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Effects of effluent from a hospital in Mexico on the embryonic development of zebrafish, *Danio rerio*

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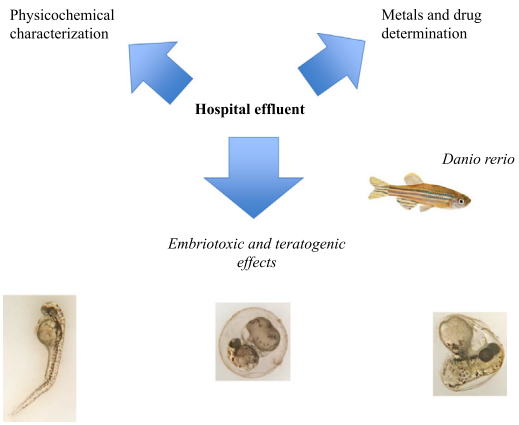
HIGHLIGHTS

- Hospital effluent studied induced alterations in embryonic development.
- The teratogenic index was 2.45%.
- Main malformations identified were yolk sac malformation and pericardial edema.
- Hospital effluent represents a risk for *Danio rerio* embryos.

GRAPHICAL ABSTRACT



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ABSTRACT

Hospitals consume a large amount of water, so they also generate large amounts of wastewater, which contain a wide variety of contaminants. It is important to consider that hospital effluents are a mixture of pollutants that can interact with each other and have a negative impact on aquatic species of water bodies. The aim of this study was to evaluate the effects induced by a hospital effluent using *Danio rerio* embryos. In this study, *Danio rerio* embryos were exposed to different concentrations of the hospital effluent and a lethality test was evaluated and the malformations present in zebrafish embryos were evaluated. The lethal concentration of effluent 50% was 6.1% and the effective malformation concentration was of 2.5%. The teratogenic index was 2.45%. The main malformations identified were yolc sac malformation, pericardial edema, hatching abnormalities, hypopigmentation, tail deformation, chorda malformation, without fin, chorion deformation and craniofacial malformation. The risks that this type of water represents for the survival of living organisms, as well as the presence of malformations in them, are reference indicators for a future regulation focused on the adequate treatment of hospital effluents.

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1. Introduction

Water pollution is one of the most frequent problems in the world and of greater severity today, because this is the most important resource for life. There are several sources of water pollution such as: agricultural activities, industry, domestic wastewater (Quintero Vargas, 2017); as well as hospital effluents (Gautam et al., 2007).

Hospitals consume a large amount of water, so they also generate large amounts of wastewater, which contain a wide variety of contaminants (Laffite et al., 2016). These compounds include chemicals, metals, disinfectants, sterilizing agents, detergents, radioactive markers, contrast agents, as well as drugs and their metabolites (Luja-Mondragón et al., 2019). The aforementioned micropollutants are not completely eliminated by the wastewater treatment plants of the hospitals; this favors the introduction of hospital effluents to municipal sewage or aquatic environments (Verlicchi et al., 2010).

It is important to consider that hospital effluents are a mixture of pollutants that can interact with each other and have a negative impact on aquatic species of water bodies (Islas-Flores et al., 2017; Neri-Cruz et al., 2015), among these damages include genetic lesions, damage to organs, reproductive problems, changes in the behavior, and/or biochemical alterations (Oliveira et al., 2017).

In Mexico, hospital effluents may contain concentrations of drugs that fluctuate between 0.018 and 4.01 μg L^{-1}. The drugs they may contain are nonsteroidal anti-inflammatory, antidiabetics, β-lactams, β-blockers and hormones (Luja-Mondragón et al., 2019). This effect is favored by poor drug disposal practices and because many health institutions lack wastewater treatment plants (Quesada et al., 2009). The continuous entry of microcontaminants into hospital effluents generates chronic exposure of hydrobionts causing serious environmental problems (Barceló and Daughton, 2003).

The wastewater treatment systems operating in Mexico were designed to purify solids, dissolved organic matter and nutrients, so many toxic compounds undergo the treatment process with little or no change in their concentration (Castro-Pastrana et al., 2015). In addition to achieve good hospital water treatment, prior knowledge of the influent is required, so it is necessary to have knowledge of the number of hospital beds, the number of inhabitants in the hospitalization area, the volume of wastewater per day per bed, the volume of wastewater per day per inhabitant and finally the flow of water from the receiving body (Verlicchi et al., 2013).

Recently, the concern for sanitation and recycling of drinking water has grown because the problem of water scarcity is increasing, along with population growth, climate change and urbanization (Richey et al., 2015). The problem is already so widespread that WHO estimates that by 2025, half of the world's population will live in areas with water scarcity (Boretti and Rosa, 2019). Everything mentioned above makes plans to improve wastewater reuse strategies a priority, since otherwise the reused water, if not receiving the appropriate treatment, will not reach the supply with the desirable quality. The poor quality of treated or untreated water can cause adverse effects on organisms and humans, when it is discharged into the environment and the supply of drinking water (Richey et al., 2015). Some of the negative effects that occur in living organisms exposed to contaminated water are teratogenic and genotoxic damage, organ damage, oxidative stress, damage to biomolecules and even death (Pérez-Alvarez et al., 2018).

Studies with aquatic organisms of embryotoxicity and teratogenicity have been conducted as a result of exposure to hospital effluent. In 2018 Pérez-Álvarez reported that a hospital effluent from Toluca, Mexico presented a teratogenic index of 3.8 for *X. laevis* and 4.0 for *L. catesbeianus*. The same effluent showed a growth inhibition, as well as various malformations such as microcephaly, facial and pericardial edema, malformations of the eye, tail and fin. On the other hand, Luja-Mondragón et al. (2019) reported embryo-lethal effects by hospital effluents in 5.65% and teratogenic effects in *Cyprinus carpio*.

Various organisms have been used for the evaluation of embryonic development disorders and teratogenic effects, however *Danio rerio* has taken importance in determining these effects, mainly because these substances affect common developmental stages in all vertebrates. In addition to the zebrafish has proven to be sensitive, easy to reproduce in the laboratory and easy to maintain under laboratory conditions (Kimmel et al., 1995).

The antecedents previously exposed allowed to establish the objective of this study, which was to evaluate the effect on embryonic development and the teratogenic effects induced by a hospital effluent using *Danio rerio* as bioindicating organisms.

