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Informe Escrito Final

Análisis transcriptómico para identificar genes biomarcadores que respondan ante la exposición al insecticida clorpirifos, en el pez dulceacuícola centroamericano *Poecilia gillii*

Trabajo Final de Graduación para optar por el grado de Licenciatura en Biotecnología

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Abreviaturas

AChE	Acetilcolinesterasa
ADNc	ADN copia o complementario
Anxa1	Proteína Anexina-1
anxa1	Gen Anexina-1
ARRIVE	Animal Research: Reporting of *In Vivo* Experiments
CAT	Catalasa
CDNB	1-cloro-2,4-dinitrobenceno
CDS	Secuencia de ADN codificante
ChE	Colinesterasa
Cish	Proteína SH2 inducible por citoquinas
cish	Gen SH2 inducible por citoquinas
CONAGEBIO	Comisión Nacional para la Gestión de la Biodiversidad
CPF	Clorpirifos
C_T	Ciclo de umbral
ECOTOX	Laboratorio de Estudios Ecotoxicológicos
ER	Retículo endoplasmático
EROD	Etoxiresorufin-O-desetilasa
GST	Proteína glutatión S-transferasas
gst	Gen glutatión S-transferasas
gstr	Gen glutatión S-transferasas rho
H_2O_2	Peróxido de hidrógeno
i.a.	Ingrediente activo
IRET-UNA	Instituto Regional de Estudios en Sustancias Tóxicas de la UNA
LAREP	Laboratorio de Análisis de Residuos de Plaguicidas
LBP-UNA	Laboratorio de Biotecnología de Plantas
LPO	Peroxidación lipídica
NADH	Ubiquinona oxidorreductasa o deshidrogenasa
Milli-Q	Agua con grado ultrapura que ha purificada por ósmosis inversa
MS-222	La tricaína metanosulfonato
NCBI	Centro Nacional de Información Biotecnológica
Ndufa2	Proteína NADH deshidrogenasa 1 subunidad 2 del subcomplejo α
ndufa2	Gen NADH deshidrogenasa 1 subunidad 2 del subcomplejo α
OECD	Organisation for Economic Co-operation and Development
PCR	Reacción en cadena de la polimerasa
PNUD	Programa de las Naciones Unidas para el Desarrollo
qPCR	PCR cuantitativa
ROS	Especies reactivas de oxígeno
RT	Retrotranscripción
Sec61g	Proteína Secretasa 61 subunidad gamma
sec61g	Gen Secretasa 61 subunidad gamma
SFE	Servicio Fitosanitario del Estado
UNDP	United Nations Development Program
UPLC	Cromatografía líquida de alto rendimiento
UV-water	Agua filtrada con sistema de Millipore, carbono activado y luz UV

Resumen

El uso excesivo de clorpirifos (CPF) en países tropicales como Costa Rica representa un posible riesgo para los ecosistemas de agua dulce. Este estudio investigó los efectos del CPF a una concentración ambientalmente relevante sobre la especie de pez nativa *Poecilia gillii*, empleando un enfoque integral que evaluó múltiples niveles de organización biológica. Utilizando RT-qPCR, se cuantificaron los cambios transcriptómicos en genes involucrados en diversos procesos biológicos, incluidos la inflamación y la apoptosis; *annexin A1* (anxa1b), regulación de citoquinas; *cytokine-inducible SH2-containing protein* (cish), reacciones redox; *NADH oxidoreductase subunit A2* (ndufa2), translocación de proteínas; *Sec61 gamma subunit* (sec61g), y biotransformación; *glutathione S-transferase rho* (gstr). Además, se evaluaron biomarcadores bioquímicos como enzimas de biotransformación de fase I; *7-ethoxyresorufin-O-deethylase* (EROD) y fase II; *glutathione S-transferase* (GST), marcadores de estrés oxidativo; *catalase* (CAT) y *lipid peroxidation* (LPO) y la neurotoxicidad midiendo la actividad de *cholinesterase* (ChE) en tejidos cerebrales y musculares. Además, se realizaron pruebas de comportamiento evaluando la natación y las reacciones de antipredación. Tras una exposición transitoria de 48 horas a 5.5 µg/L de CPF, se observó una regulación significativa a la baja de los genes *sec61g* y *gstr*, una disminución en la actividad de CAT y efectos neurotóxicos evidenciados por la reducción de la actividad de ChE en los músculos. Aunque no se detectaron cambios conductuales significativos, nuestros resultados sugieren que la exposición a corto plazo a concentraciones ambientales relevantes de CPF puede alterar la expresión génica, comprometiendo la biotransformación y la síntesis de proteínas en los juveniles de *P. gillii*. Además, la neurotoxicidad observada, consistente con el mecanismo de acción del CPF, podría conducir a cambios conductuales sutiles. Este estudio proporciona evidencia de los efectos subletales del CPF en organismos no objetivos, destacando la importancia de considerar los cambios en la expresión génica en la evaluación de la toxicidad del CPF.

Palabras clave: peces, pesticidas, expresión genética, neurotoxicidad, biotransformación, estrés oxidativo.

El siguiente artículo forma parte del requisito parcial para optar por el grado de Licenciatura en Biotecnología de la Universidad Nacional de Costa Rica.

Exposure to an Environmentally Relevant Concentration of Chlorpyrifos Induces Transcriptional Changes and Neurotoxicity in *Poecilia gillii* without Clear Behavioral Effects

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1-ABSTRACT

Overusing chlorpyrifos (CPF) in tropical countries such as Costa Rica poses a potential risk to freshwater ecosystems. This study investigated the effects of transient exposure to an environmentally relevant CPF concentration on the native fish species *Poecilia gillii*, employing a comprehensive approach that evaluated multiple levels of biological organization. Using RT-qPCR, we quantified transcript changes in genes involved in various biological processes, including inflammation and apoptosis; annexin A1 (*anxa1b*), cytokine regulation; cytokine-inducible SH2-containing protein (*cish*), redox reactions; NADH oxidoreductase subunit A2 (*ndufa2*), protein translocation; Sec61 gamma subunit (*sec61g*), and biotransformation; glutathione S-transferase rho (*gstr*). Additionally, we measured biochemical biomarkers such as phase I; 7-ethoxyresorufin-O-deethylase (EROD) and phase II; glutathione S-transferase (GST) biotransformation enzymes, oxidative stress markers; catalase (CAT) and lipid peroxidation (LPO), and conducted behavioral tests to assess swimming fitness and antipredator reactions. Neurotoxicity was assessed by measuring brain and muscle tissue cholinesterase (ChE) activity. Following 48 h of exposure to 5.5 µg/L CPF, we observed significant downregulation of the *sec61g* and *gstr* genes, decreased CAT activity, and neurotoxic effects, as indicated by reduced ChE activity in muscle. Although no significant behavioral changes were detected, our results suggest that short-term exposure to environmentally relevant CPF concentrations can disrupt gene expression, compromising biotransformation and protein synthesis in *P. gillii* juveniles. Moreover, the observed neurotoxicity, which is consistent with the mechanism of action of

CPF, may lead to subtle behavioral changes. This study provides evidence of the sublethal effects of CPF on nontarget organisms, highlighting the importance of considering gene expression changes when assessing CPF toxicity.

2-KEYWORDS: fish, pesticides, gene expression, neurotoxicity, biotransformation, oxidative stress, immunity.

3-INTRODUCTION

Agriculture is essential to Costa Rica's economy, with approximately 8.8% of the national territory dedicated to this activity (Echeverría-Sáenz et al., 2018; Ramírez-Morales et al., 2021). An estimation in 2023 indicates that close to 2 million tons of pineapples and bananas were exported, solidifying Costa Rica as one of the leading exporters of fresh fruits globally (FAO 2024a, 2024b). However, the heavy reliance of such production on agrochemicals poses significant environmental challenges (Echeverría-Sáenz et al., 2012; Rodríguez-Rodríguez et al., 2021). According to the State Phytosanitary Service (SFE, 2022a), Costa Rica applied an average of 10.18 kg of active ingredient per hectare (a.i./ha) annually during the 2020-2021 period, one of the highest rates in Latin America. Furthermore, in May 2022, the United Nations Development Program (UNDP) presented a report suggesting a potentially more alarming scenario, estimating the average product usage to escalate to 34.45 kg a.i./ha annually (UNDP, 2022).

The indiscriminate use and inadequate management of agrochemicals have led to the presence of residues of many active ingredients in water bodies adjacent to and downstream from various plantations, raising concerns about their impact on aquatic ecosystems (Echeverría-Sáenz et al., 2018; Ramírez-Morales et al., 2021). The organophosphate insecticide chlorpyrifos (CPF) is a substance of particular concern because of its environmental occurrence, high toxicity, and estimated environmental risk (Echeverría-Sáenz et al., 2021). In 2021, approximately 152.8 tons of CPF were used in various insecticide formulations in Costa Rica (SFE, 2022a). This extensive usage is evident in the annual report on pesticide residues detected in fresh vegetables by the SFE, where the CPF ranked fourth in exceeding the maximum allowable limits set by health authorities during 2020 and 2021 (SFE, 2022b). The environmental concentrations of CPF detected in local ecosystems range from 0.014 to 1.42 µg/L in water samples and from 8.05 to 12.0 µg/kg in sediment samples from areas such as the Suerte-Tortuguero river basin, the Madre de Dios

River, and the Toyogres, Reventado, and Birris microwatersheds (Castillo et al., 2000; Arias-Andrés et al., 2018; Echeverría-Sáenz et al., 2018; Ramirez-Morales et al., 2021). However, higher concentrations in surface waters have been reported in other regions, such as 7.73 µg/L in Africa, 79.7 µg/L in Asia, and 96.0 µg/L in Europe (Arain et al., 2018; Rico et al., 2021; Sishu et al., 2022).

