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Oxidative stress as regulator of neuronal impairment after exposure to hospital effluents in *Danio rerio*

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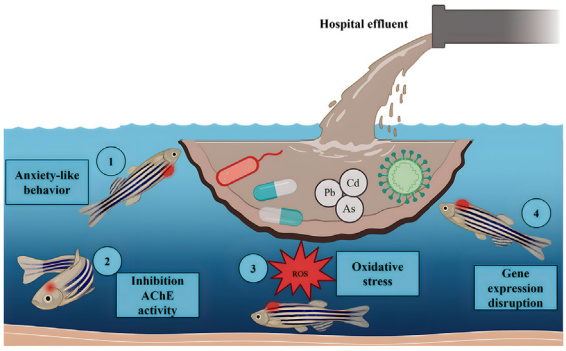
HIGHLIGHTS

- Hospital effluent under-study induced anxiety-like behavior in *Danio rerio*.
- Acetylcholinesterase activity was inhibited.
- The highly oxidative environment triggered the neurotoxicity process.
- Hospital effluent disrupted gene expression in *D. rerio*.

GRAPHICAL ABSTRACT



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ABSTRACT

The variety of activities carried out within hospitals results in their final discharges being considered hotspots for the emission of emerging pollutants. Hospital effluents contain different substances capable of altering the health of ecosystems and biota, furthermore, little research has been done to elucidate the adverse effects of these anthropogenic matrices. Taking this into account, herein we aimed to establish whether exposure to different proportions (2 %, 2.5 %, 3 %, and 3.5 %) of hospital effluent treated by hospital wastewater treatment plant (HWWTP) can induce oxidative stress, behavioral alterations, neurotoxicity, and disruption of gene expression in *Danio rerio* brain. Our results demonstrate that the hospital effluent under-study induces an anxiety-like state and alters swimming behavior, as fish exhibited increased freezing episodes, erratic movements and traveled less distance than the control group. In addition, after exposure we observed a meaningful rise in biomarkers related to oxidative damage, such as protein carbonyl content (PCC), lipoperoxidation level (LPX), hydroperoxide content (HPC), as well as an increase in enzyme antioxidant activities of catalase (CAT), and superoxide dismutase (SOD) upon short-term exposure. Moreover, we discovered an inhibition of acetylcholinesterase (AChE) activity in a hospital effluent proportion-dependent manner. Regarding gene expression, a significant disruption of genes related to antioxidant response (*cat*, *sod*, *nrf2*), apoptosis (*casp6*, *bax*, *casp9*), and detoxification (*cyp1a1*) was observed. In conclusion, our outcomes suggest that hospital effluents enhance the emergence of oxidative molecules, and promote a highly oxidative environment at the neuronal level that favors the inhibition of AChE activity, which consequently explains the anxiety-like behavior observed in *D. rerio* adults. Lastly, our research sheds light on possible toxicodynamic mechanism by which these anthropogenic matrices may trigger damage in *D. rerio* brain.

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1. Introduction

Hospital effluents represent a health risk for ecosystems and biota due to the presence of bioactive pharmaceuticals (PhACs), heavy metals and opportunistic pathogens that increase the risk associated with these anthropogenic matrices (Ramírez-Moreno et al., 2023). It is noteworthy to highlight that "hospital effluent" is the term normally used for wastewater that is discharged from hospitals. It may or may not be treated prior to being discharged into the sewer system, depending on the regulations and practices of each country (Carraro et al., 2017). Therefore, hospital effluent can mean either water just discharged from hospital or treated water discharged from a hospital wastewater treatment plant (HWWTP), depending on the context (Ramírez-Moreno et al., 2023; Verlicchi, 2021). However, it is well known that HWWTPs or conventional wastewater treatment plants (WWTPs) often employ obsolete remediation technologies focused on carbon removal. This prevents adequate removal of emerging pollutants, thus, many of them pass unchanged, reaching different environmental matrices (González-González et al., 2022; Verlicchi, 2021).

Since the concentrations of xenobiotics in both HWWTP and WWTP effluents are environmentally relevant (from ng/L to μg/L), they can be considered pollution hotspots (Sinthuchai et al., 2021; Rodriguez-Mozaz et al., 2020). For example, in Thailand, 1.49 μg/L of sulfamethoxazole was detected in HWWTP effluents (Sinthuchai et al., 2021). Additionally, ketorolac (1784.3 ng/L), and ranitidine (19.1 μg/L) have been reported in HWWTP effluents in Mexico (Ramírez-Moreno et al., 2023). In India, ciprofloxacin and sulfamethoxazole have been reported in hospital effluents from 2.2 to 236.6 μg/L, and from 36.7 to 81.1 μg/L, respectively (Diwan et al., 2009). Similarly, environmentally relevant concentrations of dexamethasone (360 ng/L and 272.12 ng/L) have been reported in hospital effluents in Spain and Mexico (Cruz-Morató et al., 2014; Ramírez-Moreno et al., 2023). However, some polar compounds like metformin have a low removal efficiency (35.4 %) in HWWTP, therefore, different PhACs persist in hospital effluents (Chiarello et al., 2016). Currently, there are several studies on the contamination of different environmental compartments by PhACs. For instance, Gómez-Regalado et al. (2023), report that paracetamol has a bioconcentration factor (BCF) of 3162 in *Epinephelus awoara*. Furthermore, Xie et al. (2020) demonstrated that diclofenac (1–1000 μg/L) has the ability to bioaccumulate in *Carassius auratus* brain, triggering oxidative damage, and alteration of detoxification-related genes.

Aerobic organisms are constantly confronted with various oxidative molecules, mainly derived from oxidative phosphorylation, inflammatory processes, and xenobiotic metabolism (Dumitrescu et al., 2018; Biswas, 2016). Oxidative stress is the insufficiency of the antioxidant system to counteract the imbalance of oxidative by-products, such as reactive oxygen species (ROS), promoting the oxidation of essential biomolecules, in particular membrane proteins and lipids, which leads to processes of protein carbonylation (PCC), hydroperoxide formation (HPC), and lipoperoxidation (LPX) (Sies et al., 2017). Regardless, organisms have an ROS neutralization system, mediated by catalase (CAT) and superoxide dismutase (SOD) activity, which act as the first line of antioxidant defence (Ighodaro and Akinloye, 2018). Some organs, such as the brain, have high O_2 demand and high content of polyunsaturated fatty acids (PUFAs), making them more susceptible to oxidative species, namely hydroxyl radicals (OH•), superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), among others (Salim, 2017). This highlights the possible negative impact of xenobiotics in hospital effluents on the normal functions of the central nervous system (CNS) in aquatic organisms.