2. Methods

2.1. Sampling

The sampling was carried out in a private hospital in the City of Puebla, Mexico, during the period of March 11–15, 2019, daily, during the hours when there was the most hospital activity (8–14 h). Samples were taken from the water that flows directly from the city sewage system after of the hospital wastewater treatment plant. Dark colored polyethylene containers were used. The procedures under which the sampling was conducted were stipulated by Mexican norm (NMX-AA-003-1980. Residual waters, 1980) The hospital under study has 26 services, including: clinical laboratory, radiotherapy unit, pediatric intensive care unit, emergency department, imaging department, oncology service, breastfeeding service, among others (Fig. 1). Once the sampling was done, the containers were transported to the laboratory. The effluent water samples were separated into three parts: the first two parts were used immediately; one part was used to determine the physicochemical parameters (water quality), the other part was used to determine the concentration of micropollutants (metals and drugs). The third part was stored at 4 °C and was previously treated to remove residual chlorine and was destined to carry out the toxicological studies.

2.2. Physicochemical characterization of hospital effluents

The physicochemical characterization of the effluents was carried out using official Mexican norms for their evaluation. The parameters analyzed were: biochemical oxygen demand, conductivity, pH, temperature, dissolved oxygen, chlorides, fluorides, hardness, ammonia, total suspended solids, total phosphorus, total nitrogen and NaClO.

2.3. Quantification of microcontaminants

The chemical characterization of the evaluated hospital effluent was performed by quantifying two larges polluting groups: 1) metals and 2) drugs. For the determination of metals, samples were filtered using Whatman paper no. 541 (Whatman, Germany). The pH of the samples was adjusted to 3.5. Subsequently, 0.5 mL of the samples were added concentrated nitric acid. Samples were digested using autoclave and at 120 °C and 15 psi for 1 h. Finally, the samples were filtered and diluted in 100 mL of deionized water and read using a Varian AA1475 atomic absorption spectrophotometer (Melbourne, Australia). The data were interpolated in a standard curve of 1 mg L^{-1} for each metal evaluated (González-González et al., 2014). The recovery percentages for the metals analyzed were between 97 and 100%. Table 1 describes some parameters used in the quantification of metals in the hospital effluent.

For drug determination, samples were filtered in a two-step process (8 μm membrane followed by a 2.5 μm membrane). Solid phase extraction (SFE) Phenomenex, Strata XL cartridges (Torrance, CA) were conditioned with 10 mL methanol followed by 10 mL water. Samples (500 mL) were added to SFE cartridges at a flow rate of 5 mL/min and then eluted with 6 mL methanol. Solvent was evaporated to dryness using nitrogen N-Evap 112 (Organomation, Haverhill, MA). Samples

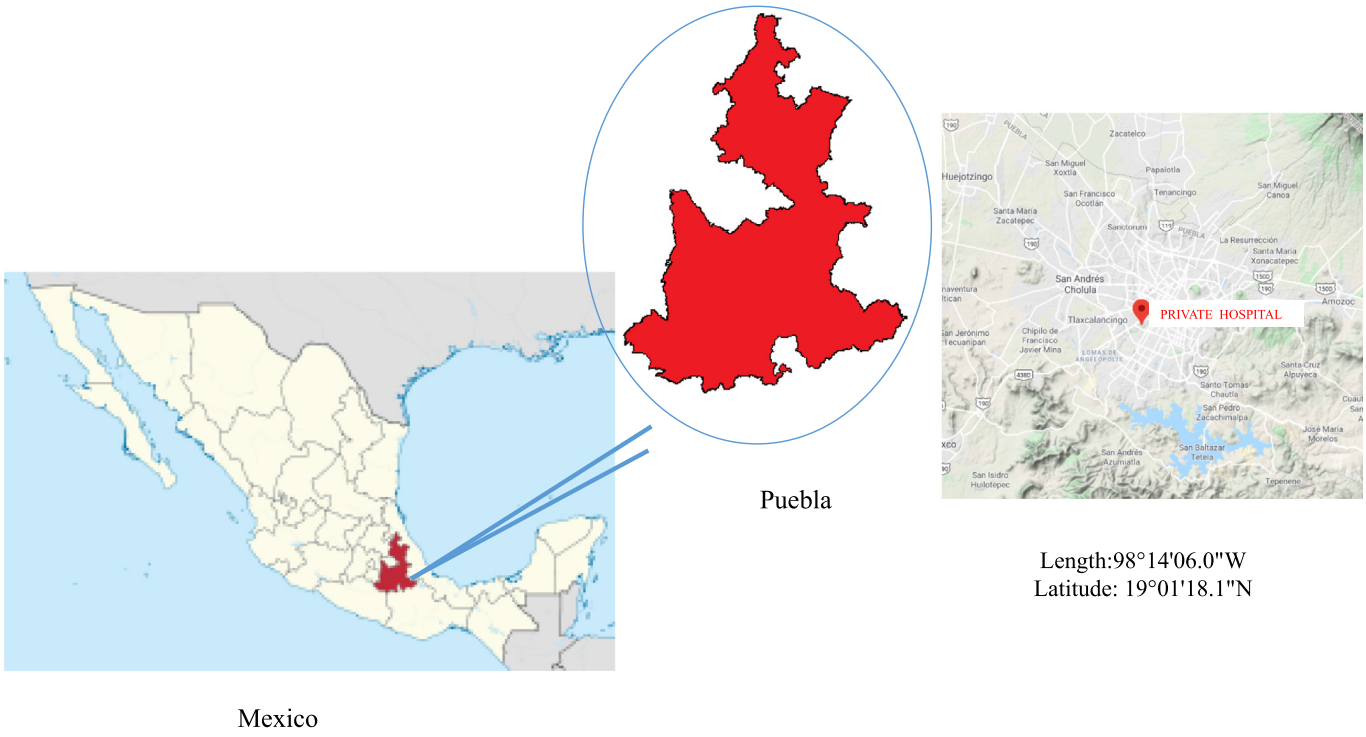


Fig. 1. Location map of the hospital sampled in Puebla, State of Puebla, Mexico.

were reconstituted with 1 mL methanol:water (50:50) and passed through a 0.2 μm syringe filter before injection.

A Waters (Mildford, MA) LC–MS/MS (liquid chromatography-mass spectrometer) was used for the identification and quantification of pharmaceutical compounds in hospital samples. An Acquity UPLC BEH C18 (2.1 × 100 mm, 1.7 μm) column was used for compounds separation. Mobile phases were 0.1% (v/v) formic acid in water (A) and 0.1% (v/v) formic acid in methanol (B). Column was maintained at 40C. A flow rate of 0.3 mL/min and injection volume of 10 μL were used. Total run time of 10 min with 90% A at 0.1 min, 10% A at 8 min and 90% A at 10 min.