In response to the critical need to assess the effects of environmentally relevant concentrations of CPF in vulnerable aquatic ecosystems, diagnostic techniques are indispensable (Mena et al., 2014; Huang et al., 2020). Evaluating sublethal responses, such as gene expression, and biochemical and behavioral changes, is crucial for describing the impacts of pollutants on biological systems (Amiard-Triquet et al., 2012; Hausen et al., 2018). Targeted gene expression quantification approaches, notably reverse transcriptase quantitative polymerase chain reaction (RT-qPCR), have been used to assess the changes in messenger RNA levels of genes related to detoxification, stress, immunity, reproduction and molting after exposure to pesticides (Liu et al., 2023; Zahran et al., 2020). Integrating these molecular techniques with traditional biochemical biomarkers and behavioral assessments contributes to the elucidation of the underlying mechanisms that lead to complex individual responses (Zhou et al., 2018). Gene expression profiling marks the initial step in the physiological response to chemical stressors, whereas biochemical biomarkers indicate functional responses such as biotransformation and adverse effects like neurotoxicity or oxidative stress within cells (Duroudier et al., 2019; Fu et al., 2021). Behavioral assessments integrate these suborganismal responses into observable impairments in motility or an organism's responsiveness to environmental cues (Amiard-Triquet, 2009; Schuijt et al., 2021).

Assessments at lower biological levels should be more specific regarding cause-effect relationships between stressors and responses (Schuijt et al., 2021). For example, the inhibition of cholinesterase (ChE) activity is considered a clear sign of neurotoxicity (Sepahi et al., 2023). However, most biochemical biomarkers related to biotransformation, such as the enzymes ethoxyresorufin-O-deethylase (EROD) of phase I and glutathione S-transferase (GST) of phase II; or oxidative stress-related markers such as the antioxidant catalase activity (CAT) or lipid peroxidation (LPO), are nonspecific processes that can be induced by a broad range of xenobiotics (Cazenave, 2021). In this context, the study by Hausen et al. (2018) represents an advance in evaluating the effects of CPF as they described transcriptomic signatures altered in the fish model *Danio rerio*, specifically by this

insecticide. These findings provide valuable insights into the regulation of response systems caused by external agents, including significantly upregulated expression of the genes encoding annexin A1 (*anxa1b*), which is related to inflammation and apoptosis; the Sec61 gamma subunit (*sec61g*), which is involved in protein translocation; the cytokine-inducible SH2-containing protein (*cish*), a cytokine regulator; and the NADH oxidoreductase subunit A2 (*ndufa2*), which is associated with redox reactions.

Considering the relevance of CPF as a contaminant in Costa Rican ecosystems, the described advances in evaluating subindividual effects should contribute to characterizing such effects of the insecticide in local aquatic biota. In this context, the freshwater fish *Poecilia gillii* (Kner & Steindachner, 1863) is a promising species for studying the effects of agrochemicals because of its widespread distribution and abundance in Costa Rican freshwater bodies, along with its role in the aquatic food web (Bussing, 1998; Jiménez-Prado et al., 2020). Furthermore, this species has been successfully used in previous ecotoxicological studies (Mena et al., 2014; Navarro et al., 2014), including a report on its responsiveness to environmentally realistic levels of CPF (Redondo-López et al., 2022).

Our study aimed to evaluate whether exposure to an environmentally relevant level of CPF would induce the expression of genes related to specific cellular processes. Additionally, we measured biochemical biomarkers (ChE, EROD, GST, CAT, and LPO) and conducted behavioral tests related to fish motility and fitness to complement the information provided by genetic markers. This comprehensive approach should increase our understanding of the early effects of exposure to CPF on the native species *P. gillii*

4-MATERIALS AND METHODS

4.1 Test animals and preparation of exposure solutions.

A group of 24 healthy juvenile specimens of *P. gillii* (3.1 ± 0.32 cm in length, 0.69 ± 0.24 g body weight) with normal morphology and no external alterations were obtained from the Laboratory of Ecotoxicological Studies (ECOTOX) of the Central American Institute for Studies on Toxic Substances, Universidad Nacional, Costa Rica (IRET-UNA). The fish were kept in a 150 L tank filled with water filtered through a 1 μm-pore cartridge, activated carbon, and treated with UV light (Millipore) (UV water). The water in the tank was constantly aerated and filtered with a biological filter. The fish were fed daily *ad libitum* with flakes (Tetra). Before the tests, the feed was suspended for 24 h.

A standard stock solution of CPF (2198.4 µg/mL) was prepared in analytical grade acetone (Sigma-Aldrich, Germany). To apply a dose with a nominal CPF concentration of 5.5 µg/L to the exposed glass jar, an intermediate solution was prepared by diluting 250 µL of the CPF stock solution in 5 mL of Milli-Q H_2O. A 40 µL aliquot of the CPF intermediate solution was added to each exposure glass container filled with 800 mL of UV water via a microsyringe. For the control group, 2 µL of acetone was added directly to the exposure container to reach a concentration of 0.00025%.

An aliquot of CPF solution from the exposure containers was taken at the beginning and end of the exposure and immediately frozen at -20° for concentration confirmation through liquid chromatography. The water samples were analyzed via direct injection into an ultra-performance liquid chromatography (UPLC) system (Acquity H, Waters), coupled with a tandem mass spectrometer (XEVO TQ-S micro, Waters). The analysis was performed with an Acquity UPLC BEH C18 column. The samples were analyzed in duplicate and quantified using standard solutions prepared in pure solvents. At the beginning of the experiment, the water content was 4.55 ± 0.07 µg/L, whereas at the end, it was 0.09 ± 0.01 µg/L. For this reason, we report it as a transient exposure to the insecticide, considering that its presence was confirmed at the start of the test and that the effects were assessed 48 h later.

4.2 Experimental Design

The 24 unsexed fish were randomly divided into two groups (n = 12). One group was exposed to 5.5 µg/L CPF, and the other was used as a control. The sample size (n) was determined on the basis of OECD Test No. 203, the Fish Acute Toxicity Testing guideline (2019), which recommends a minimum of seven fish per treatment. We increased the sample size to 12 to account for potential losses and increase the statistical robustness of our results.

Throughout the experiment, the animals were maintained in aerated UV water at a constant temperature of 22 ± 1 °C under a 16 h light and 8 h dark cycle. After 48 h of exposure, each fish was subjected to two behavioral tests, one to assess motility and the other to test their antipredation response. To minimize external interference between control and exposed individuals, all behavioral measurements were conducted consistently from 8:00 am to 11:00 am, alternating between treatments and tests. Following this, the fish were euthanized following welfare procedures. Then, the fish were dissected to obtain samples for analysis of biomarkers and molecular assessments. Additional details for each step are described in the following sections.

4.3 Behavioral evaluation

Swimming test

Each fish was placed in a glass aquarium of 8.25 L (W 55 cm x D 18 cm x H 34 cm), which was placed inside a dark tent to avoid any stimuli from the outside. The back wall of the aquarium was covered with white matte vinyl to favor the contrast of the fish during the video. A led tube was placed 15 cm above the aquarium surface to provide a light intensity of 1600 lux at the water surface. After 10 min of acclimatization, every individual was recorded for 10 min at a resolution of 1280 x 720p at 30 fps. The videos were analyzed using AnimalTA software, version 2.3.4 (Chiara & Kim, 2023), to track each fish and extract data on speed, mobility, exploration, and maeander (average angle changes per unit distance traveled) and compare them among exposed and control fish (Fig. 1A). Additionally, we used the same software to assess fish preferences for different tank areas, which were virtually divided into three vertical sections (left, middle, and right) (Fig. 1B) and two horizontal sections (bottom and top) (Fig. 1C).

Antipredator reaction

The escape response to a simulated predatory attack was evaluated following the methodology described by Sandoval-Herrera et al. (2019). The fish were placed individually in a controlled tank environment of 15 L (W 50cm x D 15cm x H 20cm). The bottom and three internal sides of the aquarium were covered with white matte vinyl to reduce visual disturbances and prevent mirror reflections. After a 5 min acclimatization period, their reactions to the release of a predator dummy were recorded on video (1920 x 1080p, 60 fps) (Fig. 1D). The time lapse between the release of the dummy (Fig. 1E) and the escape reaction was estimated by counting the frames in the video. This reaction was evidenced by a sudden and powerful contraction of the body muscles and a flexion movement of the caudal peduncle resulting in a C or S-shaped curvature (C- or S-start) (Fig. 1F).

