Science of the Total Environment 887 (2023) 164057

Contents lists available at ScienceDirect

Science of the Total Environment

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

A comprehensive approach to how hospital effluents lead to oxidative stress and shift the gene expression in target organs of *Danio rerio*

Francisco Javier Ramírez-Moreno [a], Leobardo Manuel Gómez-Oliván [a,*], Hariz Islas-Flores [a], Sandra García-Medina [b], José Félix Aguirre-Garrido [c], Luis Mario Hernández-Soto [d]

[a] Laboratorio de Toxicología Ambiental, Facultad de Química, Universidad Autónoma del Estado de México, Paseo Colón intersección Paseo Tollocan, Colonia Residencial Colón, CP 50120 Toluca, Estado de México, Mexico

[b] Laboratorio de Toxicología Acuática, Departamento de Farmacia, Escuela Nacional de Ciencias Biológicas, Instituto Politécnico Nacional, Unidad Profesional Adolfo López Mateos, Av. Wilfrido Massieu s/n y cerrada Manuel Stampa, Col. Industrial Vallejo, Ciudad de México CP 07700, Mexico

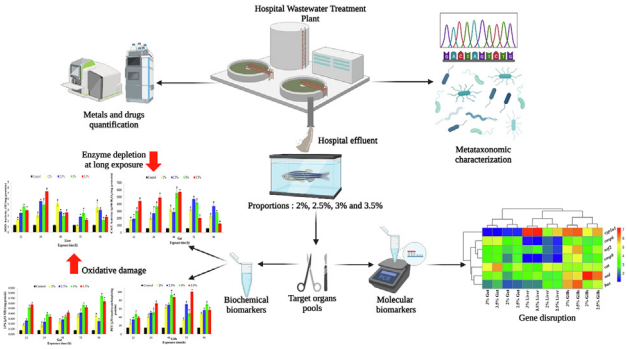
[c] Departamento de Ciencias Ambientales, Universidad Autónoma Metropolitana - Unidad Lerma, 52005 Lerma, Edo. Mex, Mexico

[d] Doctorado en Ciencias Biológicas y de la Salud, Universidad Autónoma Metropolitana-Unidad Lerma, 52005 Lerma, Edo. Mex, Mexico

HIGHLIGHTS

- Exposure to hospital effluent increases the level of oxidative biomarkers.
- Long exposure times trigger an antioxidant enzyme depletion.
- Hospital effluent disrupts gene expression in target organs of *Danio rerio*.
- Metataxonomic approach reveals pathogenic bacterial genera in hospital effluent.

GRAPHICAL ABSTRACT



ARTICLE INFO

Editor: Damià Barceló

Keywords:
Hospital effluent
Danio rerio
Oxidative stress
Gene expression
Apoptosis
Metataxonomic

ABSTRACT

Hospital effluents represent a threat to the environment owing to the content of toxic substances capable of altering the structure and function of ecosystems. Despite the available information about the impact of hospital effluents on aquatic organisms, the molecular mechanism underlying this process has received little or no attention. The present study aimed to evaluate the oxidative stress and gene expression induced by different proportions (2 %, 2.5 %, 3 % and 3.5 %) of hospital effluent treated by hospital wastewater treatment plant (HWWTP) in liver, gut, and gills of *Danio rerio* at different exposure times. Significant increases in the levels of protein carbonylation content (PCC), hydroperoxides content (HPC), lipoperoxidation level (LPX) and superoxide dismutase (SOD) and catalase (CAT) activity were observed in most of the organs evaluated at the four proportions tested with respect to the control group ($p < 0.05$). It was found that at longer exposure times there is a lower response in SOD activity, suggesting catalytic depletion due to the oxidative environment at the intracellular level. The lack of complementarity between SOD and mRNA activity patterns indicates that the activity itself is subordinated to post-transcriptional processes. Upregulation of transcripts related to antioxidant processes (*sod*, *cat*, *nrf2*), detoxification (*cyp1a1*) and apoptosis (*bax*, *casp6*, and *casp9*) was observed in response to oxidative imbalance. On the other hand, the metataxonomic approach allowed the characterization of pathogenic bacterial genera such as *Legionella*, *Pseudomonas*, *Clostridium XI*, *Parachlamydia* and

* Corresponding author at: Laboratorio de Toxicología Ambiental, Facultad de Química, Universidad Autónoma del Estado de México, Paseo Colón intersección Paseo Tollocan, Colonia Residencial Colón, CP 50120 Toluca, Estado de México, Mexico.
E-mail address: lmgomezo@uaemex.mx (L.M. Gómez-Oliván).

http://dx.doi.org/10.1016/j.scitotenv.2023.164057
Received 27 March 2023; Received in revised form 6 May 2023; Accepted 7 May 2023
Available online 12 May 2023

Mycobacterium present in the hospital effluent. Our findings indicate that although hospital effluent was treated by HWWTP, it caused oxidative stress damage and disrupted gene expression by decreasing the antioxidant response in *Danio rerio*.

1. Introduction

Water pollution is a pressing problem through the importance of water as an elementary resource for the physiological functioning of living organisms. According to UNESCO (2017), the main sources of pollution come largely from industrial, municipal, agricultural and hospital effluents (Verlicchi, 2021), which are often discharged into ecosystems without prior treatment.

Hospitals, in particular, can use a large volume of water depending on the medical services they provide, their maximum capacity, geographical location and wastewater management. It is estimated that in developed countries between 400 and 1200 L of wastewater/bed/day is generated (Kumari et al., 2020). Hospital effluent is usually the term used for wastewater that is discharged from hospitals. Hospital effluent may or may not be treated before being discharged into the sewer system or surface water, depending on the regulations and practices of each country. In this respect, hospital effluents contain a variety of potentially ecotoxic substances from clinical analyses, diagnostics, cleaning, disposal of pharmaceutical compounds and patient excreta (Verlicchi, 2021; Verlicchi et al., 2010). In addition, hospital effluents are predicted to be up to 15 times more toxic than domestic effluents (Olvera-Néstor et al., 2016) on account of the presence of heavy metals, pharmaceuticals, and their secondary metabolites, as well as antibiotic-resistant bacteria and fungi (Cuetero-Martínez et al., 2023). The most frequently reported pollutants in hospital effluents are illustrated in Fig. 1. These pollutants undergo various forms of degradation when released into surface waters, including biotic (metabolic) and abiotic (oxidation, hydrolysis, photolysis) degradation, enabling the production of metabolites that are more toxic than the original molecules (Li and Lin, 2015; Baillie and Rettie, 2011). Compounds that do not undergo some kind of transformation within wastewater treatment plants (WWTPs), hospital wastewater treatment plants (HWWTPs) or in the environment, in many cases and depending on their physicochemical characteristics (e.g. partition coefficient, bioconcentration factor, vapour pressure etc.), have the ability to accumulate in different environmental compartments, enhancing their bioavailability and bioaccumulation (Świacka et al., 2022). Evidence shows that organisms can be impacted by hospital effluents, with studies showing it can induce neurobehavioral alterations and oxidative stress (*Danio rerio* and *Cyprinus carpio*), embryotoxicity (*D. rerio* embryos), histopathological alterations (*Mus musculus*), antioxidant impairment (*Daphnia magna*) and cytotoxic and haematological damage (*Clarias gariepinus*) (Rosales-Pérez et al., 2022; Neri-Cruz et al., 2015; Wittlerová et al., 2020; Afsa et al., 2021; Afsa et al., 2022; Alimba et al., 2019).

Oxidative stress is a common challenge for aquatic organisms when exposed to various environmental pollutants. It occurs when the homeostatic balance between pro-oxidant and antioxidant mechanisms is disrupted, triggering biochemical and molecular alterations (Sies et al., 2017). The metabolism of the compounds in hospital effluents release an excess of reactive oxygen species (ROS) (Kataba et al., 2022; Liu et al., 2019; Seoane et al., 2017). Thus, the enzymatic activity of Superoxide dismutase (SOD) and Catalase (CAT) are the first line of antioxidant defence (Ighodaro and Akinloye, 2018; Claiborne, 2018; Sies et al., 2017). When the antioxidant capacity of biota exposed to xenobiotics is exceeded, oxidizing molecules such as hydrogen peroxide (H_2O_2), superoxide anion radicals ($O_2^{\bullet -}$) and hydroxyl radicals (OH•) have the possibility to interact with proteins and membrane lipids, resulting in processes of protein carbonylation (PCC), Lipoperoxidation (LPX) and generation of Hydroperoxides (HPC) (Lim et al., 2019; Levine et al., 1996; Kancheva and Angelova, 2017). It has been shown that under oxidative stress conditions Nrf-2 protein can translocate from the cytosol into the nucleus, dimerize with small Maf proteins and transactivate the antioxidant response element (ARE), which is an enhancer sequence located in the promoter region of several genes involved in antioxidant enzyme transduction (Bhakkiyalakshmi et al., 2018). Moreover, there are genes (*casp6*, *bax*, *casp9* and *cyp1a1*) related to apoptotic processes and xenobiotic metabolism, that may provide valuable information to elucidate the toxicodynamic mechanism whereby hospital effluents inflict oxidative damage in non-target organisms (Buttke and Sandstrom, 1994; Li et al., 2020; Chen and Chan, 2018).

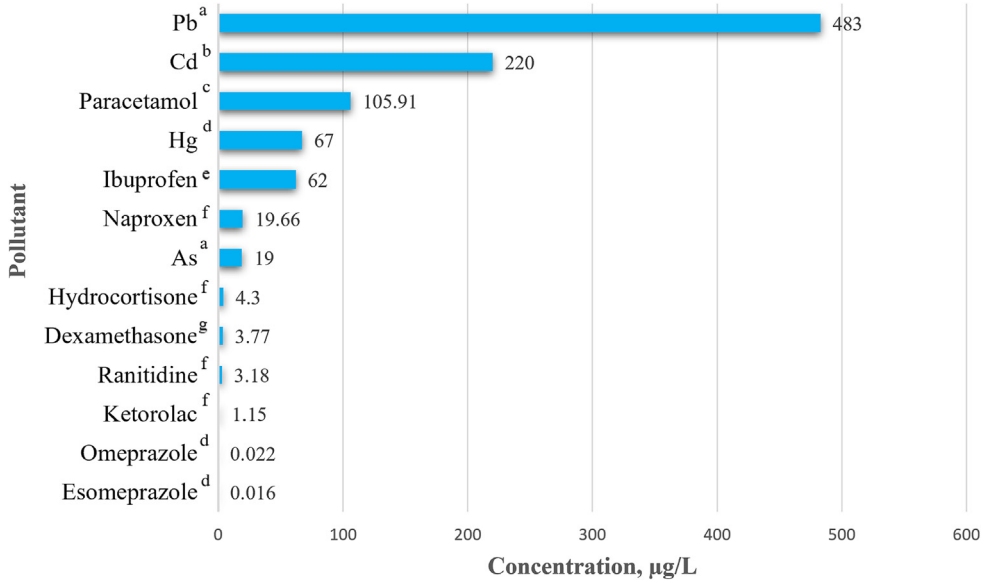


Fig. 1. Ocurrence of pollutants in hospital effluents. [a] (Gómez-Oliván et al., 2019), [b] (Deguenon et al., 2022), [c] (Niemi et al., 2020), [d] (Rosales-Pérez et al., 2022), [e] (Ajibola et al., 2021), [f] (Oliveira et al., 2015), [g] (Jha et al., 2017).

Among the great diversity of microorganisms that inhabit the biosphere, only a small part is cultivable. In view of this difficulty, new strategies have been developed to study them (Ranjan et al., 2016). Microbial ecology assesses the diversity, abundance, and distribution of microorganisms at different levels of biological organization, in order to understand their relationships with the environment. To carry out such evaluations it is necessary to extract genomic or metagenomic material from an environmental sample. Metagenomics employs two approaches, amplicon, and shotgun sequencing. The former involves metataxonomic analysis based on the amplification and sequencing of genetic markers such as 16S rRNA, 18S rRNA or the internal spacer region (ITS) on high-throughput sequencing platforms, whose main objective is to specifically characterize bacterial communities and their diversity (Cuetero-Martínez et al., 2023; Klindworth et al., 2013). Whereas the shotgun approach is mostly used to identify the metabolic potential (genes) of bacterial community members (functional metagenomics) (Ranjan et al., 2016).

Currently, the number of research addressing the potential toxicological harms of hospital effluents are still extremely low. Although it is known that hospital effluents can cause adverse effects, the molecular mechanism underlying this process has received little or no attention (Rosales-Pérez et al., 2022; Neri-Cruz et al., 2015). This is coupled with the limited use of metataxonomic approaches to characterize biological factors (e.g. bacteria and fungi) that could impact on living organisms exposed to hospital effluents. Therefore, the present investigation adopts toxicological evaluation criteria from a biochemical level, determining oxidative biomarkers and antioxidation (PCC, LPX, HPC, SOD and CAT), while also examining the relative expression of target genes (*sod*, *cat*, *nrf2*, *bax*, *casp6*, *cyp1a1* and *casp9*) at the molecular level. In this regard, target organs (livers, guts, and gills) of *D. rerio* exposed (12 h - 96 h) to four proportions (2 %, 2.5 %, 3 % and 3.5 %) of hospital effluent treated by HWWTP from a private hospital in Mexico (HPM) were analyzed. In addition, the hospital effluent was characterized at physicochemical and metataxonomic level. We speculate that the mechanism of toxicological action of hospital effluent exposure will be linked to an imbalance in oxidative status due to boosted ROS generation leading to an increase in oxidative biomarkers together with apoptosis by both intrinsic and extrinsic pathways.

2. Materials and methods

2.1. Sampling of hospital effluent

The samples were collected from HWWTP effluent, which is directly discharged to the sewer system. Fig. 2 shows the location of the HPM, this is a medium-sized general hospital containing approximately 500 beds, with a wide range of medical specialties, including oncology, cardiology, radiology, among others. Samples were collected as stipulated in the Mexican standard NMX-AA-003-1980, in October 2022, during peak work periods (8:00 am–11:00 am). The samples were deposited in a 10 L amber bottles previously sterilized and washed with 30 % nitric acid solution and deionized water, then stored at 4 °C until analysis. In addition, we decided to work with the hospital effluents treated by HWWTP, as the hospital wastewater were highly toxic and generated a mortality of about 90 % (unpublished data) in *D. rerio* larvae.