Behavior can be considered as a highly organized process based on neural networks that ensures the fitness of organisms. Thus, behavioral modifications are usually used as a sensitive measure in the identification of neurological dysfunctions (Mahapatra et al., 2023). Behavioral bioassays are essential in toxicological studies to detect and analyse external stimuli, such as exposure to a mixture of pollutants and to serve as indicators of the physiological or psychological state of model organisms (Orozco-Hernández et al., 2022). The measurement of anxiety-like behaviors using the Novel Tank Test (NTT) represents an interesting strategy that helps to understand and elucidate the mechanisms underlying various neural disorders. Furthermore, to increase the robustness of results in behavioral research, triangulation with approaches that allow the assessment of neurotoxicity is required. In this respect, the determination of acetylcholinesterase (AChE) activity depicts an excellent biomarker of neuronal damage. AChE plays a crucial role in the proper functioning of the neuromuscular system and cholinergic synapses, preventing continuous muscle contraction (Matozzo et al., 2005).

Despite the diversity of studies pointing out the harmful effects that can be induced by pollutants present in hospital effluents (Karaman et al., 2023; Wang et al., 2023; Nunes et al., 2020; Türkan et al., 2021; Chen and Chan, 2018; Félix et al., 2018; Isidori et al., 2016), toxicological studies related to mixtures of xenobiotics are still scarce (Freitas et al., 2023; da Costa Araújo et al., 2023; Freitas et al., 2022; da Costa Araújo et al., 2022; de Souza et al., 2018), and are even more so those who focus on assessing the effect that hospital effluents trigger (Rosales-Pérez et al., 2022; Afsa et al., 2022; Neri-Cruz et al., 2015). However, no study (so far) has proposed a toxicodynamic mechanism at the neuronal level in *Danio rerio* through which these anthropogenic matrices could induce harm. Therefore, the current study aimed to evaluate oxidative stress (PCC, HPC, LPX, CAT, and SOD), behavior (NTT), neurotoxicity (AChE activity), and gene expression (*cat*, *sod*, *nrf2*, *casp6*, *bax*, *casp9*, and *cyp1a1*) promoted by exposure to different proportions (2 %, 2.5 %, 3 %, and 3.5 %) of a hospital effluent previously treated by HWWTP in *Danio rerio* brain. We hypothesized that after exposure to a hospital effluent, a highly oxidative environment would be generated at the neuronal level. Thus, an increased in oxidative damage, apoptosis, and impaired AChE activity, which together would induce anxiety-like behavior that would ultimately trigger the neurotoxicity process.

2. Materials and methods

2.1. Sampling and characterization of hospital effluent

Hospital effluent samples were taken directly from water treated by a HWWTP before being discharged into the sewage system, and stored in previously sterilized amber bottles. The characterization of the hospital effluent under-study consisted of the determination of physicochemical parameters, pharmaceuticals, heavy metals, as well as bacterial communities, with results published in Ramírez-Moreno et al. (2023). The methodology proposed by Mexican standard NOM-002-SEMARNAT-1996 for physicochemical characterization was used. The parameters determined were electrical conductivity (EC), hardness (HN), total suspended solids (TSS), temperature (T. [a]), chlorides (Cl^-), sodium hypochlorite (NaClO), phosphorus and total nitrogen (TF and TN), pH, dissolved oxygen (DO), fluorides (F^-), biochemical oxygen demand (BOD), ammonia (NH_3), and chemical oxygen demand (COD). Quantification of drugs, and heavy metals consisted of non-steroidal anti-inflammatory drugs (NSAIDs), corticosteroids, proton pump inhibitors, histamine antagonists, As, Cd, Cu, Cr, Hg, Ni, Pb, and Zn. Finally, bacterial meta-taxonomic characterization was achieved using high-throughput sequencing of the 16S rRNA gene, followed by bioinformatic analysis in mothur v.1.48.0. The taxonomic approach was based on the determination of Operational Taxonomic Units (OTUs).

2.2. Danio rerio housing system

405 Wild-type four-month old *D. rerio* adults (1.33 ± 0.7 g in weight and 3.71 ± 0.12 cm in length) were preserved in 120 L tank. In order to maintain a controlled environment, different parameters were monitored during an acclimatization period of two months and throughout the experimental study. The parameters were, temperature of 27.5 °C ± 1 °C, pH of 7.4, natural photoperiod of 12:12, a carrying capacity of 1 L/fish, chlorine-free water, continuous oxygenation of 9.8 ± 0.5 mg/L, nitrate content (2.6 ± 0.2 mg/L), nitrite (0.028 ± 0.007 mg/L), conductivity (370 ± 30 S/cm) and a twice-daily feeding with commercial flake (only for

acclimatization period). Finally, only organisms ranging from 3.59 to 3.83 cm in length and free of infection and symptoms of disease, were selected for the study in a 2:1 (male: female) ratio (OECD, 2019).

2.3. Novel tank test

Experimental tanks containing six organisms were used with different proportions of a hospital effluent (2 %, 2.5 %, 3 %, and 3.5 %), as well as a control without hospital effluent. The proportions used (environmentally relevant) were selected based on embryotoxicity studies in *D. rerio* larvae (Table S1). After 96 h of exposure, fish were moved to the behavioral testing room which is an environment with acoustic isolation. Within the room, both temperature (24 °C to 26 °C) and light were controlled. Experimental organisms were maintained within the room for 30 min before each trial to avoid interference from handling stress (Orozco-Hernández et al., 2022). Subsequently, the organisms were transferred to an individual novel tank (21.2 cm × 21.2 cm × 25.2 cm) one by one, and their movements were monitored for 6 min. Throughout the trials, we documented behavior via videotape for 360 s with a GigE camera positioned in front of the Novel tank. The EthoVision XT 13.0 software was used to examine the behavior of the fish and the behavioral endpoints measured were, time spent at the top and bottom (s), total distance traveled (cm), distance traveled in the bottom and top (cm), latency to enter the top (s), freezing duration at the top and bottom (s), and total erratic movements. The NTT was executed on different days in the morning (9 am – 10 am) to evade any behavioral disorder within the experiment. Each treatment (2 %, 2.5 %, 3 %, 3.5 %, and control) was composed of three replicates ($n = 30$ fish/replica, totaling $N = 90$ throughout the experiment) and independent trials were used to compute the mean and depict it in bar charts. Lastly, it is important to emphasize that we decided to evaluate biomarkers related to behavior, neurotoxicity, and gene expression after 96 h of exposure, since we assumed that at that time the accumulated oxidative damage would be greater than at short-term exposure. In addition, OECD Guideline 203 specifies that a 96 h of exposure period is required to assess the acute toxicity of chemical compounds in fish (OECD, 2019).