Quattro Premier XE triple quadrupole mass spectrometer fitted with electrospray ionization (ESI) was used in positive mode for paracetamol, ketorolac, ranitidine, esomeprazole, omeprazole, hydrocortisone, and dexamethasone while negative mode was used for ibuprofen and naproxen. Mass spectrometer optimization was conducted by direct infusion of 200 mg L^{-1} standard solution of all nine pharmaceutical compounds analytical standards for selection of the ionization mode and precursor and product ions. The limit of detection (LOD) and the limit of quantitation (LOQ) were calculated as follows (Brubaker, 1999):

$$LOD = t_{0.99} \times S \text{ and } LOQ = 3 \times LOD$$

Table 1
Parameters used in metal quantification.

Metal	Absorption wavelength (nm)	Detection limit (LOD) μg L^{-1}	Quantification limit (LOQ) μg L^{-1}
As	193.7	2.65	5.39
Cd	228.8	1.82	6.23
Cu	324.8	2.48	5.23
Cr	357.9	1.73	5.28
Hg	253.7	0.89	3.45
Ni	232	1.12	4.67
Pb	283.3	0.94	4.17
Zn	213.9	1.03	6.78

where, $t_{0.99}$ = the one-tailed statistic at the 99% confidence level for *n* replicates, S = the standard deviation of recovery results from *n* samples fortified at the estimated LOQ.

In Table 2, some conditions of the equipment used in the evaluation of drugs in the study are presented.

2.4. Experimental set-up

2.4.1. Parent maintenance

For the production of eggs, commercially acquired breeding fish were used, those with sexual maturity, between 4 and 5 cm in length, free of infection and disease symptoms were selected. The breeding fish were kept in aquariums with a recommended loading capacity of 1 L of water per fish, separated from males and females, with a natural photoperiod and at a temperature of 27 °C. They were fed with commercial flake food until one week before spawning, when this food was changed to *Artemia* sp. Aquarium water was cleaned and replaced every three days (Test No. 236: Fish Embryo Acute Toxicity (FET) Test, 2013).

2.4.2. Egg production

Zebrafish eggs were obtained in individual spawning tanks, in which they were placed: females and males (previously sexed and isolated) in a ratio of 2: 1 respectively. The spawning tank contained 10 L of tap water with sea salts (1 mL L^{-1} of Instant Ocean) and anti-chlorine, at 27 °C. Three spawning tanks were used to perform the test. In order to collect the fertilized eggs and avoid their predation by adults, a barrier was formed between the parent organisms and the eggs; this barrier consisted of forming traps with 5 mm pore mesh boxes inside the tanks.

Once the eggs were obtained, they were observed at 4× in a Zeiss stereoscopic microscope model m40t67 and the viable eggs were separated from the non-viable. Only those embryos that were fertilized, not coagulated in blastula stage were used. To avoid genetic bias, the eggs were collected from the three breeding groups, mixed and randomly selected.

Table 2
Mass spectrometer parameters used in drug quantification.

Compound	Ionization mode	Parent ion (*m*/*z*)	Product ion (*m*/*z*)	Cone voltage (V)	Detection limit (LOD)ng L^{-1}	Quantification limit (LOD)ng/L^{-1}
Paracetamol	+	151.9	110	25	3.23	9.69
Ketorolac	+	256.2	105.5	30	2.67	8.00
Ranitidine	+	315.2	176.0	25	2.09	6.28
Esomeprazole	+	346.1	151.1	20	3.07	9.22
Omeprazole	+	346.1	198.0	20	3.31	9.93
Hydrocortisone	+	363.2	121.0	31	2.36	7.07
Dexamethasone	+	363.2	147.1	20	3.25	9.76
Ibuprofen	−	205.1	161.1	15	2.97	8.90
Naproxen	−	229.2	170.0	15	2.81	8.44

2.4.3. Exposure

The effluent water samples were previously treated to remove the NaClO present. For this process, the water was treated with sodium thiosulfate as anti-chlorine in a concentration of 1 mg L^{-1} for 20 min. Subsequently, the water was kept at rest in a dark container and protected from light with continuous oxygenation for 24 h. This process was carried out with the purpose of removing residual chlorine from the effluent, since studies previously carried out in our laboratory have shown that NaClO in concentrations of 2 mg L^{-1} affect the survival of the *Danio rerio* embryos and generate malformations.

Viable blastula embryos were exposed to various proportions of the effluent studied: 0.1, 0.5, 1.0, 1.5, 2.0, 2.3, 2.5, 3.0, and 3.5%. 60 embryos per proportion were used, which were placed in 24-well plates (one embryo per well). In each well, 2 mL of the reconstituted medium with salts, anti-chlorine and the effluent were placed in different proportions. A free hospital effluent system was used as a control and the experiments were performed in triplicate.

After 96 hpf, the live, dead and malformed embryos were counted and a maximum likelihood linear regression analysis was performed to calculate lethal concentration 50 (LC_{50}) and effective concentration of malformation (EC_{50m}) with their 95% confidence intervals ($p < 0.05$). Spearman-Karber method trimmed was used (US-EPA software ver 1.5). The teratogenic index (TI) was also calculated using the ratio LC_{50}/EC_{50m}. If the TI was >1, hospital effluent was considered as teratogenic and if it was <1 as embryolethal, according to criteria of (Weigt et al., 2011).

2.4.4. Embryonic development evaluation

The evaluation was carried out based on the visible morphology of the embryos at different cutting times at 12, 24, 48, 72 and 96 hpf, with structural anomalies and delayed symmetric development, considered the latter as a teratogenic endpoint. This evaluation was performed in each organism when compared with the reference embryo, according to (Kimmel et al., 1995). Each embryo received a score for each of the factors to be evaluated, which are the following: tail development, eye development, somite formation, movement, heartbeat, blood circulation, head-body pigmentation, tail pigmentation, appearance of pectoral fin, buccal bump and hatching; for each abnormality or delay presented in any of the characteristics to be evaluated, one unit was subtracted from the score Hermsen et al. (2011).

2.4.5. Evaluation of malformations and teratogenic effects

The count of malformations and teratogenic effects was recorded in a database for each cutting time (12, 24, 48, 72, 96 hpf). All organisms were observed in search of any of the following reported malformations in zebrafish: pericardial edema, edema of the yolk sac, ocular edema, head malformation, otolith malformation, tail malformation, heart malformation, modified rope structure (scoliosis) and spinal disease (Hermsen et al., 2011). Those most representative deformities were photographed for later description.