The process of the HWWTP is summarized below. The hospital influent coming from the different specialties is directed to HWWTP, where materials and solids that cannot be biologically processed are separated. The HWWTP's bioreactor is constantly aerated, the water enters a secondary clarifier to settle or remove suspended particles and the activated sludge

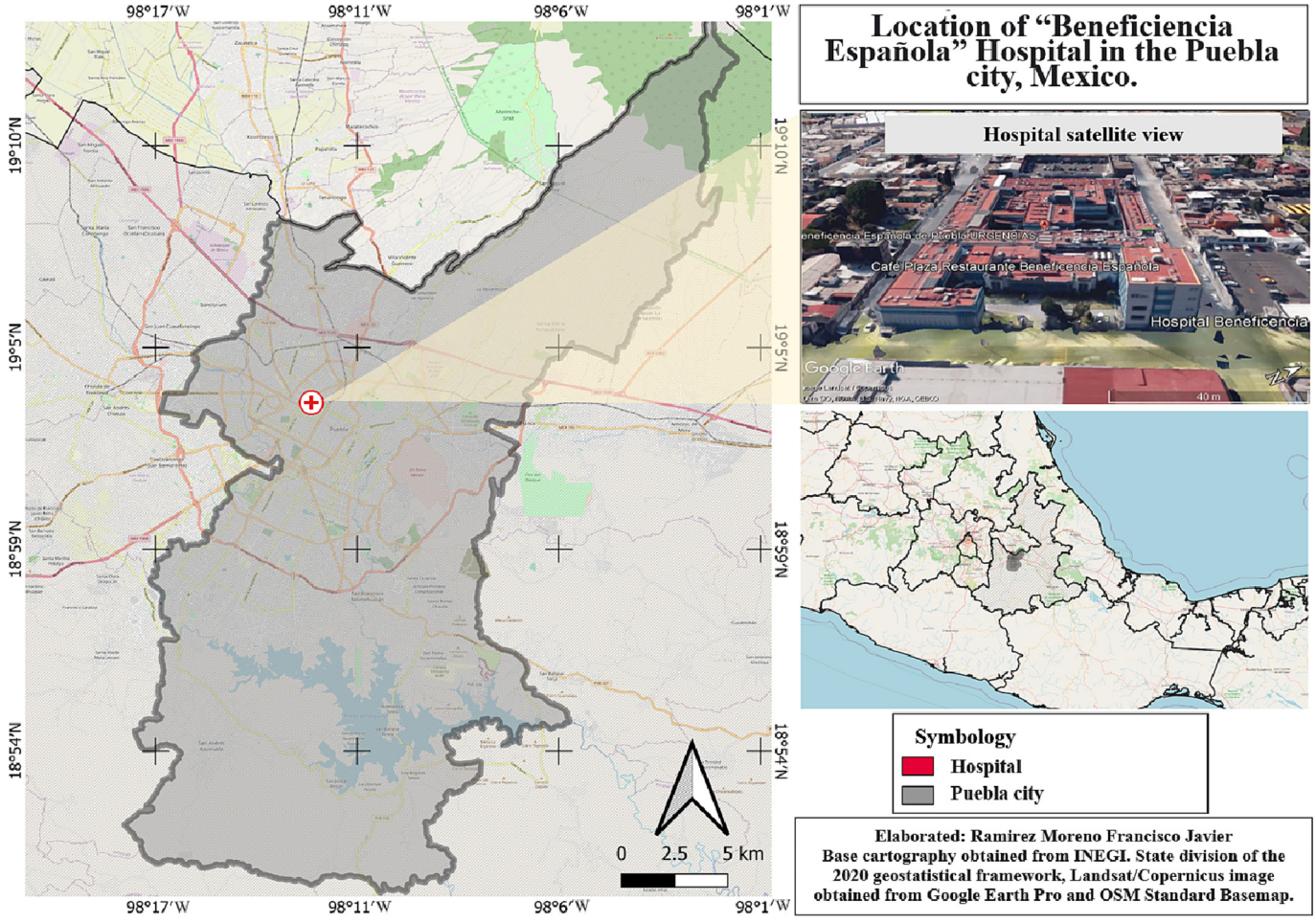


Fig. 2. Location of the private hospital, Mexico.

Table 1
Factors used in the quantification of metals in hospital effluent.

Metal	Absorption wavelength (nm)	Detection limit (LOD) μg/L	Quantification limit (LOQ) μg/L
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

(AS) is decanted. The AS must be stabilized and then dewatered in drying beds. The supernatant enters the water disinfection unit, what consists of a chlorinator with sodium hypochlorite (NaClO) tablets. Finally, the treated hospital effluent is conveyed throughout a Parshall-type channel to the municipal sewer.

2.2. Physicochemical characterization of hospital effluent

The following parameters were determined: temperature, dissolved oxygen, conductivity, pH, chlorides, fluorides, hardness, ammonia, total suspended solids, phosphorus, nitrogen, and sodium hypochlorite (NOM-001-SEMARNAT-1996; NOM-002-SEMARNAT-1996).

2.3. Quantification of pollutants in hospital effluent

The selected pharmaceuticals and metals represent the pollutants with the highest use according to HPM's activities. For the quantification of metals, the samples were filtered with Whatman No. 541 paper (Whatman, Germany) at pH 3.5, then autoclaved at 120 °C and 15 psi for 1 h. Subsequently, the samples were filtered and diluted in 100 mL of deionized H_2O and read with a Varian AA1475 atomic absorption spectrophotometer (Melbourne, Australia). The data were interpolated to a 1 mg/L standard curve for each estimated metal (González-González et al., 2014). The parameters for metal quantification are shown in Table 1.

The drugs present in the hospital effluent were determined using a serial filtration process, using an 8 μm and 2.5 μm membrane. Solid phase extraction (SFE), Strata XL cartridges (Torrance, CA) were conditioned with 10 mL CH_3OH and 10 mL H_2O. 500 mL of hospital effluent was added at a flow rate of 5 mL/min and eluted in 6 mL CH_3OH. A nitrogen evaporator N-Evap 112 (Organomation, Haverhill, MA) was used to evaporate the solvent. Samples were reconstituted with 1 mL CH_3OH: H_2O (50:50) and passed through a 0.2 μm syringe filter prior to injection.

A Waters (Mildford, MA) liquid chromatography-mass spectrometry (LC-MS/MS) system was used for the identification and quantification of drugs in hospital effluent. An Acquity UPLC BEH C18 column (2.1 × 100 mm, 1.7 μm) was used for the separation of the compounds. The mobile phases were 0.1 % (v/v) formic acid in H_2O (A) and 0.1 % (v/v) formic acid in CH_3OH (B). The column was maintained at 40 °C and a flow rate of 0.3 mL/min and an injection volume of 10 μL were used. The total run time was 10 min with 90 % (A) at 0.1 min, 10 % (A) at 8 min and 90 % (A) at 10 min. The Quattro Premier XE triple quadrupole mass spectrometer equipped with electrospray ionization (ESI) was used in positive mode for hydrocortisone, omeprazole, ranitidine, ketorolac, paracetamol, dexamethasone, and esomeprazole, while in negative mode it was used for naproxen and ibuprofen (Table 2). Mass spectrometer optimization was carried out by direct infusion of 200 mg/L standard solution of the nine analytical standards. The limit of detection (LOD) and limit of quantification (LOQ) were calculated as follows: LOD = ($t_{0.99}$) (SD) and LOQ = 3 (LOD), where $t_{0.99}$ represents the one-tailed statistic at the 99 % confidence level for n replicates and SD = the standard deviation in the estimated LOQ (Brubaker, 1999).

2.4. Metataxonomic characterization of hospital effluent

10 L of hospital effluent was filtered with the aid of a vacuum pump connected to the KG47 Glass Support kit (Advantec MFS, Inc.) and the suspended biomass was captured employing a 0.2 μm membrane. The membrane was used for metagenomic DNA extraction using the ZymoBIOMICS® DNA Miniprep kit and the manufacturer's protocol was followed. DNA was then sequenced on a MiSeq System - Illumina platform (2 × 300 bp) at the Center for Comparative Genomics and Evolutionary Bioinformatics (Dalhousie University, Canada). High-throughput sequencing of the bacterial 16S rRNA gene was performed using the 341F and 805R primer set (Klindworth et al., 2013). Sequence analysis was performed using mothur v.1.48.0 (Kozich et al., 2013). Firstly, sequences that contained an error originating from the preparation of the libraries or sequencing were eliminated. For the remaining sequences, the corresponding contigs were made and aligned with bacterial reference sequences of the 16S rRNA gene (https://www.arb-silva.de/). A maximum length of 8 homopolymers and 0 ambiguities was established. Next, chimeric sequences were removed using the Uchime command, followed by taxonomic assignment of Operational Taxonomic Units (OTUs), which was obtained by alignment with the RDP taxonomic classifier with identities ≥95 %. Eukaryotic taxa and archaea were excluded using the mothur taxon filter (Kozich et al., 2013). The α and β diversity of the two samples from the same hospital effluent, designated HB1 and HB2, was calculated. The resultant bacterial composition of hospital effluent is represented in Krona charts.

2.5. Danio rerio housing system

Wild-type seven-month old *D. rerio* organisms (1.27 ± 0.4 g in weight and 4.11 ± 0.09 cm in length) were maintained in fish tanks with a carrying capacity of 1 L/fish, natural photoperiod of 12:12, continuous oxygenation of 9.8 ± 0.5 mg/L, pH of 7.4 and temperature of 27 °C. They were fed commercial flake (Ocean Nutrition, US) and *Artemia* spp., twice-daily during an acclimatization period of three months. Several water quality parameters were monitored and controlled throughout the experiment. The water was renewed every second day to keep the nitrate (2.6 ± 0.2 mg/L) and nitrite (0.028 ± 0.007 mg/L) content low. One drop of antichlorine was added per two litres of water and Instant Ocean® salts (9 mg/L) were

Table 2
Mass spectrometer factors used in drug quantification in hospital effluent.

Compound	Ionization mode	Parent ion (m/z)	Product ion (m/z)	Cone voltaje (V)	Detection limit (LOD) ng/L	Quantification limit (LOQ) ng/L
Ketorolac	+	256.2	105.5	30	2.67	8
Paracetamol	+	151.9	110	25	3.23	9.69
Dexamethasone	+	363.2	147.1	20	3.25	9.76
Hydrocortisone	+	363.2	121	31	2.36	7.07
Esomeprazole	+	346.1	151.1	20	3.07	9.22
Omeprazole	+	346.1	198	20	3.31	9.93
Ranitidine	+	315.2	176	25	2.09	6.28
Naproxen	−	229.2	170	15	2.81	8.44
Ibuprofen	−	205.1	161.1	15	2.97	8.9

used. Finally, organisms ranging from 4 to 4.2 cm in length, free of infection and symptoms of disease, were selected for the study (OECD, 2019; Gómez-Oliván et al., 2017).

2.6. Experimental design to assess oxidative stress

Twenty 10 L systems were used with the following proportions of hospital effluent: 2 %, 2.5 %, 3 %, 3.5 % and an hospital effluent-free control. A semi-static renewal toxicity test with periodic removal (24 h) was used (OECD, 2019). The water parameters were kept constant, as was done for housing systems. Male fish were used to prevent possible alterations in hormone levels associated with spawning, in order to reduce experimental bias. The proportions were selected based on previous studies conducted in the laboratory (Rosales-Pérez et al., 2022). Exposure times were 12 h, 24 h, 48 h, 72 h and 96 h. For all tests, fish were sacrificed with the hypothermic shock method (2 °C – 4 °C). A pool of livers, guts, and gills from six *D. rerio* organisms was obtained for each system and stored in Eppendorf tubes, previously filled with 1 mL of phosphate buffer solution (PBS, pH 7.4). Tubes were frozen at −80 °C until use. The oxidative stress experiment was performed in triplicate with a total of 378 *D. rerio* organisms sacrificed.

2.7. Determination of oxidative stress biomarkers

The organ pool (≈528 μg) of *D. rerio* was homogenized for 20 s with a stator rotor (Ultra-turrax T25, IKA, Germany). Subsequently, the samples were divided into two Eppendorf tubes. Tube 1 contained 300 μL of the homogenate plus 300 μL of a 20 % trichloroacetic acid (TCA) solution. Tube 2 contained 700 μL of the homogenate. Tube 1 was centrifuged at 11495 rpm for 15 min at 4 °C and the precipitate was used to determine the protein carbonyl content (PCC) using the method of Levine et al., 1994, while the supernatant was examined to establish the degree of lipoperoxidation level (LPX) according to Buege and Aust (1978) and the hydroperoxide content (HPC) by Jiang et al. (1992). Tube 2 was centrifuged at 12500 rpm for 15 min at 4 °C, the supernatant was used to determine the activity of antioxidant enzymes: SOD and CAT by the method of Misra and Fridovich (1972) and Radi et al. (1991) respectively. The results of all biomarkers were normalized against total proteins using the method of Bradford (1976).

2.8. RT-qPCR

Four 10 L systems were used with the following proportions of hospital effluent: 2 %, 2.5 %, 3 %, 3.5 % and a control. For this analysis the exposure time was set at 96 h. The corresponding samples were obtained and stored in a stabilizing solution of RNAlater®, Qiagen. A total of 90 organisms were used for the three replicates.

Total RNA was obtained with the Qiagen RNeasy® kit. After RNA isolation, the quality (purity) was evaluated using the absorbance ratio 260/280 nm (ratio between 1.9 and 2.1), as well as the evaluation of the banding pattern in 1 % agarose gel. Quantification was performed by spectrophotometry using the THERMO Scientific NanoDrop 2000/2000c. Next, cDNA was synthesized using 1 μg of total RNA by implementing the QuantiTec® Reverse Transcription Kit, following the manufacturer's protocol (QIAGEN, Hilden, Germany, REF 205313). The cDNA was used as a template for real-time polymerase chain reaction (qPCR) based on the QuantiTec® SYBR Green Kit. The qPCR was performed using a Gentier 48 thermal cycler. Each reaction was performed in a 50 μL solution containing 0. 3 μmol of primers, 25 μL 2× SYBER Green QuantiTec® (QIAGEN, Hilden, Germany) and 500 ng of cDNA template. The reaction conditions were as follows: 94 °C for 15 s, followed by 35 cycles of 94 °C for 15 s, 60 °C for 30 s and 72 °C for 30 s. The *β-actin* gene was used as a housekeeping gene to normalize all samples. Gene expression results were calculated using the $2^{-\triangle\triangle CT}$ method (Livak and Schmittgen, 2001). The genes used for RT-qPCR are related to oxidative stress response, detoxification, and apoptosis (Table 3).

2.9. Statiscal analysis

SigmaPlot 12.3 software was used for statistical analysis. Oxidative stress data were evaluated using a Kruskal-Wallis one-way non-parametric ANOVA, followed by a post hoc test (Student Newman Keuls), and expressed as median ± interquartile range (IQR). To determine significant differences in gene expression between the treatment and control groups, a Fisher's ANOVA-1 followed by a Student's Newman Keuls (SNK) test was performed at a statistical significance of $p < 0.05$. Ultimately, a heat map was constructed using the "pheatmap" package of the R Studio software version 1.2.1335 (Kolde, 2019).

3. Results

3.1. Physicochemical characterization of hospital effluent

The physicochemical characterization of hospital effluent is shown in the table below, compared with that established in the Official Mexican Standards for wastewater discharges into national property (NOM-001-SEMARNAT-1996) and the urban-municipal sewerage system (NOM-002-SEMARNAT-1996) (Table 4). Biochemical oxygen demand, pH, chlorides, fluorides, and total suspended solids (TSS) are all observed to be above the maximum permissible limits. It should be notable that there are no standards for conductivity (347.2 μS/cm), NaClO (6.9 mg/L), dissolved oxygen (4.7 mg/L), chemical oxygen demand (190.2 mg/L) and ammonia (2.3 mg/L).