2.4. Determination of AChE activity

D. rerio organisms were exposed to different proportions of a hospital effluent (2 %, 2.5 %, 3 %, and 3.5 %) for 96 h. Each 6 L system contained three *D. rerio*. Hereafter, we dissected the head and extracted the brain of all three organisms and pooled them into one sample. The sample was stored in an Eppendorf tube with 1.5 mL of phosphate buffer solution (PBS, pH 7.4). Afterwards, it was homogenized for 20 s with a stator rotor (Ultra-273 turrax T25, IKA, Germany) and centrifugated at 10,000 rpm for 15 min (4 °C). We treated the samples as Orozco-Hernández et al., 2022 described. Briefly, we mixed 400 μL of the supernatant with 2.6 mL of phosphate buffer (pH 8, 0.1 M), 0.1 mL of 5,5-dithiobis-2-nitrobenzoate (DTNB, 0.1 M), and 25 μL of substrate (ACh iodide, 0.075 M) were added. We recorded changes in absorbance over 5 min at a wavelength of 412 nm (Ellman et al., 1961). Each treatment (2 %, 2.5 %, 3 %, 3.5 %, and control) consisted of three replicates ($n =$ 15 fish/replica, totaling $N = 45$).

2.5. Assessment of oxidative stress biomarkers

6 L systems were used with the following proportions of hospital effluent (2 %, 2.5 %, 3 % and 3.5 %), as well as a control system without hospital effluent. A semi-static renewal toxicity test (24 h) was employed (OECD, 2019). Five exposure times were selected, 12 h, 24 h, 48 h, 72 h, and 96 h. After each exposure time, *D. rerio* adults were sacrificed using the hypothermic shock method. A pool of brains from three *D. rerio* was collected for each system and stored in Eppendorf tubes, previously filled with 1 mL of phosphate buffer solution (PBS, pH 7.4). The pooled sample (≈ 135 μg) of *D. rerio* was homogenized for 20 s with a stator rotor, and centrifugated at 10000 rpm. Thereafter, the samples were split into two Eppendorf tubes. Tube 1 included 300 μL of the homogenate plus 300 μL of a 20 % trichloroacetic acid (TCA) solution, whilst tube 2 enclosed 700 μL of the homogenate. Next, tube 1 was centrifuged at 11495 rpm for 15 min at 4 °C and the precipitate was utilized to establish the protein carbonyl content (PCC) via the method of Levine et al., 1994, whilst the supernatant was examined to determine the degree of Lipoperoxidation (LPX) as stated by Buege and Aust (1978), and the Hydroperoxide content (HPC) by Jiang et al. (1992). We centrifugated tube 2 at 12500 rpm for 15 min at 4 °C, the supernatant was employed to determine the activity of antioxidant enzymes: CAT and SOD by the method of Radi et al. (1991), and Misra and Fridovich (1972), respectively. All the biomarker's results were normalized against total proteins (Bradford, 1976). Herein, each group of the oxidative stress experiment (2 %, 2.5 %, 3 %, 3.5 %, and control) was carried out in triplicate ($n = 75$ fish/replica, totaling $N = 225$ over the experiment).

2.6. Gene expression

We used four systems with the same proportions of a hospital effluent mentioned above, and a control without hospital effluent. The exposure time was set at 96 h and the analogous samples were achieved and deposited in a stabilizing solution of RNAlater®, Qiagen. As previously stated, we pooled the brains from three specimens of *D. rerio* into one sample and performed three replicates. Total RNA isolation was conducted from 45 *D. rerio* organisms using RNeasy® kit ($n = 15$ fish/replica). Further, cDNA was synthesized through 1 μg of total RNA performance the QuantiTec® Reverse Transcription Kit. Real-time PCR (RT-qPCR) was carried out utilizing SYBER Green QuantiTec®, and 500 ng of cDNA template on a Rotor-Gene Q instrument. For RT-qPCR the reaction conditions were an initial activation step 15 min at 95 °C, and 35 amplification cycles, 15 s at 94 °C, followed by 30s at 60 °C, and 30s at 72 °C. Gene expression outcomes were calculated using the $2^{-\triangle\triangle CT}$ method (Livak and Schmittgen, 2001). The target genes were normalized against the *β-actin* which was used as a housekeeping. The RT-qPCR experiment used specific primers involved in oxidative stress response (*cat*, *sod*, *nrf2*), apoptosis (*casp6*, *bax*, *casp9*), and detoxification (*cyp1a1*) (Table S2).

2.7. Statical analysis

Data were processed using SigmaPlot 12.3, and R Studio statistical software. Results of oxidative stress, and acetylcholinesterase activity were examined by Kruskal-Wallis analysis of variance (ANOVA) succeeded by a post hoc Student Newman Keuls test. The outcomes of behavioral and gene expression were evaluated using a parametric ANOVA-1, followed by the same post hoc test, significant difference was considered when $p < 0.05$. We created a heat map for the target genes utilizing the "pheatmap" package of R Studio software version 1.2.1335 (Kolde, 2019). Lastly, descriptive statistics are displayed as median with their respective Interquartile Range (IQR), and mean ± standard deviation (SD).

3. Results

3.1. Characterization of hospital effluent

The results of the characterization of hospital effluent were previously published in Ramírez-Moreno et al. (2023). The findings of physicochemical characterization disclose that Cl^- (284.3 mg/L), pH (5.7), TSS (101.2 mg/L), F^- (28.22 mg/L), BOD (92.8 mg/L), and COD (190.2 mg/L) exceed the maximum allowable limits of Mexican standards. Regarding pharmaceutical compounds, and heavy metals previously found in the hospital effluent under-study, the concentration were as follow: ketorolac (1784.3 ng/L), ibuprofen (338 ng/L), naproxen (691.7 ng/L), paracetamol (519.61 ng/L), dexamethasone (272.12 ng/L), hydrocortisone (32.13 ng/L), esomeprazole (12.43 ng/L), omeprazole (11 ng/L), ranitidine (19.1 μg/L), As (0.42 mg/L), Cd (0.39 mg/L), Cu (0.67 mg/L), Cr (0.87 mg/L), Hg (1.62 mg/L), Ni (1.46 mg/L), Pb (2.11 mg/L) and Zn (1.36 mg/L). In addition, opportunistic bacteria were also discovered in

the hospital effluent under-study. The presence of *Parachlamydia acanthamoebae*, *Pseudomonas* spp., *Mycobacterium* spp., and *Legionella* spp. was highlighted.

