Chemosphere 294 (2022) 133791

Contents lists available at ScienceDirect

Chemosphere

journal homepage: www.elsevier.com/locate/chemosphere

Brain damage induced by contaminants released in a hospital from Mexico: Evaluation of swimming behavior, oxidative stress, and acetylcholinesterase in zebrafish (*Danio rerio*)

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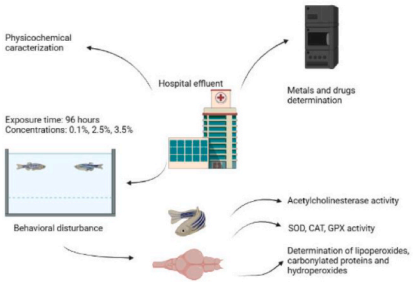
HIGHLIGHTS

- Neurotoxic effects induced by hospital effluent were evaluated.
- Several metals and drugs were identified in the effluent.
- Exposure to the effluent caused behavioral alterations in zebrafish.
- The Hospital effluent induced oxidative damage.
- A decrease in acetylcholinesterase activity was observed.

GRAPHICAL ABSTRACT



ARTICLE INFO

Handling editor: James Lazorchak

Keywords:
Hospital wastewater
Zebrafish
Oxidative status
Behavior
Neurotransmitters

ABSTRACT

Several studies have indicated that hospital effluents can produce genotoxic and mutagenic effects, cytotoxicity, hematological and histological alterations, embryotoxicity, and oxidative stress in diverse water organisms, but research on the neurotoxic effects hospital wastewater materials can generate in fish is still scarce. To fill the above-described knowledge gap, this study aimed to determine whether the exposure of adult zebrafish (*Danio rerio*) to several proportions (0.1%, 2.5%, 3.5%) of a hospital effluent can disrupt behavior or impair redox status and acetylcholinesterase content in the brain. After 96 h of exposure to the effluent, we observed a decrease in total distance traveled and an increase in frozen time compared to the control group. Moreover, we also observed a significant increase in the levels of reactive oxygen species in the brains of the fish, especially in hydroperoxide and protein carbonyl content, relative to the control group. Our results also demonstrated that hospital effluents significantly inhibited the activity of the AChE enzyme in the brains of the fish. Our Pearson correlation demonstrated that the response to acetylcholinesterase at the lowest proportions (0.1% and 2.5%) is positively related to the oxidative stress response and the behavioral changes observed. The cohort of our studies

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https://doi.org/10.1016/j.chemosphere.2022.133791
Received 10 November 2021; Received in revised form 12 January 2022; Accepted 27 January 2022
Available online 29 January 2022

demonstrated that the exposure of adult zebrafish to a hospital effluent induced oxidative stress and decreased acetylcholinesterase activity in the brain of these freshwater organisms, which can lead to alterations in their behavior.

1. Introduction

Hospitals generate a considerable quantity of wastewater with a great variety of contaminants, such as chemical products, heavy metals, disinfectants, sterilizers, detergents, radioactive markers, and active drugs and their metabolites (Verlicchi et al., 2010; Magdaleno et al., 2014; Laffite et al., 2016; Pérez-Alvarez et al., 2018). As most of them do not have proper wastewater treatment systems, their contaminated wastewater is usually released directly into the environment (Islas--Flores et al., 2017).

There is evidence that hospital effluents induce genotoxic and mutagenic effects, cytotoxicity, hematological and histological alterations, embryotoxicity, and oxidative stress in diverse water organisms. Alimba et al. (2017), for instance, showed that six concentrations (0.5–3.0%) of a hospital effluent induced mortality and cyto-genotoxicity in *Clarias gariepinus*. Analogously, Mazzitelli et al. (2018) pointed out that ten psychotropic substances from a psychiatric hospital in France decreased the number of oocytes and altered the hatching rate of *Gammarus fossarum*, *Schmidtea polychroa*, and *Radix peregra*. Moreover, Luja-Mondragón et al. (2019) indicated that seven concentrations (3–6%) of a hospital effluent from Mexico altered the embryonic development of *Cyprinus carpio* via an oxidative stress mechanism.

In the last decade, several studies have pointed out that mixtures can influence the effects of drugs on organisms, resulting in lower or higher toxicity (Lister et al., 2011). Consequently, it is worrying when substances attack the same organism as a mixture, which can cause the mechanism that the organism uses against these toxic substances to be overwhelmed or saturated (Andrade et al., 2017). Although studies on mixtures have accelerated, predicting the health consequences of multiple chemical exposures remains a challenge.

Since few studies concerning hospital effluents have evaluated the neurotoxic impact that these complex waste material mixtures may induce in aquatic organisms, this study aims to determine whether three proportions of a hospital effluent can disrupt the behavior of zebrafish (*Danio rerio*). Moreover, we also assessed whether acute exposure to this effluent could impair the redox status and acetylcholinesterase in the brains of these freshwater organisms.

2. Method

2.1. Sampling

Samples were gathered directly from the sewer system of a hospital in Puebla City, Mexico. We selected this hospital because, in our previous findings, we demonstrated that effluents from this hospital altered the embryonic development of *D. rerio* via inducing different malformations in the embryos (Tenorio-Chávez et al., 2020). For sampling, we took into account the Mexican NMX-AA.003-1980 wastewater sampling standard and used amber polyethylene containers. Sampling was performed daily between February 10 and 14 and between 8 and 14 h.

After sample collection, the samples were transported to the Environmental Toxicology Laboratory at the Autonomous University of the State of Mexico and were evaluated for all physicochemical parameters, quantities of emerging pollutants, and toxicity in the brain of *D. rerio*.

Table 1
Chromatographic conditions.

Column:	Acquity UPLC BEH C18 (2.1 × 100 mm, 1.7 μm)
Column temperature:	40 °C
Mobile phase A:	0.1% (v/v) formic acid in water
Mobile phase B:	0.1% (v/v) formic acid in methanol
Injection volume:	10 μL
Flow rate:	0.3 mL/min
Total running time:	10 min
Running conditions:	90% A at 0.1 min 10% A at 8 min 90% A at 10 min

2.2. Physicochemical determination of effluent

The parameters analyzed were pH (NMX-AA-008-SCFI-2016), temperature (NMX-AA-007-SCFI-2013), conductivity (NMX-AA-093-SCFI-2000), dissolved oxygen (NMX-AA- 012-SCFI-2001), fluoride (NMX-AA-077-SCFI-2001), chlorides (NMX-AA-073-SCFI-2001), ammonia, biochemical oxygen demand (NMX-AA-028-SCFI-2001), hardness (NMX-AA-072-SCFI-2001), total phosphorus (NMX-AA-029-SCFI-2001), total suspended solids, sodium hypochlorite, and total nitrogen (NMX-AA-026-SCFI-2010).