3.2. Quantification of pollutants in hospital effluent

Different heavy metals were identified at levels above Mexican standards (Table 5).

However, despite of the fact that there are no established upper limits for drugs, ranitidine and ketorolac had the highest concentrations found in hospital effluent. It is noteworthy that, although ranitidine has recently been restricted because of a possible carcinogenic risk, it was the drug found to have the highest concentration in hospital effluent. We detected several drugs whose concentration ranged from 11 ng/L to 19.1 μg/L. Our results are coherent with the great diversity of HPM's specialties; in this sense, the most representative pharmaceutical group corresponds to non-steroidal anti-inflammatory drugs (NSAIDs), which are often prescribed together with proton pump inhibitors to counteract the side effects produced by NSAIDs with acidic properties.

Table 3
Oligonucleotide sequences.

Gene	Access number	Forward primer	Reverse primer	Reference
sod	Y12236.1	AAG AAG CCA GTG AAG GTG ACT	ACA TTA CCC AGG TCT CCG AC	Liu et al., 2013
cat	AF170069.1	AGA TGA AAC TGT GGA AGG AGG GTC	AAA CAC TTT GGC TTT GGA GTA GCG	Liu et al., 2013
nrf2	NP_878309	ACC CAA TAG ATC TAC AGA GC	GGT GTT TGG ACA TCA TCT CG	Sant et al., 2017
bax	AF231015.1	GGC TAT TTC AAC CAG GGT TCC	TGC GAA TCA CCA ATG CTG T	Cheng et al., 2020
casp6	NM001020497.1	AGG ACA GCG CTT CAG CAG GAC A	TGA GAG CCA TTC CCC GTC TCT TGT	Félix et al., 2018
casp9	NM_001007404.2	CGG AGG AGG TGA GAA GGA TAT	TCC AGC ACA CGA TCA AGA TT	Jiang et al., 2014
cyp1a1	AF210727.2	CGCTTGTATGGGCTTGTCCT	CGCAGCTAAAACAGGCACTC	Chen and Chan, 2018
β-actin	NM_181601.4	ACT GTA TTG TCT GGT GGT AC	TAC TCC TGC TTG CTA ATC C	Félix et al., 2018

Table 4
Physicochemical characterization of hospital effluent.

Physicochemical parameter	Hospital effluent	NOM-001-SEMARNAT-1996	NOM-002-SEMARNAT-1996
Biochemical Oxygen Demand (mg/L)	92.8 ± 1.5	60	40–60
Chemical Oxygen Demand (mg/L)	190.2 ± 2.7	N.E	N.E
Conductivity (μS/cm)	347.2 ± 9.4	N.E	N.E
pH (UpH)	5.7 ± 0.1	6.5–8.5	6–9
Temperatura (°C)	22 ± 1	20–35	40
Dissolved oxygen (mg/L)	4.7 ± 0.92	N.E	N.E
Chlorides (mg/L)	284.3 ± 4.8	250	N.E
Fluorides (mg/L)	28.22 ± 0.4	0–15	N.E
Hardness (mg/L)	141.4 ± 3.2	500	N.E
Ammonia (mg/L)	2.3 ± 0.4	N.E	N.E
Total suspended solids (mg/L)	101.2 ± 2.3	40	40–60
Total phosphorus (mg/L)	7.6 ± 0.27	10	10
Total nitrogen (mg/L)	19.62 ± 1.3	25	N.E
NaClO (mg/L)	6.9 ± 0.74	N.E	N.E

N.E, not established in the standard. Data represent the mean ± SD of three independent experiments.

Table 5
Pollutants detected in hospital effluent.

Pollutant type	Metal or drug	Concentration
Metals	As (mg/L)	0.42 ± 0.06 *
	Cd (mg/L)	0.39 ± 0.02 *
	Cu (mg/L)	0.67 ± 0.09
	Cr (mg/L)	0.87 ± 0.06
	Hg (mg/L)	1.62 ± 0.07 *
	Ni (mg/L)	1.46 ± 0.05
	Pb (mg/L)	2.11 ± 0.03 *
	Zn (mg/L)	1.36 ± 0.07
NSAIDs	Ketorolac (ng/L)	1784.3 ± 5.2
	Ibuprofen (ng/L)	338 ± 1.1
	Naproxen (ng/L)	691.7 ± 3.8
	Paracetamol (ng/L)	519.61 ± 2.7
Corticosteroids	Dexamethasone (ng/L)	272.12 ± 0.71
	Hydrocortisone (ng/L)	32.13 ± 0.14
Inhibitor of proton pump	Esomeprazole (ng/L)	12.43 ± 0.14
	Omeprazole (ng/L)	11 ± 0.12
H2-antagonism	Ranitidine (μg/L)	19.1 ± 0.87

Data exceeding the values established by Mexican standards are shown with *. Data represent the mean ± SD of three independent experiments.

3.3. Metataxonomic characterization of hospital effluent

Table 6 illustrates the diversity indexes obtained for the two hospital effluent samples. The number of sequences processed was 8408 and the OTUs founded for sample HB1 and HB2 were 268 and 430, respectively. The coverage values of the samples (0.99 and 0.98) denote a good depth of sequencing and characterization. Sample HB2 had the highest bacterial diversity. Whittaker's β-diversity (0.3) indicates that ≈70 % of the bacterial communities present in the hospital effluent were characterized.

Figs. 3 and 4 depict the metataxonomic characterization of sample HB1 and HB2 from hospital effluent. The bioinformatics results allowed us to obtain the bacterial genera. However, the Krona charts are presented at the taxonomic order level for better visualization. A total of 94 bacterial genera were identified. Genera with relative abundance >1 % in HB1 and HB2 include *Aquicella* (57.15 and 7.39 %, respectively), *Clostridium XI* (2.39 %, HB2), *Clostridium* sensu stricto (5.89 %, HB2), *Parachlamydia* (1.55 and 32.36 %), *Pseudomonas* (17.07 %, HB2), *Legionella* (28.14 and 6.14 %), *Gemmatimonas* (1.72 and 4.77 %), *Mycobacterium* (1.62 %, HB2), *Dongia* (1.62 and 4.26 %), *Hyphomicrobium* (1.34 %, HB2), *Nannocystis* (1.99 %, HB2), *Nitrosospira* (1.19 %, HB2), *Nitrospira* (1.10 %, HB2), *Pseudolabrys* (1.6 and 4.52 %), *Opitutus* (1.54 %, HB1). The presence of opportunistic bacteria, namely *Legionella*, *Pseudomonas*, *Clostridium XI*, *Parachlamydia* and *Mycobacterium* is highlighted.

3.4. Oxidative stress biomarkers in liver, gut, and gill

Oxidative biomarkers are presented in Figs. 5, 6 and 7, showing significant increases relative to the control group. LPX had its highest increase at the proportion of 3.5 % (580 %) at 96 h in gills ($H(20) = 60.91, p \leq 0.001$) (Fig. 5 C), while the HPC, at the proportion of 3 % (1131 %) at 72 h in liver ($H(20) = 60.61, p \leq 0.001$) (Fig. 6A) and the PCC at the proportion of 3.5 % (876 %) at 72 h in gut ($H(20) = 60.26, p \leq 0.001$) (Fig. 7B). Similarly, the SOD and CAT antioxidant activity are shown in Figs. 8 and 9, respectively. Modifications in the antioxidant response can be observed after exposure to the four proportions of hospital effluent. For SOD the highest catalytic increase with respect to the control group is reported at the proportion of 3 % (309 %) at 24 h in gills ($H(20) = 59.63$; $p < 0.001$) (Fig. 8C). Regarding SOD, the catalytic decrease in liver and gut (Fig. 8A and B) stands out at longer exposure times (72 h and 96 h). In the case of CAT, the greatest increase with respect to the control group was observed at the proportion of 3 % (793 %) at 72 h in liver ($H(20) = 61.19, p \leq 0.001$) (Fig. 9A).

3.5. Gene expression

As can be seen in Fig. 10, the expression of *sod*, *cat*, *nrf2*, *bax*, *casp6*, *casp9* and *cyp1a1* genes in the liver, gut, and gills of *D. rerio* was disrupted after treatment with four proportions of hospital effluent. It was observed that, after 96 h of exposure, antioxidant system-related genes, *sod* and *cat*, significantly increased their expression in a hospital effluent proportion-dependent manner (*sod* liver: $F(4, 10) = 252.31$, $p \leq 0.001$; n = 3; *cat* gut: $F(4,10) = 254.94$, $p \leq 0.001$; n = 3). Nevertheless, this pattern was the opposite in the case of *sod* and *cat* in gills (*sod*: $F(4, 10) = 367.9$, $p \leq 0.001$; n = 3; *cat*: $F(4,10) = 245.84$, $p \leq 0.001$; n = 3), as they exhibited a decrease to the higher hospital effluent proportions. In the case of *nrf2*, an increase in transcripts was found at the highest proportion of hospital effluent (3.5 %), principally in gills and guts (*nrf2* gills: $F(4,10) = 503.05$; $p < 0.001$; n = 3; *nrf2* gut: $F(4,10) = 70.09$; $p < 0.001$; n = 3). Apoptosis-related genes were up-regulated in most of the organs. However, the liver showed a contrasting behaviour, since in the case of *bax*, *casp6* and *casp9* genes, a decrease in relative expression was discovered at higher proportion of hospital effluent (*bax*: $F(4,10) = 87.73$; $p < 0.001$; n = 3; *casp6*: $F(4,10) = 50.27$; $p < 0.001$; n = 3; *casp9*: $F(4,10) = 13.33$; $p < 0.001$; n = 3). Whereas the expression level of *cyp1a1* was markedly elevated in gills and liver in a hospital effluent proportion-dependent manner (Gills: $F(4,10) = 470.68$; $p < 0.001$; n = 3; Liver; $F(4,10) = 588.43$; $p < 0.001$; n = 3). Significant differences between treatment and control groups were found for almost all genes. Lastly, to visualize mRNA expression variations, a heat map was created for all genes and organs analyzed.

Table 6
Bacterial diversity indexes.

Sample	#Seqs	Coverage	OTUs	Inv-simpson (lci-hci)	Chao (lci-hci)	Shannon (lci-hci)	Whittaker's β
HB1	8408	0.99	268	4.24 (4.1–4.39)	506.39 (411.85–663.38)	2.58 (2.53–2.63)	0.3
HB2	8408	0.98	430	24.14 (23.02–25.37)	665.49 (586.77–783.74)	4.17 (4.13–4.21)	

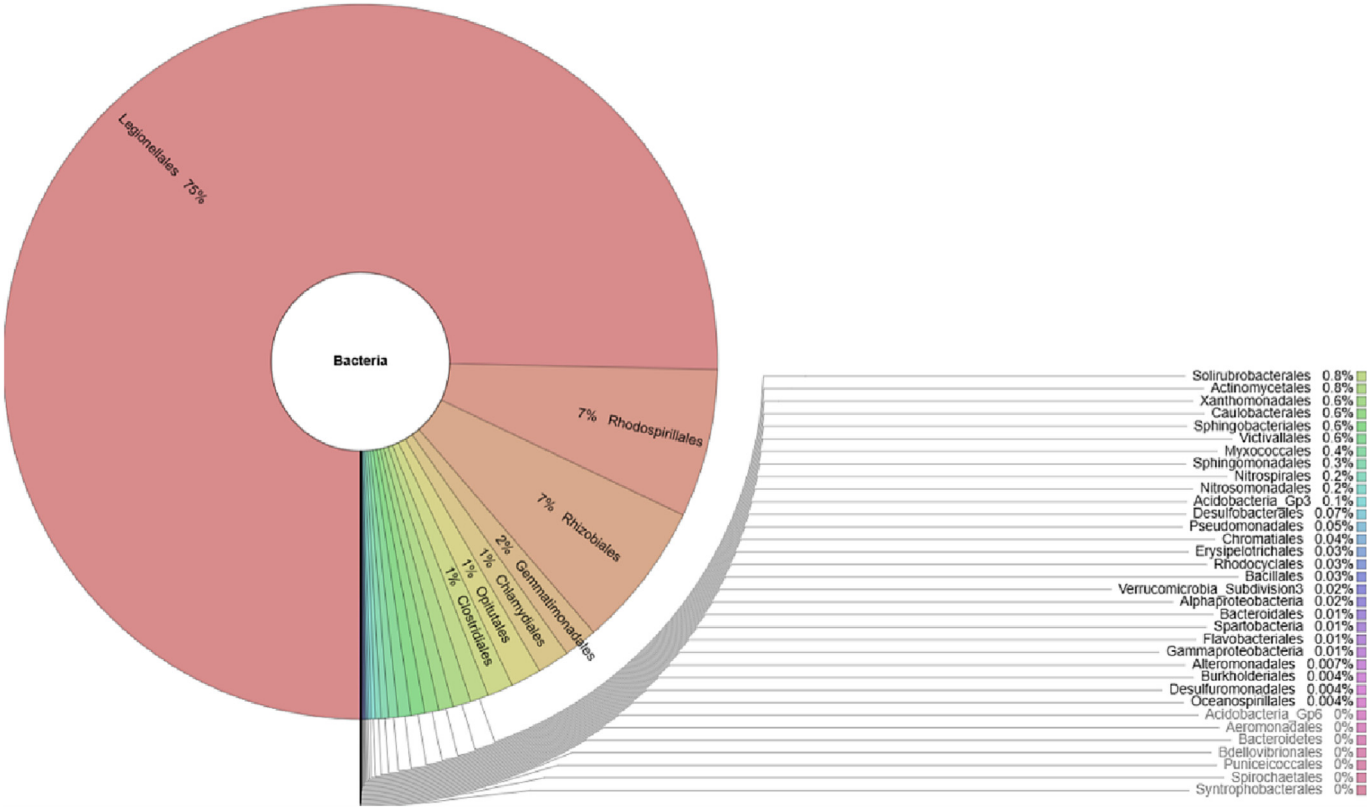


Fig. 3. Metataxonomic characterization of sample HB1 from hospital effluent.