3.2. Novel tank test

All endpoints assessed during the trial were altered after 96 h of exposure to hospital effluent (Fig. 1 A-F). We found a decrease in total distance traveled ($F(4,10) = 72.12$; $p < 0.001$), increased latency to enter the top ($F(4,10) = 47.73$; $p < 0.001$), increased time spent at the top ($F(4,10) = 38.46$; $p < 0.001$), longer duration of freezing episodes at the top ($F(4,10) = 82.51$; $p < 0.001$), and higher erratic movements ($F(4,10) = 94.27$; $p < 0.001$) compared with the control group. These findings indicate that the fish were displaying anxiety-like behavior, and episodes of stress.

3.3. Determination of AChE activity

As shown in Fig. 2, a decreased AChE activity was noted after 96 h in *D. rerio* brain exposed to four proportions of a hospital effluent. Moreover, it can be observed that AChE-inhibition was in a hospital effluent proportion-dependent manner. Therefore, significant differences were found between the treatment groups and the control ($H(4) = 41.7$, $p < 0.001$).

3.4. Oxidative stress biomarkers

Fig. 3A-C illustrates PCC, HPC, LPX, CAT, and SOD results. We found significant increases in all the experimental groups in comparison to the control group. The greatest increase in PCC and HPC occurred within the group with the highest proportion of effluent, 3.5 %, (757 % and 697 %, respectively) after 72 h (PCC: $H(24) = 205.87$, $p < 0.001$; HPC $H(24) = 200.13$, $p < 0.001$), whilst LPX also displayed the greatest increase in the 3.5 % group (630 %), however after 96 h of exposure ($H(24) = 217.42$, $p < 0.001$). Fig. 3 D-E shows that the activity levels of CAT and SOD, were higher after short-term rather than long-term exposure. Nevertheless, the highest catalytic increase of CAT and SOD in relation to the control group, was seen at 3.5 % (680 % and 129 %) after 24 h and 48 h respectively (CAT: $H(24) = 221.31$, $p < 0.001$; SOD $H(24) = 196.29$, $p < 0.001$).

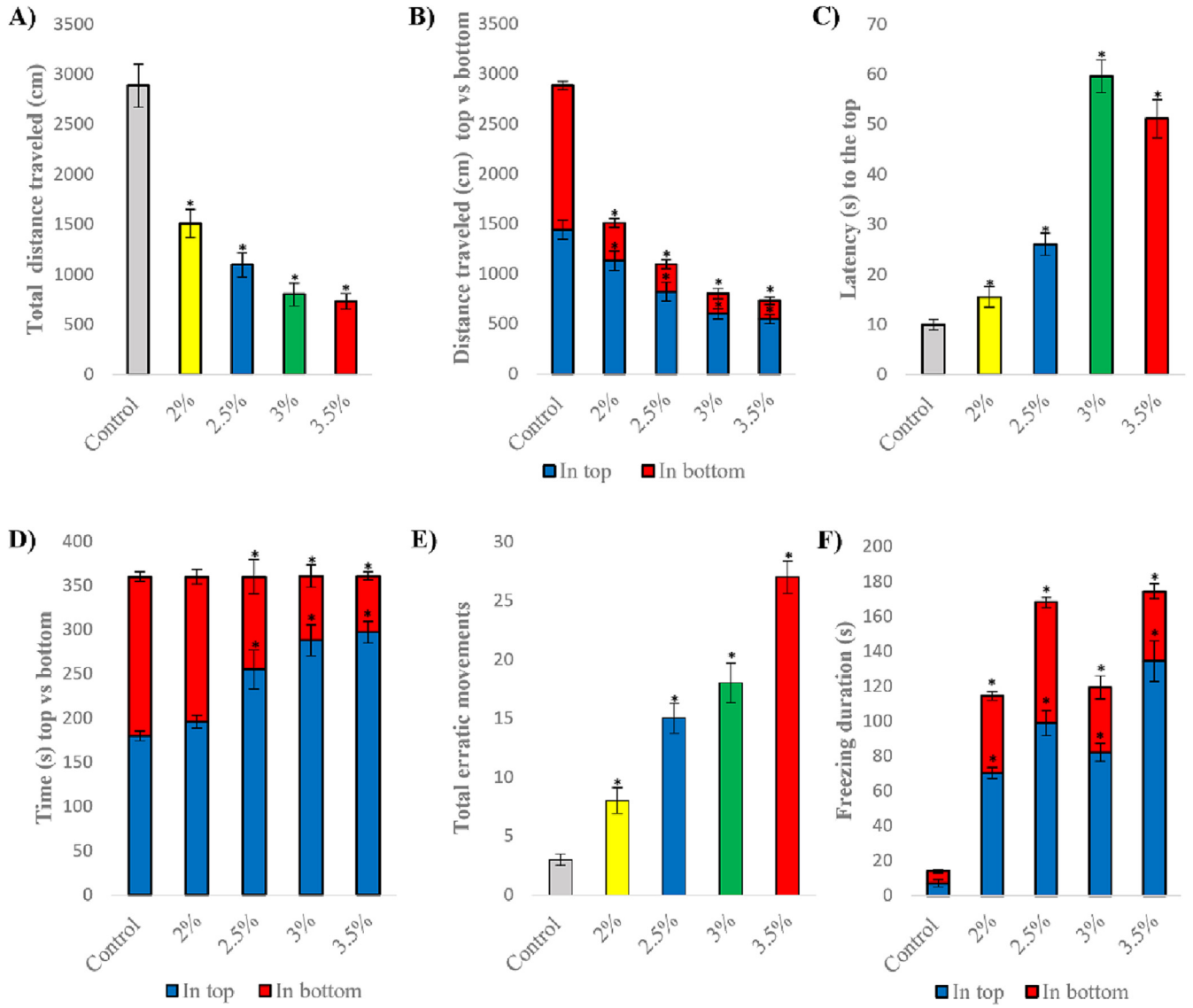

Fig. 1. NTT results of *Danio rerio* exposed to four proportion of a hospital effluent. A) Total distance traveled, B) Distance traveled top vs bottom, C) Latency to the top, D) Time top vs bottom, E) Total erratic movements, F) Freezing duration. Data represent the mean ± SD of three independent experiments, $n = 3$. Asterisks indicate significant change compared to the control group. $N = 90$ fish.