2.3. Microcontaminant quantification

To assess the drugs in the effluent, we sifted all the samples twice, first using an 8 μm membrane and then a 2.5 μm membrane. Next, we conditioned the solid-phase cartridges (Phenomenex, Strata XL) using 10 mL methanol and 10 mL water, eluted the samples (500 mL) with 6 mL of methanol, and added them to cartridges at a flow rate of 5 mL/min. To evaporate the solvent, we used nitrogen N-Evap 112 (Organization Haverhill, MA). The samples were then diluted in 1 mL of a methanol–water solution (50:50) and filtered again with a 0.2 μm syringe filter.

For drug analysis, we used a Waters ACQUITY UPLC coupled to a Quattro Premier XE Micromas with the conditions described in Table 1. Moreover, for mass spectrometer optimization, we carried out a direct infusion of 200 mg/L of the standard solution of each drug (Table 2). We used both ESI (+) and ESI (−) for the detection. The limit of detection (LOD) and the limit of quantitation (LOQ) were calculated with the formulas of Brubaker (1999): LOD = t0.99 x S and LOQ = 3 x LOD, where t0.99 represents the one-tailed statistic at a 99% confidence level for n replicates.

For metals quantification, we sifted the samples using a Whatman # 541 (Whatman, Germany) at a pH of 3.5 and then digested them using an autoclave at 120 °C and 15 psi for an hour. Then, following the above-described process, we filtered and diluted the samples in 100 mL of deionized water and read them using a Varian AA1475 atomic absorption spectrophotometer (Melbourne, Australia). For all metals evaluated, a standard curve of 1 mg/L was used (González-González et al., 2014). In Table 3, we summarize the conditions used for the quantification of metals.

To confirm the results of all tests (water quality parameters and drug and metal quantification), we carried out analyses three times in three independent experiments.

2.4. Zebrafish housing systems

We maintained *D. rerio* adults (AB-strain) in 100 L aquaria (14 h light, 10 h dark) that contained a system that supplied them with dechlorinated and UV-sterilized tap water. To optimize the conditions of fish housing, we monitored and controlled various water quality

Table 2
Mass spectrometer criteria 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

Table 3
Criteria used in metal quantification.

Metal	Absorption wavelength (nm)	LOD µg L^{-1}	LOQ µg L^{-1}
Arsenic	193.7	2.65	5.39
Cadmium	228.8	1.82	6.23
Copper	324.8	2.48	5.23
Chrome	357.9	1.73	5.28
Mercury	253.7	0.89	3.45
Nickel	232	1.12	4.67
Lead	283.3	0.94	4.17
Zinc	213.9	1.03	6.78

Table 4
Parameters evaluated during zebrafish maintenance and exposure.

Parameters	Value
Dissolved Oxygen	9.1 ± 0.3 mg/L
Nitrite	0.027 ± 0.009 mg/L
Nitrate	2.9 ± 0.3 mg/L
pH	7.21 ± 0.10
Un-ionized ammonia	0.011 ± 0.003 mg/L

Table 5
Physicochemical determination of hospital effluent.

Physicochemical Parameter	Hospital effluent evaluated	NOM-001-SEMARNAT-1996	NOM-002-SEMARNAT-1996
Temperature (°C)	21.7 ± 0.6	35	40
Dissolved oxygen (mg L^{-1})	6.8 ± 0.7	N.E.	N.E.
Conductivity (µS cm^{-1})	1091.6 ± 5.1	N.E.	N.E.
pH	6.1 ± 0.4	6.5–8.5	6–9
Chlorides (mg L^{-1})	281 ± 8.3	250	N.E.
Fluorides (mg L^{-1})	8.1 ± 0.9	0–15	N.E
Hardness (mg L^{-1})	137 ± 4.6	500	N.E.
Ammonia (mg L^{-1})	1.5 ± 0.9	N.E.	N.E.
Total Suspended Solids (mg L^{-1})	71 ± 9.8	60	40–60
Total, phosphorus (mg L^{-1})	8.3 ± 1.3	10	10
Total nitrogen (mg L^{-1})	21 ± 0.6	25	N.E.
Biochemical Oxygen Demand (mg L^{-1})	9.73 ± 0.5	60	40–60
NaClO (mg L^{-1})	6.1 ± 0.9	N.E.	N.E.

N.E. = not established in the norm. Data represent mean ± standard deviation of three independent experiments.

parameters during all experiments (Table 4). The fish were fed Spirulina flakes three times per day (Ocean Nutrition, US).

2.5. D. rerio exposure systems

For the exposure of the fish, we allocated 120 fish into four systems, each with 30 fish, that contained one of the test concentrations of the effluent (0%, 0.1%, 2.5%, and 3.5%). We selected these effluent concentrations because our previous findings indicated that low concentrations of the effluent led to different malformations in *D. rerio* embryos and eventually caused death (Tenorio-Chávez et al., 2020). In all systems, we maintained a constant temperature of 27 °C and a constant dark–light ratio (14 h:10 h). Once the exposure time (96 h) concluded, we evaluated the behavior of the remaining fish as described below.

Table 6
Micropollutants detected in hospital effluent.

