Cytogenotoxic potential and toxicity in adult *Danio rerio* (zebrafish) exposed to chloramine T

Carla Letícia Gediel Rivero-Wendt[1], Ana Luisa Miranda-Vilela[2], Luana Garcia Fernandes[1], Andreza Negrili dos Santos[3], Igor Leal Brito[3], Jeandre Augusto dos Santos Jaques[3,4], Carlos Eurico Fernandes[1*]

[1]Programa de Pós-Graduação em Biologia Animal, Instituto de Biociências (INBIO) and Laboratório de Patologia Experimental, Universidade Federal de Mato Grosso do Sul, Campo Grande, Mato Grosso do Sul, Brazil.
[2]Independent Researcher, Brasília 70.863-100, Brazil.
[3]Programa Multicêntrico de Pós-Graduação em Bioquímica e Biologia Molecular (PBBqBM), Instituto de Biociências (INBIO), Universidade Federal de Mato Grosso do Sul (UFMS), Campo Grande, Mato Grosso do Sul, Brazil.
[4]Programa de Pós-Graduação em Ciências Farmacêuticas (PPGFARM), Faculdade de Ciências Farmacêuticas, Alimentos e Nutrição (FACFAN), Universidade Federal de Mato Grosso do Sul (UFMS), Campo Grande, Mato Grosso do Sul, Brazil.

*Corresponding Author:
Carlos Eurico Fernandes[1]
Costa e Silva Avenue, s/n, Campo Grande, Mato Grosso do Sul, 79070-900, Brazil.
Email address: carlos.fernandes@ufms.br

Abstract

Background. Chloramine-T (CL-T) is a synthetic sodium salt used as a disinfectant in fish farms to combat bacterial infections in fish gills and skin. While its efficacy in pathogen control is well-established, its reactivity with various functional groups has raised concerns. However, limited research exists on the toxicity of disinfection by-products to aquatic organisms. Therefore, this study aims to assess the lethal and sublethal effects of CL-T on adult zebrafish by examining biomarkers of nucleus cytotoxicity and genotoxicity, acetylcholinesterase (AChE) enzymatic inhibition, and histopathological changes.
Methods. Males and females adult zebrafish (wildtype AB lineage) specimens were exposed to 70, 140, and 200 mg/L of CL-T and evaluated after 96 h. Cytotoxic and genotoxic effects

were evaluated by estimating the frequencies of nuclear abnormalities (NA), micronuclei (MN), and integrated optical density (IOD) of nuclear erythrocytes. Histopathological changes in the gills and liver were assessed using the degree of tissue changes (DTC). AChE activity was measured in brain samples~~The zebrafish brains were collected for AChE activity analysis~~.

Results and conclusions. At a ~~sublethal~~ concentration of 200 mg/L, NA increased, indicating the cytogenotoxic potential of CL-T in adult zebrafish. Morphological alterations in the nuclei were observed at both 70 and 200 mg/L concentrations. Distinct IOD profiles were identified across the three concentrations. There were no changes in AChE ~~enzymatic~~ activity in adult zebrafish. The DTC scores were high in all concentrations, and histological alterations suggested low to moderate toxicity of CL-T for adult zebrafish.

Keywords: ~~Chloramine-T,~~ integrated optical density ~~(IOD),~~ nuclear abnormalities, micronucleus test, gill and liver histopathology, acetylcholinesterase activity

Kommentiert [JO1]: This is already part of the title sao that it makes no sense to consider it also as a keyword

Introduction

Disinfectants play a crucial role in various industries, including agriculture, fish farming, slaughterhouses, and kitchens (Haneke, 2002), by preventing the proliferation of bacteria, viruses, and fungi and maintaining water quality. However, it is well-known that certain disinfectants and their residues can have adverse effects on the environment and human health (Alidadi Soleiman et al. 2017). When disinfectants are used for water disinfection, they react with natural organic matter, leading to the formation of disinfection by-products (DBPs), many of which are cytotoxic, genotoxic, mutagenic, and teratogenic in nature (Cui et al. 2021; Yadav et al. 2020).

Kommentiert [JO2]: Consider to delete this phrase: It is redundant.

The use of disinfectants, such as Chloramine-T (CL-T), in fish farms is common to combat various pathogens that can impact fish production. For adult fish, typical CL-T concentrations for prophylaxis or disinfection between 5-20 mg/L. It is worth noting that the baths can be used as static baths once a day for 60 minutes or as a low flow rinse treatment for three consecutive days (for intensive treatment) or alternate days with three baths of 60 min each (FDA 2014; Powell et al. 1994; Sanchez et al. 1996). However, their unintended effects on non-target species and the environment can result in pollution of aquatic ecosystems. Thus,

Kommentiert [JO3]: Incomplete sentence with missing verb. Consider to add "are used" or "are applied" at the end.

Kommentiert [JO4]: Consider to delete this phrase: It is redundant.

