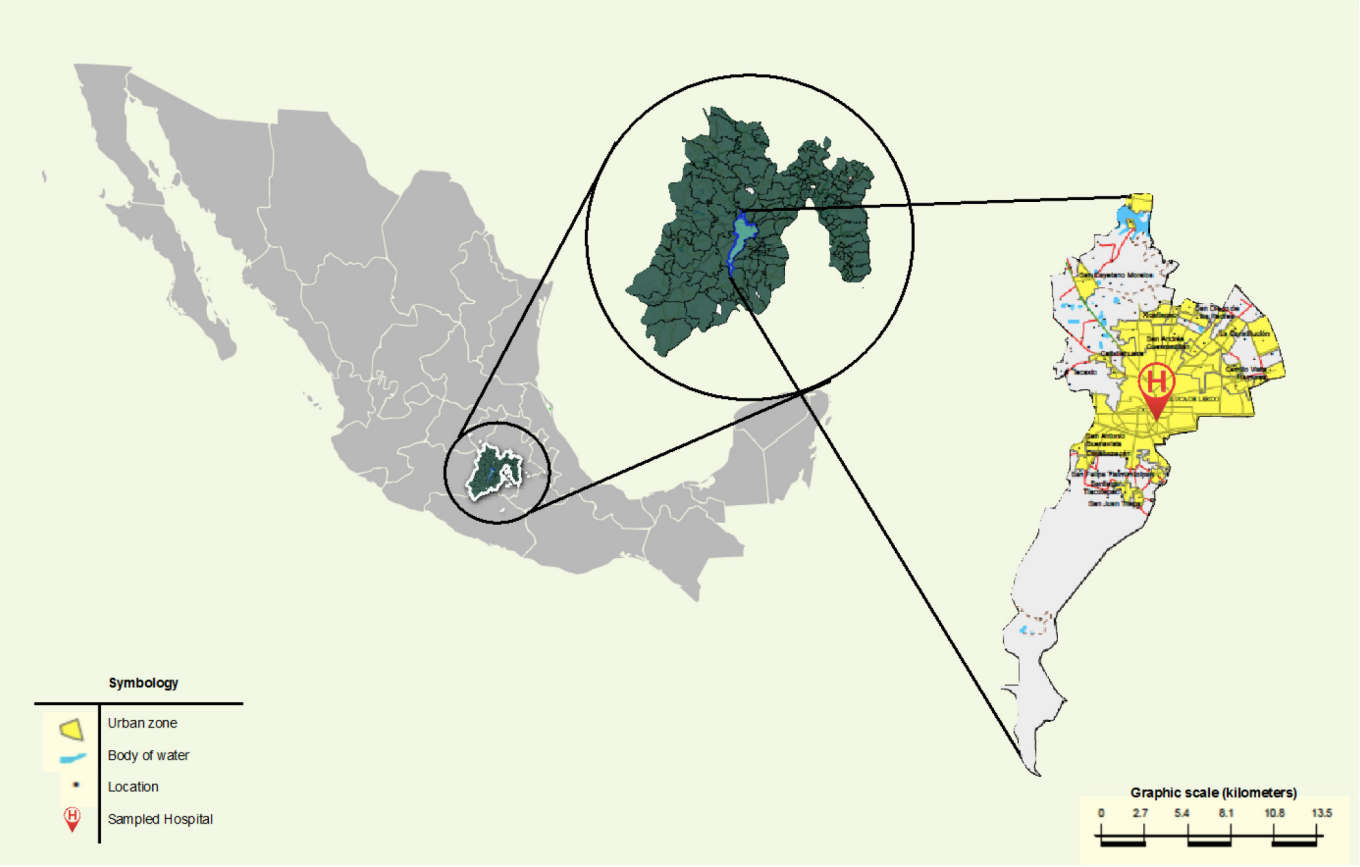


Fig. 1. Localization map of the hospital being evaluated in the city of Toluca, State of Mexico.

2.2. Quantification of microcontaminants present in the hospital effluent

The chemical characterization of the hospital effluent under study consisted in identifying two large groups of contaminants: 1) metals and 2) pharmaceutical-type emerging contaminants. In the case of metals, the methodology proposed in the *Standard Methods for the Examination of Water and Wastewater* (2017) and by González-González et al. (2014), was used. Briefly, before analysis for heavy metals, the effluent was filtered through Whatman no. 541 filter paper (Whatman, Germany) into 100 ml of prewashed plastic bottles and the analytical grade HCl was used to adjust water pH to 3.5. Later, to 0.5 mL of hospital effluent samples, 2 mL of concentrated nitric acid were added. Digestion was carried out using an autoclave at 120 °C and 15-lb of pressure for a period of an hour. Posteriorly, the samples were filtered and diluted with deionized water. The determinations were carried out in a Varian AA1475 atomic absorption spectrophotometer (Melbourne, Australia). The results were interpolated with specific standards for each one of the metals measured, which were As, Cd, Cu, Cr, Hg, Ni, Pb and Zn (Sigma-Aldrich, St. Louis, Missouri, USA). A standard solution was used for each metal 1 mg mL^{-1}. The percentage of recovery for all metals evaluated ranged between 98 and 100%. The absorption wavelength, detection limit (LOD), and quantification limit (LOQ) were respectively: 193.7 nm, 2.65 and 5.39 for As, 228.8 nm, 1.82 and 6.23 for Cd; 324.8 nm, 2.48 and 5.23 for Cu; 357.9 nm, 1.73 and 5.28 for Cr; 253.7 nm, 089 and 3.45 for Hg; 232 nm, 1.12 and 4.67 for Ni; 283.3 nm, 0.94 and 4.17 for Pb and 213.9 nm, 1.03 and 6.78 for Zn. The integration time was 0.5 s, bandwidth 0.2–1.0 nm and lamp current between 6 and 10 mA. The correlation coefficient was found between 0.998 and 0.999. Matrix matched calibration curves, reagent blanks and duplicate samples were used for quality assurance. The analytical precision of instrumental measurements was better than 10% for individual analytes.