Pollutant type	Metal or drug	Concentration
Metal	As (mg L^{-1})	0.017 ± 0.002
Metal	Cd (mg L^{-1})	0.039 ± 0.001
Metal	Cu (mg L^{-1})	0.40 ± 0.003
Metal	Cr (mg L^{-1})	0.83 ± 0.009
Metal	Hg (mg L^{-1})	0.067 ± 0.007 *
Metal	Ni (mg L^{-1})	0.83 ± 0.005
Metal	Pb (mg L^{-1})	193 ± 0.011*
Metal	Zn (mg L^{-1})	1.63 ± 0.06
NSAIDs	Ketorolac (ng L^{-1})	423.5 ± 6.9
	Ibuprofen (ng L^{-1})	18.7 ± 1.9
	Naproxen (ng L^{-1})	21.7 ± 2.3
	Paracetamol (ng L^{-1})	79.8 ± 8.1
Corticosteroids	Dexamethasone (ng L^{-1})	12.9 ± 0.97
	Hydrocortisone (ng L^{-1})	23.7 ± 1.4
Inhibitor of proton pump	Esomeprazole (ng L^{-1})	16.9 ± 1.3
	Omeprazole (ng L^{-1})	22.6 ± 1.6
H2- antagonism	Ranitidine (µg L^{-1})	16.1 ± 1.3

Data represent mean ± standard deviation of three independent experiments. * indicates data that exceed values established by Mexican regulations.

2.6. Behavioral assessment

For the behavioral assessment, we use the protocol described by Cachat et al. (2010). Briefly, to acclimatize the fish, we transferred them from the maintenance room to the behavioral room and maintained them there for 1 h. Subsequently, we selected one fish at a time and placed it into a 15 L novel tank (21.2 cm × 21.2 cm x 25.2 cm) for 8 min (2 min of acclimatization, 6 min of trial). We recorded footage of each fish, which we then used to trace their behavior with the Tox Track Ink software. Behavioral endpoints measured were freezing bouts, time frozen (s), mean speed (cm/s), total distance traveled (cm), distance traveled in the top and bottom (cm), latency to enter the top (s), the number of transitions (top-to-bottom and bottom-to-top), and time spent in top and bottom (s).

2.7. Determination of acetylcholinesterase

Following behavioral assessment, we euthanized the fish, employing the hypothermic shock method (2–4 °C) in accordance with the AVMA Guidelines on Euthanasia 2020 Edition. This method is adequate for this proof, as fish become immobilized instantly upon contact with the cold water, and behavioral markers of pain or distress hardly occur (Wallace et al., 2018). Once the fish showed no vital signs, their brains were

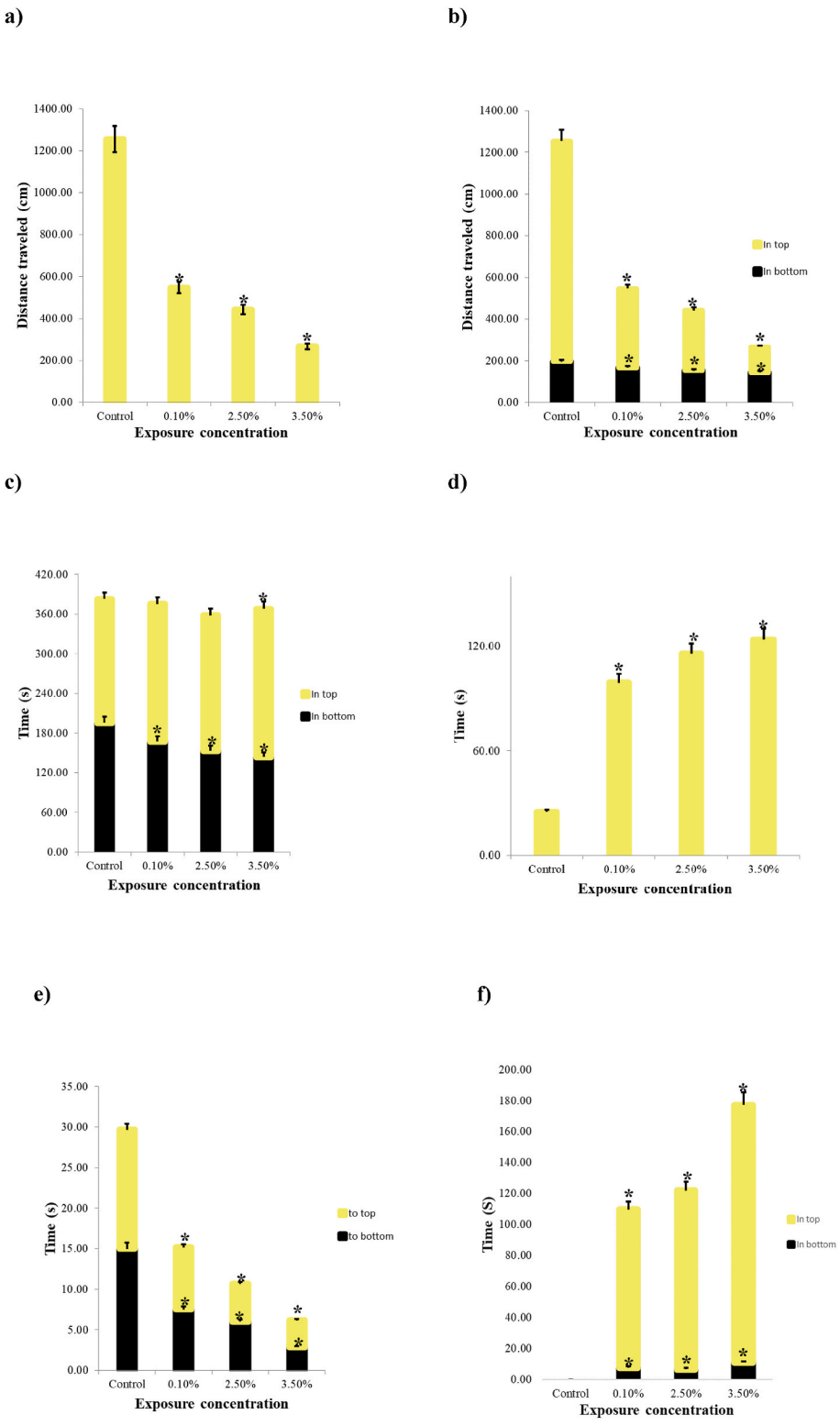

Fig. 1. *D. rerio* were exposed in groups of six to three concentrations of effluent (0.1%, 2.5%, and 3.5%) and a control environment for 96 h before behavioral testing. **a)** Total distance traveled. **b)** Distance traveled in the top versus distance traveled in the bottom. **c)** Time spent in the top versus bottom. **d)** Latency to enter the bottom. **e)** Frequency of transitions. **f)** Total duration of freezing. Significant differences between treatment groups and the control group are denoted by a star (*) in the graphics.

extracted and collected in Eppendorf tubes that we previously had refilled with 1 mL of phosphate buffer solution (PBS, pH 7.4).