2.5. Statistic analysis

LC50 and EC50 were calculated using PROBIT analysis (EPA Analysis Program v 1.5). Alterations to embryonic development and teratogenic effects data were analyzed by Fisher's exact test. Significance was accepted when $p < 0.05$, using SPSS v9 software (SPSS, Chicago, IL). The validity criteria used in this study were principal two, the first was that the fertilization rate was ≥90%, and the second was that the test was considered valid if the control groups showed no >10% of lethal teratogenic effects at 96 hpf.

3. Results

3.1. Physicochemical characterization of the effluent

Table 3 shows the values of the main physicochemical parameters that are considered to evaluate water quality in Mexican regulations. The data is an average with their respective standard deviation. Some of the parameters evaluated are not considered in Mexican regulations, but they are essential for assessing water quality. When contrasting hospital effluent results with mexican norms NOM-001-SEMARNAT-1996 and NOM-002-SEMARNAT-1996, can be observed that the total suspended solids, chlorides exceed the limits of the norms, likewise the conductivity and NaClO values are high. The results were contrasted with these norms because the first one refers, the maximum levels of pollutants allowed in wastewater discharges into domestic waters and resources and the second one refers the maximum levels of pollutants allowed in wastewater discharges into municipal or urban sewer systems. It can also be noted on the table that the norms have not been updated and have been issued for more than twenty years.

3.2. Concentration of micropollutants identified in the effluent evaluated

Table 4 shows the data of the main pollutants identified in the hospital effluent. The study effluents were sampled from a 92-bed capacity private hospital which has a wastewater treatment plant. In an interview with the hospital pharmacy staff we were provided with the general flow diagram of the treatment process. Based on the architectural complex of the hospital, there are two drainage entrances, one north and the other south, coming from different areas and buildings. Both influent sources are led to the pre-treatment unit where solids, large objects and materials that cannot be biologically treated such as plastic, glass and sand, among others, are separated. This unit aerates, homogenizes and regulates the flow variations in the supply of contaminated water entering the plant. This process unit uses pumping and regulating systems. Then the water enters the reactor where the biological treatment begins with the addition of air so that the bacteria present in the activated sludge or biomass transform the contaminated water. The resulting liquid passes to the secondary clarifier where most of the particles that are in suspension settle or are removed and the active sludge is decanted. A part of the activated sludge is returned to the bioreactor

Table 3
Physicochemical characteristics of hospital effluent.

Physicochemical parameter	Hospital effluent evaluated	NOM-001-SEMARNAT -1996	NOM-002-SEMARNAT -1996
Temperature (°C)	22 ± 0.8	40	40
Dissolved oxygen (mg L^{-1})	6.5 ± 0.3	N.E.	N.E.
Conductivity (μS cm^{-1})	1141.2 ± 3.7	N.E.	N.E.
pH	6.3 ± 0.2	6.5–8.5	6–9
Chlorides (mg L^{-1})	273 ± 6	250	N.E.
Flourides (mg L^{-1})	7.1 ± 0.8	0–15	N.E
Hardness (mg L^{-1})	123 ± 1.2	500	N.E.
Ammonia (mg L^{-1})	1.3 ± 0.6	N.E.	N.E.
Total suspended solids (mg L^{-1})	67 ± 13.2	60	40–60
Total phosphorus (mg L^{-1})	5.8 ± 1.0	10	10
Total nitrogen (mg L^{-1})	17 ± 0.5	25	N.E.
Biochemical oxygen demand (mg L^{-1})	5.67 ± 0.3	60	40–60
NaClO (mg L^{-1})	4.1 ± 0.5	N.E.	N.E.

N.E. = not established in the norm.

to maintain the balance between bacteria and contaminating particles, the other part of the sludge is taken to a digester for its total stabilization and safety. After several days, the stabilized sludge is dehydrated in drying beds and its final disposal is carried out.

For its part, the supernatant that leaves the clarification chamber passes to the water disinfection unit, which consists of a chlorinator of calcium hypochlorite tablets that dissolve slowly, eliminating pathogens for human health and reducing the biological demand for oxygen at levels within the national ecological regulations. Then, a treated water tank sends the disinfected water to a Parshall-type metering system for discharge to the municipal sewer system. It was at this point that the samples were taken for the present work.

In the case of lead and mercury, concentrations were found above the permissible limit set by Mexican regulations. On the other hand, it is observed that concentrations of various pharmacological groups were found in concentrations of 0.02 and up to 373.8 ng L^{-1}. It should be mentioned that in Mexico no regulations have been established in reference to the permissible limits of drugs in water. The most important groups identified in the effluent were NSAIDs, corticosteroids, inhibitor of proton pump, H2-antagonism, which are related to the most prescribed medications in the hospital.

3.3. Embryo-lethality data of hospital effluent

Table 5 shows the data on the percentages of mortality and malformations of the embryos affected to the hospital effluent. The number of malformations and dead embryos increased proportionally with the increase in concentration. The LC_{50} value was 6.1% with 95% confidence intervals (3.6–17.4) and the EC_{50} of malformations was 2.5% with intervals between 1.7 and 4.5%. With these data the teratogenic index that was 2.4 was calculated. According to the criteria established by Weigt et al. (2011), hospital effluent was classified as teratogenic.

In Fig. 2, live, dead and malformed embryos are observed in each proportion of the hospital effluent. From the proportion of 2.3 to 3.5%, the embryos presented a similar number of malformations. The number of live embryos decreased proportionally as the proportion of effluent increased. On the 3.5% proportion of hospital effluent, live embryos were no longer observed.

Table 4
Micropollutants detected in hospital effluent.

Pollutant type	Metal or drug	Concentration
Metal	As (mg L^{-1})	0.014 ± 0.001
Metal	Cd (mg L^{-1})	0.048 ± 0.001
Metal	Cu (mg L^{-1})	0.36 ± 0.001
Metal	Cr (mg L^{-1})	0.71 ± 0.02
Metal	Hg (mg L^{-1})	0.041 ± 0.001[a]
Metal	Ni (mg L^{-1})	0.79 ± 0.001
Metal	Pb (mg L^{-1})	180 ± 0.08[a]
Metal	Zn (mg L^{-1})	0.53 ± 0.02
NSAIDs	Ketorolac (ng L^{-1})	373.8 ± 5.8
	Ibuprofen (ng L^{-1})	10 ± 1.2
	Naproxen (ng L^{-1})	9.3 ± 1.1
	Paracetamol (ng L^{-1})	52 ± 5.3
Corticosteroids	Dexamethasone (ng L^{-1})	9.8 ± 0.79
	Hydrocortisone (ng L^{-1})	14.4 ± 0.93
Inhibitor of proton pump	Esomeprazole (ng L^{-1})	12.6 ± 0.90
	Omeprazole (ng L^{-1})	11.5 ± 0.78
H2- antagonism	Ranitidine (μg L^{-1})	0.023 ± 0.002

[a] Above what is established in Mexican regulations.