In the case of the quantification of pharmaceutical-type emerging contaminants, 12 pharmaceuticals belonging to the groups of non-steroidal anti-inflammatory drugs, beta-lactam antibiotics, antidiabetic drugs, beta-blockers and hormones, were evaluated. The groups consist of the pharmaceuticals that are most prescribed in the hospital under study. The determination was carried out employing that stipulated by López-Serna and Petrović (2012). For this purpose, the chromatographic separation was carried out using an Acquity UHPLC system and a reverse phase BEH C18 column (2.1 mm of 100 mm, with a particle size of 1.7 mm), both from the Waters Corporation. The characteristics of the method are found stipulated in Pérez-Alvarez et al. (2018). Briefly, the pharmaceutical analyzed in positive ionization (PI) mode were eluted employing a mobile phase [acetonitrile (ACN)-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, drugs 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 mL. 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. The LOD and LOQ for each drug were respectively: 0.02 and 0.08 for diclofenac; 0.01 and 0.05 for ibuprofen; 0.75 and 5.82 for naproxen; 0.38 and 1.56 for paracetamol; 0.12 and 0.40 for

penicillin G; 0.22 and 0.74 for penicillin V; 13.54 and 45.13 for glibenclamide; 0.54 and 2.51 for metformin; 0.04 and 0.12 for atenolol; 0.12 and 0.39 for metoprolol and 0.01 and 0.05 for 17 β-estradiol.

2.3. Obtention and oocyte analysis

C. carpio oocytes were employed, obtained by natural fertilization. The process of fertilization was carried out *in situ* in the Tiacaque carp-breeding center in the State of Mexico. For this process, two adult females and four adult males in reproductive stages were placed inside a fertilization pond. The carps were in their natural environment in the ponds and were not subject to changes in their photoperiod. The deep portion of the pond was covered with casuarina branches in order to support the carp's oocytes and aid in their hatching. For the embryotoxicity and teratogenicity tests, only fecundated oocytes analyzed by a stereomicroscope and found in the blastula stage at 2 h post-fertilization were employed. Posteriorly, the fecundated oocytes were exposed to different proportions of the hospital effluent under study.

2.4. Exposure

These tests were carried out following the directives established by the OCDE, in its test guideline *Test No. 236: Fish Embryo Acute Toxicity (FET) Test* (2013), with some modifications for the common carp *C. carpio*. For the tests, the proportions of hospital effluent used were the following: 3, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0 and a control, free of hospital effluent. These proportions were selected based on previous studies done in the laboratory. It is important to indicate that since the total effluents were very toxic, the proportions mentioned above were used, which consisted of the proportions of the effluent indicated and diluted with tap water. The systems consisted of fertilized oocytes randomly selected, which were placed in 24-well microplates. An oocyte was place in each well until it had formed a batch of 20 oocytes. These systems were employed for each of the proportions of effluent and tests were carried out in triplicate. The microplates were maintained for five days at 24 ± 1 °C and natural light-dark periods in the Toxicology laboratory of the Autonomous University of the State of Mexico. Observations were done employing the stereoscopic microscope at 12, 24, 48, 72 and 96 h post-fertilization (hpf), taking photographs and using the Zeiss program for Windows.

2.4.1. Embryolethality test

For the determination of oocyte LC_{50}, systems such as the aforementioned were used. The oocytes were observed with the stereoscopic microscope at 96 h. Lethality was considered when oocytes were coagulated or no heartbeat was detected. Dead and live oocytes were counted and, for the calculation of LC_{50}, a maximum likelihood for linear regression analysis was carried out. LC_{50} was determined with its confidence limits at 95% ($p < 0.05$).

2.4.2. Calculation of LC50, EC50malformations and teratogenic index (TI)

For the determinations of LC_{50} and CE_{50}, the "trimmed" Spearman-Karber method was used (Hamilton et al., 1977), employing the US-EPA software (see 1.5). In order to determine the teratogenic potential of the hospital effluent, the teratogenic index was determined through the quotient between CL_{50} and CE_{50} of malformations. If the TI of a substance is >1, the substance is considered teratogenic; if the substance is <1, the substance mainly produces embryo lethal effects (Weigt et al., 2011).

2.4.3. Evaluation of embryotoxicity and teratogenesis

For the evaluation of embryotoxicity, systems such as those mentioned in Section 2.4, "Materials and Methods", were used. The live embryos were observed under the microscope at 12, 24, 48, 72 and 96 h. The alterations in embryonic development were registered according to the scoring system established by Kimmel et al. (1995) and Hermsen et al. (2011), with the modifications shown in Fig. 2.

The embryonic alterations and teratogenic malformations identified were used to create a histogram of frequencies; they were: developmental delay, delay in the hatching process, fin deformity, hypopigmentation, hemorrhaging in the head, hemorrhaging in the tail, hemorrhaging in the yolk, modified chorda structure, malformation of the head, malformation of the heart, mouth hyperplasia, malformation of the otoliths, malformation of the tail, pericardial edema, rachischisis, scoliosis, yolk deformation and yolk edema.

2.5. Validity criteria of the test and statistical analysis

In order to guarantee the traceability of the results, the oocyte batches were used only if the fertilization rate was ≥90%. It is also important to mention that the test is considered valid if the control groups do not show >10% of the lethal teratogenic effects at 96 hpf.

Statistical analysis was performed with IBM SPSS Statistics 25. One-way analysis of variance (ANOVA) followed by *post hoc* multicomparison with the Bonferroni's test was used to analyze homogeneous data of the continuous variables. Kruskal–Wallis test was used to analyze non-homogeneous data. The frequency of abnormal oocytes or embryos was evaluated with Fisher's exact test. Significance was accepted when $P < 0.05$.