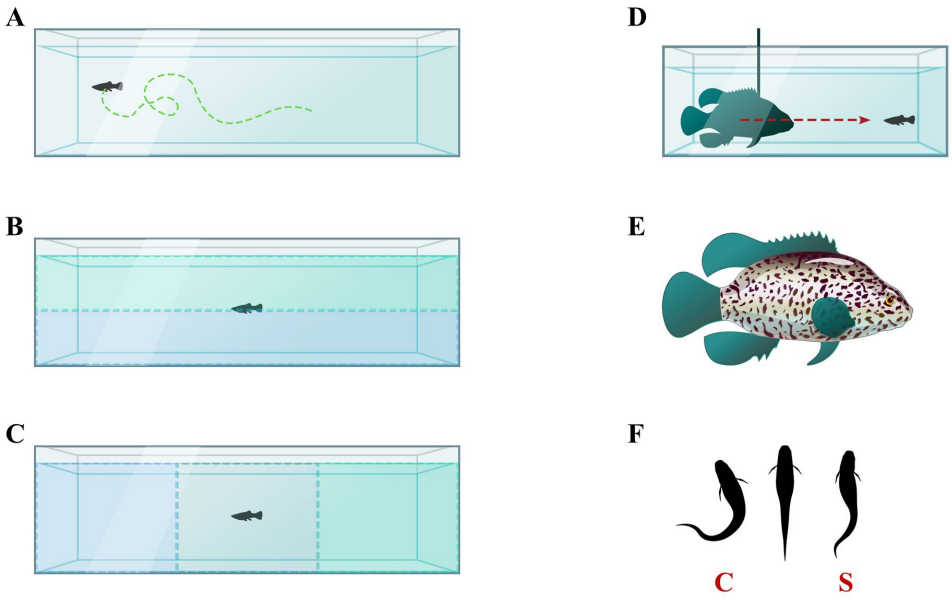

Figure 1. Swimming test of *P. gillii* after 48 h of treatment. Movement tracking (A), horizontal sections (B), vertical sections (C), antipredator reaction (D), dummy fish (E), C- or S-start (F).

4.4 Dissection and sampling

After the behavioral tests were conducted, each fish was anesthetized with 200 mg/L tricaine methane sulfonate (MS-222) in UV water. Subsequently, their weight and standard length were recorded promptly, followed by euthanasia via transection of the spinal cord behind the opercula. Each fish´s liver was then extracted and divided into two portions: one portion was immediately stored at -80 °C for measurement of EROD, CAT, LPO, and GST activities (Fig. 2A), while the remaining half of the liver and a sample of gills (at least three arches) were preserved in 400 µl of RNAlater (QIAGEN, Germany) at -80 °C until they were used for RNA isolation (Fig. 2B). Additionally, samples of the brain (whole) and sections of the lateral muscle (approx. 50 mg) were dissected, placed in microtubes, and promptly stored at -80 °C to measure ChE activity (Fig. 2A) muscle (approx. 50 mg) were dissected, placed in microtubes, and promptly stored at -80 °C to measure ChE activity.

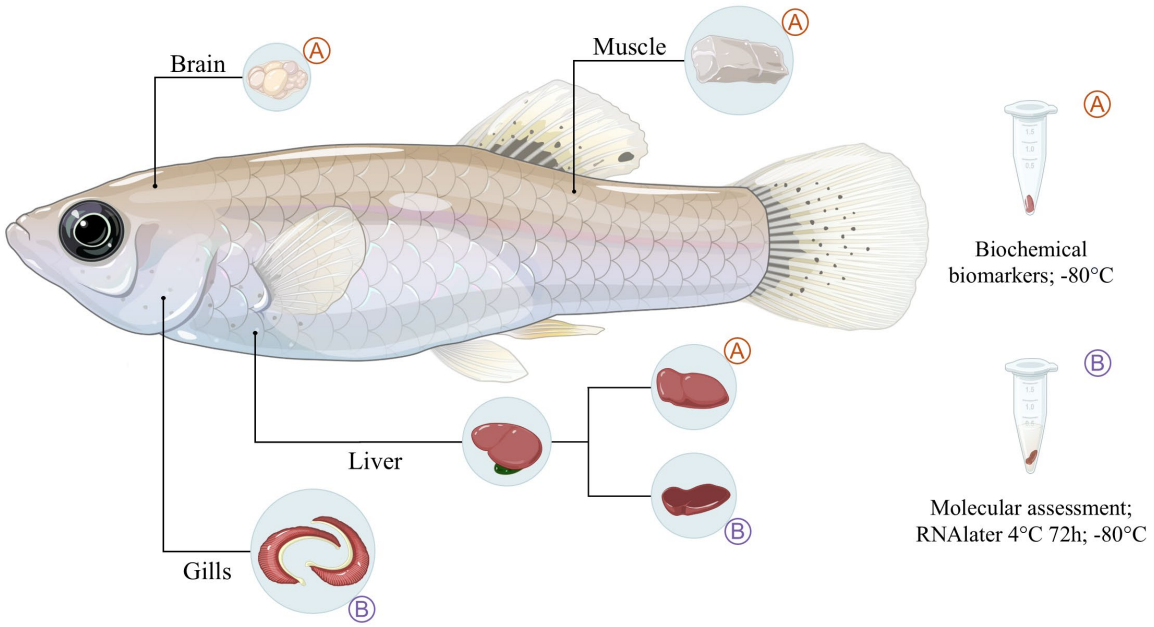

Figure 2. Dissection and sampling of *P. gillii* after 48 h of exposure. Brain, muscle, and a section of the liver for molecular measurements (A). Gills and a section of the liver for biochemical biomarkers (B).

4.5 Biochemical biomarkers

Protein quantification and biomarker determination were conducted following the methodologies outlined by Mena et al. (2014). The tissue samples were homogenized via sonication (Branson SLPT, Danbury, USA) in 1.5 mL tubes immersed in crushed ice to maintain stable sample temperatures. Liver samples were homogenized in 0.1 M phosphate buffer (KH_2PO_4/K_2HPO_4) at pH 7.4. An aliquot was immediately separated, mixed with 0.2 mM butylated hydroxytoluene and preserved at -80 °C for LPO analysis. The rest of the homogenate was centrifuged (15300 rcf, 4 °C, 20 min) and the supernatant was used to measure EROD, GST and CAT. Brain and muscle samples were homogenized in 0.1 M KH_2PO_4/K_2HPO_4 at pH 7.2 and centrifuged (10600 rcf, 4 °C, 5 min) to measure ChE in the supernatant.

To standardize the enzymatic activities on the basis of protein concentration, the protein content in the samples was quantified via the Bradford (1976) method, with bovine serum albumin serving as the standard. All spectrophotometric and fluorometric measurements were conducted via a Fluoroskan microplate fluorometer (Thermo Fisher Scientific, USA).

The activity of EROD was determined in the liver following modified protocols by Peters et al. (1994) and Jiménez et al. (2021). The samples were exposed to a reaction mixture prepared with 0.1 M phosphate buffer (pH 7.4), NADPH (0.10 mM), and 7-ethoxyresorufin (0.415 μM) as the substrate. Reaction kinetics were measured for 3 min, and the EROD activity unit was defined as the formation of 1 nmol of resorufin/min/mg of protein.

GST activity was measured according to Habig et al. (1976), using 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate in a reaction mixture prepared with 0.2 M phosphate buffer (pH 6.5) and 1 mM CDNB, along with 1 mM reduced L-Glutathione. The reaction kinetics were monitored at 340 nm for 3 min and expressed as nmol/min/mg of protein.

CAT activity was determined via the Aebi (1984) method, measuring the decrease in absorbance at 240 nm for 20 s due to the consumption of 6 mM H_2O_2 and is expressed as μmol/min/mg of protein. The reaction occurred in a 0.05% phosphate buffer (pH 7.0).

LPO levels were assessed via a thiobarbituric acid reactive substances assay, following the protocol of Ohkawa et al. (1979). The samples were mixed with 12% trichloroacetic acid, Tris-HCl (60 mM, pH 7.4) containing 0.1 mM diethylenetriaminepentaacetic acid, and 0.73% thiobarbituric acid and then heated at 100 °C for 1 h. After centrifugation (14000 rcf, 25 °C, 5 min), the absorbance of the supernatant was measured at 535 nm. LPO levels were quantified as TBARS nmol/mg of protein.

Finally, ChE activity was measured via the Ellman et al. (1961) method adapted for microplates (Guilhermino et al., 1996). The reaction occurred in 0.1 mM phosphate buffer (pH 7.2) in the presence of 1 mM acetylthiocholine as a synthetic substrate and 0.1 mM 5,5′-dithiobis-2-dinitrobenzoic acid as a chromogenic reagent, with kinetics measured at 415 nm for 10 min and activity expressed as nmol/min/mg of protein.

4.6 Molecular assessment

Total RNA isolation and cDNA synthesis

Total RNA was isolated from liver and gill tissues via the PureLink RNA Mini Kit (Invitrogen, USA) according to the manufacturer's instructions. The gill samples were mechanically disrupted using a digital disruptor genie (Scientific Industries, USA). The RNA concentration in the samples was measured via a Qubit 4 fluorometer (Invitrogen, USA) with an RNA broad-range assay kit.