With the help of a rotor-stator homogenizer (Ultra-turrax T25, IKA, Germany), we homogenized the previously extracted brains from each treatment group during 20 s at 10,000 rpm and used the homogenate to evaluate acetylcholinesterase and oxidative stress biomarkers. For the determination of AChE activity, we used the method established by Ellman et al. (1961). Briefly, we mixed 400 μL of the supernatant with 2.6 mL of phosphate buffer (pH 8.0, 0.1 M) and 0.1 mL of DTNB (5, 5-dithiobis-2-nitrobenzoate, 0.1 M). We measured all changes in absorbance every minute at 412 nm for 5 min.

2.8. Determination of oxidative stress

To measure oxidative stress biomarkers, we treated the samples as Elizalde-Velázquez et al. (2021a,b) described. Briefly, we split up the

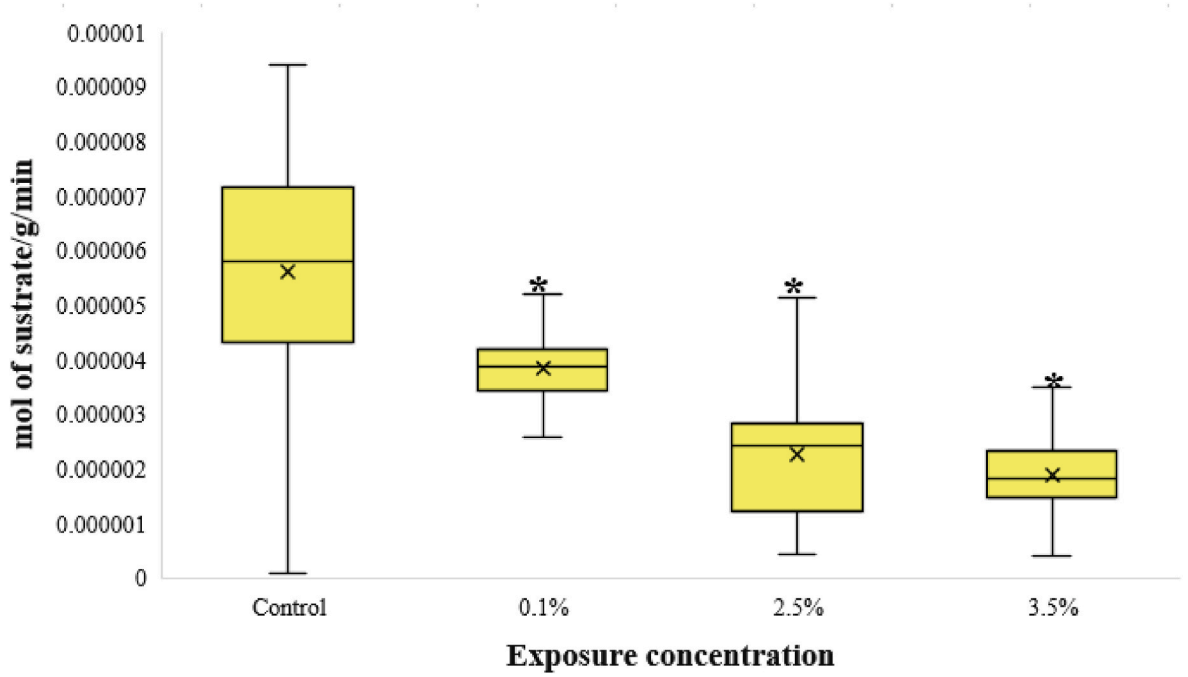

Fig. 2. Acetylcholinesterase activity in *D. rerio* adults exposed to effluent. Significant differences between treatment groups and the control group are denoted by a star (*) in the graphics.

homogenate into two Eppendorf tubes. One of the tubes contained 300 μL of trichloroacetic acid (20%) and the same amount of the homogenate, while the other only contained 700 μL of the latter. Finally, we centrifuged tubes 1 and 2 at 11,495 and 12,500 rpm, respectively, and used supernatant and precipitate to evaluate the whole oxidative stress biomarker battery. The colorimetric methods used for the evaluation of oxidative stress were as follows: 1) Misra and Fridovich (1972) for SOD; 2) Radi et al. (1991) protocol for CAT; 3) Gunzler and Florhe-Clairborne (1985) technique for GPx; 4) Buege and Aust (1978) process for LPx; 5) Jiang et al. (1992) procedure for HPx; 6) Levine et al. (1994), Parvez and Raisuddin (2005), and Burcham (2007) methods for POx.

2.9. Ethical statement

This study was approved by the Ethics and Research Committee of the Autonomous University of the State of Mexico (UAEMex) (approval ID: RP.UAEM.ERC.145.2020).

2.10. Statistical analysis

For statistical analysis, we assessed all results in the Sigma Plot 12.3 software using a non-parametric ANOVA followed by a post-hoc Student Newman Keuls test and expressed them as the mean ± standard deviation (SD). We also performed a Pearson correlation among behavioral endpoints and biomarkers tested using the R software; p-values less than 0.05 were considered significant.

3. Results

3.1. Physicochemical determination

For the physicochemical determination of the water, we took into account the parameters established by the Mexican regulations (Table 5). As can be seen in this table, the dissolved oxygen value in the effluent is low. Although the Mexican regulations do not have reference values for dissolved oxygen, we consider a normal range to be 7–8 mg/L (Roldan, 2003). In addition, we found that chlorides, total suspended solids, and sodium hypochlorite values were above the allowed limits.

3.2. Micropollutant concentration

In the quantification of micropollutants in the effluent, we found a variety of drugs and metals; nonetheless, as can be seen in Table 6, only mercury and lead exceeded the values established by Mexican regulations. Even though no drugs exceeded the limits permitted by Mexican regulations, we found that NSAIDs like ketorolac and paracetamol were the most abundant drugs in the hospital effluent. Our results are in agreement with the medication used in the hospital, as the main specialties of this medic unit include oncology, orthopedics, orthodontics, rheumatology, neurology, pneumology, and echocardiography. Moreover, as the consumption of NSAIDs in diseases or indications related to these specialties is huge, physicians usually prescribe them with inhibitors of proton pumps or H2- antagonists to avoid secondary effects of these drugs in the gastric system.