3. Results

3.1. Physicochemical characterization of the effluent

In Table 1, the values of the mean and the standard deviations of the physicochemical characteristics of the hospital effluent are shown. As can be observed in Table 1, many of the parameters are not established in Mexican norms; however, these parameters are essential in order to be able to evaluate the quality of the water. All the physicochemical characteristics evaluated are within the values stipulated in Mexican regulation. However, as can be seen, said norms are not updated and have more than twenty years in use.

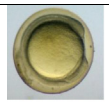
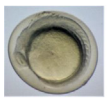
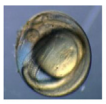
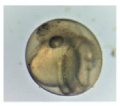
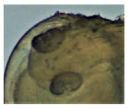
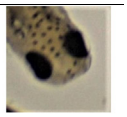
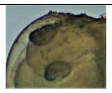
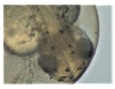
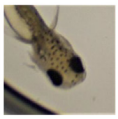
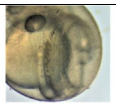
Although the physicochemical characterization is important in order to evaluate water quality, it is not the only parameter that must be considered to guarantee that the water from a hospital effluent is adequate and safe to be disposed of in the city sewage system. For this purpose, the chemical characterization of the effluent evaluated was carried out as follows.

3.2. Metals and pharmaceutical-type emerging contaminants found in the hospital effluent

In Table 2, data obtained from the chemical characterization of the effluent studied is presented. In the case of metals such as Hg and Pb, these are higher than that permitted by Mexican norms. In the case of drugs, this type of micropollutants is not included in the Mexican regulations, however, the concentrations found in the effluent have been associated to different toxic effects, such as genotoxicity, cytotoxicity and oxidative stress in *C. carpio* juveniles (Guadalupe Martínez-Viveros et al., 2018; Nava-Álvarez et al., 2014; Olvera-Néstor et al., 2016; Orozco-Hernández et al., 2018).

Fig. 2. General morphology scoring system showing normal development of *C. carpio*, up to 96 hpf, with different scores assigned to specific developmental endpoints in time. Adapted from Hermsen et al. (2011), for *C. carpio*.

General Morphology Score (GMS)

	12 hpf	24 hpf	48 hpf	72 hpf	96 hpf
Detachtment of tail	 0 				
	1	2	3	3 + 1 for pigment	3 + 1 for pigment
Somite formation	No = 0	Yes = 1	Yes = 1	Yes = 1	Yes = 1
Eye development	 1	 2	 2 + 1 for pigment	 2 + 1 for pigment	 2+ 1 for pigment
Movement	No = 0	Yes = 1	Yes = 1	Yes = 1	Yes = 1
Heartbeat	No = 0	Yes = 1	Yes = 1	Yes = 1	Yes = 1
Blood circulation	No = 0	No = 0	Yes = 1	Yes = 1	Yes = 1
Pigmentation head-body	0	0	  1	 1	 1
Pigmentation tail	0	0	 1	 1	 1
Pectoral fin	0	0	0	 1	 1
Protruding mouth	0	0	0	 1	 2
Hatching	No = 0	No = 0	No = 0	 Yes = 1	 Yes = 1
GMS	2	7	12	16	17

3.3. Embryolethality and teratogenicity data of the hospital effluent evaluated

In Table 3, the data related to the embryolethality and teratogenicity studies, is presented. On one side, exposed oocytes are shown, as well as percentages of mortality and malformations that were employed to calculate lethal concentration 50 and malformation effective concentration. These values are presented with their confidence intervals at 95% ($p < 0.05$). Taking into account the relationship between $LC_{50}/CE_{50\text{-malformations}}$, the teratogenic index was determined, which was of 1.46 in this study, reason for which the hospital effluent in this study was considered as "teratogenic".

In Fig. 3, the data of the oocytes exposed to the different proportions of hospital effluent under study and the different percentages of oocytes (those that were "normal", those that presented some teratogenic alteration and those that died), is shown. As can be seen, the oocytes with teratogenesis were between 20 and 34%, and the dead between 17 and 29%. It is important to note that, in the highest proportions of hospital effluent, the number of deaths increased because the teratogenic alterations risked the integrity of the oocyte. In the controls, the development of the oocytes stayed normal up to 96 hpf and no death or teratogenic alteration took place.

3.4. Main teratogenic alterations and frequency of the same by exposure to the hospital effluent

In Fig. 4, the teratogenic alterations observed in the oocytes of *C. carpio* exposed to different proportions of hospital effluent, are shown. As can be observed, the most frequent were: yolk deformation, scoliosis, modified chorda structure, malformation of the tail, fin deformity and mouth hyperplasia. To the different proportions of effluent, the number of malformations was between 20 and 34% and, as the exposure time increased, the malformations became more severe, risking the integrity of the carp oocyte.

In Fig. 5, the different malformations which appeared in the oocytes of common carp by exposure to the hospital effluent can be seen more clearly. In the control, the development of the carp oocyte at different hpf can be seen. As can be observed at 72 and 96 hpf, the most severe alterations in the oocyte were generally skeletal malformations with severe cases of scoliosis, notochord alterations, mouth hyperplasia and pericardial edema. These alterations were very severe in some oocytes, even causing death.

In Fig. 6, the score obtained by each one of the proportions of hospital effluent evaluated at the different exposure times employed, can be seen. As can be observed in the case of the control, the score obtained was the maximum of each exposure time, given that the development of the oocyte was normal; however, as can be observed, as the proportions of hospital effluent increased, the score decreased due to all the alterations presented during embryonic development ($p < 0.05$). At 6%, the score was the most drastic. In all proportions of hospital effluent at 12, 24, 48, 72 and 96 h were observed significant differences respect to control group ($p < 0.05$). In the case of the 3% proportion of the effluent, decreases were observed between 20% and up to 50% with respect to time, for the proportion of 3.5% of the effluent were observed decreases in the score between 28.5 and up to 50%, for the proportion of 4% decreased the scores between 50 and 64.7%, for 4.5% of the effluent between 72 and 78.5%, for 5% of the effluent between 42.8 and 76.5%, for 5.5% of the effluent between 41.6 and 82.4% and for the last proportion 6%, a decrease between 48 and 88%. All the decrements were statistically significant with respect to the control score and at all exposure times.