Reverse transcription (RT)

The RT reaction for cDNA synthesis was performed using the RevertAid RT-Kit (Thermo Scientific, USA), starting with 5 µL of total RNA, following the manufacturer's instructions. The mixturewas incubated at 25 °C for 5 min, followed by 60 min at 42 °C, and a final step at 70 °C for 5 min. The cDNA was then stored at -80 °C until qPCR amplification.

Primer design

Considering the genes of *D. rerio* that exhibited differential expression during CPF exposure in the study by Hausen et al. (2018), primer sets were designed to amplify regions of these genes via qPCR in the *P. gillii*. (Table 1). As biotransformation processes are expected to be induced by many xenobiotics, an isoform of glutathione S-transferase (*gst rho*) was also included in this list. All the genetic sequences used for the designs are available in the National Center for Biotechnology Information (NCBI) database (https://www.ncbi.nlm.nih.gov/). The NCBI web platform was utilized to employ the blastn algorithm (https://blast.ncbi.nlm.nih.gov/Blast.cgi), which uses mRNA sequences corresponding to the genes of interest from *D. rerio* as queries, to identify sequences of homologous genes in different members of the *Poecilia* genus. Once these sequences were selected, they were downloaded and contrasted against the assembly of *P. gillii* (GCA_903068115.1) through local blastn alignments via the discontinuous megablast algorithm within Geneious Prime software, v.2022.2. This alignment aimed to identify regions of interest within the contigs of the *P. gillii* assembly, including the coding sequences (CDS) and exon-intron boundaries for each gene.

Table 1. Sequences and contigs used for primer design for *P. gillii* target genes

Gene	*D. rerio* sequences	*P. mexicana* sequences	*P. gillii* contigs
anxa1b	NM_181759	XM_014968977	CAEGAJ010123909.1
			CAEGAJ010013974.1
cish	NM_001076617	XM_014998984	CAEGAJ010042873.1
ndufa2	NM_001024420	XM_014988418	CAEGAJ010083974.1
sec61g	NM_001002499	XM_014992915	CAEGAJ010088011.1
gstr	NM_001045060	XM_014996038	CAEGAJ010050275.1

Annexin A1b (*anxa1b*), cytokine inducible SH2-containing protein (*cish*), NADH dehydrogenase (*ubiquinone*) alpha subcomplex subunit 2 (*ndufa2*), Sec61 gamma subunit (*sec61g*) and glutathione transferase rho (*gstr*).

For primer design, the online tool IDT PrimerQuest (https://www.idtdna.com/PrimerQuest/Home/Index) was used. The genomic DNA regions identified in the contigs of the *P. gillii* assembly were used as inputs. Among the generated primers, five sets were selected for each gene. Through Geneious Prime, a new alignment was created between the target genes and the generated primers. Finally, one pair of primers for each gene was selected for qPCR on the basis of the position of both the forward and reverse primers within the CDS or at the exon-intron boundaries (Table 2). In addition, for each gene a primer set was designed to amplify the majority of the CDS region to confirm the gene sequences via Sanger sequencing. All the primers were synthesized by Macrogen Inc. (Seoul, South Korea).

Table 2. Primers used for qPCR and sequencing target genes in the livers and gills of *P. gillii*. One primer pair was used for qPCR and another one for sequencing (CDS).

Primer ID	Sequences (5′→3′; forward/reverse)	Amplicon size (bp)
anxa1b_CD	F-CAAGCCTTCTTGCAGCAGAC	938
	R-AATGTCCTGGTAGAGCGCTT	
anxa1b	F-GTCTTGGGACAGATGAGGATAC	114
	R-TCCTCCTCCAGATCCTTCTT	
cish_CD	F-GTGAACGAGGACTCAGGTCT	604
	R-GGCGCTGTGTCTGTTGAT	
cish	F-CCGTGCTTTCAGAGCAAAC	99
	R-CCCAGTACCAGCCTGAATTT	
gstr_CD	F-ATGGCCAAGGACATGACTCT	662
	R-CTTCAGTGCATCCATTCCCG	
gstr	F-GCAACTGAAGGACGAGATCAA	93
	R-GATGACATCGGCCAGAGAAA	
ndufa2_CD	F-GCGAGAAGCCTGGGC	276
	R-ACTGGGCCAGAGTCTTAAGG	
ndufa2	F-CCATCCTGATTCGAGAGTGTTC	100
	R-CTCAGCTGACATGTTGTCCA	
sec61g_CD	F-GATGCAGTTTGTTGAGCCCA	192
	R-ACCACCGACAATGATGTTGT	
sec61g	F-AGTTCGTCAAGGACTCCATAAG	109
	R-GAAGCCCATAATGGCAAATCC	
β-actin	F-AGATCATTGCCCCACCAGAG	111
	R-ACTCATCGTACTCCTGCTTGCTG	

Internal reference gene (*β-actin*, housekeeping), annexin A1b (*anxa1b*), cytokine inducible SH2-containing protein (*cish*), NADH dehydrogenase (ubiquinone) alpha subcomplex subunit 2 (*ndufa2*), glutathione transferase rho (*gstr*), and Sec61 gamma subunit (*sec61g*).

Quantitative real-time PCR (qPCR)

To evaluate the relative mRNA expression of *anxa1b*, *cish*, *gstr*, *ndufa2*, and *sec61g*, quantitative real-time PCR (qPCR) was performed via a CFX96 Real-Time System (Bio-Rad, USA). Reactions were conducted in a 10 μL mixture containing 5 μL of Power Track SYBR Green Master Mix (Thermo Fisher Scientific, USA), 0.40 μL of each primer (10 μM) (Macrogen Inc., Seoul, South Korea), 0.25 μL yellow sample buffer (Thermo Fisher Scientific, USA), 2.95 μL of RNase-free water, and 1 μL of cDNA as a template. Water was used as a negative control. Thermal cycling was carried out under the following conditions:

95.0 °C for 5 min, 45 cycles of 95.0 °C for 10 s, 60.0 °C for 20 s and 72.0 °C for 20 s. The melting curve was generated from 65.0 °C to 95.0 °C, with temperature increments of 0.5°C every 5 s. All reactions were performed in duplicate.

Melt curves were analyzed during the relative expression analysis to identify and exclude potential primer-dimer formations and nonspecific amplifications. Only threshold cycle (C_t) values within the range of 15 - 30 were considered valid. The relative mRNA expression of the target genes was calculated according to the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001).

To amplify the CDS, the previously described conditions were repeated with specific primers (Table 1). The resulting product was subjected to Sanger sequencing at Macrogen, Inc. (Seoul, South Korea). The sequences were subsequentlyassembled in Geneious Prime and compared to known *Poecilia genus* sequences in the NCBI database using blastn.

4.7 Statistical Analysis

The statistical analyses were conducted via the R programming language, version 4.3.2 (R Core Team, 2023). The outliers were identified with the function “identify_outliers” from the package rstatix, version 0.7.2 (Kassambara, 2023). With this function, values beyond greater than 1.5 times the interquartile range from Q1 or Q3 were identified as outliers and excluded. The resulting data were subjected to a Shapiro–Wilk test to assess normality. The parametric t-test then compared control and CPF-exposed fish, assuming a normal distribution. In cases of nonnormal distribution, the nonparametric Wilcoxon test (Mann-Whitney U) was used for comparisons. Differences between treated and control fish were considered statistically significant when the *p*-value was lower than or equal to 0.05 ($p \leq 0.05$). Visualization of the results was performed via the ggplot2 package, version 3.4.4 (Wickham, 2016).

5-RESULTS

5.1 Behavioral assessment

Following the exposure period, no deaths were recorded in either the exposed group or the control group, so the mobility of each fish was assessed in the swimming aquarium. No significant differences were found between groups concerning average speed, distance traveled, or exploration parameters during the 10 min analysis. Nevertheless, there was a

tendency toward an increase (226.11%) in the meander for the group exposed to CPF (Fig. 3D) compared with the control group. With respect to the spatial distribution within the experimental tank, no discernible pattern of preference for the left or right sections was found (Fig. 4A), but exposed fish tended to spend more time at the bottom of the tank (Fig. 4B).