Kommentiert [JO5]: Consider to delete this adjective. It is a doubling to "non-target species" because effects on these species are by definition not intendend.

it is essential to control the use of chemical products to minimize environmental contamination (Agoba et al. 2017; Bahadir et al. 2019; Gustavino et al. 2005).

CL-T, a disinfectant employed since the 1990s for treating intensively cultured fish for human consumption (Bullock et al. 1991), is a synthetic sodium salt known as N-chloro-r-toluenesulfonamide, with the chemical formula r-$CH_3C_6H_4SO_2NClNa.3H_2O$ (Nayak et al. 2022). It readily dissolves in water and is also used for chlorination in water treatment due to its reactivity with a broad range of functional groups. CL-T reacts with oxidizable materials, including amines, amino acids, humic substances, and other organic and inorganic compounds present in the treated water and subsequent dilution waters (Nayak et al. 2022; Schmidt et al. 2007).

Furthermore, it is important to consider (i) the continuous discharge of chlorine-treated waters in wastewater treatment plants; (ii) the intermittent discharge of aquaculture effluents containing CL-T in aquaculture facilities (averaging approximately 40 discharges per year per facility employing CL-T); and (iii) the scarcity of studies reporting the toxicity of DBPs to aquatic organisms (Schmidt et al. 2007).

Therefore, a comprehensive evaluation of the effects of CL-T on aquatic organisms is necessary to better understand its impact. This assessment would enable the investigation of ecotoxicological effects, examining both target and non-target species within fish farms and aquaculture systems.

In this context, freshwater aquatic organisms, such as *Danio rerio* (zebrafish), are used to assess the toxicity of chemicals. Zebrafish share 70-80% genetic homology with humans and possess vertebrate-like structures (Bertotto et al. 2020; Gunnarsson et al. 2008). Adult zebrafish and embryos serve as valuable model organisms for screening the toxicity of drugs and chemicals (Brannen et al. 2010). Recent studies have reported the lethality and sublethal toxicity associated with CL-T exposure using zebrafish embryos as model organisms. In a short-term acute toxicity test at 96 hours, CL-T-induced abnormalities were observed in zebrafish embryos. Concentrations above 64 mg/L resulted in cardiac oedema and reduced heart rate. Furthermore, acetylcholinesterase (AChE) inhibition is consistent with the morphological and equilibrium disturbances observed. Therefore, the use of CL-T in zebrafish embryos should be used with caution and the effects on adults need to be assessed (Rivero-Wendt et al. 2023).

~~Recent studies have reported the lethality and sublethal toxicity associated with CL-T exposure using zebrafish embryos as model organisms (Rivero-Wendt et al. 2023).~~ Therefore, to compare the findings obtained from zebrafish embryos and examine alternative biomarkers

of effects that could potentially indicate threats to this species. In the present study, our objective is to evaluate the sublethal ~~lethal and sublethal~~ effects of CL-T in adult zebrafish by examining biomarkers of nucleus cytotoxicity and genotoxicity, enzymatic inhibition of ~~acetylcholinesterase (~~AChE~~)~~, and histopathological changes.

Material & Methods

Experimental design

The zebrafish (wildtype AB lineage) used in this study were obtained from a quality-certified aquarium (Ogawa e Sato LTDA) acquired at 3 months of age, with a length of 2-3 cm and acclimated at the Aquaculture Station of the Federal University of Mato Grosso do Sul (CEUA establishment license no. 3.128/2021). They were housed in 10 L aquariums for the acclimatization period. In the test, a total of 40 specimens were used, males and females, 10 specimens were used per treatment (control, 70, 140 and 200 mg/L) and divided into three replicates. All samples were collected from treated and control specimens, only from individuals that survived until the end of the 96 h exposure. ~~All samples were taken from the exposed and survival specimens.~~ The water conditions were maintained with a waterfall filter, a temperature of 28 ºC, pH of 7 ± 0.5, conductivity of 55 ± 50 µS/cm, and dissolved oxygen levels equal to or above 95% saturation. A 12:12 h photoperiod cycle was followed. The adult zebrafish were fed twice daily with a commercial artificial diet (TetraMin flakes fish food, Germany). Sublethal concentrations of CL-T ($C_7H_7ClNNaO_2S.\ 3H_2O$) (Halamid®) for adult zebrafish were determined based on the LC_{50} values obtained from previous evaluations in zebrafish embryos (143.05 ± 3.11 mg/L at 24 h and 130.97 ± 7.40 mg/L at 96 h) (Rivero-Wendt et al. 2023). ~~The concentrations selected for this study were 70, 140, and 200 mg/L. These concentrations were used to assess the effects of CL-T on genotoxicity markers, including NA, MN frequency, and IOD of erythrocyte nuclei. Additionally, brains AChE activity and morphological effects on the gills and livers of adult zebrafish were examined.~~ CL-T (Halamid®) with 98% purity was obtained from Western Chemical Inc. Exposure to CL-T was static for 96 h. The animals were euthanized following Concea guidelines (2018). All experimental procedures were conducted following the guidelines and regulations approved by the Ethics Committee for the Experimental Use of Animals (CEUA; approval no. 3.128/2021).