4. Discussion

At present hospitals have been described in different works as important sources of pollutants that are discharged into the aquatic environment, so the characterization of the toxicity of these is important in risk management and to ensure the establishment of environmental regulations to protect the aquatic environments.

The toxic effects of hospital effluents in different parts of the world have been reported in various studies. In these works a great variety of biomarkers has been evaluated as oxidative stress, cytotoxicity, mutagenicity and teratogenicity in different organisms, such as in the case of Boillot et al. (2008) in France, who demonstrated ecotoxicity (EC_{20}) in *D. magna*, *V. fischeri*, *P. subcapitata* and *C. dubia*. In Argentina, Magdaleno et al. (2014) reported toxicity in *P. subcapitata* (inhibition of growth) and genotoxicity in *A. cepa* (mitotic index, frequency of chromosomic aberrations and micronuclei). Also, in Argentina, Paz et al. (2008) demonstrated a mutagenic effect (Ames test) in *S. cerevisiae*. In Taiwan, Li and Lin (2015) concluded that a hospital effluent generates acute toxicity (LC_{50}) in *C. carpio*. In Mexico, Neri-Cruz et al. (2015) reported the induction of OS (lipid and protein damage, modifications in superoxide dismutase (SOD) and catalase (CAT) antioxidant enzyme activity) in *C. carpio*. Olvera-Néstor et al. (2016) demonstrated genotoxicity (micronuclei) and cytotoxicity (caspase 3 and lactate dehydrogenase activity; TUNEL test) in the same species. These authors have attributed the toxicity to the complex mixture of compounds that are present in this type of effluents. Few studies have shown the ability of effluents to generate embryotoxic and teratogenic effects, such as Pérez-Alvarez et al. (2018) who observed alterations in embryonic development, teratogenesis (FETAX test) and induction of OS (lipid and protein damage, modifications in SOD and CAT antioxidant enzyme activity) in *Xenopus laevis* and *Lithobates cataesbeianus* after exposure to a hospital effluent. However, the literature reviewed does not show signs that hospital effluents generate alterations to embryonic development or teratogenic effects in fish and in a species of economic interest such as *C. carpio*, this is one of the reasons for carrying out this work.

Another one of the main reasons for the necessity of toxicologic evaluation, which is prompted by hospital effluents on aquatic organisms, is that the release of compounds which are contained in these wastewaters go directly into the city sewage system (Langford and Thomas, 2009) and into the bodies of water, leading to deleterious effects for aquatic life. As previously mentioned in the introduction, through metabolic activity or abiotic conditions of the systems, metabolites or degradation products can be obtained, which can be even more toxic than the original compounds and that add on to the toxicity already produced by these (Trawiński and Skibiński, 2017).

This study, along with others that our research group has carried out concerning hospital effluents, has the main objective of characterizing the toxic response and its magnitude in wastewaters which the hospital under evaluation produces, with the finality of informing authorities of this medical facility, the ecological impact on the environment its waste is having, and to propose some alternative in order to avoid further ecologic deterioration.

The hospital under study does not have a wastewater treatment plant and, as the only treatment measure, employ chlorination, as this is an easy and cheap method; however, there are different reports which have established that there is an increment in toxicity in diverse aquatic species after treatment of wastewaters through the chlorination method (Pignata et al., 2012; Gopal et al., 2007).

As can be observed in the results, once the physicochemical characterization of the hospital effluent had been done, most of the physicochemical properties were within the values permitted by Mexican regulation. The parameters that are not contemplated in said regulation were dissolved oxygen, conductivity, ammonia and NaClO. Nonetheless, the values of dissolved oxygen, conductivity and ammonia found in the effluent are appropriate for the survival of *C. carpio* and have not been correlated with physiological or biochemical alterations in aquatic organisms (Tchobanoglous et al., 2003; Postma et al., 2002).

However, in the case of NaClO, we found a concentration of 1.3 mg L^{-1}; in literature, there are reports with concentrations between

Table 1
Physicochemical characteristics of the hospital effluent.

Physicochemical parameter	Hospital effluent evaluated	NOM-001-SEMARNAT-1996	NOM-002-SEMARNAT-1996
Temperature (°C)	15.3 ± 0.5	40	40
Oxygen dissolved (mg L^{-1})	13.6 ± 0.3	N.E.	N.E.
Conductivity (μS cm^{-1})	7.3 ± 0.7	N.E.	N.E.
pH	8.1 ± 0.2	6.5–8.5	6–9
Chlorides (mg L^{-1})	203 ± 4	250	N.E.
Flourides (mg L^{-1})	6.7 ± 0.9	0–15	N.E
Hardness (mg L^{-1})	2.45 ± 0.1	500	N.E.
Ammonia (mg L^{-1})	0.97 ± 0.2	N.E.	N.E.
Total suspended solids (mg L^{-1})	42 ± 1.2	60	40–60
Total phosphorus (mg L^{-1})	8.3 ± 0.8	10	10
Total nitrogen (mg L^{-1})	19 ± 0.5	25	N.E.
Biochemical oxygen demand (mg L^{-1})	43 ± 0.3	60	40–60
NaClO (mg L^{-1})	1.3 ± 0.1	N.E.	N.E.

N.E. = not established in the norm.