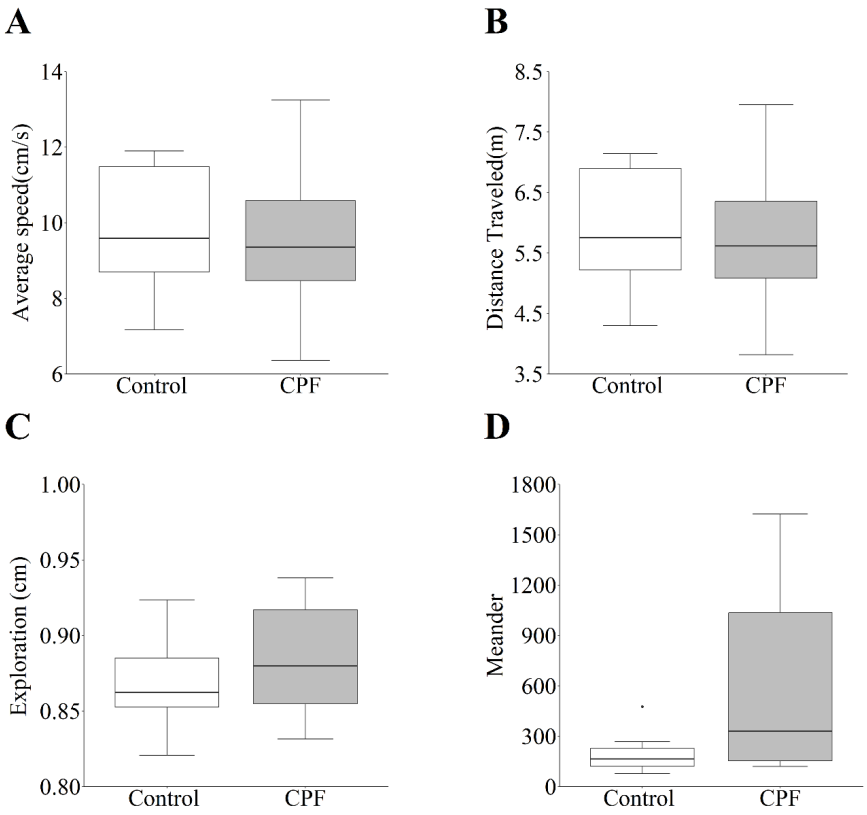

Figure 3. Swimming parameters assessed in *P. gillii* (n = 12) for 10 min, after a 48 h exposure to CPF (5.5 µg/L) and in the control group. Average speed (A), distance traveled (B), exploration absolute(C), meander (D). No significant difference between groups.

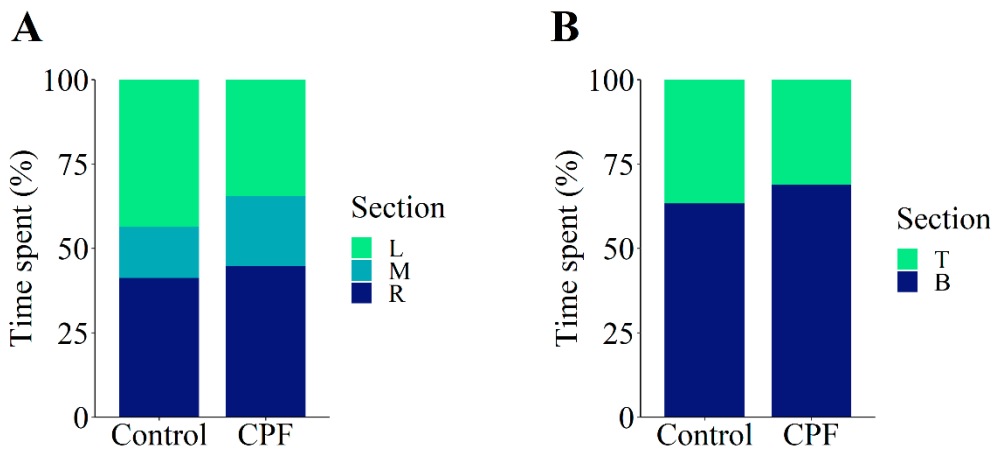

Figure 4. Location preference of *P. gillii* (n = 12) assessed for 10 min after a 48 h exposure to CPF (5.5 µg/L) and in the control group. A: Position across the horizontal axis, left (L), middle (M) and right (R) of the experimental tank. B: Position regarding the vertical axis, top (T) and bottom (B) of the experimental tank. No significant difference between groups.

With respect to the predation escape response, the results indicated that within the control group, 8 out of the 12 fish presented an evident C or S-start upon the release of the dummy, whereas in the group treated with CPF, this reaction was observed in 10 out of the 12 individuals. Nevertheless, there was no statistically significant difference in the reaction time between the stimulus and flexion conditions between the two groups (Fig. 5).

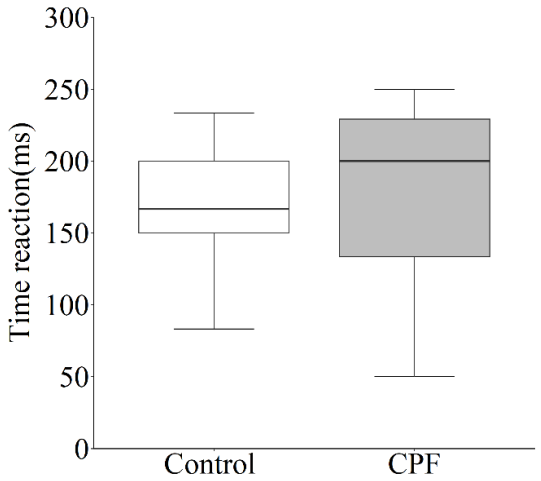

Figure 5. Average reaction time from stimulus to C or S-start in *P. gillii* (n=12) subjected to a simulated predatory attack after a 48-h exposure to CPF (5.5 µg/L) and in the control group. No significant difference between groups.

5.2 Biochemical biomarkers

Regarding the biomarkers of biotransformation and oxidative stress assessed, individuals of *P. gillii* exposed to CPF presented a significant reduction ($p \leq 0.05$) in liver CAT activity, with an inhibition of 19.66%, compared with the control group (Fig. 6A). Even though the remaining biomarkers showed no statistically significant differences, there were apparent responses observed in terms of a decrease in EROD activity (Fig. 6B) and an increase in LPO levels (Fig. 6D). No change in GST activity was observed (Fig. 6C).

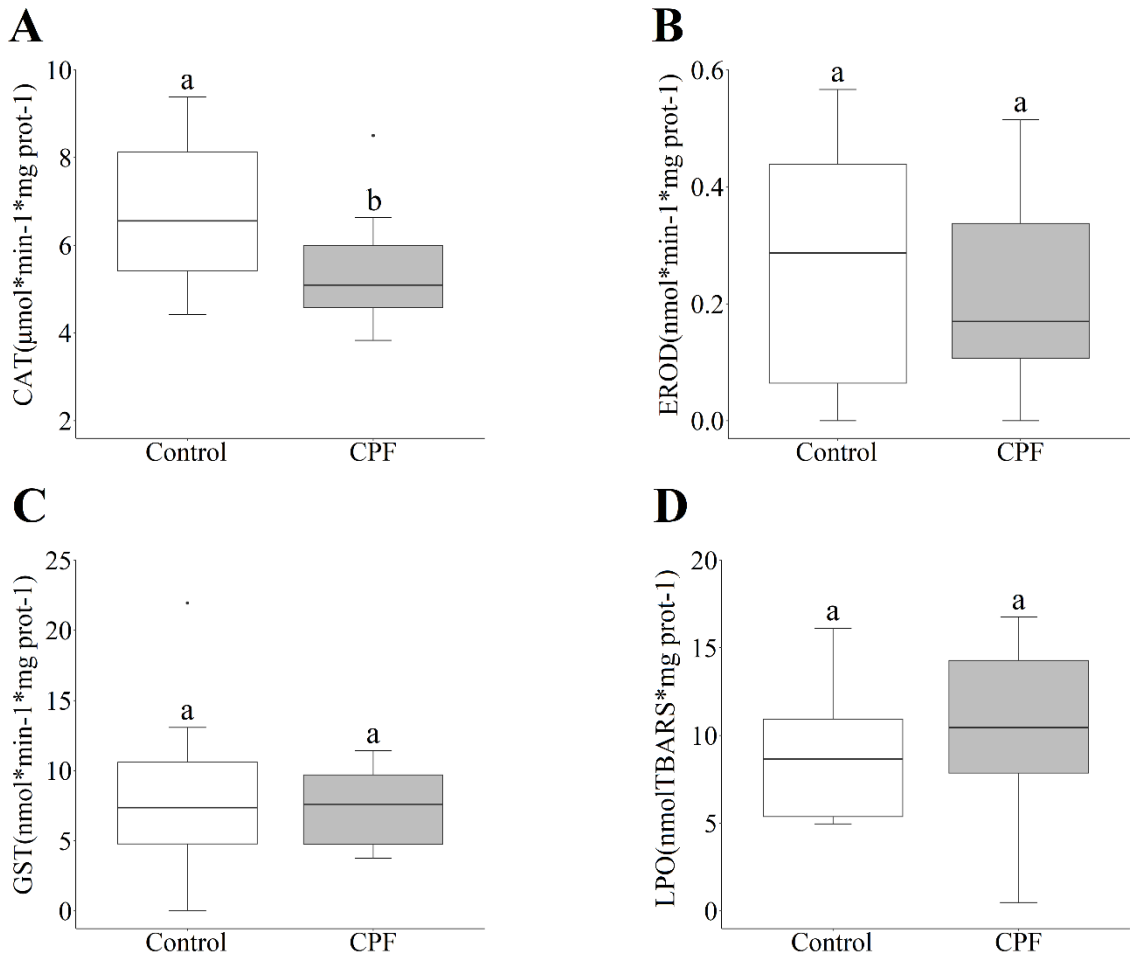

Figure 6. Activities of catalase (CAT) (A), ethoxyresorufin O-deethylase (EROD) (B), glutathione S-transferase (GST) (C), and lipid peroxidation (LPO) (D) in livers of juveniles *P. gillii* (n = 12), after 48 h of exposure to CPF (5.5 µg/L) and in the control group. Boxplot with different letters indicates statistically significant differences ($p \leq 0.05$) among groups.