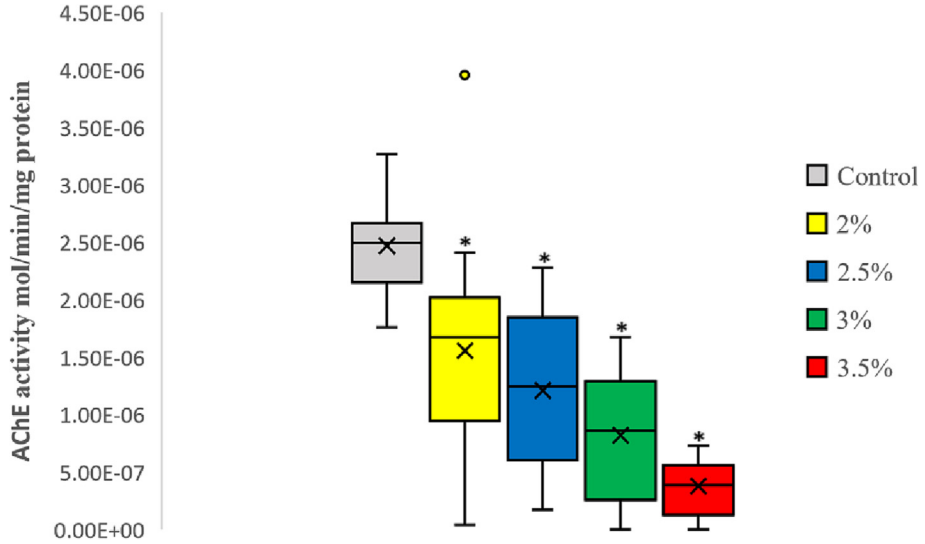

Fig. 2. Inhibition of AChE triggered by four proportions of a hospital effluent in *Danio rerio* brain. Data represent the median ± IQR of three independent experiments, $n = 3$. * Significantly different from control group. $N = 45$ fish.

3.5. Effects of hospital effluent on mRNA expression

Expression patterns can be seen in Fig. 4. The mRNA levels of *sod* increased by about 3.2-fold and 5.52-fold, in the 2 % and 3.5 % hospital effluent treatment groups, respectively ($F(4, 10) = 344.25$, $p \leq 0.001$; $n = 3$). However, the expression behavior of *cat* was slightly different since relative expression decreases at 3.5 % (1.9 fold) ($F(4, 10) = 72.81$, $p \leq 0.001$; $n = 3$). In addition, the expression of *casp9*, *casp6* and *bax* increased around 2.09-fold, 2.45-fold and 3.67-fold at the highest proportion tested (*casp9*: $F(4, 10) = 86.12$, $p \leq 0.001$; $n = 3$; *casp6*: $F(4,10) = 97.63$, $p \leq 0.001$; $n = 3$; *bax*: $F(4,10) = 214.49$, $p \leq 0.001$; $n = 3$). It is notable that, *nrf2* and *cyp1a1* genes were strongly upregulated at 3.5 % of hospital effluent with nearly 7-fold and 4.5-fold, respectively (*nrf2*: $F(4, 10) = 478.85$, $p \leq 0.001$; $n = 3$; *cyp1a1*: $F(4,10) = 411.23$, $p \leq 0.001$; $n = 3$). Finally, for almost all the genes the transcripts increased in a hospital effluent proportion-dependent manner.

4. Discussion

Currently, insight into the toxicological effect that can be induced by hospital effluents in aquatic organisms is scarce. Moreover, most research does not consider relevant aspects of these anthropogenic matrices (e.g. presence of pathogenic microorganisms, gene expression disruption, etc.) that could mediate damage on aquatic organisms. In this regard, the current study is pioneering in its field, since it aims to combine the results obtained herein, and those obtained by different authors, in order to purpose a toxicodynamic mechanism that in the near future could be firmly validated, and thus help to clarify the existing knowledge gaps about how hospital effluents impair neuronal capacity in *D. rerio*.

The molecules that can be found in hospital effluents are widely diverse; specifically, the hospital effluent studied in this research which was constituted by complex mixtures of heavy metals, NSAIDs, corticosteroids, proton pump inhibitors, and histamine antagonists to name but a few (Ramírez-Moreno et al., 2023; Verlicchi, 2021). One of the main issues raised by hospital effluents is the concentration of xenobiotics that they contain. In this regard, it is necessary to take into account the variability of the ecotoxicity values of the pollutants in question, whose Predicted No Effect Concentration (PNEC) is close to 0.01 pg/L and a maximum of 1 mg/L (Perrodin and Orias, 2018). Previous studies have reported adverse effects after exposure to hospital effluents. Isidori et al. (2016) reported DNA fragmentation in *D. rerio* hepatocytes in response to the presence of high concentrations of antineoplastic drugs and their corresponding metabolites in hospital effluents from Slovenia and Spain. Furthermore, chromosomal aberrations, sperm, and histopathological alterations have been detected in mice exposed to hospital effluents in Nigeria and Tunisia (Afsa et al., 2021; Alabi and Shokunbi, 2011). The toxicological damage in *D. rerio* (LC50, 29.25 %) caused by hospital laundry wastewater, usually combined with discharges from various medical specialties, has been investigated in detail (Kern et al., 2015). In Afsa et al. (2022), the authors reported catalytic decreases of CAT, AChE, and Glutathione S-transferase (GST) in *D. magna* neonates after 48 h of exposure to hospital effluent. In addition, Rosales-Pérez et al. (2022) and Neri-Cruz et al. (2015) have shown that these anthropogenic matrices have the potential to cause an imbalance in the redox status of fish. However, none of the above-mentioned research points out how these effects may occur.

In this context, we found a meaningful rise in oxidative biomarkers, such as PCC, HPC, and LPX (Fig. 3 A-C) after exposure to different proportions of a hospital effluent (2 %, 2.5 %, 3 %, and 3.5 %) in *D. rerio* brain. Our findings are in agreement with those established by different authors that support the capacity of hospital effluents to generate oxidative modifications in *D. rerio* and *Cyprinus carpio* brains (Rosales-Pérez et al., 2022; Neri-Cruz et al., 2015). Moreover, da Costa Araújo et al. (2022) have depicted that mixtures of xenobiotics induce DNA damage in *D. rerio* erythrocytes, as well as a redox imbalance at the brain level. We found that CAT and SOD antioxidant activities were enhanced after short-term exposures due to the increase of ROS, such as H_2O_2 and $O_2^{\bullet -}$ at the cellular level. However, at long-term exposures, the antioxidant defence was depleted (Fig. 3 D-E). Our results reinforce the discussion promoted by Ramírez-Moreno et al. (2023), who suggest that biochemical activity of the antioxidant system may be depleted by exposure to a cocktail of xenobiotics. On the other hand, it is paramount to mention that, not only a mixture of chemical compounds can be found in hospital effluents, but also SARS-CoV-2 peptide fragments (PSPD) (Freitas et al., 2023). In this regard, Freitas et al. (2022) report that *Cloeon dipterum* exposed to mixtures of pollutants (e.g. ibuprofen, ranitidine, nitrogen, detergent etc.), and PSPD (alone or in combination with pollutant mix) has the ability to suppress SOD and CAT activity, as well as decrease the content of thiol groups. Nevertheless, organisms have alternative non-enzymatic defence systems that help them to cope with ROS, for instance, Glutathione (GSH), selenium, β,γ-carotene, ferritin, melatonin, lutein, α-tocopherol, which act as scavengers by giving up or accepting an electron, becoming by definition a free radical. However, they have the ability to adapt to the electron exchange without becoming reactive (Afsa et al., 2022; Moniruzzaman et al., 2022; Atta et al., 2017).