Nuclear cytotoxicity/genotoxicity and ~~Integrated~~ integrated optical density

Kommentiert [JO6]: Incomplete sentence. Please revise.

Kommentiert [JO7]: You have to state the sex ratio in the treatments or that the sex ratio was not recorded.

Kommentiert [JO8]: Either here or in the result chapter you have to provide the number of specimens per treatment that did not survive the 96 h exposure (or the percentage). The information in line 176 (n = 38) indicates that a total of 2 specimens died.

The methodology employed for the test followed the procedure outlined by ~~(~~Hooftman & de Raat (1982). After euthanasia, p~~P~~eripheral blood samples were collected from the caudal vein of the zebrafish using a 10 ~~-~~µL micropipette containing 3% EDTA. Immediately after collection, slide smears were prepared from the blood samples. The slides were fixed in methanol for 5 min, air-dried, and then stained with May-~~Grunwald~~Grünwald-Giemsa-Wright (MGGW) solution using the modified Rosenfeld method to enhance the visualization of erythrocyte nuclei for morphological analysis (Ranzani-Paiva et al. 2013).

~~Nuclear abnormalities (~~Nuclear abnormalities (NA)~~)~~ were defined according to Carrasco et al. (1990). Erythrocytes with two nuclei were considered binuclei; blebbed nuclei, when they exhibited a relatively small evagination of the nuclear membrane, containing euchromatin; lobed nuclei, when evaginations larger than the blebbed nuclei, which can have several lobes; and vacuolated nuclei, when an appreciable depth chromatin failure without nuclear material was observed. In this work, one thousand erythrocytes cells with complete cytoplasm were scored per fish and specific NA types were not discriminated (Carrasco et al. 1990).

For the analysis of micronucleus (~~micronuclei (~~MN)~~)~~, three thousand erythrocyte cells with complete cytoplasm were scored per fish. ~~Micronuclei~~ MN were identified when they were smaller than one-third of the main nuclei (a), did not touch the main nuclei (b), and exhibited a non-refractive circular or ovoid chromatin body with a similar staining pattern as the main nucleus (c) (Rivero-Wendt et al. 2020).

To calculate the IOD of the erythrocyte nuclei, ten images (RGB 4096 x 3286 pixels) were randomly captured from each blood smear slide at a magnification of 1000x using bright-field microscopy (Opticam 500R®) equipped with LOPT1001® optics. The images were transformed into 8-bit type and the threshold tool in ImageJ software version 1.53a (Ferreira & Rasband 2012)~~.~~ was used for image analysis. In the "Analyze/Set Measurements" menu, the area (μm^2), circumference (µm), roundness (0-1), and Ferret's diameter (µm) of 15 randomly selected nuclei per image were measured. The nuclear erythrocyte volume (Vn) was estimated using the formula $Vn = (4/3\ \pi) \times r^3$, in which Vn represents the nuclear volume (μm^3) and r represents the nuclear radius (Ferret's diameter / 2). The calculation of IOD followed the method described by Hardie et al. (2002), as follows:

$$\sum_{i=0}^{n} - log\ 10\ (\frac{IFi}{IBi})$$

in which *n* = total number of pixels in the nucleus; *IFi* = intensity of the nuclear pixels; and *IBi* = intensity of background image pixel (Hardie et al. 2002).

Acetylcholinesterase (~~Acetylcholinesterase (~~AChE)~~)~~ activity inhibitory assay

The inhibition of ~~acetylcholinesterase (~~AChE~~)~~ was assessed following the Ellman's assay protocol (Ellman et al. 1961). The primary modifications involved the use of a phosphate/HEPES buffer (50/5 mM, pH 7) to enhance the stability of 5,5-dithiol-bis-(2-nitrobenzoic acid) (DTNB), which was added after catalysis to minimize potential interference with AChE activity. Tetraisopropyl pyrophosphoramide (iso-OMPA) was employed as a selective inhibitor of butyrylcholinesterase (BuChE). After conducting the fish toxicity test, the brains of ~~surviving~~ adult fish (n= 38) were crushed in 10 mM Tris buffer (pH 7.2) using a mortar and pestle to create homogenates, while keeping them on ice. The enzymatic activity analysis was performed in 96-well plates using the following experimental procedure, with a final volume of 300 µL. Briefly, 150 µL of phosphate/HEPES buffer (100/10 mM, pH 7), 80 µL of distilled water, 20 µL of homogenates, and 20 µL of 292 µM iso-OMPA were mixed. The plate was preincubated at 37°C for 30 min. To initiate the reaction, 30 µL of 10 mM acetylthiocholine (ACSCh) was added and incubated at 37°C for 30 min. Subsequently, 20 µL of 51 mM neostigmine bromide prepared in phosphate/HEPES buffer (100/10 mM, pH 7) was added to complete the reaction. Finally, 20 µL of 8.5 mM DTNB prepared in phosphate/HEPES buffer (100/10 mM, pH 7) was promptly added to induce the production of thiocholine. The hydrolysis of acetylthiocholine (ACSCh) by ~~acetylcholinesterase (~~AChE~~)~~ leads to the formation of 5-thio-2-nitrobenzoate anion (TNB) through the reaction with DTNB. The concentration of TNB was determined at λmax = 412 nm using a SpectraMax Plus 384 Microplate Spectrophotometer (Molecular Devices LLC, USA) at room temperature. The activity of AChE was expressed as µmol hydrolyzed ACSCh/hr/mg protein. The protein content of the samples was determined using the method described by Bradford (1976) with bovine serum albumin (BSA) as the standard (Bradford 1976).