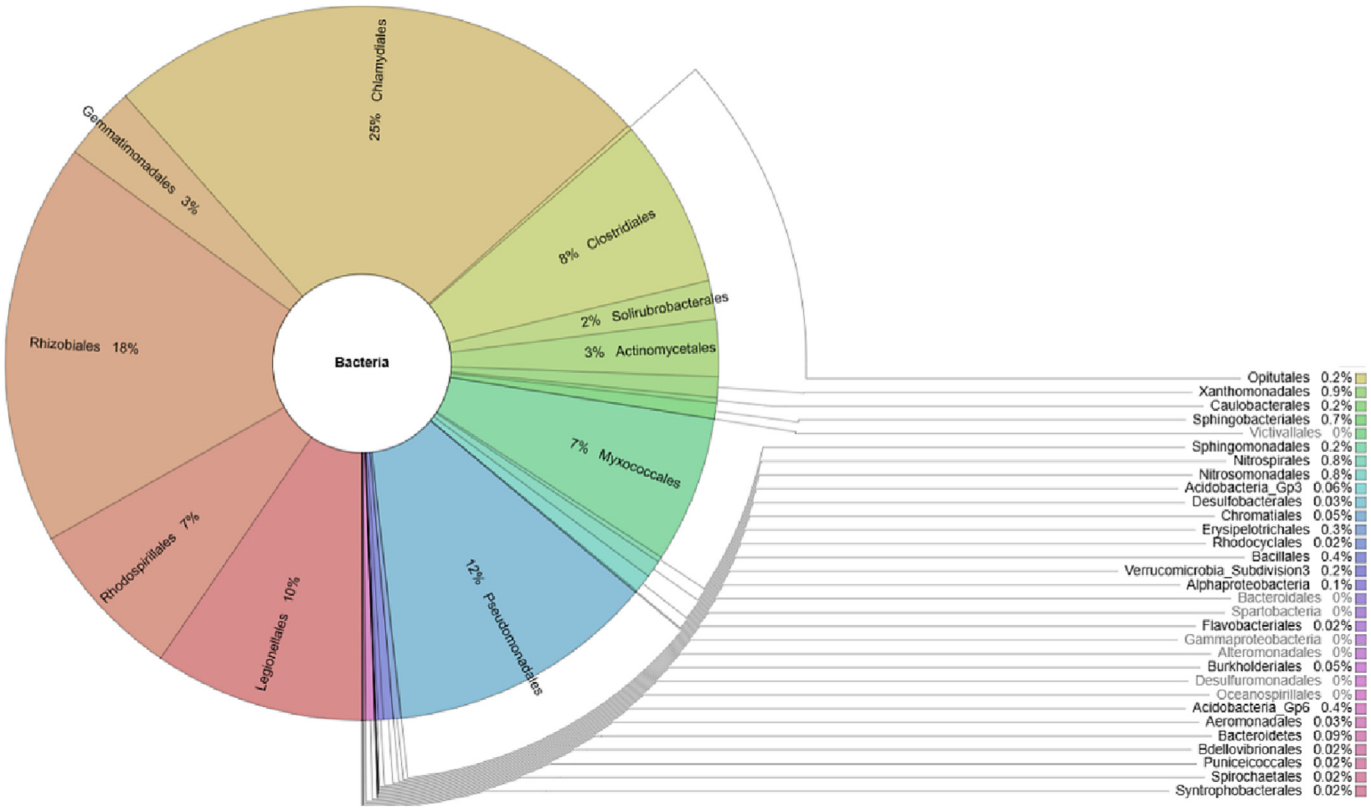


Fig. 4. Metataxonomic characterization of sample HB2 from hospital effluent.

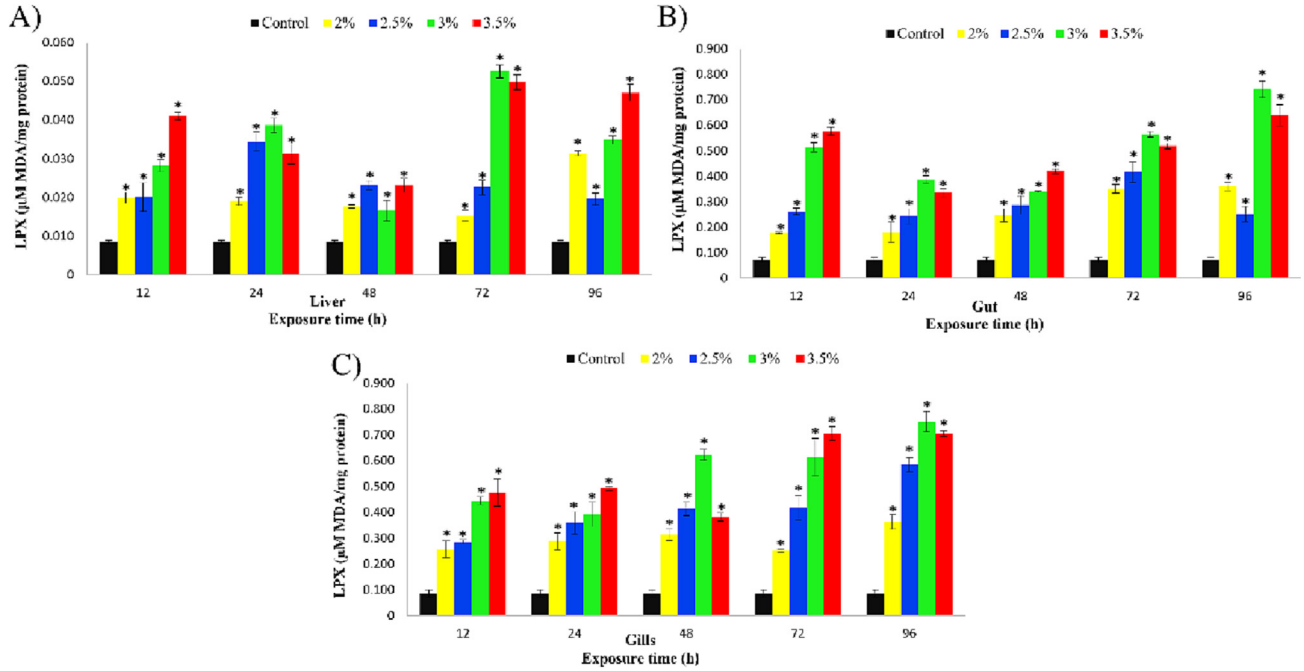


Fig. 5. Lipoperoxidation level (LPX) in Liver (A), Gut (B), Gill (C) of *D. rerio* exposed to 4 proportions of hospital effluent for 12, 24, 48, 72 and 96 h. Values are median ± interquartile range, n = 3. * Significantly different from control group, Kruskal-Wallis ANOVA and SNK ($p < 0.05$). N = 378 fish.

4. Discussion

In view of the imminent contribution of pollutants discharged into the environment by hospital effluents, they are of major concern at the international level (Verlicchi, 2021). It is important to note that the effluent studied was previously treated by HWWTP. In this regard, despite some of the water quality parameters of hospital effluent being within the maximum limits allowed by Mexican standards, the presence of NaClO and chlorides derived from the treatment at the HWWTP may promote the creation of organochlorine compounds (e.g. trihalomethanes, haloacetic acids, bromodichloromethane etc.) (Srivastav et al., 2020; Emmanuel et al., 2004). An example is paracetamol, whose chemical transformation is

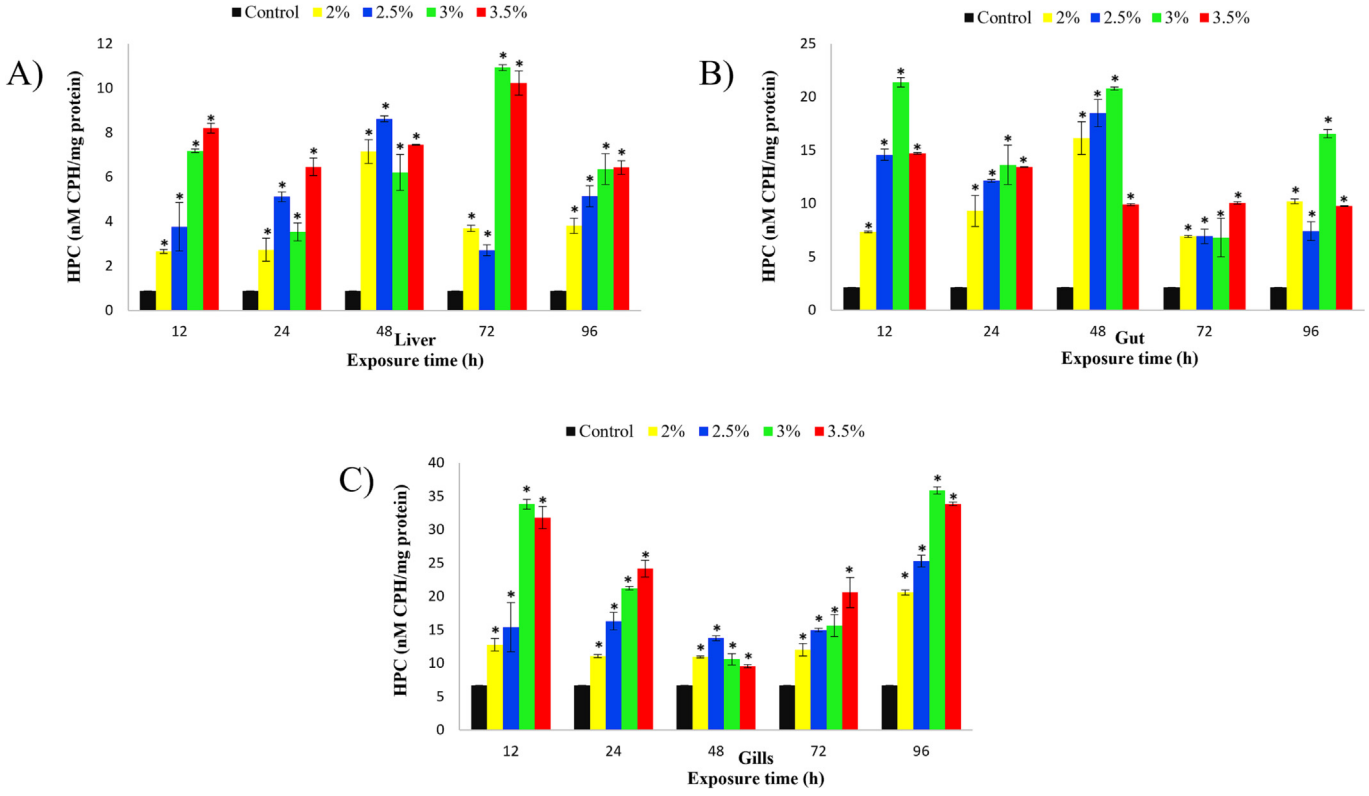


Fig. 6. Hydroperoxide content (HPC) in Liver (A), Gut (B), Gill (C) of *D. rerio* exposed to 4 proportions of hospital effluent for 12, 24, 48, 72 and 96 h. Values are median ± interquartile range, n = 3. * Significantly different from control group, Kruskal-Wallis ANOVA and SNK ($p < 0.05$). N = 378 fish.

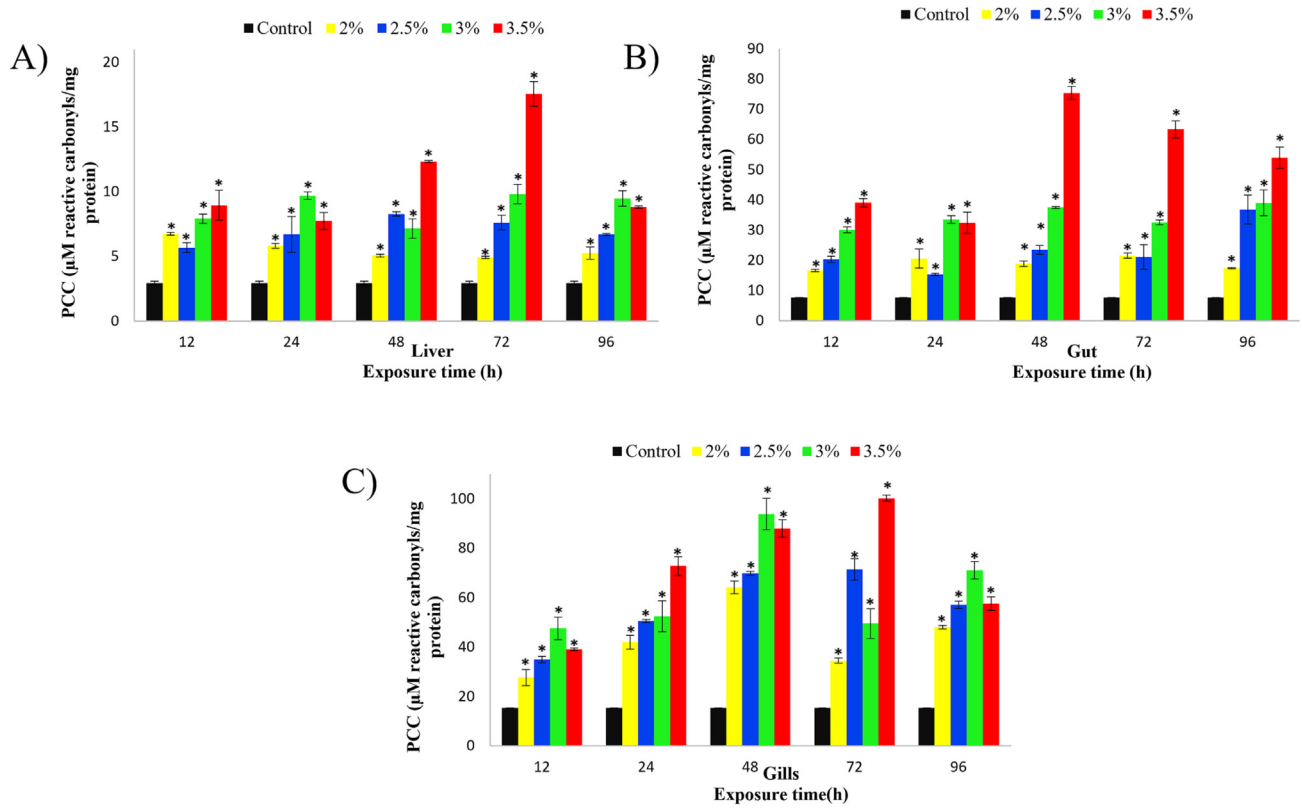


Fig. 7. Carbonyl protein content (PCC) in Liver (A), Gut (B), Gill (C) of *D. rerio* exposed to 4 proportions of hospital effluent for 12, 24, 48, 72 and 96 h. Values are median ± interquartile range, n = 3. * Significantly different from control group, Kruskal-Wallis ANOVA and SNK ($p < 0.05$). N = 378 fish.