0.55 mg L^{-1} and 1.24 mg L^{-1}, which are capable of generating genotoxic effects in erythrocytes of *C. carpio* (Buschini et al., 2004). Likewise, this compound has induced alterations in hepatic enzymes of the same species (Elia et al., 2006).

Once the chemical characterization of the microcontaminants present in the hospital effluent had been carried out, it was observed that metals such as As, Cd, Cu, Cr, Hg, Ni, Pb and Zn were present in concentrations between 0.017 and 0.478 mg L^{-1}. Some studies have demonstrated that metals are capable of producing embryotoxicity and teratogenicity in aquatic organisms as *Crassostrea gigas*, *Mytilus trossolus* and *Strongylocentrotus purpuratus* (Mai et al., 2012; Nadella et al., 2009; Phillips et al., 2003). One study carried out by our research team demonstrated that the same metals present in the hospital effluent under study during 2017 induced oxidative stress, embryotoxicity and teratogenicity in *L. catesbeianus* and *X. laevis* (Pérez-Alvarez et al., 2018). Also, Mai et al. (2012) demonstrated that embryotoxic and genotoxic effects appeared due to exposure to Cu (0.1 μg L^{-1}) and Cd (10 μg L^{-1}) at the first stages of life of the Pacific oyster (*Crassostrea gigas*). Also, it has been demonstrated that concentrations of Cr between 30 μg L^{-1} and 88.2 mg L^{-1} are capable of inducing alterations such as cytotoxicity, affectation to the immune system, DNA damage and embryonic alterations in species such as *Labeo rohita*, *Pimephales promelas*, *Salmo gardnerii*, *C. carpio* and *Oreochromis nilitica*, among others (Bakshi and Panigrahi, 2018).

Table 2
Microcontaminants detected in the hospital effluent.

Type of contaminant	Metal or compound	Concentration
Metal	As	0.017 ± 0.001 mg L^{-1}
Metal	Cd	0.052 ± 0.001 mg L^{-1}
Metal	Cu	0.34 ± 0.001 mg L^{-1}
Metal	Cr	0.63 ± 0.02 mg L^{-1}
Metal	Hg	0.037 ± 0.001 mg L^{-1} [a]
Metal	Ni	0.67 ± 0.001 mg L^{-1}
Metal	Pb	0.478 ± 0.03 mg L^{-1} [a]
Metal	Zn	±0.02 mg L^{-1}
NSAIDs	Diclofenac	0.60 ± 0.08 μg L^{-1}
	Ibuprofen	0.72 ± 0.12 μg L^{-1}
	Naproxen	1.83 ± 0.30 μg L^{-1}
	Paracetamol	2.84 ± 0.09 μg L^{-1}
ß-lactams	Penicillin G	4.01 ± 0.30 μg L^{-1}
	Penicillin V	0.56 ± 0.10 μg L^{-1}
Antidiabetics	Glibenclamide	2.03 ± 0.08 μg L^{-1}
	Metformin	1.42 ± 0.06 μg L^{-1}
ß-blockers	Atenolol	0.023 ± 0.002 μg L^{-1}
	Metoprolol	2.8 ± 0.16 μg L^{-1}
Hormones	17 βestradiol	0.018 ± 0.001 μg L^{-1}

[a] Exceeds the limits of Mexican regulations (NOM-001-SEMARNAT-1996 and NOM-001-SEMARNAT-1996); LOD: limit of detection; LOQ: Limit of quantification; NSAIDs: Non-steroidal anti-inflammatory drugs. The limits of detection and quantification are expressed in μg L^{-1} for metals and ng/L for pharmaceuticals.

With regard to the pharmaceuticals identified in the effluent, NSAIDs were the main pharmaceuticals found, such as diclofenac, ibuprofen, naproxen and paracetamol, in concentrations between 0.023 and 4.01 μg L^{-1}. A study carried out by Xia et al. (2017) demonstrated that exposure to 5, 50 and 500 μg L^{-1} of diclofenac and ibuprofen produced a delay in the hatching process of the embryos, as well as affectation to the motor activity of the larvae of the zebra fish. Also, Ji et al. (2013) demonstrated that concentrations ≥ 1 μg L^{-1} of ibuprofen affected reproduction, delayed hatching and the production of eggs of *Danio rerio*. In the hospital effluent evaluated, penicillin G and V, antidiabetics such as glibenclamide and metformin, beta-blockers such as atenolol and propranolol and hormones such a 17-β-estradiol, were found. Hermsen et al. (2011) informed of diverse effects due to exposure to carbamazepine, diclofenac and metoprolol, at concentrations of 30.6, 1.5 and 12.6 mg L^{-1}, respectively, exhibiting growth delay, delay in the hatching process, deformation of the tail and yolk sac, and scoliosis in embryos of *Danio rerio*. Likewise, 17-β-estradiol is capable of inducing oxidative stress, DNA damage and alterations to embryonic development in *C. carpio* and *Dendraster excentricus* at concentrations of 0.001, 0.01, 0.1 and 10 mg L^{-1} (Orozco-Hernández et al., 2018; Gutiérrez-Gómez et al., 2016; Rempel et al., 2009).

The results of embryolethality found in this study demonstrated that the hospital effluent evaluated is very toxic for the oocytes of *C. carpio*, since a proportion of 5.64% of the hospital effluent provoked a mortality of 50% of the oocytes exposed. Different studies carried out throughout the world have established the occurrence and toxic effects on some aquatic organisms due to hospital effluents containing a variety of contaminants (Pérez-Alvarez et al., 2018; Azuma et al., 2016; Verlicchi et al., 2010; Neri-Cruz et al., 2015; Oliveira et al., 2017; Santos et al., 2013).

The studies cited have demonstrated the presence of the same types of contaminants that were found in the present study, mainly drugs, metals and disinfectants. Besides having shown that the concentrations of the different contaminants found in the hospital effluent are toxic to

Table 3
Mortality and malformation data in oocytes of *C. carpio* exposed to hospital effluent.