Gills and liver histopathology

Following euthanasia, the head of the fish was separated from the body for ~~the fish were~~ immediately immersed in Davidson's solution for 24 h and subsequently subjected to decalcification for three days using a solution containing 0.7 g EDTA, 8 g sodium and potassium tartrate, 0.14 g sodium tartrate, 120 mL HCl, and distilled H_2O to a final volume of

900 mL. The fish specimens were then processed for histological analysis by embedding them in paraffin, cutting them into 3 µm-thick sections, and staining with hematoxylin and eosin for examination under bright-field microscopy (Suvarna et al. 2019).

Histopathological findings were classified based on the degree of tissue changes ~~(degree of tissue changes (~~DTC~~)~~ as described by (Bernet et al. 2001), which adopted the standard reaction (*a*) features and assigned importance scores (*w*) to these changes. Table 1 shows the histological alterations considered in this study and their corresponding importance degrees. The DTC was estimated using the following formula:

$$DTC = \sum_{alt} (a \times w),$$

in which *a* represents the distribution of damage (0 = absent; 1 = minor; 2 = moderate; and 3 = marked occurrence) and *w* represents the reversibility degree of the damage (1 = easily reversible; 2 = moderate alterations with probable reversion after exposure; and 3 = irreversible alterations). The frequency (%) of each *w* value, adjusted according to the respective values, was calculated based on the overall sum ($\sum_{alt}$) for each alteration using the following formula: $F\% = [(a \times w) / \sum_{alt}] \times 100$ (Silva et al. 2021).

Statistical analysis

The normal distribution of continuous variables was assessed using the Shapiro-Wilk test. Differences between the groups under analysis were evaluated using either ANOVA or the Kruskal-Wallis test, depending on the data distribution. When ANOVA yielded significant results, the Bonferroni's post-hoc test was used for pairwise comparisons between the treatments. For significant Kruskal-Wallis results, the Mann-Whitney U test was employed to examine differences between the treatments through pairwise comparisons.

Thus, the effect of CL-T concentrations on the erythrocyte ~~micronucleus~~ MN~~(MN~~ test~~)~~ and histopathological findings ~~(degree of tissue changes~~ DTC~~)~~ was compared using Kruskal-Wallis analysis of variance with the Mann-Whitney U test, as the data were not normally distributed. Total ~~nuclear abnormalities (~~NA~~)~~, erythrocyte nuclear morphometric data (area, circumference, roundness, and volume), and AChE activity inhibitory assay were compared using a one-way ANOVA (general linear model – GLM). The intensity over distance (IOD) curve fit was generated from the normalized values (y/mean). Box plots were used to represent the median, 25th and 75th quartiles, and the minimum and maximum interquartile intervals between concentrations. The IBM SPSS Statistics version 22.0, GraphPad Prism 8.1 (San Diego, California, USA), and OriginPro version 2022 (OriginLab

Corporation, Northampton, MA, USA) software packages were utilized for statistical analyses. A significance level of $p < 0.05$ was considered statistically significant.

Results

Nuclear cytotoxicity/genotoxicity and Integrated Optical Density OD

The response pattern of CL-T exhibited was concentration dependence. After 96 h, a The effects of sublethal concentrations (mg/L) of CL-T on cytotoxicity/genotoxicity, nuclear optical density, and histopathological alterations in the gills and liver of adult zebrafish were investigated. sSignificant differences increase were observed in total NA between was observed at a concentration of 200 mg/L compared to the control group and at a concentration of 200 mg/L (Fig. 1). the treatment groups. (p = 0.014 0.0162) . However, after 96 h, significant differences in NA only occurred at a concentration of 200 mg/L (Fig. 1).

The assessment of genotoxicity using the MN assay showed no genotoxic effects of CL-T. However, IOD decreased at concentrations of 70, 140, and 200 mg/L. At 200 mg/L, the curve fit displayed a different profile compared to 70 and 140 mg/L (Fig. 2 and 23). Figure 3 shows the presence of two distinct wavelengths for the cell populations exposed to CL-T concentrations. Notably, the increase in IOD was only detected in a specific pixel wave (3.5 - 5.8) at a concentration of 200 mg/L.