3.4. Morphological alterations in embryos of D. rerio due to exposure to a hospital effluent

Fig. 3 shows the main morphological alterations observed in embryos of *D. rerio* exposed to different proportions of the effluent. The most frequent malformations were: hatching alterations, tail deformation, pericardial edema, yolk sac malformation, tail malformation and hypopigmentation. At the highest proportions of effluent, the number of malformations was increasing and they became more serious, putting the life of the embryos at risk. In this case, the malformations increased with mortality as the concentration increased.

Table 6 shows photographs of the main malformations at different times 12, 24, 48, 72 and 96 hpf in the *Danio rerio* embryos exposed to different proportions of the hospital effluent. By increasing the exposure time and the proportion of the effluent, an increase in malformations and their severity is observed. In the highest concentrations of the effluent deaths were observed due to the severity of malformations presented.

Table 5
Percentages of dead and teratogenic embryos due to exposure to hospital effluent.

Proportion of effluent (%)	Total embryos exposed	Total dead embryos	Mortality (%)	Total embryos with teratogenic effects	Embryos with teratogenic effects (%)
0	180	0	0	0	0
0.1	180	21	11.67	42	23.33
0.5	180	30	16.67	51	28.33
1.0	180	30	16.67	57	31.67
1.5	180	42	23.33	75	41.67
2.0	180	54	30.00	78	43.33
2.3	180	66	36.67	87	48.33
2.5	180	66	36.67	96	53.33
3.0	180	66	36.67	102	56.67
3.5	180	72	40.00	108	60.00

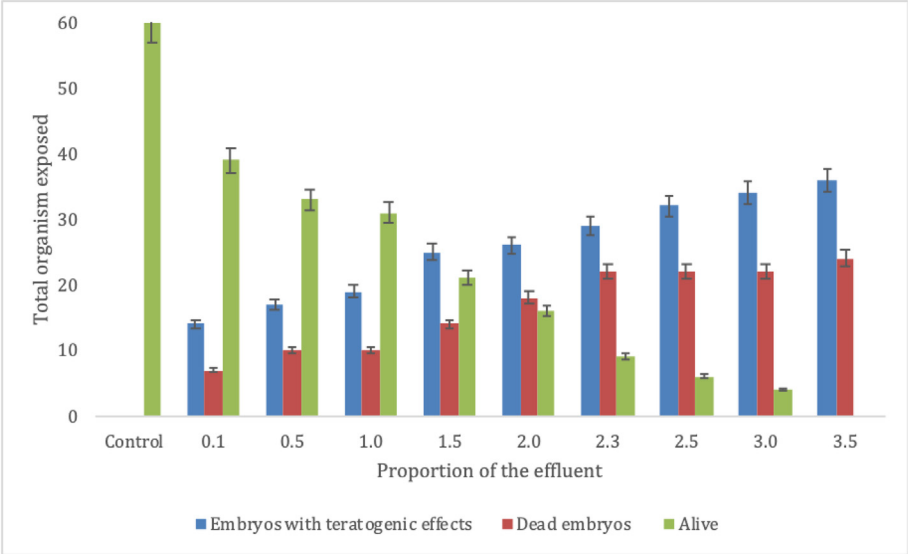


Fig. 2. Effects on embryos exposed to hospital effluent in different proportions.

3.5. Scoring due to the malformations of D. rerio exposed to a hospital effluent

In Fig. 4, the averages of the score obtained by the embryos of *D rerio* in each of the effluent proportions are shown. The average and standard error are presented. For the control group, the maximum score is observed due to the adequate development of the embryo with respect to time. In the different proportions of the effluent significant differences were observed with respect to the control ($p < 0.05$). There were differences at 12 hpf with respect to the control, only in the groups exposed to 0.5, 1.5, 2.3, 3.0 and 3.5 of the hospital effluent ($p < 0.05$). After 24 hpf differences were presented in each of the concentrations with respect to the control group ($p < 0.05$).

4. Discussion

The present study showed that the hospital effluent under study was able to generate alterations to embryonic development and teratogenic effects at very low proportions on *Danio rerio* embryos. It is important to mention that the effluent evaluated, previously passed through a wastewater treatment plant, in which a biological reactor was used and subsequently through a chlorination tank. However, as can be seen in the physicochemical analysis, although most of the parameters are within the values of Mexican regulations, parameters such as chlorides, suspended solids, $NaClO^{-}$ and conductivity are high, in addition to highlighting the presence of metals such as Hg and Pb, in addition to drugs from different therapeutic groups. In Mexico there is no regulation in reference to the presence of emerging contaminants and especially the drug type.

As can be seen in Table 3, the conductivity value was very high (1141.2 μS cm^{-1}). In a study by Kumar and Sinha (2010), they mention that conductivity is an important parameter to determine water quality. High conductivity values are related to water pollution. In addition, these authors mention that the conductivity is significantly related to other physicochemical parameters such as total dissolved solids and chloride, parameters that were also high in this study. Also, Postma et al. (2002) mention that high values of ammonia (>60 mg L^{-1}) and conductivity (>650 μS cm^{-1}), can be confusing parameters in the evaluation of ecotoxicity.

Also, Buschini et al. (2004) demonstrated that $NaClO^{-}$ values between 0.55 and 1.24 mg L^{-1} generate genotoxic effects in *Cyprinus*