Proportion of effluent (%)	Number of embryos exposed	Mortality (%)	Malformations (%)
0	60	0	0
3	60	26.6	41.6
3.5	60	30.0	45.0
4	60	33.3	45.0
4.5	60	38.3	51.6
5	60	41.6	61.6
5.5	60	46.6	73.3
6	60	58.3	88.3
		LC50 = 5.65% CI = [4.99%–7.47%] Teratogenic index 1.46	EC50 = 3.85% CI = [3.47%–4.14%]

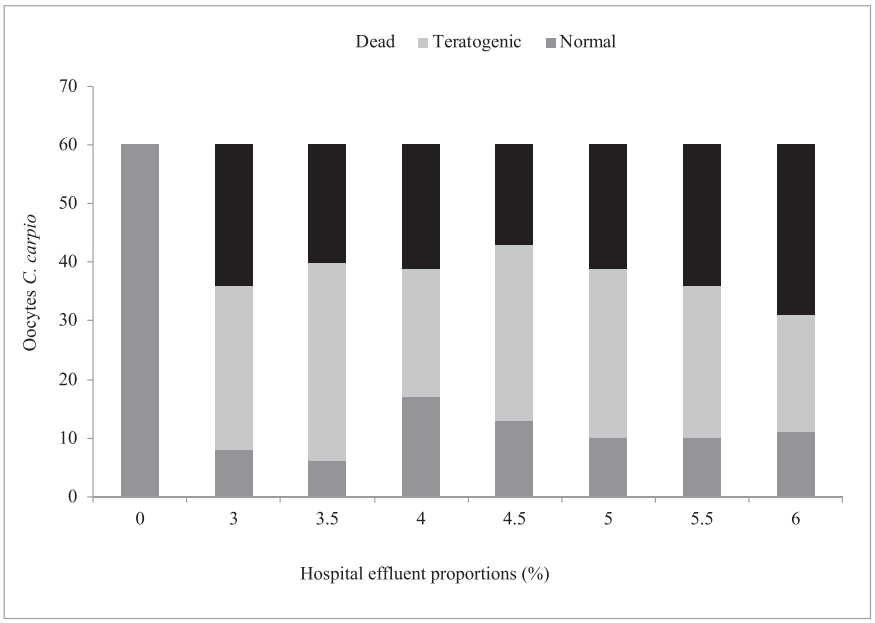


Fig. 3. Percentage of normal oocytes, oocytes with teratogenesis and dead oocytes exposed to the different resources of the hospital effluent used.

aquatic organisms, the problem with this type of effluent is that the compounds are found in complex mixtures that allow them to interact with each other, altering their toxicity (Carraro et al., 2016; Emmanuel et al., 2005; Olvera-Néstor et al., 2016). For example, metals can be found in low concentrations or in quantities within the values established by regulations; however, the synergic interaction that can exist between them can potentiate their toxic effects (Kosmehl et al., 2012).

In the present study, the hospital effluent showed the capability of inducing alterations to embryonic development and having teratogenic effects in *C. carpio* oocytes exposed to different proportions of the hospital effluent. The results obtained can be explained by the action mechanism of the metals present in the hospital effluent which, in general, are related to osmotic alterations and in the synthesis and enzymatic activity ("An introduction to metals in fish physiology and toxicology: basic principles", 2011). For example, Cd, Zn and Pb alter the absorption of calcium through the reduction of the activity of Ca^{2+}-ATPase (Baldisserotto et al., 2004). Copper induces osmoregulatory failure through the alteration of the Na^{+}/K^{+}-ATPase pump (Grosell et al., 2004). Also, lead and cadmium have the capacity of bonding to calmodulin and can affect different cell activities (BIRCEANU et al., 2008). Besides osmotic alterations, metals can affect the enzymatic activity of different enzymes such as citrate synthase, succinate dehydrogenase, lactate dehydrogenase, among others (Shaw and Handy, 2011). Metals have also been associated to oxidative stress in aquatic organisms (Gómez-Oliván et al., 2017; González-González et al., 2014; Craig et al., 2007). As catalysts in the Fenton reaction within cells (Di Giulio et al., 1989); the free radicals that are formed are capable of inducing lipoperoxidation, carbonylation of proteins and DNA damage (Lushchak, 2011). With these different action mechanisms, the metals