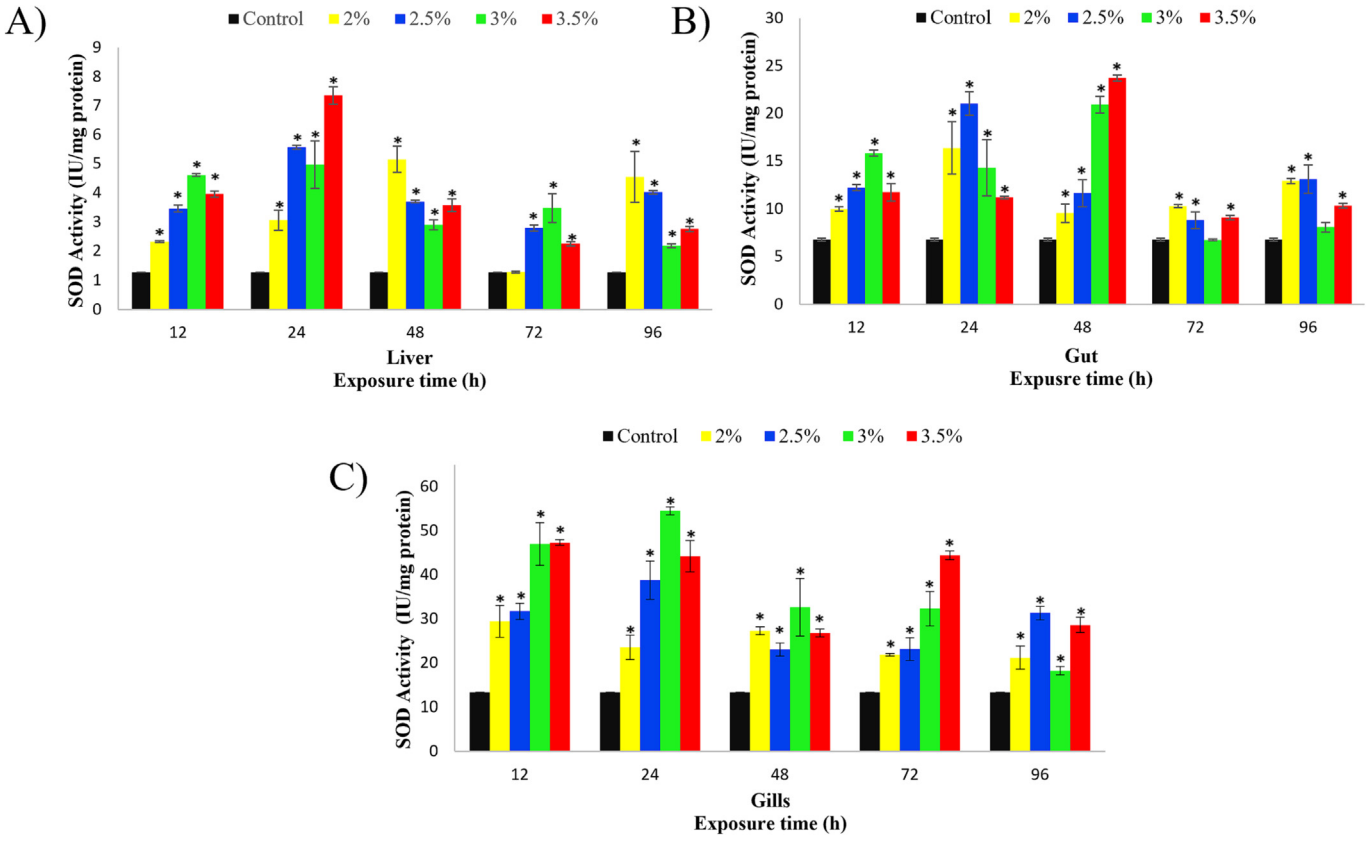


Fig. 8. Superoxide dismutase activity (SOD) in Liver (A), Gut (B), Gill (C) of *D. rerio* exposed to 4 proportions of hospital effluent for 12, 24, 48, 72 and 96 h. Values are median ± interquartile range, n = 3. * Significantly different from control group, Kruskal-Wallis ANOVA and SNK ($p < 0.05$). N = 378 fish.

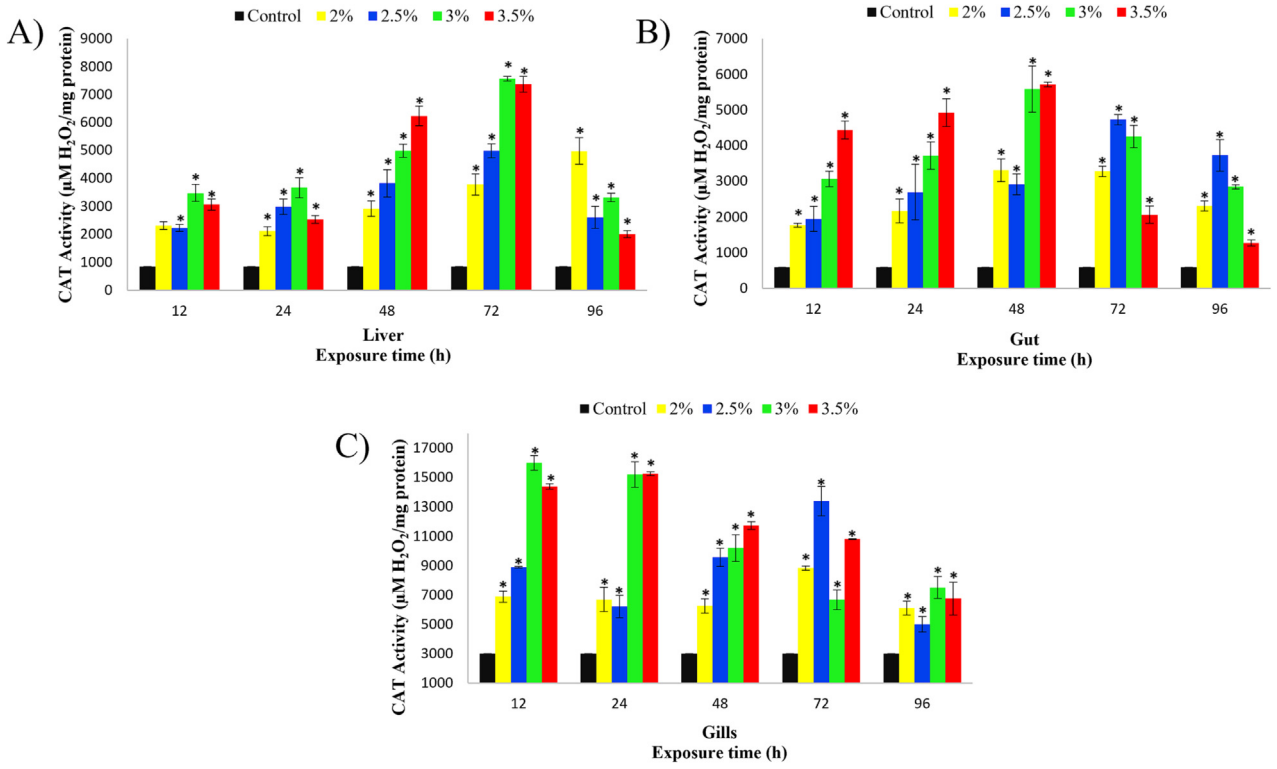


Fig. 9. Catalase activity (CAT) in Liver (A), Gut (B), Gill (C) of *D. rerio* exposed to 4 proportions of hospital effluent for 12, 24, 48, 72 and 96 h. Values are median ± interquartile range, n = 3. * Significantly different from control group, Kruskal-Wallis ANOVA and SNK ($p < 0.05$). N = 378 fish.

mediated by its interaction with NaClO, leading to at least 11 by-products with a higher toxicological potential than the original molecule, in particular 1,4-benzoquinone and *N*-acetyl-p-benzoquinone imine (Cerro-López et al., 2021). Other parameters such as TSS and pH influence the bioavailability of heavy metals through adsorption processes and by the presence of bioactive metal ions (e.g. $H_2AsO_4^-$) (Cloran et al., 2010).

In addition to the above, the activation of antioxidant defence is expected when living organisms are subjected to xenobiotics. The SOD and CAT results (Figs. 8 and 9) indicate the effort made by *D. rerio* to cope with the complex mixture of drugs, heavy metals, and antibiotic-resistant pathogenic bacterial genera (Figs. 3 and 4) (Cuetero-Martínez et al., 2023). Similar results have been stated in *D. rerio*, *Hyalella azteca* and *Cyprinus carpio* (Rosales-Pérez et al., 2022; Gómez-Oliván et al., 2019; González-González et al., 2014). Nonetheless, our results show a decrease in SOD activity as exposure time increases (48 h–96 h) (Fig. 8A and C). Three hypotheses are put forward to explain the enzymatic behaviour of SOD. Firstly, SOD is the only enzyme capable of biotransforming $O_2\bullet^-$ to H_2O_2. Thus, despite there being different isoforms of SOD (cytosolic or mitochondrial), its performance is likely to be impaired by enzyme depletion owing to exposure to complex mixtures (Wang et al., 2021; Neri-Cruz et al., 2015). The second hypothesis concerns protein carbonylation (Fig. 7) of certain amino acids (e.g. methionine, cysteine, lysine, etc.) near to the active site, which would encourage a conformational and functional change of SOD that prevents the passage of the $O_2\bullet^-$ anion to the catalytic site (Lim et al., 2019; Gómez-Oliván et al., 2014; Levine et al., 1996). It is worth mentioning that *sod* transcripts were strongly upregulated in gills and liver (7.17 and 4.04, respectively) (Fig. 10). Similar results have been recorded for exposure to Cu (8 µg and 15 µg) and Cd (1.9 µg and 2.9 µg) (Craig et al., 2007; Gonzalez et al., 2006). This implies that the gene order is processed, but the mainly oxidative environment at the intracellular level may mediate a loss of function. This is related to the upregulation of the transcriptional factor *nrf2* in all organs, which acts as a redox sensor in the endoplasmic reticulum to maintain cellular homeostasis (Bhakkiyalakshmi et al., 2018). Mondal et al. (2019) show that the *nrf2* gene is upregulated in liver when exposed to fluorides (6.8 mg/L). Previous studies reveal that *nrf2* regulates the expression of enzymes involved in both phase I and II detoxification processes, including Heme oxygenase-1 (HO-1), SOD, Glutathione peroxidase (GPX), Sulfotransferases (SULFs), γ-Glutamyl cysteine ligase (GCL) and Glutathione-S-Transferases (GSTs) (Mondal et al., 2019; Bhakkiyalakshmi et al., 2018; Sant et al., 2017; Loboda et al., 2016). Lastly, reduced glutathione (GSH) could play a crucial role in the decrease of SOD activity, as it acts as a chelating agent, possibly contributing to decrease the concentration of trace metals (Cu and Zn) essential for this metalloprotein (Steinbrueck et al., 2020). It is suggested that the catalytic activity of SOD is subordinated to post-transcriptional processes, given the lack of complementarity between biochemical and molecular biomarkers at 96 h of exposure. Alternatively, CAT activity would be stimulated by an excessive increase in H_2O_2 from SOD or by means of the activation of signalling pathways that use it as a second messenger to trigger apoptosis (Xiang et al., 2016). The catalytic decrease of CAT was less pronounced than SOD, possibly on account of the presence of several enzymes that help to deal with the generation of H_2O_2 or other peroxides, namely GPX, Thioredoxins or Peroxiredoxins (Liu et al., 2019; Hanschmann et al., 2013).

During LPX, different polyunsaturated fatty acids (PUFAs) in the plasma membrane (e.g. arachidonic acid, docosahexaenoic acid, linoleic acid, eicosapentaenoic acid etc.) react chiefly with OH•. This process starts with the abstraction of a hydrogen atom from PUFAs, generating a chain reaction that drives the formation of hydroperoxides and their degradation compounds, for example epoxides, pentane, or reactive aldehydes like malondialdehyde (MDA), 4-hydroxy-2-nonenal (HNE) and 4-hydroxy-2-hexenal (HHE) (Kancheva and Angelova, 2017). The presence of MDA (Fig. 5A) seems to be related to CAT activity at 48 h in liver (Fig. 9A), so that an increase in CAT activity may indirectly decrease the presence of OH• (by the transformation of H_2O_2 to H_2O and O_2) giving rise to a decrease in LPX (MDA). It should be noted that OH• levels are also influenced by the presence of H_2O_2 and its interaction with Fe^{2+} via the Fenton reaction and the interplay between

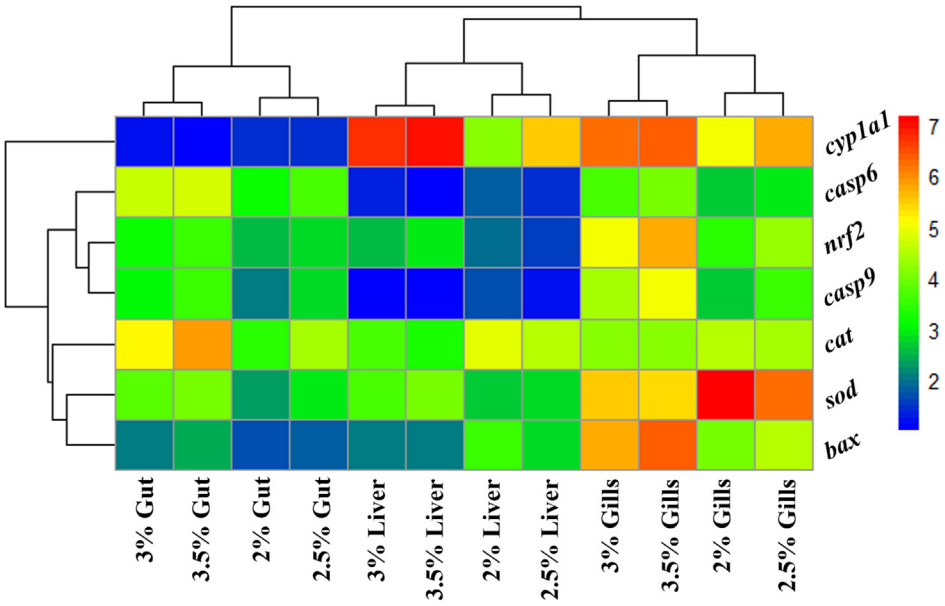


Fig. 10. Heat map to evaluate variations in transcript expression levels of 7 genes after exposure to 4 proportions of hospital effluent at 96 h in liver, gut, and gills of *D. rerio*. Values are mean, n = 3. Fisher's ANOVA-1 and SNK ($p < 0.05$) were used. N = 90 fish.

H_2O_2 and $O_2^{\bullet -}$ via the Haber-Weiss reaction (Ighodaro and Akinloye, 2018; Claiborne, 2018).