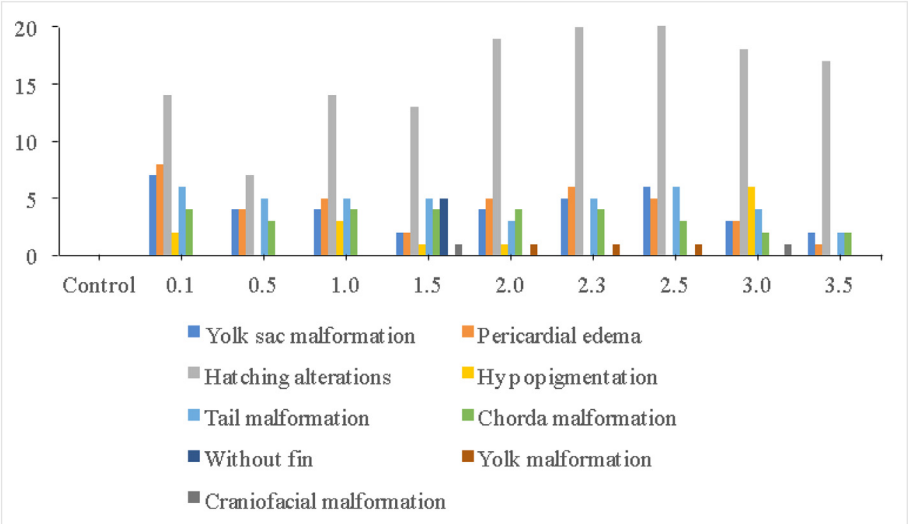
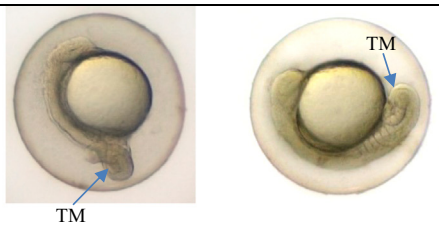
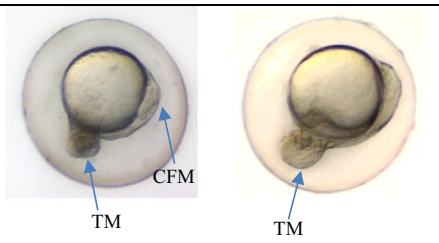
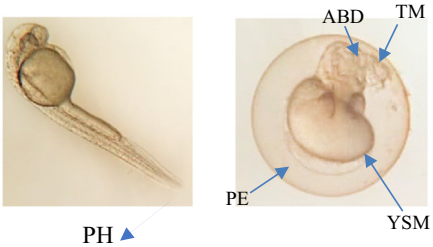
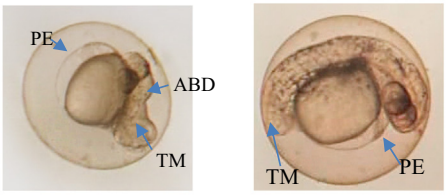
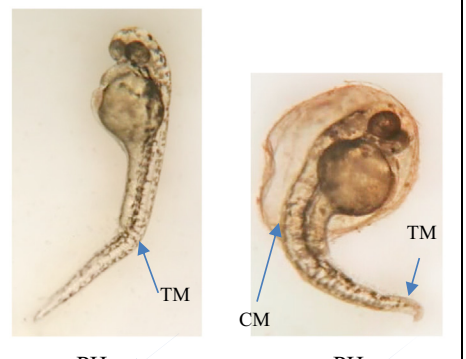
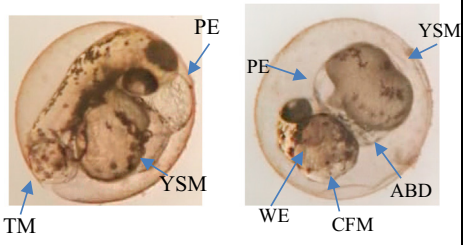
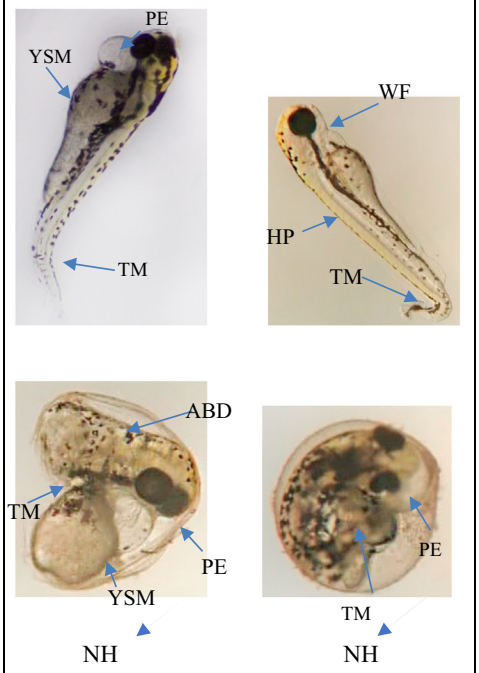
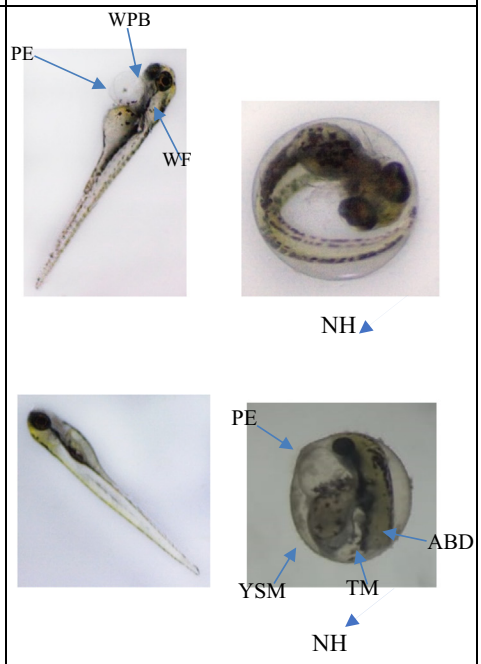


Fig. 3. Frequency of malformations in *D rerio* presented in different proportions of hospital effluent.

Table 6
Main malformations in embryos of *Danio rerio* identified by exposure to hospital effluent at different times.

Exposure time	Malformations
12 pfh	
	
24 pfh	
	
48 pfh	
	
72pfh	
96pfh	

CFM = craniofacial malformation; **TM** = tail malformation; **YSM** = yolk sac malformation; **PE** = pericardial edema; **CM** = chorda malformation; **ABD** = abnormal body development; **WE** = without eyes; **HP** = hypopigmentation; **WF** = without fin; **pH** = premature hatching; **WBP** = without buccal protuberance; **NH** = No hatching.

carpio erythrocytes. In this study the values of this parameter were found above 4 mg L^{-1}. The chlorination process used in water treatment of hospital effluent may increase this parameter. However, for toxicological tests this parameter was eliminated, since the effluent waters were previously treated to eliminate residual chlorine as previously mentioned in the methodology.

In this study, values of metals in magnitudes mg L^{-1} and drugs in ng L^{-1} were observed in hospital effluent. Studies conducted by (Coz et al., 2004), showed that pH < 6.5 favors soluble forms of metals. In this study, we observed that metals such as As, Cd, Cu, Cr, Hg, Ni, Pb and Zn

were present in the effluent studied, which could favor their absorption by the zebrafish. On the other hand in Table 4, it can also be seen that the drugs identified in the effluent were ketorolac, ibuprofen, naproxen, paracetamol, dexamethasone, hydrocortisone, esomeprazole, omeprazole and ranitidine.