3.3. Behavioral assessment

Our results indicate that exposure of *D. rerio* to effluents causes an alteration in their behavior. From Fig. 1, we see how exposure to the effluent considerably decreased the total distance traveled by the zebrafish ($F(3,23) = 20.940$; $p<0.001$), the distance traveled in the top ($F(3,23) = 20.929$; $p<0.001$), the distance traveled in the bottom ($F(3,232) = 33.516$; $p<0.001$), the time spent in the top ($F(3,23) = 5.385$; $p = 0.007$), the time spent in the bottom ($F(3,23) = 48.968$; $p<0.001$), latency to enter the top ($F(3,23) = 264.803$; $p<0.001$), the number of transitions top-to-bottom ($F(3,23) = 18.329$; $p<0.001$), the number of transitions bottom-to-top ($F(3,23) = 18.706$; $p<0.001$), the time frozen in the top ($F(3,23) = 21.086$; $p<0.001$), and the time frozen in the bottom ($F(3,23) = 16.9971$; $p<0.001$) (see Fig. 2).

3.4. Acetylcholinesterase activity

The activity of the enzyme acetylcholinesterase was significantly decreased in fish in the 0.1% concentration group compared to those in

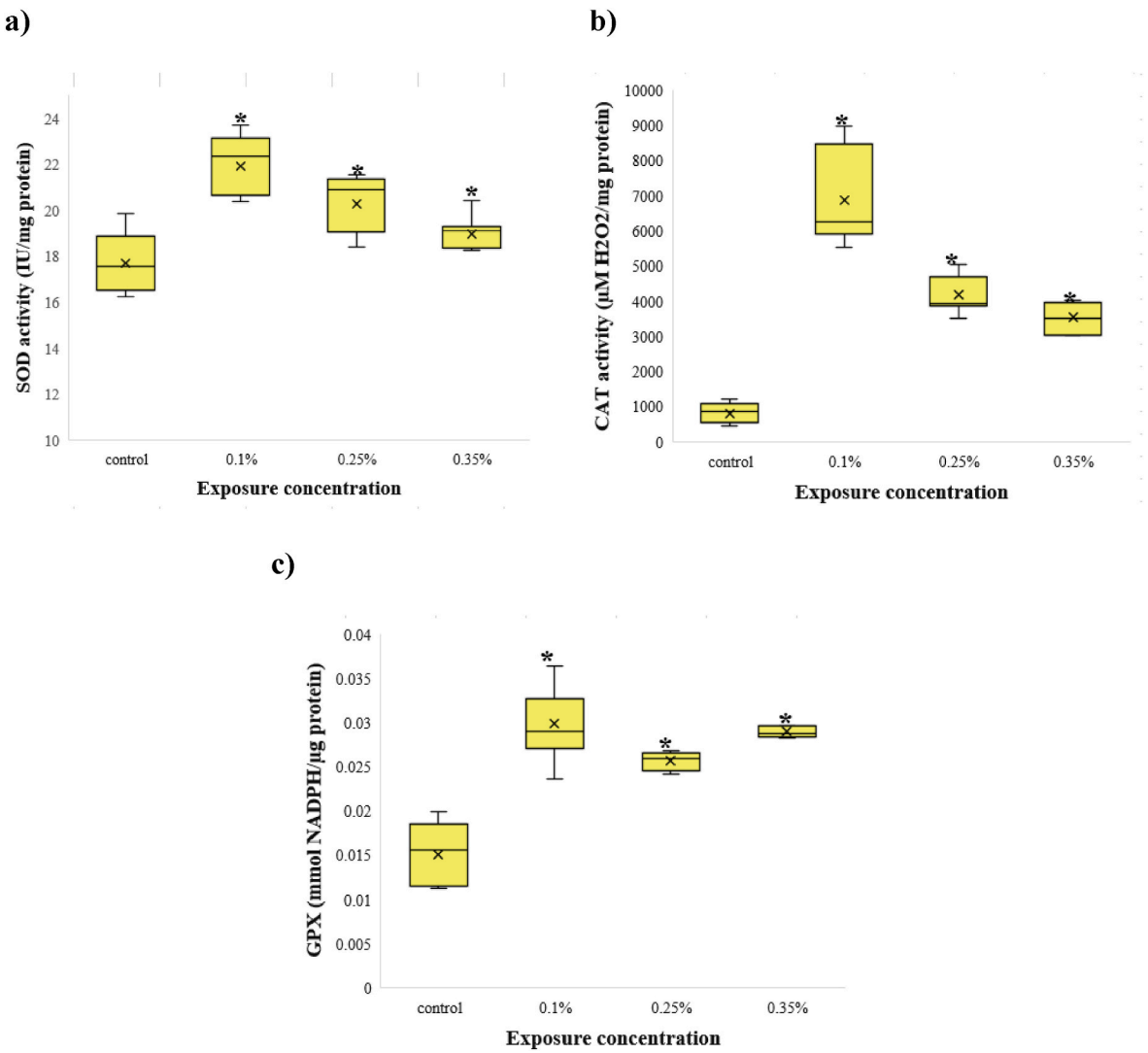

Fig. 3. Brain levels of **a)** SOD, **b)** CAT, and **c)** GPX in *D. rerio* adults exposed to effluent. Significant differences between treatment groups and the control group are denoted by a star (*) in the graphics.

the control group. Moreover, we observed the same decrease in the activity of this enzyme for proportions of 2.5% and 3.5% ($F(3,23) = 132.736$; $p<0.001$).

3.5. Oxidative stress biomarkers in the brain

Based on the results, we demonstrated that the enzymatic activity of SOD ($F(3,23) = 21.252$; $p<0.001$), CAT ($F(3,23) = 30.395$; $p<0.001$), and GPX ($F(3,23) = 27.078$; $p<0.001$) (Fig. 3) increased compared to the control group. Similarly, we observed that levels of LPX ($F(3,23) = 29.685$; $p<0.001$), HPx ($F(3,23) = 31.801$; $p<0.001$), and POx ($F(3,23) = 20.288$; $p<0.001$) (Fig. 4) were augmented in comparison to the control group.