A high amount of MDA was detected in the gut following 12 h of exposure (Fig. 5B); which interestingly, saw higher levels than 24 h or 48 h of exposure. This could be related to an alteration in fatty acid catabolism by early activation of enzymes such as lipoxygenases 5, 12 and 15 or cyclooxygenases I and II in fish exposed to drugs and metals, which has been shown to favor a microbial dysbiosis that might intensify at longer exposure times (72 h and 96 h) (Zheng et al., 2022; Zang et al., 2019; Sheng et al., 2018: Xia et al., 2018). With respect to the aforementioned, gut was displayed to be the organ with the highest upregulation of *cat* and *casp6* with an increase of 5.86 and 3.49 at the proportion of 3.5 %. Stancová et al. (2015) advise that *cat* gene plays a crucial detoxification role in the elimination of ROS produced by naproxen (1 and 100 μg). Moreover, several studies support that exposure to complex mixtures of xenobiotics and opportunistic bacterial pathogens induce microbial dysbiosis in zebrafish (Zheng et al., 2022; Wang et al., 2021; González-Penagos et al., 2020; Xia et al., 2018). Therefore, *D. rerio* may suffer from an imbalance in the abundance of species of the phylum *Firmicutes* and *Bacteroidetes* by pathogens such as *Mycobacterium* spp. and *Pseudomonas* spp. whose main route of infection is at the intestinal level, with dissemination to other visceral organs for instance liver, spleen, and kidney (Zheng et al., 2022; González-Penagos et al., 2020; Harriff et al., 2007) (Figs. 3 and 4). Concerning *Clostridium* spp., they are known to have the ability to produce toxins that cause necrotizing enteritis (Lopetuso et al., 2013). Additionally, *Legionella* spp. can cause a serious multisystemic disease (Legionellosis) (Wang et al., 2019). Furthermore, *Parachlamydia acanthamoebae* (single sp. for *Parachlamydia* genus) is an obligately intracelular bacteria, which has the capacity to infects macrophages cells, and then inducing apoptosis (Greub et al., 2003). Moreover, in line with our outcomes, the genus *Aquicella* has been previously reported together with other intracellular pathogens such as *Legionella* spp. and *Stenotrophomonas* spp. in sediments from cave in France (García Sánchez et al., 2013). Lastly, it is paramount to consider that biochemical and molecular biomarkers were possibly modified not only by the presence of xenobiotics in the hospital effluent, but also by the attendance of pathogenic bacteria and resistomes associated with these anthropogenic matrices. Therefore, microbial community (e.g. *Legionella*, *Pseudomonas*, *Parachlamydia*, *Mycobacterium* etc.) could infect *D. rerio* and promote immunomodulatory responses in the host, due to the release of endotoxins (lipopolysaccharides) that eventually trigger oxidative processes which largely explain the damage detected in target organs of zebrafish. For these reasons, hospital effluent has an additional biohazard risk (Figs. 3 and 4).

The increase of oxidative biomarkers (LPX, HPC and PCC), mainly in gills, are typical of oxidative damage caused by ROS. The gills represent an important target organ in teleost fish, as they are responsible for gas exchange, excretion of nitrogenous waste compounds, acid-base regulation, ion exchange, and are implicated in osmoregulatory processes (Hwang, 2009; Evans et al., 2005). The maximum level of expression in nearly all genes was discovered in the gills. Regarding *cyp1a1* gene, we found a hospital effluent proportion-dependent expression. This is in agreement with investigations that support the key role of *cyp1a1* in the metabolism of chemical compounds that prompt a detoxification response at the hepatic and gill level (Derikvandy et al., 2020; Chen and Chan, 2018). Moreover, NSAIDs present in hospital effluent are associated with mitochondrial dysregulation and increased $O_2^{\bullet -}$ production resulting from the Oxy-Cyp1a1-Fe^{+3} intermediate (Galar-Martínez et al., 2016; Neri-Cruz et al., 2015). Even if ranitidine is not found in sufficient concentrations to generate toxicity (>1000 mg/L), it has the ability to bind to cytochrome P450 (Godoy et al., 2020), resulting in inhibition of NSAIDs metabolism or catalyzed drugs (multiple xenobiotics) by the mixed-function oxygenase system, driving to inefficiency in phase I detoxification processes (Derikvandy et al., 2020; Chen and Chan, 2018).

Kataba et al. (2022) noted that *D. rerio* larvae exposed to Pb (1, 10, 25 and 50 μg) increased embryonic activity, such as involuntary muscle twitching, more movements/min and an antioxidant response mediated by CAT and GST activity. Otherwise, higher concentrations (100 and 500 μg) cause a decrease in activity (bradycardia, dysregulation of pro-apoptotic genes and decreased antioxidant response). Arsenic (As) intermediate metabolites can inhibit approximately 200 proteins and enzymes. These include DNA repair enzymes, antioxidant defence enzymes and enzymes involved in glycolysis. Inhibition occurs by As binding to sulfhydryl groups of cysteines located at key positions in the protein structure (Gökalp et al., 2022). Valles et al. (2020) concluded that exposure to between 0.05 mg/L and 0.5 mg/L of As was capable of causing transgenerational alterations in *D. rerio* (Fo and F2) and epigenetic changes at the brain level by increasing DNA packaging histone methylation (H3K4me3). In combination, Cd (II) and Cu (II) might exhibit synergistic toxicity. Pilehvar et al. (2020) depicted a high mortality rate on adult zebrafish exposed to mixtures of Cd (0.022481 mg) and Cu (0.006345 mg) whose toxicity increases

with decreasing water hardness (<100 mg $CaCO_3$/L). This occurs because Cd can displace Cu from its intracellular complexes. This would support the catalytic reduction of Cu/Zn SOD at longer exposure times. Our results are in accordance with Ajitha et al. (2021) who described that gills of *D. rerio* exposed to Zn (160 μg/L) and Hg (90 μg/L) promotes apoptotic events.

Hospital effluents report a high occurrence of dexamethasone (DEX) as consequence of implementation in severe COVID-19 patients. DEX in the hospital effluent is found to be environmentally relevant since the Predicted No Effect Concentration (PNEC) on fish is 1 ng/L (Musee et al., 2021). Moreover, it is known that DEX and diclofenac might cause adverse effects via a synergistic effect (Ukić et al., 2019). Furthermore, exposure to DEX at 50 pM ([693.3 pM] in hospital effluent) brings forth an upregulation of *fbxo32* gene in zebrafish (Chen et al., 2017). In particular, the *fbxo32* gene in fish is related to the processing and presentation of antigens by MHC-I and protein metabolism, because it mediates the ubiquitination and subsequent proteasomal degradation of target proteins (Shi et al., 2018). The *fbxo32* gene is known to be upregulated in the catfish gut when exposed to opportunistic pathogens (Li et al., 2012; Shi et al., 2018).

Evidence is growing that heavy metals such as Pb, As and Cd have an impact on double-stranded DNA repair processes and activity of certain chaperones. Thereby, unrepaired carbonyl proteins could be ubiquitinated and metabolized by the 26S proteosome (Carlson and Van Beneden, 2014; Canedo and Rocha, 2021; Doganlar et al., 2016). This could in turn cause fragments that may be processed and presented as epitopes by MHC-I to $CD4^+$ or Natural Killer (NK) lymphocytes, which would ultimately trigger the release of TNF or other cytokines and would stimulate the extrinsic apoptotic pathway via the activation of transmembrane death receptors (Fig. 11). Similarly, the positive transcriptional regulation of the *casp9* gene in gut and gills at 3.5 % of hospital effluent (3.59 and 4.96, respectively) implies activation of the intrinsic pathway (Figs. 10 and 11) (Li et al., 2020; Luzio et al., 2013). This arises by permeabilization of the mitochondrial outer membrane (MOMP) and subsequent release of cytochrome C and binding to Apaf-1 to originate the apoptosome and activate Casp9 (Félix et al., 2018; Jiang et al., 2014; Luzio et al., 2013). This hypothesis is supported by the upregulation of *bax*, which is directly responsible for the MOMP (Cheng et al., 2020; Li et al., 2020; Gonzalez et al., 2006). This pathway would be mediated by imminent damage to DNA through the presence of ROS that would favor the appearance of adducts with MDA, oxidation at position 8′ of guanines or direct damage by heavy metals that engender base modifications, pyrimidine dimers, single/double strand breaks, and intra/inter-strand crosslinking (Valles et al., 2020; Doganlar et al., 2016; Ajitha et al., 2021). Finally, Casp6 must be the turning point of apoptosis prompting to the dismantling of the cytoskeleton and formation of apoptotic bodies (Li et al., 2020; Félix et al., 2018).

Considering the findings highlighted above and as noted in previous studies, it is essential to rethink the technological effectiveness of HWWTPs in removing pollutants present in hospital effluents, and in turn, the contemporary need to implement ad hoc remediation strategies for these recalcitrant compounds is pressing. The physicochemical characteristics of hospital effluents and its chemical compounds per se condition the transfer of them to different environmental compartments and, thus, pose a greater risk to the environment and public health. Eventually, it is considered desirable that future research should evaluate and contrast the oxidative alterations that could be induced by hospital wastewater and treated hospital water in order to demonstrate the efficiency of the HWWTPs or WWTPs.

5. Conclusions

Biomarkers related to oxidative stress (LPX, HPC, PCC), antioxidation system (*sod*, *cat*, *nrf2*), detoxification (*cyp1a1*) and apoptosis (*bax*, *casp6*, *casp9*) confirm the damage triggered in *D. rerio* adults after exposure to

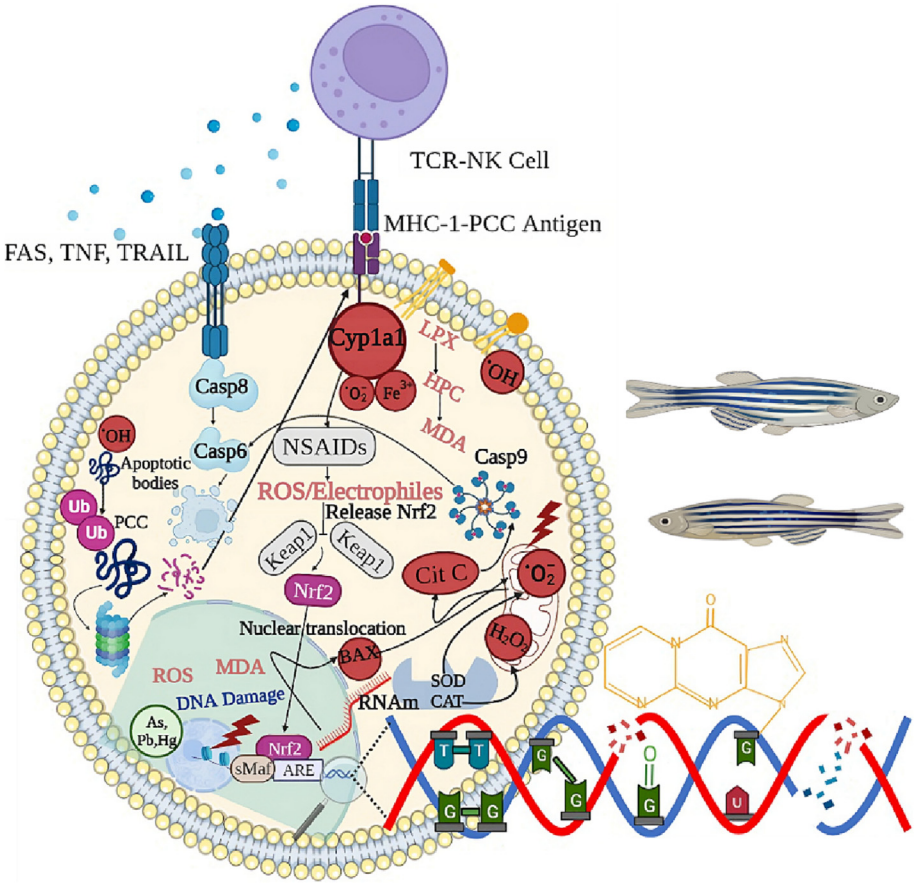


Fig. 11. Proposed toxicodynamic mechanism whereby hospital effluent induces damage in *D. rerio*.

different proportions of hospital effluent treated by HWWTP (2 %, 2.5 %, 3 % and 3.5 %). Our outcomes suggest that oxidative damage is exacerbated when antioxidant defence decreases at longer exposure times. The diversity of pollutants and pathogenic bacterial genera found in hospital effluent, allows the emergence of toxic interactions that largely explain the homeostatic alterations detected. Finally, more research with a holistic and multifactorial approach is needed to gain access to a broader panorama, and thereby consolidate the reliable mechanisms by which these complex anthropogenic matrices promote harm to non-target organisms.

CRediT authorship contribution statement

Francisco Javier Ramírez-Moreno 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 Hariz Islas-Flores, Sandra García-Medina, José Félix Aguirre-Garrido, and Luis Mario Hernández-Soto.

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.

Acknowledgments

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

References

Afsa, S., Sallem, O.F., Abdeljelil, N.B., Feriani, A., Najjar, M.F., Mansour, H.B., 2021. In vivo toxicities of the hospital effluent in Mahdia Tunisia. J. Water Health 19 (3), 499–511. https://doi.org/10.2166/wh.2021.024.

Afsa, S., Vieira, M., Nogueira, A.F., Mansour, H.B., Nunes, B., 2022. A multi-biomarker approach for the early assessment of the toxicity of hospital wastewater using the freshwater organism *Daphnia magna*. Environ. Sci. Pollut. Res. 1–16. https://doi.org/10.1007/s11356-021-16977-7.

Ajibola, A.S., Adebiyi, A.O., Nwaeke, D.O., Ajibola, F.O., Adewuyi, G.O., 2021. Analysis, occurrence and ecological risk assessment of diclofenac and ibuprofen residues in wastewater from three wastewater treatment plants in south-western Nigeria. J. Appl. Sci. Environ. Manag. 25 (3), 330–340. https://doi.org/10.4314/jasem.v25i3.5.

Ajitha, V., Sarasan, M., Sreevidya, C.P., Aswathy, C., Kachiprath, B., Mohandas, A., Singh, I.S.B., Hameed, A.S.S., Schlenk, D., Magnuson, J.T., Puthumana, J., 2021. Cytotoxic impacts of treated electroplating industrial effluent and the comparative effect of their metal components (Zn, Hg, and Zn + Hg) on *Danio rerio* gill (DrG) cell line. Sci. Total Environ. 793, 148533. https://doi.org/10.1016/j.scitotenv.2021.148533.

Alimba, C.G., Adekoya, K.O., Soyinka, O.O., 2019. Exposure to effluent from pharmaceutical industry induced cytogenotoxicity, hematological and histopathological alterations in *Clarias gariepinus* (Burchell, 1822). EXCLI J. 18, 63. https://doi.org/10.17179/excli2018-1916.

Baillie, T.A., Rettie, A.E., 2011. Role of biotransformation in drug-induced toxicity: influence of intra-and inter-species differences in drug metabolism. Drug Metab. Pharmacokinet. 26 (1), 15–29. https://doi.org/10.2133/dmpk.DMPK-10-RV-089.

Bhakkiyalakshmi, E., Sireesh, D., Ramkumar, K.M., 2018. Redox sensitive transcription via Nrf2-Keap1 in suppression of inflammation. Immunity and Inflammation in Health and Disease. Academic Press, pp. 149–161 https://doi.org/10.1016/B978-0-12-805417-8.00012-3.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72 (1–2), 248–254. https://doi.org/10.1016/0003-2697(76)90527-3.

Brubaker, K.L., 1999. Handbook of environmental analysis. Environ. Prog. Sustain. Energy 18 (2), S7. https://doi.org/10.1002/ep.670180205.

Buege, J.A., Aust, S.D., 1978. Microsomal lipid peroxidation. Methods in Enzymology. vol. 52. Academic press, pp. 302–310. https://doi.org/10.1016/S0076-6879(78)52032-6.