AChE inhibition

During the 96-h exposure to sublethal concentrations of CL-T, AChE activity was not significantly inhibited in the brain of adult zebrafish (Fig. 34).

Gill and liver histopathology

Figure 45 illustrates the degree of tissue changes (DTC) in the gills and liver, along with the frequency of respective importance scores (w1, w2, and w3). Figures 56 and 67 depict the control and exposed specimens, respectively. All fish exposed to CL-T exhibited higher DTC. In the gills, although the concentrations differed from each other, the occurrence of w2 changes was approximately 50% across all concentrations. W3 changes were observed in 25% of the samples exposed to a concentration of 200 mg/L. The most frequently observed alterations included dilatation of lamellar capillaries, edema in the secondary lamellae (sl) (Fig. 56-B), and adhesion and complete fusion of the secondary lamellae (Fig. 56-C and D).

In the liver, a high DTC was observed at ~~the concentration of~~ 200 mg/L. Furthermore, a w2 degree of reversibility was found in all concentrations, with a frequency of approximately 50%. Liver structural changes ranged from vascular hyperemia to focal and diffuse necrosis, although these occurred less frequently. Hepatocellular changes were characterized by hypertrophy, vacuolar degeneration (hydropic and fatty), and the presence of typical degenerative nuclear figures (Fig. 6~~6~~).

Discussion

The presence of ~~nuclear abnormalities (~~NA~~)~~ in our study indicates a possible cytogenotoxic effect of CL-T on adult zebrafish (Harabawy & Mosleh 2014). ~~Nuclear abnormalities~~NA have been widely used as biomarkers for cytogenotoxicity and are associated with the replication of damaged cells (Erbe et al. 2011). The significant increase in NA observed, particularly at the sublethal dose of 200 mg/L of CL-T, suggests a possible cytogenotoxic effect on adult zebrafish. Exposure to xenobiotics at different stages of cell division can result in nuclear protrusions or invaginations in erythrocytes, leading to impaired protein formation (Fasulo et al. 2010; Mitchelmore & Chipman 1998). Additionally, DNA damage can be heritable, causing mutations and cell proliferation (Francisco et al. 2019).

Our findings demonstrate that CL-T at a concentration of 200 mg/L has significant effects on zebrafish erythrocyte DNA. ~~Similar effects have been observed in *Cyprinus carpio* fingerlings, including biochemical and behavioral changes such as abnormal skin secretions, slow movements, complete arrest, and death. Concentrations higher than 27 mg/L have resulted in mortality, and the observed effects can be compared to the effects of exposure to mercuric chloride and the pesticide diazinon (Imanpoor et al. 2011).~~ Disinfectants used in water disinfection, such as chlorine, ozone, UV, and chloramines, can react with natural organics to produce trihalomethanes and disinfection byproducts (DBPs), which have genotoxic effects and potential carcinogenic properties (Gustavino et al. 2005).

The cytogenotoxic effects of CL-T may be attributed to the cross-linking of DNA within and between strands, occurring when exogenous or endogenous substances react with two DNA nucleotides, forming covalent bonds. These adducts interfere with cell metabolism and can trigger cell death, while also providing insights into how proteins interact with DNA. Chloramines can be formed through the interaction of hypochlorous acid with DNA bases, subsequently decomposing into aminyl radicals. N-radicals located at the exocyclic amino

positions of cytosine and adenine are the major radical adducts of a nucleoside mixture (Cadet & Wagner 2013; Gustavino et al. 2005).

Although our results did not show a significant increase in ~~micronucleus (~~MN~~)~~ frequency in fish erythrocytes exposed to CL-T for 96 h, ~~micronucleus~~ MN formation typically arises from chromosomal breaks or mitotic anomalies, which require the occurrence of the mitosis process (Shi et al. 2009). However, CL-T was observed to affect the chromatin of zebrafish peripheral erythrocytes, as indicated by the increase in nuclear density observed in the ~~integrated optical density (~~IOD~~)~~ analysis. This effect of CL-T could influence chromatin condensation, organization, and positioning, as well as mitotic spindles (Hübner & Spector 2010). Variations in the density of chromatin attachment sites could explain the observed differences in chromatin mobility during the cell cycle and cellular development (Vazquez et al. 2001). While major chromosomal rearrangements or translational mobility at the level of individual chromosomes are not apparent during interphase, chromatin dynamics are rapid enough to allow intrachromosomal interactions, such as the cis or trans association of an enhancer and a promoter, occurring within seconds and spanning distances of less than 1 µm (Hübner & Spector 2010).

~~Furthermore, a~~Analysis of erythrocyte maturation stages in *Cyprinus carpio* revealed low- and high-density chromatin domains, as well as an interchromatin domain with high light transmittance and low optical density. CL-T could not only alter chromatin organization and condensation but also affect the cell cycle, leading to the detection of mature erythrocytes only after 96 h of exposure (Rothmann et al. 2000).