The oxidative imbalance that occurs at the neuronal level in *D. rerio* largely explains the up-regulation of *cat*, *sod*, and *nrf2* transcripts (Fig. 4).

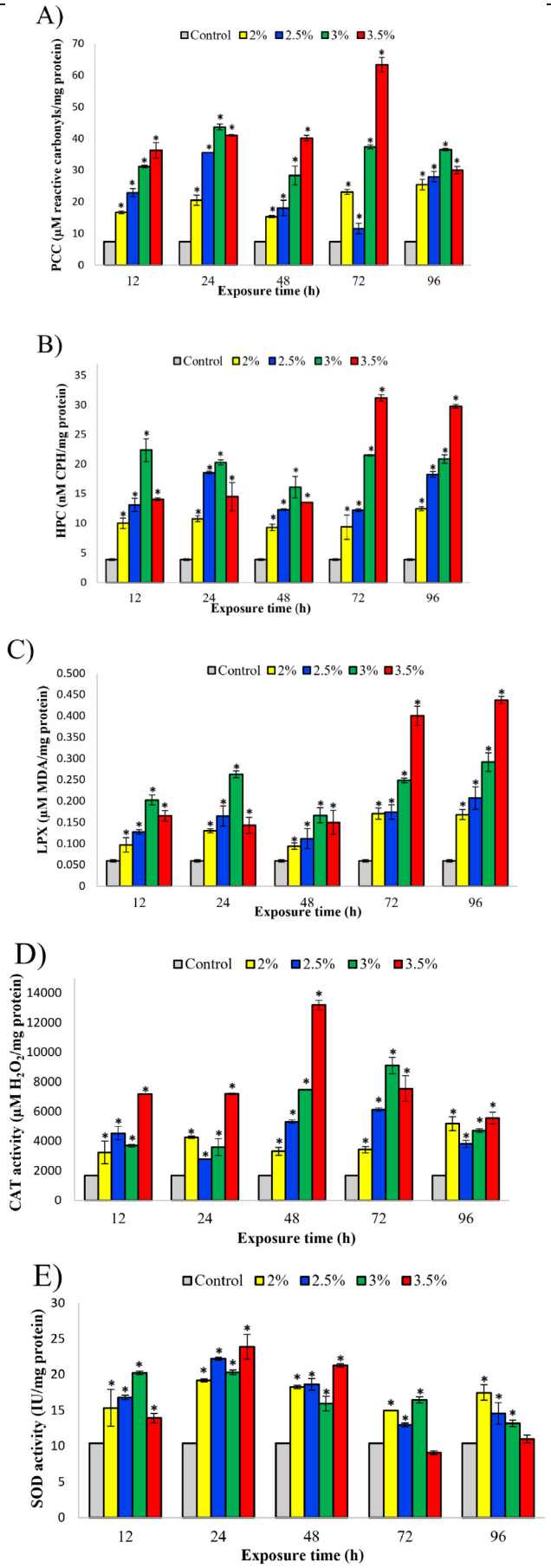

Fig. 3. Oxidative stress biomarkers (A PCC, B HPC, C LPX, D CAT, and E SOD) in *Danio rerio* brain exposed to four proportions of a hospital effluent. Data represent the median ± IQR of three independent experiments, $n = 3$. Asterisks denote a significant change compared to the control group. $N = 225$ Fish.

In particular, the *nrf2* gene is related to the transactivation of the antioxidant response element (ARE) located in the promoter region of different genes involved in cytoprotective defence, and, in turn, participates in the interaction with the aryl hydrocarbon receptor (AhR) to regulate the transcription of phase I and II enzymes (Wakabayashi et al., 2010). Up-stream activation of AhRs induces the expression of cytochrome P450 enzymes, such as *cyp1a1* (Chen and Chan, 2018). It is well known that *cyp1a1* plays a regulatory role as a detoxifying and metabolizing enzyme. Furthermore, it is intimately linked to the processes of cellular inflammation and neuronal death in response to environmental pollutants (Wójtowicz et al., 2019). The up-regulation of *casp6*, *bax*, and *casp9* detected in *D. rerio* reveals that there are apoptotic processes at the neuronal level, both by intrinsic and extrinsic pathways, largely owing to the excessive increase of ROS, PCC, HPC, and LPX (Ramírez-Moreno et al., 2023; Cheng et al., 2020; Félix et al., 2018; Jiang et al., 2014).

The CNS is one of the major targets of different pollutants. Notably, the heavy metal Nickel (Ni) at concentrations of 10–1000 µM has the ability to promote Ferroptosis at the neuronal level in *D. rerio*, which is a recently described form of iron-mediated cell death (Wang et al., 2023). This occurs due to an increased concentration of LPX end-products, namely Malondialdehyde (MDA) or 4-Hydroxynonenal (4-HNE), high concentrations of ROS, a decrease in intracellular GSH, as well as depletion of enzymes involved in HPC decomposition, such as Glutathione peroxidase (GPX), and CAT primarily (von Krusenstiern et al., 2023; Wang et al., 2023).