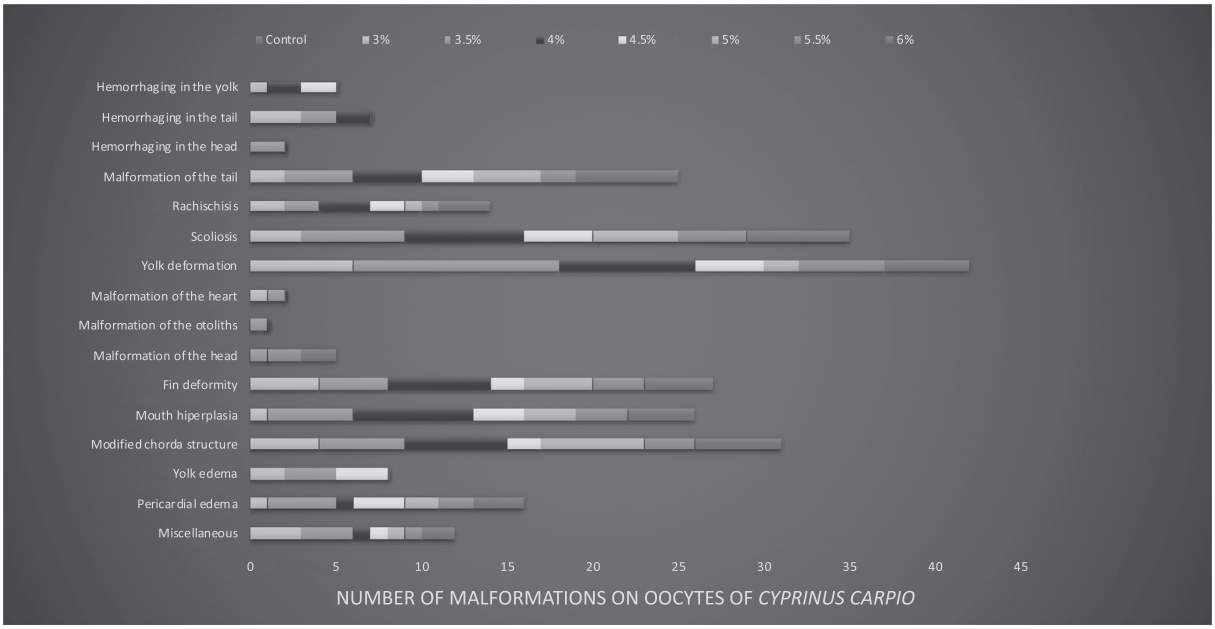
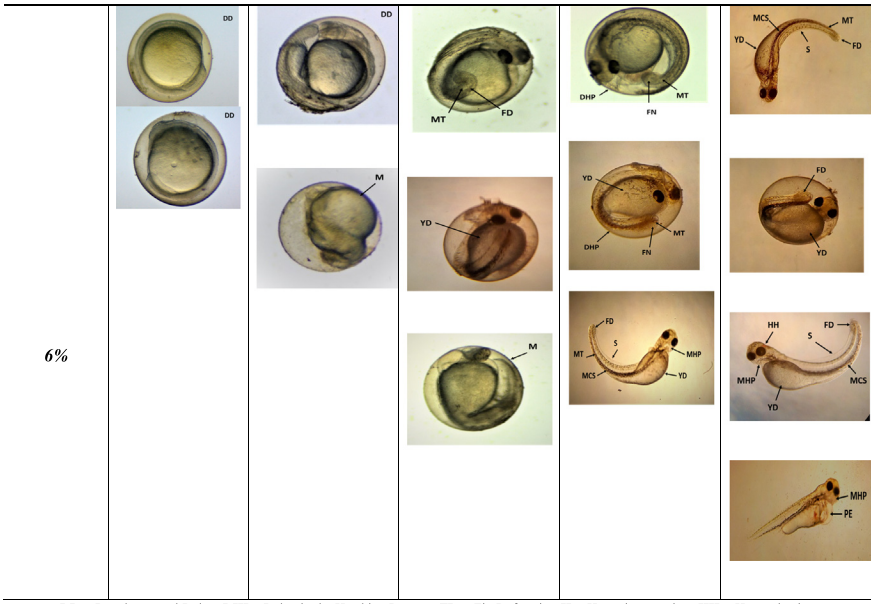


Fig. 4. Teratogenic malformations induced by exposure of oocytes of *C. carpio* to different proportions of the hospital effluent.

Fig. 5. Malformations induced by exposure to hospital effluent at12, 24, 48, 72 and 96 h at the following proportions: 0, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5 and 6%, on *C. carpio*.

Fig. 5 (*continued*).



DD = Developmental Delay; **DHP=** Delay in the Hatching Process**; FD =** Fin Deformity; **H** = Hypopigmentation**; HH** = Hemorrhaging in the Head; **HT** = Hemorrhaging in the Tail; **HY** = Hemorrhaging in the Yolk**; M** = Miscellaneous; **MCS** =Modified Chorda Structure; **MH** = Malformation of the Head; **MHE** =Malformation of the Heart; **MHP** = Mouth Hyperplasia; **MO** =Malformation of the Otoliths; **MT** = Malformation of the Tail; **PE** = Pericardial Edema; **R** =Rachischisis**; S** = Scoliosis; **YD** =Yolk Deformation; **YSE** = Yolk Edema

Fig. 5 (*continued*).

can affect a great variety of physiological, biochemical and metabolic processes during the development of the oocytes of fish such as *C. carpio* resulting in growth delay, morphologic and anatomic anomalies or the death of the least-resistant individuals (Jezierska et al., 2009). In addition to these findings, there are different studies which establish that the metals have the capability of penetrating the oocytes, especially during the swelling phase, and to accumulate (Jezierska et al., 2009). For example, lead can bond to mucopolysaccharides of the oocyte membrane, modifying its permeability, generating alterations in the ionic exchange between the perivithel fluid of the oocyte and the external environment (Stouthart et al., 1994).

It has been observed that metals such as Pb, Cd, Cu and Zn are capable of generating delays in organogenesis, delay in the eye and body pigmentation process, and alterations in heartbeat and delay in the hatching process of species such as *C. carpio* and *Melanotaenia fluviatilis* have also been observed (Witeska et al., 2014;(Williams and Holdway, 2000).

Also, metals are capable of generating diverse teratogenic effects such as deformation of the notochord, scoliosis, dorsal fin edema, eye, mouth and head edema, and malformation of the eyes, hypopigmentation, deformations in the yolk sac, among others, in species such as *Pimephales promelas*, *Clarias gariepinus*, *Danio rerio* and *C. carpio* (Cheng et al., 2000; Flik et al., 2002; Fraysse et al., 2006; Hallare et al., 2005; Nguyen and Janssen, 2002; Osman et al., 2007).