~~Acetylcholinesterase (~~AChE, EC 3.1.1.7~~)~~ is a crucial enzyme in the nervous system that is responsible for terminating nerve impulses through the hydrolysis of the neurotransmitter acetylcholine (ACh). It plays a vital role in cholinergic neurotransmission processes (Lionetto et al. 2013; Rang et al. 2016). ~~Although our results did not indicate significant differences in AChE inhibition, it is important to note that evaluating only one enzyme involved in neurotransmission processes, such as AChE, cannot rule out the neurotoxicity of CL-T.~~ Previous studies have shown significant AChE inhibition in embryos exposed to CL-T concentrations of 64 and 128 mg/L (Rivero-Wendt et al. 2023). Additionally, considering that AChE expression starts early, before synapse formation, and increases with embryo age (Teixidó et al. 2013), the seemingly contradictory results may suggest that embryos are more sensitive to the effects of CL-T compared to adults. A similar effect was confirmed in a study conducted with ~~C~~cetylpyridinium chloride on juveniles and adult zebrafish, where the impact of the product on neurotransmitters (serotonin, dopamine,

and acetylcholine) was less in adults than in juveniles of the species (Dong et al. 2022). Although our results did not indicate significant differences in AChE inhibition, it is important to note that evaluating only one enzyme involved in neurotransmission processes, such as AChE, cannot rule out the neurotoxicity of CL-T.

CL-T induced an acute irritant response in gill mucosal and epithelial cells, consistent with previous reports (Quezada-Rodriguez et al. 2022). Teleost fish gills consist of four pairs of gill arches, each containing numerous filaments, and each filament is comprised of multiple folded lamellae forming a complex cellular layer. Gills are in direct contact with the environment and are vulnerable to morphological changes when exposed to substances that harm their tissues. Toxic interactions with different stages of branchial transport or infusion patterns can significantly affect the fish's ability to regulate osmotic balance in fresh water environments ~~Toxic interactions with various stages of branchial transport or infusion patterns can have marked effects on the fish's osmoregulatory capacity in freshwater environments~~ (Evans 1987; Fernandes et al. 2020; Tavares-Dias 2021).

In the present study, the effects of CL-T on the ~~degree of tissue changes (~~DTC~~)~~ in the gills varied depending on the concentration. However, all concentrations showed significant effects compared to the control ~~specimens~~. The most prevalent lesions classified as W2 included the rupture of the lamellar epithelium and lamellar thrombosis. Several studies have highlighted the impact of various drugs on the gill morphology of teleosts. For instance, exposure to Halamid® at concentrations of 15 ~~, 30, 50, 100, and~~ 200 mg/L for 96 h in *Danio rerio* resulted in changes such as congestion, edema, epithelial detachment, hyperplasia, cellular hypertrophy, telangiectasia, and necrosis of the respiratory epithelium (Alidadi Soleiman et al. 2017). Similarly, in Atlantic salmon (*Salmo salar*), acute exposure to Halamid® (CL-T) at concentrations of 25 and 50 mg/L for 12 h led to desquamation of epithelial cells, multifocal hyperemia, and extensive epithelial necrosis in the primary and secondary lamellar segments (Powell, 2004). In juveniles of *Arapaima gigas*, concentrations ranging from 50 to 100 mg/L resulted in hyperplasia, sinus dilation, epithelial detachment, and lamellar fusion, which are nonspecific defense mechanisms in the presence of irritants (Cordeiro Bentes et al. 2022). Our findings demonstrate that the histopathological effects observed in the gills were reversible, characterized by epithelial responses without progression to necrosis or structural dissociative processes. In contrast to the gill histopathological findings, the liver showed higher frequencies classified as W3. The liver's metabolism and morphology are known to be sensitive to various environmental toxins (Gu & Manautou 2012). In the present study, reversible changes such as hydropic swelling, atypical

vacuoles, hepatocellular lipidosis, and irreversible changes were observed. Focal and diffuse necrotic areas were more common in specimens exposed to 200 mg/L of CL-T. Additionally, degenerative nuclear figures were frequently observed at concentrations of 140 and 200 mg/L. Limited data are available regarding safe treatment concentrations of CL-T for freshwater fish. Baths of 180 min at concentrations of 20, 50, and 80 mg/L of CL-T were insufficient to cause liver damage in *Ictalurus punctalus* (Gaikowski et al. 2009). Conversely, our results suggest that liver injury following CL-T exposure may be attributed to elevated levels of oxidative stress, indicating disruption of homeostatic and functional membrane disruption (Bilzer & Lauterburg 1991; Tatsumi & Fliss 1994; Tkachenko et al. 2013).