3.6. Pearson correlation between behavioral assessment, acetylcholinesterase, and oxidative stress biomarkers

We performed a Pearson correlation to establish the intensity by which the variables are related (Fig. 5). This correlation uses color: blue indicates a strong positive relationship between variables, while red indicates a strong negative relationship. From our results, we can observe that for the lowest proportions of the effluent (0.1% and 2.5%), there is a strong positive correlation between all variables (acetylcholinesterase activity, oxidative stress biomarkers, and behavioral endpoints). Nevertheless, at the highest proportion of the effluent (3.5%), we observe that the biomarkers of stress and acetylcholinesterase are inversely related to the number of transitions, freezing time, and latency.

4. Discussion

In this study, we aimed to evaluate whether three proportions (0.1%, 2.5%, 3.5%) of a hospital effluent from Mexico may alter the oxidative status, the acetylcholinesterase activity, and the swimming behavior of *D. rerio* adults. Our results revealed that the hospital effluent, in all studied proportions, significantly altered the swimming behavior of *D. rerio* adults after 96 h of exposure. Moreover, this waste material also altered the redox status and the AChE activity in the brain of this freshwater organism. Thus, hospital effluents may adversely impact the fitness and survival of aquatic species. In agreement with our results, previous studies have evidenced that hospital effluents induced oxidative stress in *Cyprinus carpio* adults (Neri-Cruz et al., 2015) and cyto-genotoxicity in *Clarias gariepinus* (Alimba et al., 2017), as well as altered the reproduction and embryonic development of *Gammarus fossarum*, *Schmidtea polychroa*, *Radix peregra*, and *C.carpio* (Mazzitelli et al., 2018; Luja-Mondragón et al., 2019).

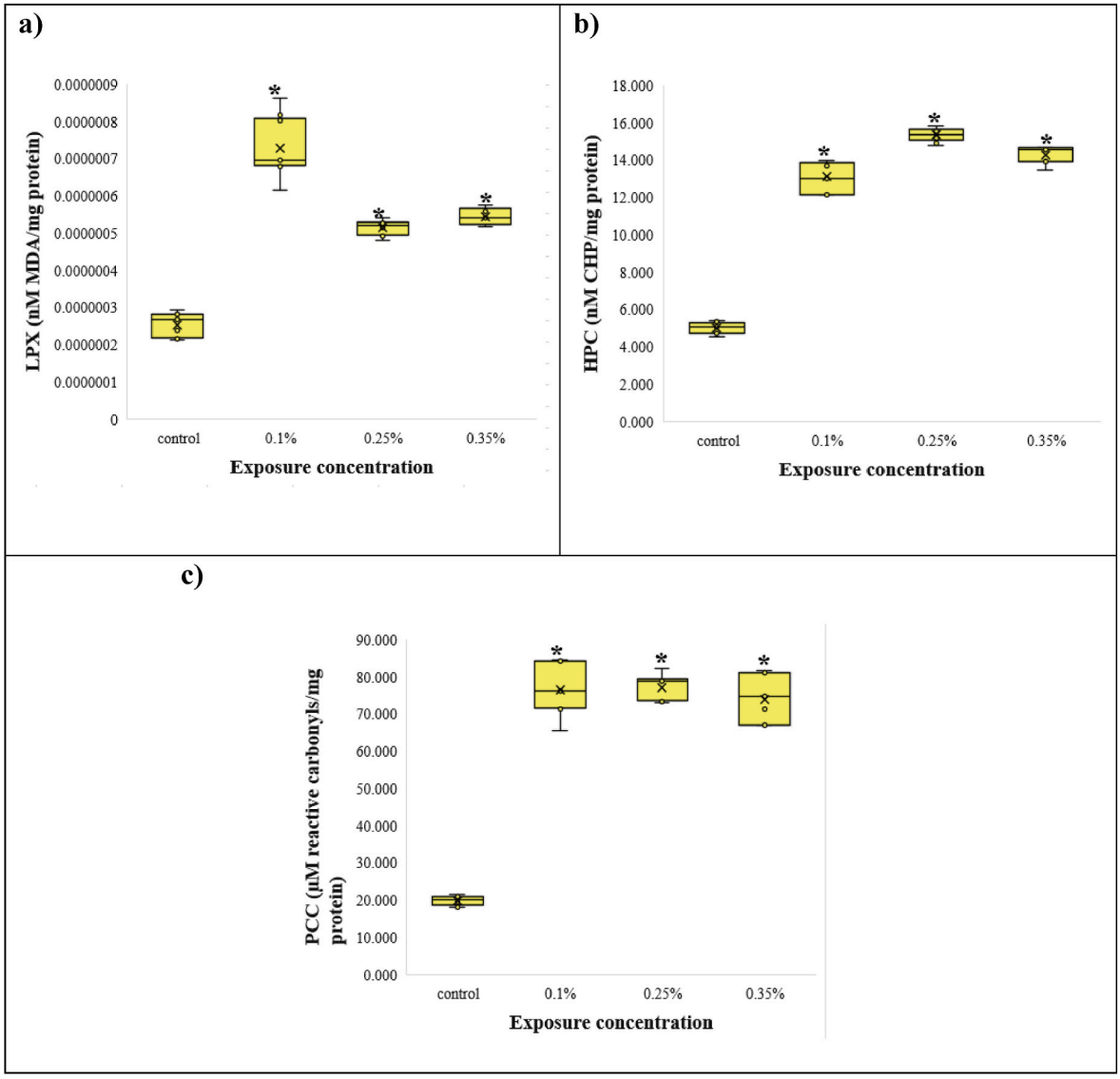

Fig. 4. Brain levels of **a)** lipoproteins (LPX), **b)** hydroperoxide content (HPC), and **c)** protein carbonyl content (PCC) in *D. rerio* adults exposed to effluent. Significant differences between treatment groups and the control group are denoted by a star (*) in the graphics.