The embryotoxic and teratogenic effects presented in the oocytes of *C. carpio* can be explained by the presence of pharmaceuticals in the hospital effluent. For example, non-steroidal anti-inflammatory drugs such as IBP, at concentrations ranging from 10 to 100 μg L^{-1}, have demonstrated their capacity to induce some alterations such as pericardial edema, decrease in heart rate, malformations and loss of the pectoral fins in embryos of *Danio rerio* (David and Pancharatna, 2009). The authors of this previous work explain that said effects can be attributed to the action mechanism of IBP, which is the inhibition of the cyclooxygenase 1 enzyme (COX-1), since the prostaglandins obtained from this enzyme are necessary for the egg segmentation process. Also, the inhibition of COX-1 has been associated with defects in the formation of the heart and shortening of the intersomitic vessels (Cha et al., 2005). It has also been demonstrated that there is an increase in hatching time due to exposure to DCF and IBP at different concentrations in the zebra and medaka fish (David and Pancharatna, 2009; Hallare et al., 2004; Lee

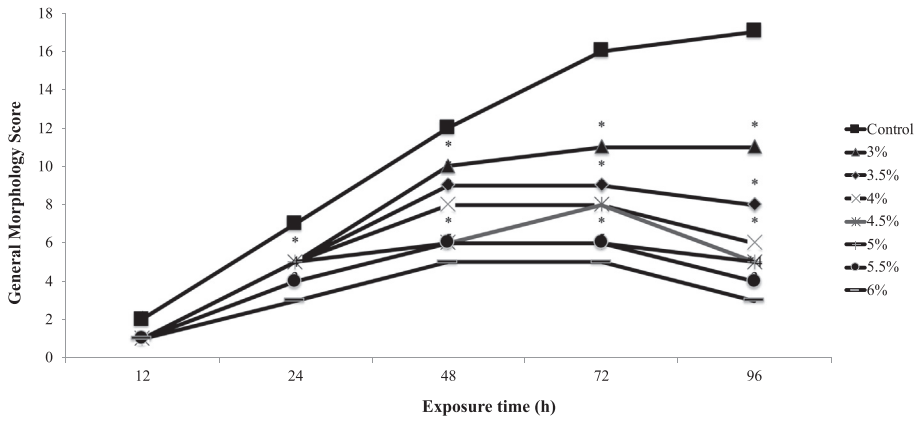


Fig. 6. Exposure time-response curve of the general morphology score of the hospital effluent on oocytes of *C. carpio*.

et al., 2011). Another of the pharmaceuticals found in this study has been associated to the increase in the mortality of *Danio rerio* embryos and the appearance of severe malformations: 17-β-estradiol (Westerlund et al., 2000), as well as teratogenic alterations, delay in the hatching process and increase in vitellogenin in embryos of *Zoarces viviparus* (Morthorst et al., 2014).

Also, beta-blockers such as metoprolol (found in this study) have been associated to the decrease in heart rate, delay in the hatching process and increase in the mortality of *Danio rerio*, in concentrations ranging from 8 to 16 mg L^{-1} (Sun et al., 2014). Additionally, the same pharmaceutical has generated alterations in behavior such as swimming activity (Bittner et al., 2018). In general, our research team has demonstrated that pharmaceuticals present in the hospital effluent evaluated have the common characteristic of being capable of inducing oxidative stress in species such as *Hyalella azteca*, *Daphnia magna*, *C. carpio*, *X. laevis* and L. *catesbeianus* (Martínez-Rodríguez et al., 2018; Martínez-Rodríguez et al., 2018; Pérez-Alvarez et al., 2018; Cardoso-Vera et al., 2017; Gutiérrez-gómez et al., 2016; García-Medina et al., 2015; Saucedo-Vence et al., 2015; Gómez-Oliván et al., 2014a, 2014b, 2014c; Islas-Flores et al., 2013, 2014; Oviedo-Gómez et al., 2010). Some studies have demonstrated that oxidative stress damages the oocytes, as well as causes mitochondrial alterations, depletion of ATP, DNA damage, apoptosis and delay in embryonic development in mammal and human embryos (Aitken and Krausz, 2001). In a manner similar to the findings in mammals, the role of oxidative stress in the alteration of embryos and larvae of fish such as *Pleuragramma antarcticum*, *Oncorhynchus mykiss* and *Macrobrachium rosenbergii*, has been established (Dietrich et al., 2005; Regoli et al., 2005; Dandapat et al., 2003). In general, it has been demonstrated that oxidative stress is a mechanism that can generate embryonic and teratogenic alterations. These phenomena are essentially produced by modifications in genetic expression, alterations in the signaling of the transcription factor and the cell cycle (Dennery, 2007; Hansen, 2006). In summary, all the contaminants present in the effluent have the capacity of generating deleterious effects on the oocytes of *C. carpio*, leading to alterations in embryonic development and teratogenesis. These effects are correlated to the specific mechanisms of toxicity of these compounds; however, the present work demonstrates that the mixture of all the contaminants of the effluent potentiates toxicity, given that very low proportions of the effluent manifested themselves in the alterations observed.

It is important to emphasize that in complex mixtures of pollutants, such as hospital effluents, the toxic effects cannot be estimated directly from individual responses of each component of the effluent or from the sum of the biological responses. In this case the toxic effects are the reflection of all the components that are present in the effluent, their interactions, besides considering that due to the biotic and abiotic characteristics of the system, metabolites can be formed that are even more toxic than the original compounds, and that can interact with these.

5. Conclusions

The physicochemical properties of the effluent were found to be within permitted Mexican regulation values; however, it is important to consider that some parameters were not contemplated within the official norms due to the fact that these norms have not been updated in over 20 years. The main microcontaminants found in the effluent were metals and pharmaceuticals. The main alterations found in this study were yolk deformation, scoliosis, modified chorda structure, malformation of the tail, fin deformity and mouth hyperplasia. The data obtained in this study clearly show that the hospital effluent studied induces alterations to embryonic development and teratogenic effects in *C. carpio* oocytes, despite the low concentrations of micropollutants that were identified and quantified in this study. However, it is necessary to make a determination of other contaminants that were not detected in this work, this will be useful for designing programs to control the treatment of wastewater generated in this hospital unit. Additionally, the oocytes of *C. carpio* have demonstrated to be a good bioindicator of toxicity, since they are highly sensible to the effects of the effluent evaluated.

Conflict of interest

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

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