Conclusions

Overall, our results indicate that changes in the erythrocyte nuclear chromatin condensation profile can be observed in association with branchial and hepatic toxicity, as determined by NA and IOD measurements. In the case of short-term exposure, tissue changes suggest low to moderate toxicity responses for adult samples. In the gills, nonspecific reversible lesions can be observed, whereas in the liver, irreversible lesions with a more pronounced harmful potential are present.~~Overall, our results demonstrate that NA and IOD are potential biomarkers to analyze non-lethal concentrations of CL-T in zebrafish. Changes in the erythrocyte nuclear chromatin condensation profile may be observed in association with gill and liver toxicity. At a short-time exposition, tissue changes indicate responses of low to moderate toxicity for adult specimens. Nonspecific reversible lesions might be observed in the gill in contrast to the liver, which presents irreversible injuries with more pronounced harmful potential.~~

Acknowledgments

This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Finance Code 001, and financed in part by the Fundação Universidade Federal de Mato Grosso do Sul – UFMS/MEC – Brazil; CEF has been continuously supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (grant #310058/2010-1).

References

Agoba E, Adu F, Agyare C, and Boamah V. 2017. Antibiotic Use And Practices In Selected Fish Farms In The Ashanti Region Of Ghana. *J Infect Dis Treat* 03:1-6. 10.21767/2472-1093.100036

Alidadi Soleiman T, Sattari A, Kheirandish R, and Sharifpour I. 2017. Safety evaluation of chloramine –T on ornamental zebra fish (Danio rerio) using LC50 calculation and organ pathology. *Iran J Fish Sci* 16:26-37.

Bahadir T, Çelebi H, Şimşek İ, and Tulun Ş. 2019. Antibiotic applications in fish farms and environmental problems. *Turkish J Eng* 3:60-67. 10.31127/tuje.452921

Bernet D, Schmidt-Posthaus H, Meier W, Burkhardt-Holm P, and Wahli T. 2001. Histopathology in fish: Proposal for a protocol to assess aquatic pollution. *J Fish Dis* 22:25-34. 10.1046/j.1365-2761.1999.00134.x

Bertotto LB, Catron TR, and Tal T. 2020. Exploring interactions between xenobiotics, microbiota, and neurotoxicity in zebrafish. *NeuroToxicology* 76:235-244. https://doi.org/10.1016/j.neuro.2019.11.008

Bilzer M, and Lauterburg BH. 1991. Effects of hypochlorous acid and chloramines on vascular resistance, cell integrity, and biliary glutathione disulfide in the perfused rat liver: modulation by glutathione. *J Hepatol* 13:84-89. 10.1016/0168-8278(91)90868-c

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72:248-254. 10.1006/abio.1976.9999

Brannen KC, Panzica-Kelly JM, Danberry TL, and Augustine-Rauch KA. 2010. Development of a zebrafish embryo teratogenicity assay and quantitative prediction model. *Birth Defects Res B Dev Reprod Toxicol* 89:66-77. 10.1002/bdrb.20223

Bullock GL, Herman RL, and Waggy C. 1991. Hatchery Efficacy Trials with Chloramine-T for Control of Bacterial Gill Disease. *J Aquat Anim Health* 3:48-50. 10.1577/1548-8667(1991)003<0048:HETWCT>2.3.CO;2

Cadet J, and Wagner JR. 2013. DNA base damage by reactive oxygen species, oxidizing agents, and UV radiation. *Cold Spring Harb Perspect Biol* 5. 10.1101/cshperspect.a012559

Carrasco KR, Tilbury KL, and Myers MS. 1990. Assessment of the Piscine Micronucleus Test as an in situ Biological indicator of Chemical Contaminant Effects. *Can J Fish Aquat Sci* 47:2123-2136. 10.1139/f90-237