Hospital effluents are complex mixtures of chemical and biological substances that are continually spilled out into the aquatic environment (Laffite et al., 2016). In our study, for instance, the effluent contained a variety of metals and drugs (Table 4). This is noteworthy, as most of the metals found in the effluent (Hg, Ni, Pb, Cu, As, Cd) may lead to cumulative impairments in the cognitive functions of fish. Kienle et al. (2009), for instance, demonstrated that Ni (7.5 mg/L) significantly decreased the locomotor activity of *D. rerio* larvae. Analogously, Chen et al. (2012) showed that zebrafish larvae exposed to 0.025–0.1 mg/L of Pb exhibited a significant decrease in swimming speed under the light condition. Moreover, Bakar et al. (2017) pointed out that chronic exposure to 100 nM of Hg induced motor deficiencies and increased the anxiety-like behaviors in *D. rerio* larvae. Similarly, Valles et al. (2020) established that As (50–500 ppb) altered the motor activity and increased anxiety-like behaviors in *D. rerio*, and these effects were transmitted to the F2 generation. Unlike metals, to our knowledge, only two drugs from the effluent (ibuprofen and paracetamol) may induce behavioral changes in aquatic organisms. Xia et al. (2017) proved that ibuprofen and diclofenac at 500 µg L^{-1} significantly reduced spontaneous movement, free-swimming distance duration, and speed under dark conditions in *D. rerio* embryos.

Concerning alterations to the redox status, we demonstrated that the antioxidant activity of SOD, CAT, and GPx in the brains of fish exposed to the effluent was significantly increased compared to the control group. Like antioxidant enzymes, levels of oxidative damage biomarkers (LPX, HPx, and POx) showed a significant increase compared to the control group. Thus, the hospital effluent induced oxidative damage in the brain of *D. rerio*. In general, there are three mechanisms by which metals found in the effluent produce reactive oxygen species (ROS) in cells (Sevcikova et al., 2011; Samet et al., 2020). Redox-active metals (Cu and Cr) generate ROS through redox cycling (Kalinowski et al., 2016). Redox cycling is a phenomenon in which a free radical is formed when the chemical accepts an electron. The free radical then reacts with oxygen, generating a superoxide anion radical, reforming the parent compound and becoming toxic to the organism (Koppenol and Hider, 2019). Cell antioxidant defenses can be affected by metals that do not have redox potential (Hg, Ni, Pb, Zn, and Cd), especially those defenses that involve enzymes that contain thiol (Qu et al., 2014; Trevisan et al., 2014; Islam et al., 2019; Wu et al., 2019). Trevisan et al. (2014), for instance, demonstrated that Zn altered the antioxidant system of mussels, decreasing glutathione reductase activity and glutathione levels in the gills of *Perna perna*. Moreover, Wu et al. (2019) showed that the

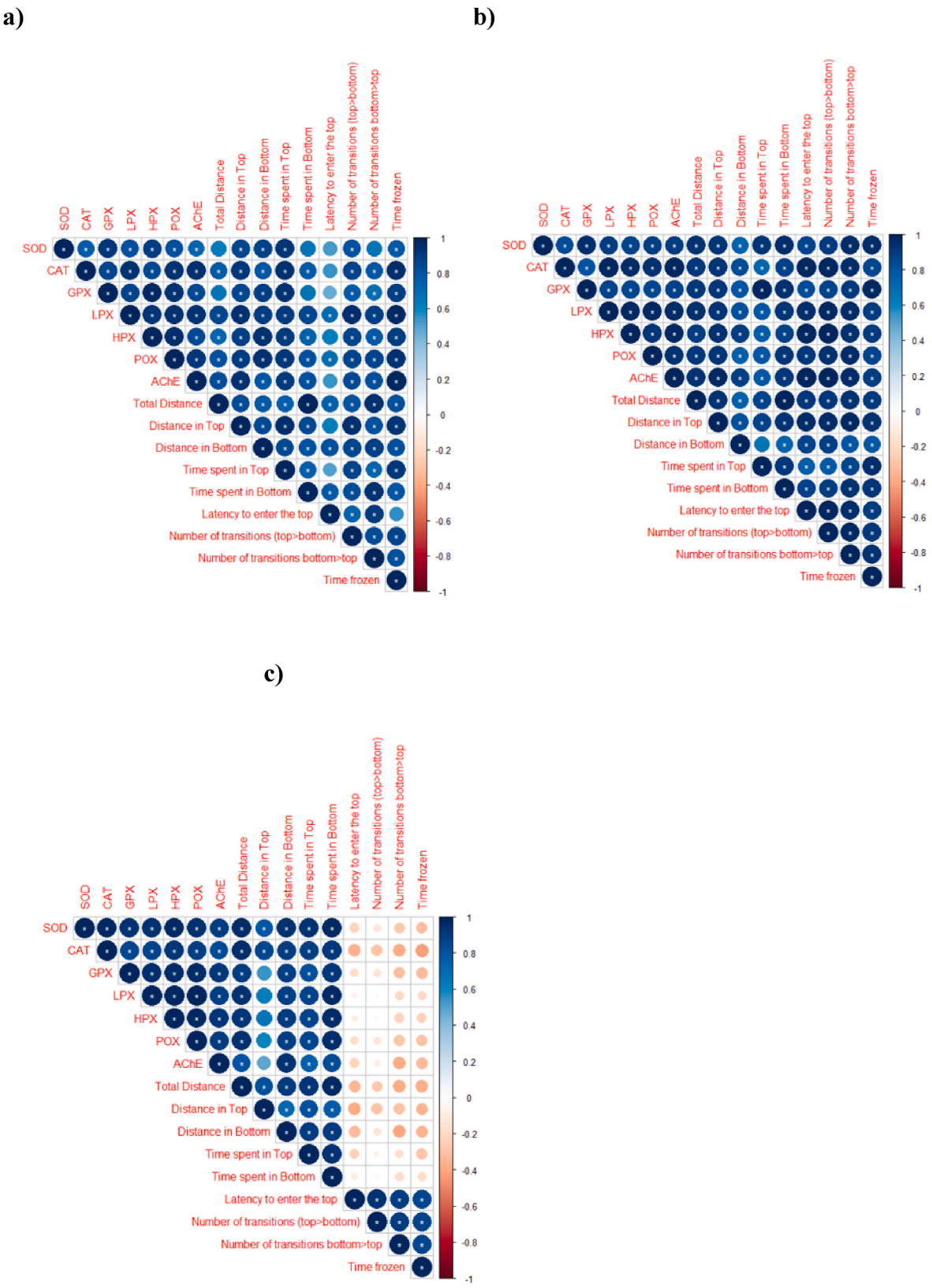

Fig. 5. Correlation between behavioral changes, acetylcholinesterase, and oxidative stress for all the three proportions of the effluent **a)** 0.1%, **b)** 2.5%, and **c)** 3.5%.

enzymatic activity of SOD was significantly inhibited in *Oxya chinensis*. In our results, we observed that the activity of SOD, CAT, and GPx decreased in the brains of fish exposed to the higher proportions of the effluent (Fig. 3). This decrease may be related to the negative effect that metals without redox potential have on the antioxidant enzymes. Finally, the third mechanism of ROS production is the Fenton reaction, by which Fe^{2+} is oxidized by H_2O_2 to become Fe^{3+}, a hydroxyl radical, and a hydroxyl anion (Valko et al., 2005; Zhong et al., 2014). Other

metals, such as Cu and Cr, are also involved in the Fenton reaction (Lushchak, 2011; Sevcikova et al., 2011; Milnerowicz et al., 2015).