Buttke, T.M., Sandstrom, P.A., 1994. Oxidative stress as a mediator of apoptosis. Immunol. Today 15 (1), 7–10. https://doi.org/10.1016/0167-5699(94)90018-3.

Canedo, A., Rocha, T.L., 2021. Zebrafish (*Danio rerio*) using as model for genotoxicity and DNA repair assessments: historical review, current status and trends. Sci. Total Environ. 762, 144084. https://doi.org/10.1016/j.scitotenv.2020.144084.

Carlson, P., Van Beneden, R.J., 2014. Arsenic exposure alters expression of cell cycle and lipid metabolism genes in the liver of adult zebrafish (*Danio rerio*). Aquat. Toxicol. 153, 66–72. https://doi.org/10.1016/j.aquatox.2013.10.006.

Cerro-López, M., Castro-Pastrana, L.I., Toledo-Wall, M.L., Gómez-Oliván, L.M., Saldívar-Santiago, M.D., 2021. Análisis de fármacos en aguas residuales de tres hospitales de la ciudad de Puebla, México. Ingeniería del agua 25 (1), 59–73. https://doi.org/10.4995/ia.2021.13660.

Chen, Y.Y., Chan, K.M., 2018. Modulations of TCDD-mediated induction of zebrafish *cyp1a1* and the AHR pathway by administering Cd^{2+} in vivo. Chemosphere 210, 577–587. https://doi.org/10.1016/j.chemosphere.2018.07.032.

Chen, Q., Li, C., Gong, Z., Chan, E.C.Y., Snyder, S.A., Lam, S.H., 2017. Common deregulated gene expression profiles and morphological changes in developing zebrafish larvae exposed to environmental-relevant high to low concentrations of glucocorticoids. Chemosphere 172, 429–439. https://doi.org/10.1016/j.chemosphere.2017.01.036.

Cheng, B., Zhang, H., Jia, K., Li, E., Zhang, S., Yu, H., Cao, Z., Xiong, G., Hu, C., Lu, H., 2020. Effects of spinetoram on the developmental toxicity and immunotoxicity of zebrafish. Fish Shellfish Immunol. 96, 114–121. https://doi.org/10.1016/j.fsi.2019.11.066.

Claiborne, A.L., 2018. Catalase activity. CRC Handbook of Methods for Oxygen Radical Research. CRC press, pp. 283–284.

Cloran, C.E., Burton, G.A., Hammerschmidt, C.R., Taulbee, W.K., Custer, K.W., Bowman, K.L., 2010. Effects of suspended solids and dissolved organic carbon on nickel toxicity. Environ. Toxicol. Chem. 29 (8), 1781–1787. https://doi.org/10.1002/etc.226.

Craig, P.M., Wood, C.M., McClelland, G.B., 2007. Oxidative stress response and gene expression with acute copper exposure in zebrafish (*Danio rerio*). Am. J. Phys. Regul. Integr. Comp. Phys. 293 (5), R1882–R1892. https://doi.org/10.1152/ajpregu.00383.2007.

Cuetero-Martínez, Y., de Los Cobos-Vasconcelos, D., Aguirre-Garrido, J.F., Lopez-Vidal, Y., Noyola, A., 2023. Next-generation sequencing for surveillance of antimicrobial resistance and pathogenicity in municipal wastewater treatment plants. Curr. Med. Chem. 30 (1), 5–29. https://doi.org/10.2174/0929867329666220802093415.

Deguenon, E., Dougnon, V., Houssou, V.M.C., Gbotche, E., Ahoyo, R.A., Fabiyi, K., Agbankpe, J., Mousse, W., Lougbegnon, C., Klotoe, J.R., Tchobo, F., Bankole, H., Boko, M., 2022. Hospital effluents as sources of antibiotics residues, resistant bacteria and heavy metals in Benin. SN Appl. Sci. 4 (8), 1–12. https://doi.org/10.1007/s42452-022-05095-9.

Derikvandy, A., Pourkhabbaz, H.R., Banaee, M., Sureda, A., Haghi, N., Pourkhabbaz, A.R., 2020. Genotoxicity and oxidative damage in zebrafish (*Danio rerio*) after exposure to effluent from ethyl alcohol industry. Chemosphere 251, 126609. https://doi.org/10.1016/j.chemosphere.2020.126609.

Doganlar, O., Doganlar, Z.B., Muranlı, F.D.G., Guner, U., 2016. Genotoxic effect and carcinogenic potential of a mixture of as and cd in zebrafish at permissible maximum contamination levels for drinking water. Water Air Soil Pollut. 227 (3), 1–16. https://doi.org/10.1007/s11270-016-2779-1.

Emmanuel, E., Keck, G., Blanchard, J.M., Vermande, P., Perrodin, Y., 2004. Toxicological effects of disinfections using sodium hypochlorite on aquatic organisms and its contribution to AOX formation in hospital wastewater. Environ. Int. 30 (7), 891–900. https://doi.org/10.1016/j.envint.2004.02.004.

Evans, D.H., Piermarini, P.M., Choe, K.P., 2005. The multifunctional fish gill: dominant site of gas exchange, osmoregulation, acid-base regulation, and excretion of nitrogenous waste. Physiol. Rev. 85 (1), 97–177. https://doi.org/10.1152/physrev.00050.2003.

Félix, L.M., Vidal, A.M., Serafim, C., Valentim, A.M., Antunes, L.M., Monteiro, S.M., Matos, M., Coimbra, A.M., 2018. Ketamine induction of p53-dependent apoptosis and oxidative stress in zebrafish (*Danio rerio*) embryos. Chemosphere 201, 730–739. https://doi.org/10.1016/j.chemosphere.2018.03.049.

Galar-Martínez, M., García-Medina, S., Gómez-Olivan, L.M., Pérez-Coyotl, I., Mendoza-Monroy, D.J., Arrazola-Morgain, R.E., 2016. Oxidative stress and genotoxicity induced by ketorolac on the common carp *Cyprinus carpio*. Environ. Toxicol. 31 (9), 1035–1043. https://doi.org/10.1002/tox.22113.

García Sánchez, A., Ariza Astolfi, C., Úbeda Ontiveros, J.M., Martín Sánchez, P.M., Jurado, V., Bastian, F., Alabouvette, C., Saiz-Jimenez, C., 2013. Free-Living Amoebae in Sediments From the Lascaux Cave in France. https://doi.org/10.5038/1827-806X.42.1.2.

Godoy, A.A., Domingues, I., De Carvalho, L.B., Oliveira, Á.C., de Jesus Azevedo, C.C., Taparo, J.M., Assano, P.K., Mori, V., H, V. de A.V., Nogueira, A.J.A., Kummrow, F., 2020. Assessment of the ecotoxicity of the pharmaceuticals bisoprolol, sotalol, and ranitidine using standard and behavioral endpoints. Environ. Sci. Pollut. Res. 27 (5), 5469–5481. https://doi.org/10.1007/s11356-019-07322-0.

Gökalp, F.D., Doğanlar, O., Doğanlar, Z.B., Güner, U., 2022. The genotoxic effects of mixture of aluminum, arsenic, cadmium, cobalt, and chromium on the gill tissue of adult zebrafish (*Danio rerio*, Hamilton 1822). Drug Chem. Toxicol. 45 (3), 1158–1167. https://doi.org/10.1080/01480545.2020.1810260.

Gómez-Oliván, L.M., Galar-Martínez, M., Islas-Flores, H., García-Medina, S., SanJuan-Reyes, N., 2014. DNA damage and oxidative stress induced by acetylsalicylic acid in *Daphnia magna*. Comp. Biochem. Physiol. C: Pharmacol. Toxicol. Endocrinol. 164, 21–26. https://doi.org/10.1016/j.cbpc.2014.04.004.

Gómez-Oliván, L.M., Mendoza-Zenil, Y.P., SanJuan-Reyes, N., Galar-Martínez, M., Ramírez-Durán, N., Martín-Doimeadios, R.D.C.R., Rodríguez-Fariñas, N., Islas-Flores, H., Elizalde-Velázquez, A., García-Medina, Borja, R.P.P., 2017. Geno-and cytotoxicity induced on *Cyprinus carpio* by aluminum, iron, mercury and mixture thereof. Ecotoxicol. Environ. Saf. 135, 98–105. https://doi.org/10.1016/j.ecoenv.2016.09.037.

Gómez-Oliván, L.M., Islas-Flores, H., Juan-Reyes, N.S., Galar-Martínez, M., García-Medina, S., Elizalde-Velázquez, A., 2019. Oxidative stress induced by water from a hospital effluent of the city of toluca, mexico, on *Hyalella azteca*. Pollution of Water Bodies in Latin America: Impact of Contaminants on Species of Ecological Interest, pp. 79–95. https://doi.org/10.1007/978-3-030-27296-8_6.

Gonzalez, P., Baudrimont, M., Boudou, A., Bourdineaud, J.P., 2006. Comparative effects of direct cadmium contamination on gene expression in gills, liver, skeletal muscles and brain of the zebrafish (*Danio rerio*). Biometals 19 (3), 225–235. https://doi.org/10.1007/s10534-005-5670-x.

González-González, E.D., Gómez-Oliván, L.M., Galar-Martínez, M., Vieyra-Reyes, P., Islas-Flores, H., García-Medina, S., Jiménez-Vargas, J.M., Razo-Estrada, C., Pérez-Pastén, R., 2014. Metals and nonsteroidal anti-inflammatory pharmaceuticals drugs present in water from Madín Reservoir (Mexico) induce oxidative stress in gill, blood, and muscle of common carp (*Cyprinus carpio*). Arch. Environ. Contam. Toxicol. 67 (2), 281–295. https://doi.org/10.1007/s00244-014-0048-0.

González-Penagos, C.E., Zamora-Briseño, J.A., Cerqueda-García, D., Améndola-Pimenta, M., Pérez-Vega, J.A., Hernández-Nuñez, E., Rodríguez-Canul, R., 2020. Alterations in the gut microbiota of zebrafish (*Danio rerio*) in response to water-soluble crude oil components and its mixture with a chemical dispersant. Front. Public Health 8, 584953. https://doi.org/10.3389/fpubh.2020.584953.

Greub, G., Mege, J.L., Raoult, D., 2003. *Parachlamydia acanthamoeba* enters and multiplies within human macrophages and induces their apoptosis. Infect. Immun. 71 (10), 5979–5985. https://doi.org/10.1128/IAI.71.10.5979-5985.2003.

Hanschmann, E.M., Godoy, J.R., Berndt, C., Hudemann, C., Lillig, C.H., 2013. Thioredoxins, glutaredoxins, and peroxiredoxins—molecular mechanisms and health significance: from cofactors to antioxidants to redox signaling. Antioxid. Redox Signal. 19 (13), 1539–1605. https://doi.org/10.1089/ars.2012.4599.

Harriff, M.J., Bermudez, L.E., Kent, M.L., 2007. Experimental exposure of zebrafish, *Danio rerio* (Hamilton), to *Mycobacterium marinum* and *Mycobacterium peregrinum* reveals the gastrointestinal tract as the primary route of infection: a potential model for environmental mycobacterial infection. J. Fish Dis. 30 (10), 587–600. https://doi.org/10.1111/j.1365-2761.2007.00839.x.

Hwang, P.P., 2009. Ion uptake and acid secretion in zebrafish (*Danio rerio*). J. Exp. Biol. 212 (11), 1745–1752. https://doi.org/10.1242/jeb.026054.

Ighodaro, O.M., Akinloye, O.A., 2018. First line defence antioxidants-superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX): their fundamental role in the entire antioxidant defence grid. Alexandria J. Med. 54 (4), 287–293. https://doi.org/10.1016/j.ajme.2017.09.001.

Jha, R.R., Singh, N., Kumari, R., Patel, D.K., 2017. Ultrasound-assisted emulsification microextraction based on a solidified floating organic droplet for the rapid determination of 19 antibiotics as environmental pollutants in hospital drainage and Gomti river water. J. Sep. Sci. 40 (13), 2694–2702. https://doi.org/10.1002/jssc.201700170.

Jiang, Z.Y., Hunt, J.V., Wolff, S.P., 1992. Ferrous ion oxidation in the presence of xylenol orange for detection of lipid hydroperoxide in low density lipoprotein. Anal. Biochem. 202 (2), 384–389. https://doi.org/10.1016/0003-2697(92)90122-N.

Jiang, J., Wu, S., Wu, C., An, X., Cai, L., Zhao, X., 2014. Embryonic exposure to carbendazim induces the transcription of genes related to apoptosis, immunotoxicity and endocrine disruption in zebrafish (*Danio rerio*). Fish Shellfish Immunol. 41 (2), 493–500. https://doi.org/10.1016/j.fsi.2014.09.037.

Kancheva, V.D., Angelova, S.E., 2017. Synergistic effects of antioxidant compositions during inhibited lipid autoxidation. Lipid Peroxidation: Inhibition, Effects and Mechanisms. Nova Science Publishers Inc., New York, pp. 49–81.

Kataba, A., Botha, T.L., Nakayama, S.M., Yohannes, Y.B., Ikenaka, Y., Wepener, V., Ishizuka, M., 2022. Environmentally relevant lead (Pb) water concentration induce toxicity in zebrafish (*Danio rerio*) larvae. Comp. Biochem. Physiol. C: Pharmacol. Toxicol. Endocrinol. 252, 109215. https://doi.org/10.1016/j.cbpc.2021.109215.

Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M., Glöckner, F.O., 2013. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. Nucleic Acids Res. 41 (1), e1. https://doi.org/10.1093/nar/gks808.

Kolde, R., 2019. pheatmap: Pretty Heatmaps. R package version 1.0, p. 12. https://rdrr.io/cran/pheatmap/.

Kozich, J.J., Westcott, S.L., Baxter, N.T., Highlander, S.K., Schloss, P.D., 2013. Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina sequencing platform. Appl. Environ. Microbiol. 79 (17), 5112–5120. https://doi.org/10.1128/AEM.01043-13.

Kumari, A., Maurya, N.S., Tiwari, B., 2020. Hospital wastewater treatment scenario around the globe. Current Developments in Biotechnology and Bioengineering. Elsevier, pp. 549–570 https://doi.org/10.1016/B978-0-12-819722-6.00015-8.

Levine, R.L., Williams, J.A., Stadtman, E.P., Shacter, E., 1994. Carbonyl assays for determination of oxidatively modified proteins. Methods in Enzymology. vol. 233. Academic press, pp. 346–357. https://doi.org/10.1016/S0076-6879(94)33040-9.

Levine, R.L., Mosoni, L., Berlett, B.S., Stadtman, E.R., 1996. Methionine residues as endogenous antioxidants in proteins. Proc. Natl. Acad. Sci. 93 (26), 15036–15040. https://doi.org/10.1073/pnas.93.26.1503.

Li, S.W., Lin, A.Y.C., 2015. Increased acute toxicity to fish caused by pharmaceuticals in hospital effluents in a pharmaceutical mixture and after solar irradiation. Chemosphere 139, 190–196. https://doi.org/10.1016/j.chemosphere.2015.06.010.