In terms of neurotoxicity, a significant ChE inhibition of 28.6% was detected in the muscle of the fish exposed to CPF compared with that of the control fish ($p \leq 0.001$) (Fig. 7A). In the brain, an apparent decrease in ChE activity was observed; however, it was not significantly different from that in the controls (Fig. 7B).

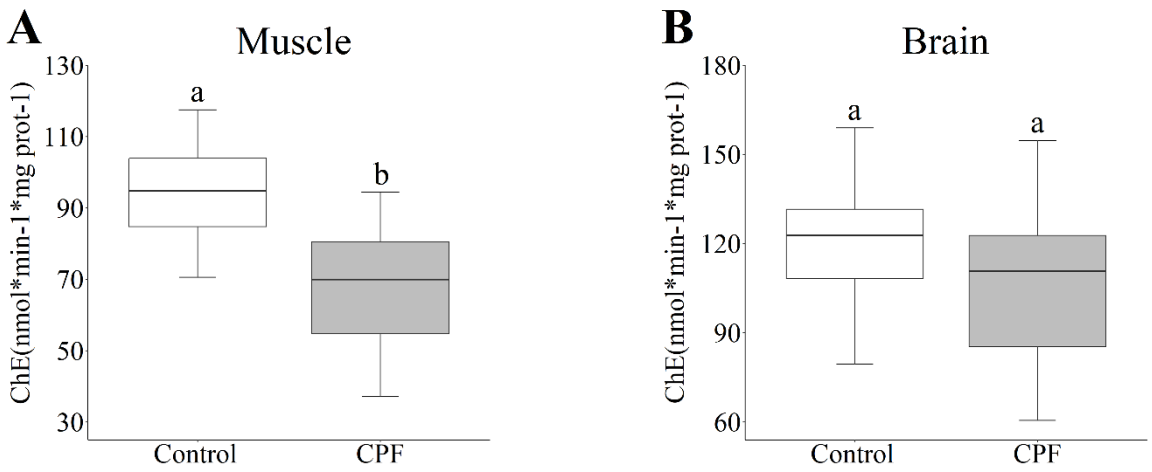

Figure 7. Cholinesterase (ChE) activity in muscle tissue (A) and the brain (B) of *P. gillii* (n = 12) under control conditions and after a 48 h exposure to CPF (5.5 µg/L). Boxplot with different letters indicates statistically significant differences ($p \leq 0.001$) among groups.

5.3 Gene expression

For each target gene, two pairs of primers were tested. Here we present the results of those pairs that yielded relatively high levels of expression in each tissue (pair 1 for each gene in Table 2). With respect to differences in their expression, *sec61g* and *gstr* were the only genes whose expression significantly changed after exposure to CPF. The expression of these genes was suppressed in the livers of exposed fish. In the case of the *sec61g* gene, the decrease in expression was 71.19% (Fig. 8A). For *gstr*, a reduction of 51.74% was observed (Fig. 8B). Even though no significant changes in expression of the other genes were observed, there were interesting trends in some of them. For example, the expression of *anxalb* tended to increase in the livers of exposed fish and decrease in their gills (Fig. 8C). Additionally, the expression of *ndufa2* tended to increase in both tissues of exposed fish (Fig. 8E).

With respect to blastn results for the assembled sequences, the identity percentages ranged from 98.7% to 100.0% compared with sequences from *P. mexicana*, *P. formosa*, and *P. reticulata*. The obtained sequences are available in GenBank under the following accession numbers: PQ299144 (*anxa1b*), PQ299145 (*cish*), PQ299146 (*gstr*), PQ299147 (*ndufa2*), and PQ299148 (*sec61g*).

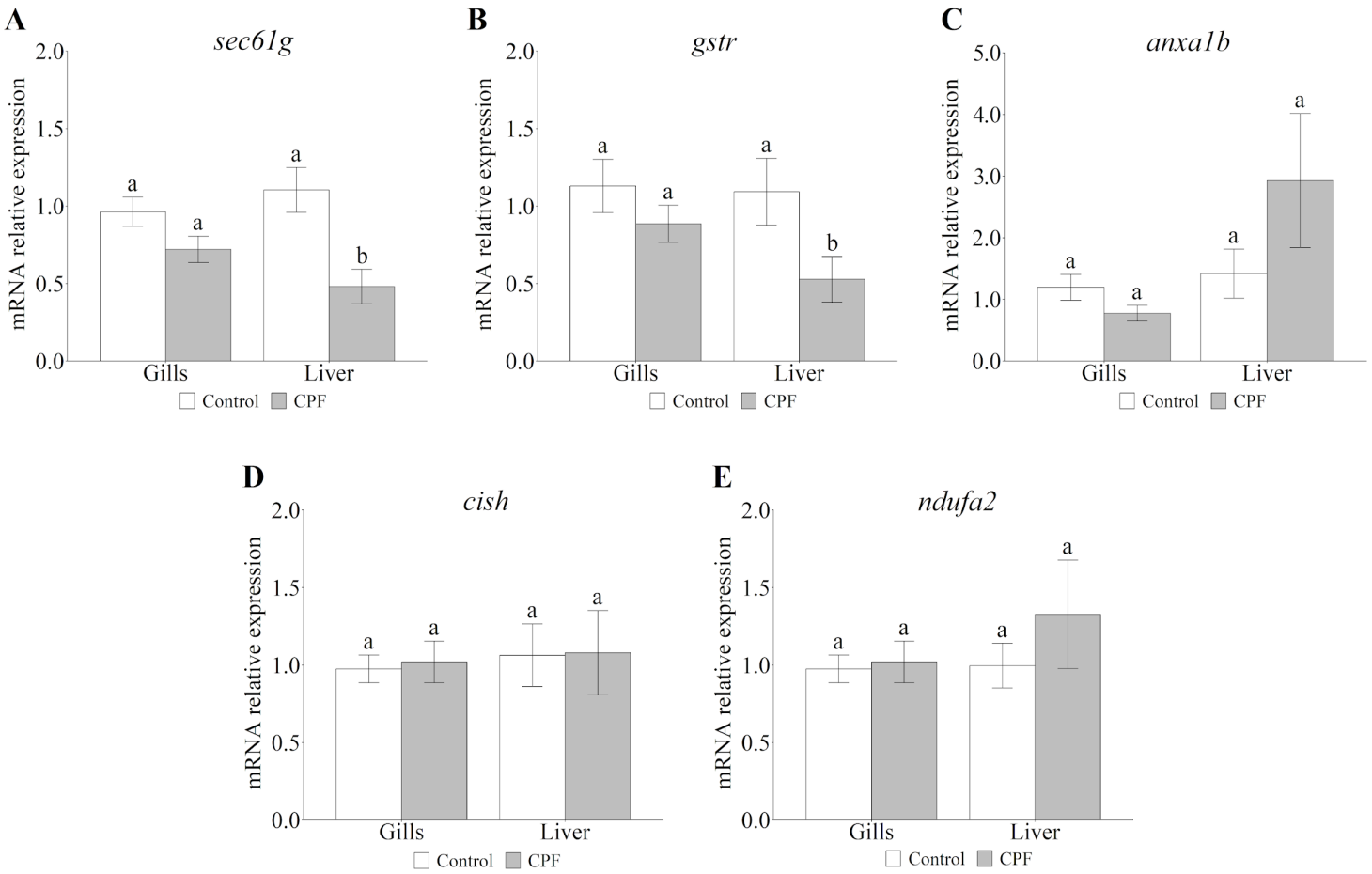

Figure 8. Relative mRNA expression levels of *sec61g* (A), *gstr* (B), *anxalb* (C), *cish* (D), and *ndufa2* (E) of *P. gillii* after exposure to CPF for 48 h (5.5 µg/L) in comparison to controls. Bars represent the means ± SD of the control and experimental groups (n = 12 fish per group). Values were normalized against *β-actin* as a housekeeping gene. Bars with different superscript letters indicate statistically significant differences ($p \leq 0.05$) between treatments.

6-DISCUSSION

Our study demonstrated that transient exposure to an environmentally relevant concentration of CPF was sufficient to induce clear subindividual effects on *P. gillii*. The initial response of the fish to CPF occurred at the level of gene expression transcription, even before physiological and behavioral changes became evident. However, the magnitude of gene expression changes was less pronounced than that previously reported in *D. rerio* by Hausen et al. (2018), who reported alterations in 42 genes following CPF exposure, with a majority (64%) exhibiting decreased expression, while those showing the most significant changes tended to be upregulated. In contrast, only *sec61g* showed a significant difference in liver tissue expression among the four genes analyzed, and was downregulated in the

exposed group, contrary to Hausen's findings. These discrepancies may be attributed to methodological differences, such as the higher CPF exposure concentration used in the reference study (7 mg/L vs. 5.5 μg/L), the different developmental stages (embryos vs. juveniles), and the species used in the bioassay.