Another important issue that must be considered is that there are endogenous danger molecules, for example ATP, LPX-adducts, and PCC termed damage-associated molecular patterns (DAMPs) that stimulate adaptive immunity through the activation of brain macrophages (microglia) (Krysko et al., 2011; Moghaddam et al., 2011; Uchida, 2013). Although the apoptotic processes obey a series of well-orchestrated steps, apoptotic cells that are blebbing are known to release ATP into the extracellular space, resulting in immunomodulatory activity. Successively, ATP release is known to be caspase-dependent. ATP released by damaged brain cells acts as a chemoattractant, is recognized by P_2Y_2 receptors on microglia and induces their recruitment and activation (Krysko et al., 2011; Elliott et al., 2009). Additionally, microglia can be activated by a vast range of oxidatively modified biological molecules, which bind to pattern-recognition receptors (PRRs) (Uchida, 2013; Miller et al., 2011). LPX-derived adducts, and PCC are usually recognized as DAMPs at the extracellular level (Moghaddam et al., 2011; Uchida, 2013; Miller et al., 2011). Activation of microglia favors the production, and release of proinflammatory molecules, such as interleukin (IL)-1β, IL-6, tumor necrosis factor (TNF-α), chemokines and reactive oxygen and nitrogen species, which together may promote the emergence of highly oxidative environments in *D. rerio* brain (Mahapatra et al., 2023; Kreisl, 2022; Biswas, 2016; Nakajima and Kohsaka, 2001). In this circumstance, the release of pro-inflammatory interleukins perpetuates inflammatory processes following extrinsic activation of apoptosis receptors (Ramírez-Moreno et al., 2023) (Fig. 5).

Aquatic organisms are constantly surrounded by water that, by default, usually contains various pathogens. It should be noted that the hospital effluent studied herein, has opportunistic bacterial genera with a high infectivity capacity, namely *Parachlamyda*, *Pseudomonas*, *Mycobacterium*, and *Legionella*. Specially, *Parachlamyda acanthamoebae* (single sp. of *Parachlamydia* genus) can parasitise macrophages and subsequently induce apoptosis (Greub et al., 2003; Kebbi-Beghdadi et al., 2011). Moreover, bacteria contain different endotoxins capable of inducing inflammatory responses and immunomodulation in fish (Moniruzzaman et al., 2022). The most studied endotoxin is lipopolysaccharide (LPS), which is found in the outer membrane of most gram-negative bacteria (Teles et al., 2011;

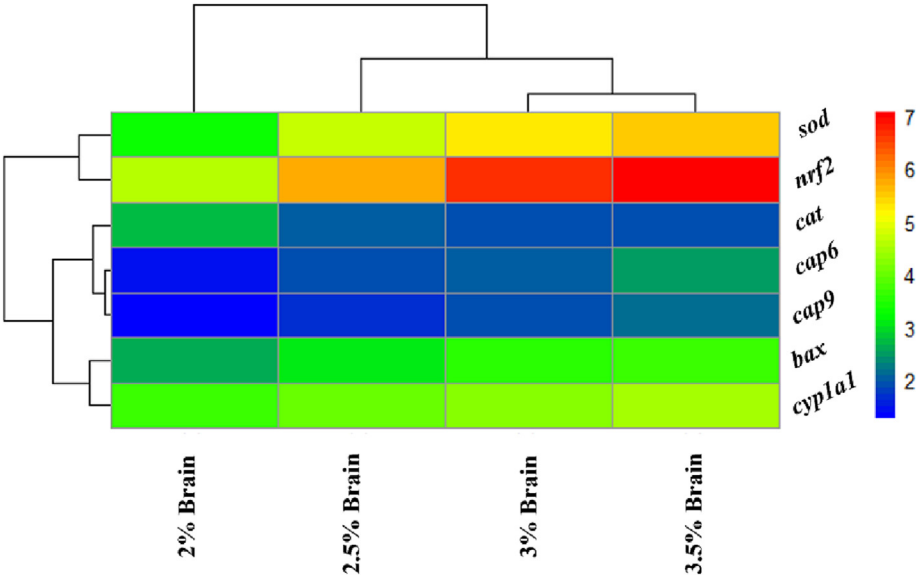

Fig. 4. Heat map for target genes related to oxidative stress, apoptosis, and detoxification after exposure to four proportions of a hospital effluent at 96 h in *Danio rerio* brain. Values correspond to the mean, n = 3. *N* = 45 fish.

Swain et al., 2008). It has been demonstrated that LPS exposure can cause oxidative stress, tissue damage and an innate immune response that triggers inflammatory processes in the host (Moniruzzaman et al., 2022; Leng et al., 2014).

Notably, extracellular traps (ETosis) are part of the first line of defence of the innate immune system and are induced by the presence of bacteria or their corresponding antigens (e.g. LPS) (Yam-Puc et al., 2012). ETosis have been demonstrated in neutrophils, macrophages, and recently in *D. rerio* erythrocytes (Isles et al., 2021; Rinaldi et al., 2021; Rinaldi et al., 2023). The process involves the release of mitochondrial reactive oxygen species (mROS), antimicrobial proteins, and nuclear material (DNA and histones) packed with granular proteins, which, all together, serve to trap and neutralize invading microorganisms, in particular bacteria and fungi (Rinaldi et al., 2023; Yam-Puc et al., 2012) (Fig. 5).

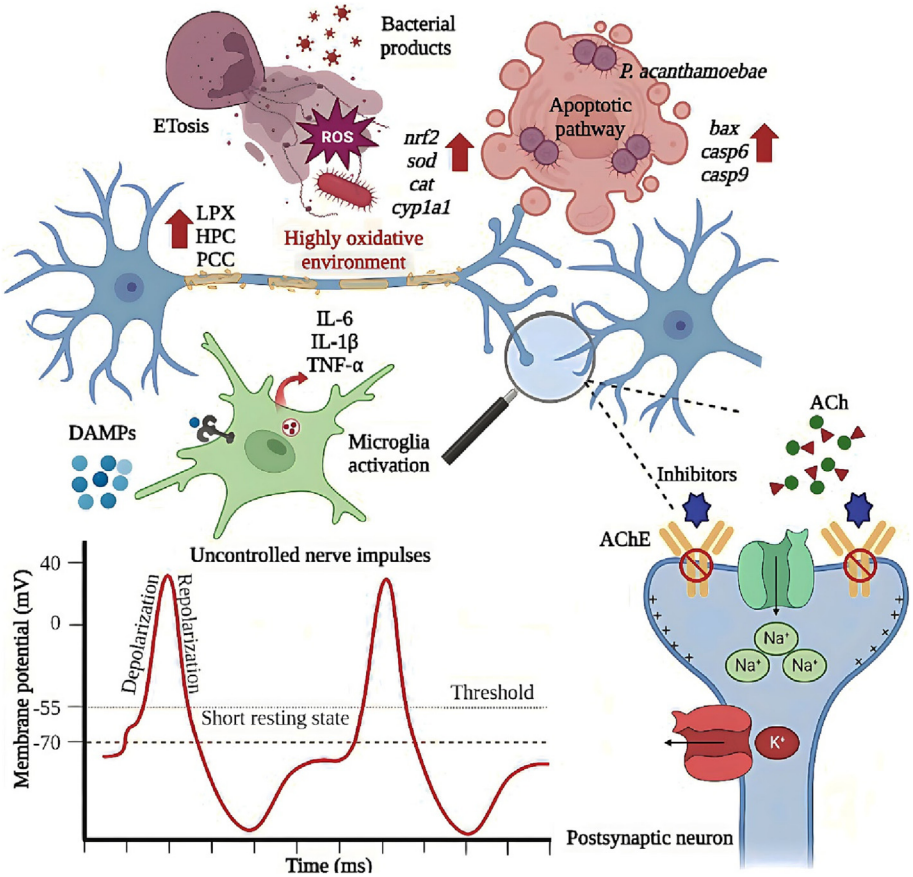

Fig. 5. Suggested mechanism whereby a hospital effluent induces harm in *D. rerio brain*.