Li, C., Zhang, Y., Wang, R., Lu, J., Nandi, S., Mohanty, S., Terhune, J., Liu, Z., Peatman, E., 2012. RNA-seq analysis of mucosal immune responses reveals signatures of intestinal barrier disruption and pathogen entry following *Edwardsiella ictaluri* infection in channel catfish, *Ictalurus punctatus*. Fish Shellfish Immunol. 32 (5), 816–827. https://doi.org/10.1016/j.fsi.2012.02.004.

Li, M., Cao, J., Zhao, Y., Wu, P., Li, X., Khodaei, F., Han, Y., Wang, J., 2020. Fluoride impairs ovary development by affecting oogenesis and inducing oxidative stress and apoptosis in female zebrafish (*Danio rerio*). Chemosphere 256, 127105. https://doi.org/10.1016/j.chemosphere.2020.127105.

Lim, J.M., Kim, G., Levine, R.L., 2019. Methionine in proteins: it's not just for protein initiation anymore. Neurochem. Res. 44 (1), 247–257. https://doi.org/10.1007/s11064-017-2460-0.

Liu, C., Xu, H., Lam, S.H., Gong, Z., 2013. Selection of reliable biomarkers from PCR array analyses using relative distance computational model: methodology and proof-of-concept study. PLoS One 8 (12), e83954. https://doi.org/10.1371/journal.pone.0083954.

Liu, S., Ding, R., Nie, X., 2019. Assessment of oxidative stress of paracetamol to *Daphnia magna* via determination of Nrf1 and genes related to antioxidant system. Aquat. Toxicol. 211, 73–80. https://doi.org/10.1016/j.aquatox.2019.03.014.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2-\Delta\Delta CT$ method. Methods 25 (4), 402–408. https://doi.org/10.1006/meth.2001.1262.

Loboda, A., Damulewicz, M., Pyza, E., Jozkowicz, A., Dulak, J., 2016. Role of Nrf2/HO-1 system in development, oxidative stress response and diseases: an evolutionarily conserved mechanism. Cell. Mol. Life Sci. 73 (17), 3221–3247. https://doi.org/10.1007/s00018-016-2223-0.

Lopetuso, L.R., Scaldaferri, F., Petito, V., Gasbarrini, A., 2013. Commensal Clostridia: leading players in the maintenance of gut homeostasis. Gut Pathog. 5, 1–8. https://doi.org/10.1186/1757-4749-5-23.

Luzio, A., Monteiro, S.M., Fontaínhas-Fernandes, A.A., Pinto-Carnide, O., Matos, M., Coimbra, A.M., 2013. Copper induced upregulation of apoptosis related genes in zebrafish (*Danio rerio*) gill. Aquat. Toxicol. 128, 183–189. https://doi.org/10.1016/j.aquatox.2012.12.018.

Misra, H.P., Fridovich, I., 1972. The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. J. Biol. Chem. 247 (10), 3170–3175. https://doi.org/10.1016/S0021-9258(19)45228-9.

Mondal, P., Shaw, P., Bandyopadhyay, A., Bhowmik, A.D., Chakraborty, A., Sudarshan, M., Chattopadhyay, A., 2019. Mixture effect of arsenic and fluoride at environmentally relevant concentrations in zebrafish (*Danio rerio*) liver: expression pattern of Nrf2 and related xenobiotic metabolizing enzymes. Aquat. Toxicol. 213, 105219. https://doi.org/10.1016/j.aquatox.2019.06.002.

Musee, N., Kebaabetswe, L.P., Tichapondwa, S., Tubatsi, G., Mahaye, N., Leareng, S.K., Nomngongo, P.N., 2021. Occurrence, fate, effects, and risks of dexamethasone: ecological implications post-COVID-19. Int. J. Environ. Res. Public Health 18 (21), 11291. https://doi.org/10.3390/ijerph182111291.

Neri-Cruz, N., Gómez-Oliván, L.M., Galar-Martínez, M., del Socorro Romero-Figueroa, M., Islas-Flores, H., García-Medina, S., Jiménez-Vargas, J.M., SanJuan-Reyes, N., 2015. Oxidative stress in *Cyprinus carpio* induced by hospital wastewater in Mexico. Ecotoxicology 24 (1), 181–193. https://doi.org/10.1007/s10646-014-1371-y.

Niemi, L., Taggart, M., Boyd, K., Zhang, Z., Gaffney, P.P., Pfleger, S., Gibb, S., 2020. Assessing hospital impact on pharmaceutical levels in a rural 'source-to-sink' water system. Sci. Total Environ. 737, 139618. https://doi.org/10.1016/j.scitotenv.2020.139618.

NMX-AA-003-1980. Norma Oficial Mexicana. Aguas residuales, muestreo. Procuraduría Federal de Protección al Ambiente. Diario Oficial de la Federación 25-03-1980.

NOM-001-SEMARNAT-1996. Norma Oficial Mexicana que establece los límites máximos permisibles de contaminantes en las descargas residuales en aguas y bienes nacionales. Procuraduría Federal de Protección al Ambiente. Diario Oficial de la Federación 06-01-1997.

NOM-002-SEMARNAT-1996. Norma Oficial Mexicana que establece los límites máximos permisibles de contaminantes en las descargas de aguas residuales a los sistemas de alcantarillado urbano o municipal. Secretaría del Medio Ambiente y Recursos Naturales. Diario Oficial de la Federación 18-10-1993.

OECD, 2019. Test no. 203: fish, acute toxicity testing, section 2: effects on biotic systems. Guidel. Test. Chem 10.

Oliveira, T.S., Murphy, M., Mendola, N., Wong, V., Carlson, D., Waring, L., 2015. Characterization of pharmaceuticals and personal care products in hospital effluent and waste water influent/effluent by direct-injection LC-MS-MS. Sci. Total Environ. 518, 459–478. https://doi.org/10.1016/j.scitotenv.2015.02.104.

Olvera-Néstor, C.G., Morales-Avila, E., Gómez-Olivan, L.M., Galár-Martínez, M., García-Medina, S., Neri-Cruz, N., 2016. Biomarkers of cytotoxic, genotoxic and apoptotic effects in *Cyprinus carpio* exposed to complex mixture of contaminants from hospital effluents. Bull. Environ. Contam. Toxicol. 96 (3), 326–332. https://doi.org/10.1007/s00128-015-1721-3.

Pilehvar, A., Cordery, K.I., Town, R.M., Blust, R., 2020. The synergistic toxicity of cd (II) and cu (II) to zebrafish (*Danio rerio*): effect of water hardness. Chemosphere 247, 125942. https://doi.org/10.1016/j.chemosphere.2020.125942.

Radi, R., Turrens, J.F., Chang, L.Y., Bush, K.M., Crapo, J.D., Freeman, B.A., 1991. Detection of catalase in rat heart mitochondria. J. Biol. Chem. 266 (32), 22028–22034. https://doi.org/10.1016/S0021-9258(18)54740-2.

Ranjan, R., Rani, A., Metwally, A., McGee, H.S., Perkins, D.L., 2016. Analysis of the microbiome: advantages of whole genome shotgun versus 16S amplicon sequencing. Biochem. Biophys. Res. Commun. 469 (4), 967–977. https://doi.org/10.1016/j.bbrc.2015.12.083.

Rosales-Pérez, K.E., Elizalde-Velázquez, G.A., Gómez-Oliván, L.M., Orozco-Hernández, J.M., Cardoso-Vera, J.D., Heredia-García, G., Islas-Flores, H., García-Medina, Sandra, Galar-Martínez, M., 2022. Brain damage induced by contaminants released in a hospital from Mexico: evaluation of swimming behavior, oxidative stress, and acetylcholinesterase in zebrafish (*Danio rerio*). Chemosphere 294, 133791. https://doi.org/10.1016/j.chemosphere.2022.133791.

Sant, K.E., Hansen, J.M., Williams, L.M., Tran, N.L., Goldstone, J.V., Stegeman, J.J., Hahn, M.E., Timme-Laragy, A., 2017. The role of Nrf1 and Nrf2 in the regulation of glutathione and redox dynamics in the developing zebrafish embryo. Redox Biol. 13, 207–218. https://doi.org/10.1016/j.redox.2017.05.023.

Seoane, M., Esperanza, M., Cid, Á., 2017. Cytotoxic effects of the proton pump inhibitor omeprazole on the non-target marine microalga *Tetraselmis suecica*. Aquat. Toxicol. 191, 62–72. https://doi.org/10.1016/j.aquatox.2017.08.001.

Sheng, Y., Ren, H., Limbu, S.M., Sun, Y., Qiao, F., Zhai, W., Du, Z.Y., Zhang, M., 2018. The presence or absence of intestinal microbiota affects lipid deposition and related genes expression in zebrafish (*Danio rerio*). Front. Microbiol. 9, 1124. https://doi.org/10.3389/fmicb.2018.01124.

Shi, H., Zhou, T., Wang, X., Yang, Y., Wu, C., Liu, S., Bao, L., Li, N., Yuan, Z., Jin, Y., Tan, S., Wang, W., Zhong, X., Qin, G., Geng, X., Gao, D., Dunham, R., Liu, Z., 2018. Genome-wide association analysis of intra-specific QTL associated with the resistance for enteric septicemia of catfish. Mol. Gen. Genomics. 293 (6), 1365–1378. https://doi.org/10.1007/s00438-018-1463-0.

Sies, H., Berndt, C., Jones, D.P., 2017. Oxidative stress. Annu. Rev. Biochem. 86, 715–748. https://doi.org/10.1146/annurev-biochem-061516-045037.

Srivastav, A.L., Patel, N., Chaudhary, V.K., 2020. Disinfection by-products in drinking water: occurrence, toxicity and abatement. Environ. Pollut. 267, 115474. https://doi.org/10.1016/j.envpol.2020.115474.

Stancová, V., Ziková, A., Svobodová, Z., Kloas, W., 2015. Effects of the non-steroidal anti-inflammatory drug (NSAID) naproxen on gene expression of antioxidant enzymes in zebrafish (*Danio rerio*). Environ. Toxicol. Pharmacol. 40 (2), 343–348. https://doi.org/10.1016/j.etap.2015.07.009.

Steinbrueck, A., Sedgwick, A.C., Brewster, J.T., Yan, K.C., Shang, Y., Knoll, D.M., Vargas-Zuñiga, G.I., He, X.P., Tian, H., Sessler, J.L., 2020. Transition metal chelators, pro-chelators, and ionophores as small molecule cancer chemotherapeutic agents. Chem. Soc. Rev. 49 (12), 3726–3747. https://doi.org/10.1039/C9CS00373H.

Świacka, K., Maculewicz, J., Kowalska, D., Caban, M., Smolarz, K., Świeżak, J., 2022. Presence of pharmaceuticals and their metabolites in wild-living aquatic organisms–current state of knowledge. J. Hazard. Mater. 424, 127350. https://doi.org/10.1016/j.jhazmat.2021.127350.

Ukić, Š., Sigurnjak, M., Cvetnić, M., Markić, M., Stankov, M.N., Rogošić, M., Rasulev, B., Božić, A.L., Kušić, H., Bolanča, T., 2019. Toxicity of pharmaceuticals in binary mixtures: assessment by additive and non-additive toxicity models. Ecotoxicol. Environ. Saf. 185, 109696. https://doi.org/10.1016/j.ecoenv.2019.109696.

UNESCO, 2017. ¿Son las aguas residuales el nuevo “oro negro”? Presentación pública del Informe de las Naciones Unidas sobre el Desarrollo de los Recursos Hídricos

Valles, S., Hernández-Sánchez, J., Dipp, V.R., Huerta-González, D., Olivares-Bañuelos, T.N., González-Fraga, J., Bardullas, U., 2020. Exposure to low doses of inorganic arsenic induces transgenerational changes on behavioral and epigenetic markers in zebrafish (*Danio rerio*). Toxicol. Appl. Pharmacol. 396, 115002. https://doi.org/10.1016/j.taap.2020.115002.

Verlicchi, P., 2021. Trends, new insights and perspectives in the treatment of hospital effluents. Curr. Opin. Environ. Sci. Health 19, 100217. https://doi.org/10.1016/j.coesh.2020.10.005.

Verlicchi, P., Galletti, A., Petrovic, M., Barceló, D., 2010. Hospital effluents as a source of emerging pollutants: an overview of micropollutants and sustainable treatment options. J. Hydrol. 389 (3–4), 416–428. https://doi.org/10.1016/j.jhydrol.2010.06.005.

Wang, C., Chuai, X., Liang, M., 2019. Legionella feeleii: pneumonia or Pontiac fever? Bacterial virulence traits and host immune response. Med. Microbiol. Immunol. 208, 25–32. https://doi.org/10.1007/s00430-018-0571-0.

Wang, C., Yuan, Z., Sun, Y., Yao, X., Li, R., Li, S., 2021. Effect of chronic exposure to textile wastewater treatment plant effluents on growth performance, oxidative stress, and intestinal microbiota in adult zebrafish (*Danio rerio*). Front. Microbiol. 12. https://doi.org/10.3389/fmicb.2021.782611.

Wittlerová, M., Jirova, G., Vlkova, A., Kejlova, K., Maly, M., Heinonen, T., Wittlingerová, Z., Zimova, M., 2020. Sensitivity of zebrafish (*Danio rerio*) embryos to hospital effluent compared to *Daphnia magna* and *Aliivibrio fischeri*. Physiol. Res. 69 (Suppl. 4), S681. https://doi.org/10.33549/physiolres.934616.

Xia, J., Lu, L., Jin, C., Wang, S., Zhou, J., Ni, Y., Fu, Z., Jin, Y., 2018. Effects of short term lead exposure on gut microbiota and hepatic metabolism in adult zebrafish. Comp. Biochem. Physiol. C: Pharmacol. Toxicol. Endocrinol. 209, 1–8. https://doi.org/10.1016/j.cbpc.2018.03.007.

Xiang, J., Wan, C., Guo, R., Guo, D., 2016. Is hydrogen peroxide a suitable apoptosis inducer for all cell types? Biomed. Res. Int. 2016. https://doi.org/10.1155/2016/7343965.

Zang, L., Ma, Y., Huang, W., Ling, Y., Sun, L., Wang, X., Zeng, A., Dahlgren, R.A., Wang, C., Wang, H., 2019. Dietary *lactobacillus plantarum* ST-III alleviates the toxic effects of triclosan on zebrafish (*Danio rerio*) via gut microbiota modulation. Fish Shellfish Immunol. 84, 1157–1169. https://doi.org/10.1016/j.fsi.2018.11.007.

Zheng, Y., Wang, Y., Zheng, M., Wang, G., Zhao, H., 2022. Exposed to sulfamethoxazole induced hepatic lipid metabolism disorder and intestinal microbiota changes on zebrafish (*Danio rerio*). Comp. Biochem. Physiol. C: Pharmacol. Toxicol. Endocrinol. 253, 109245. https://doi.org/10.1016/j.cbpc.2021.109245.