As seen in Table 4, one of the metals that was above the threshold limit value was Hg. The embryotoxic and teratogenic effects induced by the hospital effluent found in this study could be related to the presence of Hg. Studies conducted by (Dong et al., 2016) showed that Hg in 0.1 μM concentrations is capable of crossing the pores of chorion the *Oryzias latipes* and *Danio rerio* embryos. The chorion is important because it is a semipermeable membrane that provides protection to the embryo from its surroundings and when the hatching time approaches the membrane becomes more permeable. Once Hg is inside the chorion, it binds to protein thiols and induces the formation of reactive oxygen species (ROS) responsible for embryotoxic effects (Weil et al., 2009).

On the other hand, the Pb that was also found above the threshold limit value could contribute to the embryolethal and teratogenic effects found in this study. Lead is known to be an electropositive metal with affinity for the sulfhydryl groups of proteins and has the ability to inhibit the delta-ALAD enzyme that has the ability to induce malformations in zebrafish embryos (Bartzke et al., 2010). In addition, being a divalent metal the Pb, it can compete with Ca^{++} in cellular processes such as the respiration at the level of mitochondria. Once Pb replaces Ca^{++}, Pb acts as a second intracellular messenger, altering the distribution of Ca^{++} in the different compartments of the cell. This generates that the Ca^{++} available for the development of the bone system is limited, causing malformations such as scoliosis, kyphosis and lordosis (Brannen et al., 2010).

Another of the effects observed in this study was premature hatching that occurred in the highest proportions of the hospital effluent. (Hollert et al., 2003), indicates that Pb interferes with neurotransmission and vascular tone, which leads to involuntary contractions that favor larvae to hatch early. Likewise, the same authors mention that the Pb lodged in the endoneural space produces edemas, increases the pressure and generates hemorrhages in the embryo (Bartzke et al., 2010; Hollert et al., 2003).

Zn has been associated with delayed hatching in zebrafish, Ansari et al. (2015) showed that this metal at concentrations of 1 m L^{-1} was able to delay the hatching of the embryos up to 7 days, or death of the embryos at 11–12 days without hatching.

Another of the metals identified in the hospital effluent were nickel, arsenic and chromium. In accordance with Factor and Chavez (2012), determined that Al, As, Cr and Ni in concentrations of 1.88, 1.01, 0.02 and 1.59 mg L^{-1}, respectively, were able to generate developmental delay, pericardial edema and hatching delay in *Radix quadrasi* embryos. These results coincide with the findings identified in this study for zebrafish embryos.

These results could be explained because the As binds to the mitochondrial enzymes of the embryos, which causes blockage of the synthesis of proteins and oxidative phosphorylation (Ansaldo et al., 2009). On the other hand, has been determined that chromium is capable of binding to the phosphodiester skeleton of DNA bases causing mutations and death of freshwater species (Marchese et al., 2008). Also, Cu at 100 μg L^{-1} in zebrafish embryos is capable of delaying or avoid hatching, induces tail and chorda malformations, pericardial edema and increases mortality. These effects are produced by Cu ability to generate free radicals, which attack the fatty acids of the membrane and modify changes in the structure of the larva (Luzio et al., 2013).

In addition to metals, another group of contaminants identified in the hospital effluent studied were drugs. Santos et al. (2010), identified that drugs are an important group of pollutants that have been associated with different toxic effects, including alterations in embryonic development and teratogenic effects. In our study, one of the identified subgroups was the non-steroidal anti-inflammatory drugs. These have been associated with alterations in embryonic development and teratogenic effects in aquatic species (Santos et al., 2010). For example, Li et al. (2016), found effects such as pericardial edema, delayed hatching, hypopigmentation and stunted growth due to exposure to naproxen at concentrations of 20 mg L^{-1} in *Danio rerio* embryos. These same authors determined that due to the ability of naproxen to interact with the hydrophilic groups of the membrane, its absorption is facilitated and causing alteration of important biomolecules in the development of the embryo and generating hypopigmentation, hemorrhage, condensation of the yolk, and craniofacial abnormalities.

Also studies carried out by Sehonova et al. (2017) demonstrated that naproxen sodium at concentrations of 0, 50, 100 and 200 μg L^{-1} in subchronic exposure of up to 32 days is capable of inducing alterations in growth, delay in hatching and high mortality in *Cyprinus carpio* embryos.

Pérez-Alvarez et al. (2018), demonstrated that a hospital effluent containing paracetamol in a concentration of 2.66 μg L^{-1} was able to induce some effects such as growth inhibition, microcephalia, facial and pericardial edema, eye malformations, and damage to the notochord, tail, fin and intestine of the frogs *Lithobates catesbeianus* and *Xenopus laevis*.

Some morphological alterations such as pericardial blood accumulation, pericardial and peritoneal edema, and kyphosis have been associated with the exposure of paracetamol in concentrations of 2.5 and 4.9 mM at different exposure times (24–96 hpf) in *Danio rerio* embryos (Cedron et al., 2020).

Paracetamol has been related to delays in hatching and reduction in length and body mass, deformation of tail and caudal fin at concentrations of 1–100 mg L^{-1} in embryos of *Danio rerio* (A. David and Pancharatna, 2009a).

In another study by the same authors, it was shown that ibuprofen at concentrations between 10 and 100 μg L-1 induced pericardial edema, decrease in heart rate and loss of pectoral fins in *Danio rerio* embryos (Anuradha David and Pancharatna, 2009b). Also, ibuprofen at concentrations of 0.0001 mg L^{-1} was able to alter some important parameters in the reproduction of *Oryzias latipes* such as induction of vitellogenin in male fish, fewer broods per pair, and more eggs per brood (Han et al., 2010). Gutiérrez-Noya et al. (2020), determined that concentrations of 1.5–11.5 μg L^{-1} of ibuprofen, generated delayed hatching, hypopigmentation, pericardial edema, yolk deformation, and developmental delay in *Cyprinus carpio* embryos.

Another of the NSAIDs identified in this study was ketorolac, although this drug has not been identified as embryotoxic or teratogenic, if it has been shown that at concentrations of 1 and 60 mg L-1 it was able to induce an increase in bioindicators of cellular oxidation as lipoperoxidation level, hydroperoxide content and carbonylated protein content and altering antioxidant enzymes such as superoxide dismutase, catalase and glutathione peroxidase in liver and brain of *Cyprinus carpio*. Likewise, in common carp blood, it induced an increase in the frequency of micronuclei at the same concentrations (Galar-Martínez et al., 2016).