Currently, the SEC61G protein subunit has been documented as a prognostic biomarker that is overexpressed in cancer patients (Liu et al., 2019; Ma et al., 2021; Zeng et al., 2023), but its role in toxicology has not been widely investigated, and the exact pathway through which CPF affects *sec61g* expression remains unclear. SEC61G is an essential subunit of the SEC61 translocon complex. This complex is crucial for mediating the translocation of polypeptides into the lumen or membrane of the endoplasmic reticulum (ER). It is involved in posttranslational modifications and retrotranslocation during the unfolded protein response (UPR) (Greenfield & High, 2000; Meng et al., 2021; Fan et al., 2022). Dysfunction of SEC61G could disrupt protein translocation, leading to reduced efficiency in synthesizing and folding critical cellular proteins, thereby compromising various functions, including the detoxification capacity of the liver.

Consequently, this disruption may lead to the accumulation of misfolded proteins, causing a homeostasis imbalance in the ER, also known as ER stress, ultimately compromising cell viability. Indeed, ER stress has been linked to the action of CPF in *Cyprinus carpio* and mammalian cell lines (Reyna et al., 2017; Anderson et al., 2021; Kim., et al., 2022; Li et al., 2023), triggering the UPR, which can lead to the generation of reactive oxygen species (ROS) (Li et al., 2023). During CPF biotransformation, the action of oxidoreductase enzymes can result in an overproduction of ROS, causing an imbalance between oxidant concentration and antioxidant enzyme activity. This imbalance can lead to irreversible damage to essential molecules, including membrane lipids, structural and nuclear proteins, and the DNA itself (Hilscherova et al., 2003; van de Wetering et al., 2021). Therefore, it is speculated that the induction of oxidative stress could be the primary pathway of CPF toxicity in certain organisms (Saulsbury et al., 2009; Fu et al., 2021).

Furthermore, studies in other organisms have demonstrated varying responses of SEC61 expression to different stressors. For example, Yan et al. (2017) reported that the gene expression of sec61 was significantly downregulated in oyster hybrids (*Crassostrea sikamea* × *Crassostrea angulata*) under low salt stress, suggesting an impact on cellular immunity via translocation events. At the proteomic level, Li & Wang (2021) reported a decrease in the SEC61 protein in *Crassostrea hongkongensis* samples exposed to high

concentrations of Cu and Zn. In contrast, Poynton et al. (2014) reported an increase in sec61 in *Mytilus edulis* after exposure to sublethal concentrations of Cd and Pb. Additionally, Yuan et al. (2018) reported a significant upregulation of *sec61a* subunit expression in *Daphnia magna* following an exposure to triphenyl phosphate (TPHP), an organophosphate flame retardant. Alterations in the expression of SEC61 could affect the efficiency of its translocation and, consequently, the elongation of peptides that require cotranslational translocation into the ER (Yuan et al. 2018; Li & Wang 2021). These findings underscore the relevance of SEC61 in cellular responses to environmental stressors, which have been identified principally through untargeted omics approaches.

Owing to the significant downregulation of gstr expression, this insecticide, like other organophosphoruses, undergoes biotransformation and detoxification processes within organisms. The GSTr protein isoform belongs to the ubiquitous GST isoenzyme family and is an exclusive aquatic subtype found in teleost fish, from an evolutionarily distinct branch compared with that of mammalian GSTs (Konishi et al., 2005; Tierbach et al., 2018). GSTr is particularly significant because of its potential protective role against ROS-induced damage. Research with recombinant GSTr enzymes, from the livers of *Anguilla anguilla* and *Micropterus salmoides*, suggests that they can rapidly detoxify electrophilic byproducts generated during oxidative stress, thereby reducing their reactivity and potential damage to cellular components (Doi et al., 2004; Carletti et al., 2008; Espinoza et al., 2012).

Furthermore, GSTs have been found to regulate processes beyond substrate binding, including stress responses, apoptosis, and cell proliferation, through protein-protein interactions (Laborde, 2010). The underexpression of *gstr*, as observed in our study, could decrease the efficient biotransformation of CPF, where it conjugates CPF metabolites to endogenous compounds such as amino acids, sugars, or sulfates, to increase their water solubility and reduce their toxicity (John & Shaike, 2015; De Anna et al., 2018). In another study, Xing et al. (2012) exposed *C. carpio* samples to three concentrations of CPF for 40 days. They noted a significant downregulation in GSTr activity in the liver, kidneys, and gills of the fish exposed to 11.6 g/L and 116 g/L CPF. In the case of the 1.16 g/L treatment, there was an increase in the expression of GSTr. They also quantified the activity of GSTm and GSTt and reported that there was no consistent pattern of regulated activity across the different CPF treatments among the three isoforms. These results underscore the variability in the transcriptional responses of different GST isoforms even within the same organism under identical treatment conditions. Several factors contribute to this variability, such as

tissue-specific concentrations of GST isoforms and xenobiotics, variations in subunit structures, and intricate regulation at the transcriptional and posttranscriptional levels influenced by substrate availability and the buildup of toxic conjugation products (Konishi et al., 2005; Contreras-Vergara et al., 2007;).

At the biochemical level, in contrast to the decrease observed in the molecular assessment, the GST enzyme activity did not significantly change after CPF treatment. Among the biomarkers of biotransformation and oxidative stress, only CAT showed a significant decline in enzymatic activity. Similar decreases have been reported in other fish species exposed to CPF, such as *Cnesterodon decemmaculatus* (Bonifacio et al., 2017), *Oreochromis niloticus* (Zahran et al., 2018; Abdel-Daim et al., 2020), *Heteropneustes fossilis* (Tripathi & Shasmal 2010; Mishra et al., 2020), *Clarias gariepinus* (Mokhbatly et al., 2020), *C. carpio* (Yonar et al., 2012; Yonar et al., 2018; Ural et al., 2013), and *Capoeta umbla* (Kirici et al., 2022). However, there are many reports of increased in CAT activity in fishes exposed to this insecticide, such as *Parachromis dovii* (Jimenez et al., 2021), *Poecilia reticulata* (Sharbidre et al., 2011), *Oncorhynchus mykiss* (Ali et al., 2020), *O. niloticus* (Özkan et al., 2012; Fırat & Tutus, 2020) and *C. carpio* (Georgieva et al., 2021). These results suggest that CAT activity in fish exposed to CPF varies significantly depending on the concentration, exposure time, and specific tissue, highlighting the complexity of the antioxidant response of aquatic organisms to contaminant exposure. This diminished CAT activity observed in *P. gillii*, combined with the downregulation of s*ec61g* expression discussed earlier, suggests that in addition to neurotoxicity, CPF can induce oxidative stress-related impairments even at short-term exposure and low doses.

Regarding the ChE activity inhibition in the muscle, this observation is consistent with a previous report by Redondo-López et al. (2022) on the same species exposed to a mixture of CPF and the fungicide difenoconazole, confirming that an environmentally relevant level of the insecticide is enough to cause significant neurotoxicity. However, in this study, we found a greater reduction in ChE activity in muscle tissue than in the brain. Similarly, Bonifacio et al. (2017) reported a significant decrease in ChE activity in the muscle and no effect on the brain of *Cnesterodon decemmaculatus* after 48 h of exposure to relevant environmental CPF concentrations. The inhibition of ChE, particularly acetylcholinesterase (AChE), by CPF is generally considered irreversible, as once the inhibitor binds, breaking the bond between phosphorus and the enzyme's active site can take

from a few hours to several days, making recovery dependent on the synthesis of new replacement enzymes (Fulton & Key, 2001; Bodnar et al., 2021).

At the behavioral level, an apparent increase in meander was observed. Similar effects have been documented in *D. rerio* exposed to various stressors. For example, Biswas et al. (2018) reported increased meandering following mercury exposure, along with other indicators of swimming impairment and stress. Similarly, Sarasamma et al. (2019) noted a dose-dependent rise in this behavior in response to nanoparticles. Furthermore, Razali et al. (2022) reported an increase in the meander pattern, turn angle, and erratic movement in individuals injected with the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. These findings suggest that increased meandering could be a sensitive behavioral endpoint in fish and might serve as an early indicator of neurotransmission dysfunction. Given the neurotoxic effects of CPF, swimming behavior in aquatic organisms is commonly monitored in toxicity studies, as alterations in locomotor activity can signal neural disruptions (Little & Finger, 1990; Toni et al., 2023). Previous bioassays with other members of the *Poecilia* genus have shown that CPF-treated fish exhibit abnormal behaviors, including aggression, rapid gulping, increased opercular movement, and erratic swimming (Sharbidre et al., 2011).

Overall, our results demonstrate that the exposure of juvenile *P. gillii* to an environmentally relevant concentration of CPF can downregulate the transcription of genes related to biotransformation and protein synthesis, impairing their ability to metabolize xenobiotics and maintain the cellular oxidative balance. Furthermore, the neurotoxicity associated with the insecticide's mechanism of action can significantly compromise the fitness of these organisms which could manifest as incipient behavioral impairment. Although interactions with CPF in surface waters may be sporadic and short-term, the adverse effects of CPF are persistent and necessitate extended recovery periods (Williams et al., 2014; Qiu et al., 2017). Notably, behavioral alterations can occur at exposure levels far below those associated with lethal effects, up to 10 to 100 times lower (Gerhardt, 2007; Sandoval-Herrera et al., 2019; Redondo-López et al., 2022). These findings underscore the significant ecological implications of even low-level exposure, and the potential risks associated with CPF exposure in natural ecosystems, which is worrisome in highly biodiverse countries such as Costa Rica.