In recent years, information has emerged concerning a new cell death process, which is still being explored. Pyroptosis is a cell death pathway marked by cell lysis as well as release of proinflammatory cytokines, DAMPs, and pathogen-associated molecular patterns (PAMPs), which involves the activation of inflammasomes through recognition by related PPRs (Cao et al., 2023). This mechanism constitutes an integral component of the innate immune system of fish (Song et al., 2022). It can be activated by various extracellular signals, including LPS and nucleotides, as well as by intracellular signals, such as oxidative stress (Cao et al., 2023; Brokatzky and Mostowy, 2022). Zhang et al. (2020) depicted that Cd (40 μM) increase ROS in *Cyprinus carpio* lymphocytes and induced the occurrence of pyroptosis. Furthermore, it is well known that infection of pathogens intracellular, such as *Pseudomonas*, *Salmonella*, and *Mycobacterium* can trigger pyroptosis in *D. rerio* (Brokatzky and Mostowy, 2022; Chen et al., 2021). Therefore, it is tempting to speculate that this process may occur and would trigger an inflammatory response, which would explain the oxidative environment found in *D. rerio* brain.

PUFAs are abundant in the brain and are very vulnerable to oxidation by ROS (Salim, 2017). Therefore, we speculate that the myelin sheath synthesized by oligodendrocytes could deteriorate due to the increase of LPX and HPC discovered in *D. rerio* brain (Fig. 5). Our results demonstrate that oxidative stress is intimately related to behavioral alterations in *D. rerio* after exposure to different proportions of a hospital effluent. NTT results showed fish suffered alterations in their swimming behavior and displayed anxiety-like behaviors. We found a decrease in total distance traveled, increased latency to enter the top, increased time spent at the top, an increase in total erratic movements and longer duration of freezing episodes at the top compared with the control group, which are essential parameters for assessing anxiety (Fig. 1). da Costa Araújo et al. (2023) stated that *Physalaemus cuvieri* tadpoles exposed to mixtures of pollutants exhibited a decrease in swimming activity, which could be related to possible sensorimotor disturbances, altering the reception and neural processing of information. Consistent with our outcomes, Karaman et al. (2023) report that *D. rerio* adults exposed to 1.5 ppm–100 ppm F^- display anxiety-like behavior and disruptions to their circadian rhythm. Furthermore, Rosales-Pérez et al. (2022) concluded that hospital effluents alter the behavior and AChE activity in *D. rerio* adults. AChE inhibition may correlate closely with detected changes in anxiety behaviors (Figs. 1 and 2). This neurotransmitter performs essential functions for neuronal communication by transmitting electrical activity at the synapse. Inhibition of AChE causes an accumulation of acetylcholine (ACh) in the neuronal cleft, and an uninterrupted influx of Na^+ into the postsynaptic membrane, which depolarizes the membrane and generates uncontrolled action potentials (Fig. 5). Firstly, membrane depolarization may be compensated by partial activation of voltage-gated K^+ channels that attempt to polarize the membrane towards its resting state (≈ -90 mV). However, in the long-term, erratic action potentials would trigger an impairment of nerve transmission that would explain the observed behavioral alterations (Čolović et al., 2013; Hong and Chang, 1993). In this respect, exposure to hospital effluents prompts inhibition of AChE together with anxiety-like behavior, which could have serious repercussions on the fitness of *D. rerio*, since the ability to escape from predators in the wild may be reduced. For instance, de Souza et al. (2018) concluded that mice exposed to a mix of pollutants had a decrease in their defence behavior when confronted by predators.

There are different PhACs that may be present in hospital effluents capable of inhibiting AChE activity by direct oxidation of the -SH groups available in the enzyme, or by competitive or non-competitive kinetics. Neurotoxic compounds, such as fluoxetine, salicylic acid, paracetamol, carbamazepine, and tamoxifen, among others, could inhibit per se activity (Orozco-Hernández et al., 2022; Nunes et al., 2020; Daniel et al., 2022; Türkan et al., 2021). Finally, we strongly believe that the highly oxidative environment it raises at the neuronal level encompasses a set of complexes, and interconnected cellular interactions involving oxidative stress, apoptosis, detoxification, synaptic, inflammatory, and immunological processes among many other mechanisms that still need to be elucidated to obtain a holistic view of the toxicological effects that hospital effluents trigger in aquatic organisms (Fig. 5).

5. Conclusions

Our experiments uncovered that exposure to different proportions of a hospital effluent treated by HWWTP (2 %, 2.5 %, 3 %, and 3.5 %) disrupted *Danio rerio* brain homeostasis and caused a neurotoxic effect. The oxidative imbalance was confirmed by the increase of oxidative biomarkers (PCC, HPC, and LPX). In addition, we found a positive regulation of genes related to oxidative stress response (*cat*, *sod*, *nrf2*), apoptosis (*casp6*, *bax*, *casp9*) and detoxification (*cyp1a1*). The increase in ROS promotes a highly oxidative environment at the neuronal level that is conducive to the inhibition of AChE activity, and is congruent with the anxiety-like behavior observed in *D. rerio* adults. Finally, based on our findings, we encourage further research into the mechanisms underlying the adverse effects triggered by these anthropogenic matrices on aquatic organisms.

CRediT authorship contribution statement

Francisco Javier Ramírez-Moreno, Luis Alberto Orozco- Hernández & José Manuel Orozco- Hernández performed all the exposure experiments.

Leobardo Manuel Gómez-Oliván and Francisco Javier Ramírez-Moreno were involved in the conception

Leobardo Manuel Gómez-Oliván and Francisco Javier Ramírez-Moreno, were involved in the design and interpretation of the data and the writing of the manuscript with input from Sandra García-Medina & Marcela Galar-Martínez

Data availability

Data will be made available on request.

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2023.164906.

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