The results identified in this study of alterations to embryonic development and teratogenic effects can be explained by the mechanism of action of NSAIDs, which is the inhibition of cyclooxygenase (COX-1 or COX-2), which leads to the inhibition of Prostaglandin (PG) synthesis. PG are related to the process of reproduction, as well as in the immune and circulatory systems, and finally in osteo and chondrogenesis (Peltzer et al., 2019). Likewise, studies conducted by Cha et al. (2005, 2006) showed that inhibition of cyclooxygenase 1 produces defective formation of the vascular had, shortened intersomitic vessels, and causes growth arrest. This phenomenon occurs because the PG produced by COX-1 are necessary during gastrulation and segmentation.

The other drugs identified in the effluent and that have been related to alterations to embryonic development and teratogenic effects was dexamethasone. This drug at 5 μM caused the reduction of the yolk sac size, malformations in the pericardium, craniofacial malformations in embryos of *Danio rerio* (Teixidó et al., 2019). These effects can be

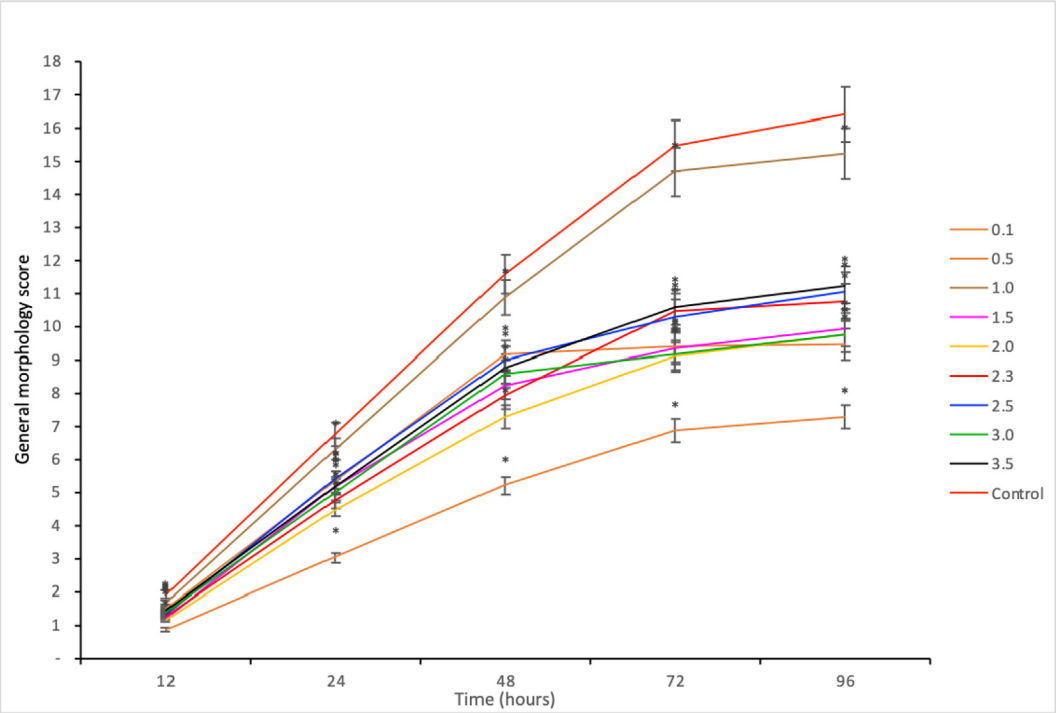


Fig. 4. Concentration-response curves of hospital effluent in *D. rerio* embryos.

related with the ability of dexamethasone to mobilize reserves of energy substrates in embryos.

Also, LaLone et al. (2012), identified that dexamethasone at 500 μg L^{-1} produced a decrease in vitellogenin messenger ribonucleic acid (mRNA) expression in liver tissue from females and this drug can interfere with fish reproduction systems and with normal growth, thus negatively affecting embryonic development, increasing deformities in *Pimephales promelas*.

Likewise, omeprazole at concentrations of 0.09 μM induced a teratogenic index of 1.93. The main malformations of this drug are delayed hatching and skeletal malformations in zebrafish embryos (Nikfar et al., 2002; Selderslaghs et al., 2012).

Rocco et al. (2010) demonstrated that furosemide at a concentration of 611.08 ng L^{-1} and ranitidine at a concentration of 245.55 ng L^{-1} are capable of inducing genotoxic effects in Danio rerio after 5 days and up to 21 days of exposure. These effects were verified using the random amplified polymorphic DNA (RAPD-PCR) technique, diffusion assay and comet assay.

Many of the drugs identified in this study have been shown to generate oxidative stress and genotoxicity in aquatic organisms such as *Cyprinus carpio, Xenopus laevis* and *Lithobates catesbeianus* (Gutiérrez-Noya et al., 2020; SanJuan-Reyes et al., 2020; Luja-Mondragón et al., 2019; Pérez-Alvarez et al., 2018), these mechanisms may be related to the teratogenic and embryotoxic effects of these drugs identified in this study.

In summary, the pollutants present in the evaluated hospital effluent act at different levels, one of them as metals alter the permeability of the chorion, and together with the drugs they are capable of generating reactive oxygen species that modify the functions of biomolecules in embryos of zebra fish.

5. Conclusions

Metals and drugs present in the hospital effluent evaluated were the cause of the alterations to embryonic development and teratogenic effects in embryos of *Danio rerio*. LC_{50} and EC_{50} of malformations were respectively 6.1 and 2.5. The teratogenic index was 2.45. The main malformations identified were yolk sac malformation, pericardial edema, hatching abnormalities, hypopigmentation, tail deformation, chorda malformation, without fin, chorion deformation and craniofacial malformation. The evaluated hospital effluent represents a risk for *Danio rerio* embryos.

CRediT authorship contribution statement

Paulina Tenorio-Chávez: Investigation, Methodology, Data curation, Writing - original draft. **Mónica Cerro-López:** Conceptualization, Methodology, Data curation, Writing - original draft. **Lucila Isabel Castro-Pastrana:** Writing - original draft. **Milena María Ramírez-Rodrigues:** Writing - original draft. **José Manuel Orozco-Hernández:** Investigation, Writing - original draft. **Leobardo Manuel Gómez-Oliván:** Investigation, Conceptualization, Methodology, Data curation, Writing - original draft.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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