7-CONCLUSIONS

The genes *sec61g* and *gstr* can serve as biomarkers for the exposure of *P. gillii* to CPF. As their suppression is related to impairments in key cellular processes, these biomarkers can contribute to the description of the cellular stress caused by the insecticide. The observation of significant gene expression changes only in liver tissue confirms the importance of this organ as a target for assessing the cellular effects of pollutants.

Environmentally relevant exposure of *P. gillii* to CPF induced neurotoxicity, accompanied by signs of an impaired antioxidant response and apparent behavioral changes previously associated with xenobiotic exposure. This evidence highlights the hazardous effects of this insecticide on aquatic biota.

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ARRIVE Statement

This study adheres to the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines. All procedures complied with the current animal welfare standards set by UNA and were conducted in alignment with international guidelines and regulations for animal research. Authorization to access the animals for molecular and biochemical analyses was granted by the National Biodiversity Management Commission (CONAGEBIO) under permit number R-CM-UNA-012-2022-OT-CONAGEBIO.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the authors used ChatGPT 3.5 in order to improve the writing, ensuring greater clarity and fluency in the manuscript. After using this tool/service, the authors reviewed and edited the content as needed and take full responsibility for the content of the published article**.**

Declaration of interests

☒ 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.

Author contributions

Daniel Sánchez-González: Conceptualization, Formal analysis, Investigation, Methodology, Visualization, Writing - original draft.

Kinndle Blanco-Peña: Funding acquisition, Conceptualization, Resources, Project administration, Methodology, Investigation, Formal analysis, Writing - original draft.

Frank Solano-Campos: Conceptualization, Resources, Methodology, Formal analysis, Writing - original draft.

Karla Solano: Resources, Methodology, Investigation, Formal analysis, Writing - original draft.

Freylan Mena: Funding acquisition, Conceptualization, Resources, Methodology, Investigation, Formal analysis, Supervision, Writing - original draft.

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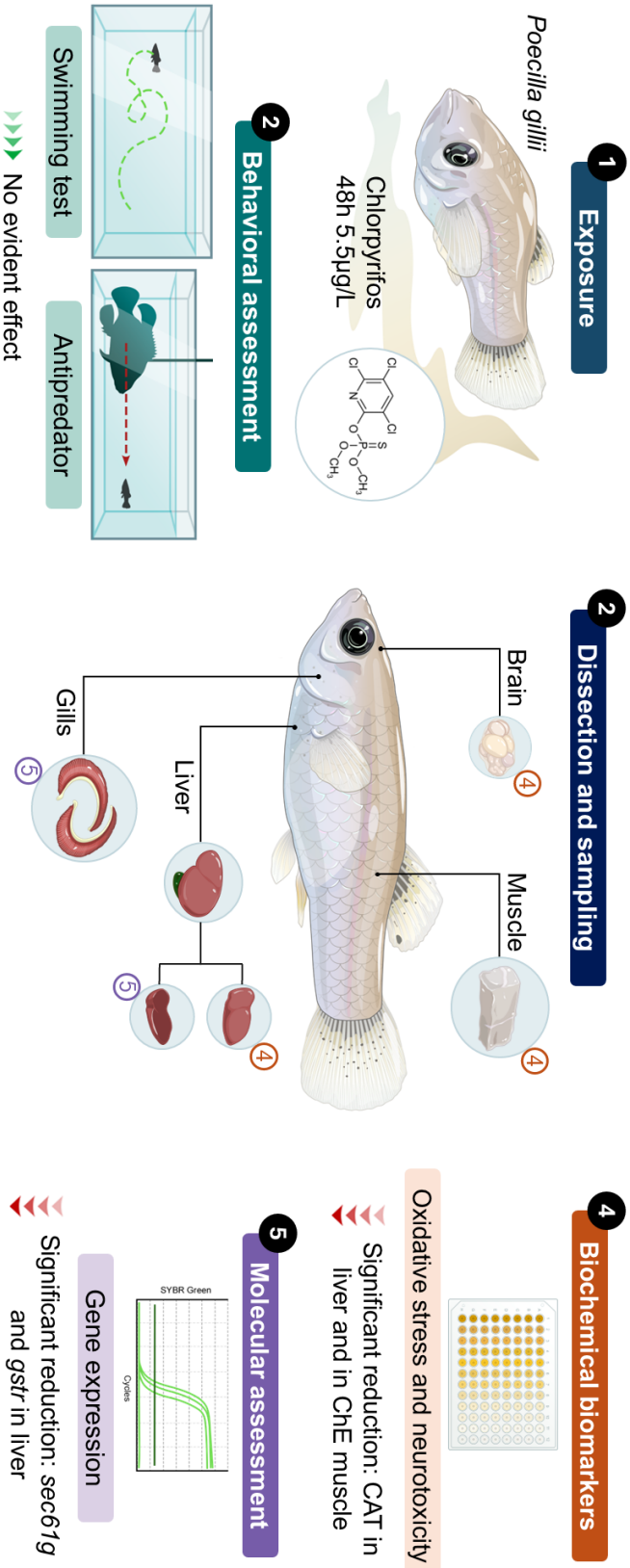
8-ANEXOS

Figure S1. Graphical abstract



Conclusiones

Los resultados de esta investigación representan un avance en el estudio de los efectos del clorpirifos (CPF) en la especie nativa *Poecilia gillii*. Por primera vez, se evaluó un conjunto de genes involucrados en diversos procesos biológicos mediante el uso de técnicas moleculares e informáticas, lo que permitió ampliar la visión sobre los procesos metabólicos afectados por la exposición a este insecticida. Específicamente, se demostró que una exposición transitoria a una concentración ambientalmente relevante de CPF puede alterar la transcripción de genes clave como *gstr* y *sec61g*. Estos genes no solo ofrecen una herramienta valiosa para la comprensión de las interacciones entre los contaminantes y los organismos, sino que también tienen el potencial de servir como biomarcadores para diagnosticar la salud ambiental en ecosistemas acuáticos.

El gen *sec61g* se destacó como un biomarcador para la exposición al CPF, ofreciendo una nueva perspectiva sobre su función en los procesos de biotransformación y respuesta celular. Hasta ahora, la mayor parte de las investigaciones relacionadas con este gen se han centrado en su papel en enfermedades como el cáncer, lo que enfatiza la relevancia de este estudio al remarcar su aplicación en un contexto de ecotoxicología. Por su parte, el gen *gstr* también sobresale como indicador sensible en exposiciones de corta duración, reafirmado su papel como adaptación en este grupo de organismos durante los procesos de biotransformación.

Además, se corroboró el valor de *P. gillii* como especie bioindicadora debido a su sensibilidad a concentraciones ambientalmente relevantes de CPF, lo cual se reflejó en alteraciones significativas en la expresión génica y el evidente efecto neurotóxico. Su amplia distribución en la región y su relevancia en la red trófica refuerzan su utilidad para evaluar los impactos de los agroquímicos en ambientes acuáticos.

Finalmente, la información recabada en este trabajo sienta las bases para futuras investigaciones destinadas a profundizar en los efectos de los plaguicidas sobre la regulación de la expresión génica. Esto permitirá desarrollar enfoques más efectivos para proteger a los organismos acuáticos, que son especialmente vulnerables a la contaminación por agroquímicos, contribuyendo así a la conservación de los ecosistemas.

Recomendaciones

El hallazgo de cambios significativos en la expresión génica únicamente en el tejido hepático de *P. gillii* reafirma la relevancia de este órgano como objetivo principal para evaluar los efectos celulares de los contaminantes. Por lo tanto, se recomienda que futuras investigaciones continúen explorando este enfoque, dado el papel central del hígado en la biotransformación de xenobióticos.

Aunque los genes *anxa1*, *cish* y *ndufa2* no mostraron cambios estadísticamente significativos en su expresión, su evaluación en estudios posteriores con periodos de exposición más prolongados o concentraciones mayores de CPF podría proporcionar información adicional sobre su posible transcripción diferencial bajo distintas condiciones de estrés.

Asimismo, es necesario analizar la respuesta de los genes *sec61g* y *gstr* frente a otros estresores individuales y en mezclas, con el fin de identificar posibles patrones diferenciales específicos para cada xenobiótico. Esto facilitaría la identificación o clasificación de los grupos de contaminantes que estén afectando el medio, ampliando su aplicabilidad en la monitorización de la salud ambiental.

Se sugiere continuar con un enfoque de evaluación multinivel que permita visualizar de manera integral los efectos de los estresores. Esta metodología permite un mayor aprovechamiento de las muestras e información que se genera en cada ensayo para una valoración más completa de los impactos celulares y fisiológicos.

Además, la implementación en posteriores estudios de técnicas de secuenciación de alto rendimiento como ARNseq, que permitan evaluar de manera más exhaustiva los cambios en el transcriptoma, podría facilitar la identificación de nuevos genes que tengan el potencial como marcadores de estrés inducido por CPF.

La observación de efectos claros a nivel individual en peces, utilizando biomarcadores a nivel molecular y bioquímico, debe contribuir a validar la aplicación de estos métodos en esquemas regulatorios sobre el riesgo de la contaminación química en los ambientes acuáticos.