Drugs are also responsible for oxidative damage in the brain of *D. rerio* adults. Nonsteroidal anti-inflammatory drugs (NSAIDs), for instance, may increase the production of ROS in cells via four different mechanisms: 1) mitochondrial impairment (Nagano et al., 2012; van Leeuwen et al., 2012), 2) activation of NADPH oxidases (Li et al., 2008; Vázquez-Meza et al., 2013), 3) increased production of xanthine oxidase and arachidonic acid (Sagi et al., 2003; Yun et al., 2010; Ghosh et al., 2015), and 4) abiotic and biotic transformations (Agúndez et al., 2009; González-González et al., 2014). Corticosteroids (dexamethasone) and proton pump inhibitors (esomeprazole) have also been associated with increased production of ROS (You et al., 2009; Marino et al., 2010; Mutsaers and Tofighi, 2012; Flaherty et al., 2017; Liu et al., 2018). Nonetheless, the pathways by which these drugs induce oxidative stress in humans and animals have not been completely elucidated. Concerning dexamethasone, it has been suggested that this drug may increase the production of ROS either through a mechanism that employs glucocorticoid receptor-mediated genomic regulation (You et al., 2009; Flaherty et al., 2017) or via the dysfunction of the mitochondria and endoplasmic reticulum (Mutsaers and Tofighi, 2012; Liu et al., 2018). In addition, for esomeprazole, Marino et al. (2010) reported that mitochondrial and NADPH oxidase seemed to be involved in triggering ROS production.

AChE is an important enzyme that terminates nerve impulses by catalyzing the hydrolysis of acetylcholine (Lionetto et al., 2013). Measurement of AChE activity has been increasingly used as a biomarker of nervous system effects following exposure to toxicants. According to our results, AChE activity in the brain of *D. rerio* was significantly inhibited by the hospital effluent. The inhibition of this enzyme may be related to the oxidative stress response that this waste material induced in the brain of *D. rerio* adults. Recent studies have shown that H_2O_2 may decrease the activity of AChE through shifts in the composition and structure of the lipid bilayer (Molochkina et al., 2005) and via oxidation of methionine, cysteine, and tryptophan related to the active center (Schallreuter and Elwary, 2007), and by modifying its isoform profile (Garcimartín et al., 2017). Based on the studies mentioned above, Phyu et al. (2014) suggested that oxidative brain damage of *D. rerio* induced by Hg, Pb, and Cd triggered a significant decrease in AChE activity. Nonetheless, other studies have indicated that metals may decrease AChE activity by binding to functional groups of the protein (Najimi et al., 1997; Pohanka, 2014) or by a denaturing of the protein when metal is in direct contact with the enzyme (de Lima et al., 2013).

Low dissolved oxygen levels in water have been closely related to behavioral changes in aquatic species. Saloom and Scot Duncan (2005), for instance, demonstrated that the anti-predation behaviors of *Corbicula fluminea* decreased as oxygen availability decreased. Moreover, Abdel-Tawwab et al. (2015) pointed out that growth performance and feed intake of *Oreochromis niloticus* were adversely affected by low dissolved oxygen concentrations. Dissolved oxygen was not the only physicochemical parameter altered by the effluent; chlorides and hypochlorite were affected as well. This is noteworthy, as these two parameters may also negatively affect the fitness, growth, and survival of fish. For example, Elia et al. (2006) reported that the hepatic antioxidant enzymes and total glutathione of *C. carpio* significantly decreased following exposure to two chlorine disinfectants (chlorine dioxide and sodium hypochlorite). Moreover, López-Galindo et al. (2010) demonstrated that sodium hypochlorite generated oxidative stress and diverse gill pathologies in juveniles of *Solea senegalensis*. Thus, sodium hypochlorite discharges from industrial and hospital treatment plants can alter the xenobiotic metabolism of fish and generate oxidative stress.

5. Conclusions

The lack of efficient technologies and the poor removal rates achieved in hospital wastewater treatment plants lead to the spill-out of drugs and metals that can be harmful to water species. In this study, we demonstrated that Puebla hospital effluents contain a huge amount of NSAIDs and metals that can disrupt the swimming behavior of fish (Novel Tank Test). Moreover, our findings indicated that the anxiety-like behavior described above could be related to the increased production of ROS and the inhibition of AChE activity in the brains of fish. We believe behavioral changes and AChE inhibition are mainly related to the excessive production of ROS in the brain; however, since hospital effluents are complex mixtures, we do not discard the possibility that the neurotoxic effects observed here may be related to the different of compounds present in them. Thus, more studies are needed to comprehend the mechanism by which effluents and complex mixtures affect the health and fitness of fish. Furthermore, our results clearly show that effluents can disrupt the swimming behavior of fish, induce oxidative stress, and alter AChE homeostasis. We suggest that future studies continue assessing the neurotoxic effects of complex mixtures on water organisms.

Author contribution statement

Karina Elisa Rosales-Pérez performed all the exposure experiments. Leobardo Manuel Gómez-Oliván and Karina Elisa Rosales-Pérez were involved in the conception. Leobardo Manuel Gómez-Oliván, Karina Elisa Rosales-Pérez and Gustavo Axel Elizalde-Velázquez, José Manuel Orozco-Hernández, Jesús Daniel Cardoso-Vera, Gerardo Heredia-García and Sandra García-Medina were involved in the design and interpretation of the data and the writing of the manuscript with input from Hariz Islas-Flores and Marcela Galar-Martínez.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This study was made possible by financial support from the Consejo Nacional de Ciencia y Tecnología (CONACyT, Project 300727).

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