427 Concea. 2018. Diretriz da Prática de Eutanásia do CONCEA. Controle Nacional de Controle
428 de Experimentação Animal, 1–49
429 Cordeiro Bentes SP, Gomes da Cruz M, Jerônimo GT, Coimbra FC, and Gonçalves LU. 2022.
430 Chloramine-T application for Trichodina sp. in Arapaima gigas juveniles: Acute
431 toxicity, histopathology, efficacy, and physiological effects. *Vet Parasitol*
432 303:109667. 10.1016/j.vetpar.2022.109667
433 Cui H, Chen B, Jiang Y, Tao Y, Zhu X, and Cai Z. 2021. Toxicity of 17 Disinfection By-
434 products to Different Trophic Levels of Aquatic Organisms: Ecological Risks and
435 Mechanisms. *Environ Sci Technol* 55:10534-10541. 10.1021/acs.est.0c08796
436 Dong K, Li L, Chen C, Tengbe M S, Chen K, Shi Y, Wu X, and Qiu X. 2022. Impacts of
437 cetylpyridinium chloride on the behavior and brain neurotransmitter levels of juvenile
438 and adult zebrafish (Danio rerio). *Comp. Biochem. Physiol. Part - C: Toxicol.*
439 *Pharmacol.*, 259, 109393.
440
441 Ellman GL, Courtney KD, Andres V, Jr., and Feather-Stone RM. 1961. A new and rapid
442 colorimetric determination of acetylcholinesterase activity. *Biochem Pharmacol* 7:88-
443 95. 10.1016/0006-2952(61)90145-9
444 Erbe MC, Ramsdorf WA, Vicari T, and Cestari MM. 2011. Toxicity evaluation of water
445 samples collected near a hospital waste landfill through bioassays of genotoxicity
446 piscine micronucleus test and comet assay in fish Astyanax and ecotoxicity Vibrio
447 fischeri and Daphnia magna. *Ecotoxicol* 20:320-328. 10.1007/s10646-010-0581-1
448 Evans DH. 1987. The fish gill: site of action and model for toxic effects of environmental
449 pollutants. *Environ Health Perspect* 71:47-58. 10.1289/ehp.877147
450 Fasulo S, Marino S, Mauceri A, Maisano M, Giannetto A, D'Agata A, Parrino V, Minutoli R,
451 and De Domenico E. 2010. A multibiomarker approach in Coris julis living in a
452 natural environment. *Ecotoxicol Environ Saf* 73:1565-1573.
453 https://doi.org/10.1016/j.ecoenv.2010.01.008
454 FDA. 2014. HALAMID ® Aqua Chloramine-T, powder for immersion, freshwater-reared
455 salmonids, walleye and freshwater- reared warmwater finfish. New Anim. Drug Appl.
456 56.
457 Fernandes CE, da Silveira AW, do Nascimento Silva AL, de Souza AI, Povh JA, dos Santos
458 Jaques JA, dos Santos EdA, Koshi A. Yonekawa M, de Barros Penteadoall B, and
459 Franco-Belussi L. 2020. Osmoregulatory profiles and gill histological changes in Nile

tilapia (Oreochromis niloticus) exposed to lambda-cyhalothrin. *Aquatic Toxicology* 227:105612. https://doi.org/10.1016/j.aquatox.2020.105612

Ferreira T, and Rasband W. 2012. ImageJ User Guide (IJ 1.45 m). Image J Softw. *Available at https://imagej.nih.gov/ij/docs/guide/user-guide.pdf*.

Francisco CM, Bertolino SM, De Oliveira Júnior RJ, Morelli S, and Pereira BB. 2019. Genotoxicity assessment of polluted urban streams using a native fish Astyanax altiparanae. *J Toxicol Environ Health A* 82:514-523. 10.1080/15287394.2019.1624235

Gaikowski MP, Densmore CL, and Blazer VS. 2009. Histopathology of repeated, intermittent exposure of chloramine-T to walleye (Sander vitreum) and (Ictalurus punctalus) channel catfish. *Aquaculture* 287:28-34. https://doi.org/10.1016/j.aquaculture.2008.10.020

Gu X, and Manautou JE. 2012. Molecular mechanisms underlying chemical liver injury. *Expert Rev Mol Med* 14:e4. 10.1017/s1462399411002110

Gunnarsson L, Jauhiainen A, Kristiansson E, Nerman O, and Larsson DG. 2008. Evolutionary conservation of human drug targets in organisms used for environmental risk assessments. *Environ Sci Technol* 42:5807-5813. 10.1021/es8005173

Gustavino B, Buschini A, Monfrinotti M, Rizzoni M, Tancioni L, Poli P, and Rossi C. 2005. Modulating effects of humic acids on genotoxicity induced by water disinfectants in Cyprinus carpio. *Mutat Res* 587:103-113. 10.1016/j.mrgentox.2005.08.009

Harabawy AS, and Mosleh YY. 2014. The role of vitamins A, C, E and selenium as antioxidants against genotoxicity and cytotoxicity of cadmium, copper, lead and zinc on erythrocytes of Nile tilapia, Oreochromis niloticus. *Ecotoxicol Environ Saf* 104:28-35. 10.1016/j.ecoenv.2014.02.015

Hardie D, Gregory TR, and Hebert P. 2002. From Pixels to Picograms: A Beginners' Guide to Genome Quantification by Feulgen Image Analysis Densitometry. *J Histochem Cytochem* 50:735-749. 10.1177/002215540205000601

Hooftman RN, and de Raat WK. 1982. Induction of nuclear anomalies (micronuclei) in the peripheral blood erythrocytes of the eastern mudminnow Umbra pygmaea by ethyl methanesulphonate. *Mutat Res* 104:147-152. 10.1016/0165-7992(82)90136-1

Hübner MR, and Spector DL. 2010. Chromatin dynamics. *Annu Rev Biophys* 39:471-489. 10.1146/annurev.biophys.093008.131348

~~Imanpoor M, Ahmadi A, and Kabir M. 2011. Effects of sub lethal concentration of Chloramin T on growth, survival, haematocrit and some blood biochemical parameters in common carp fry (Cyprinus carpio). *AACL Bioflux* 4:280-291.~~

494 Lionetto MG, Caricato R, Calisi A, Giordano ME, and Schettino T. 2013.
495 Acetylcholinesterase as a biomarker in environmental and occupational medicine: new
496 insights and future perspectives. *Biomed Res Int* 2013:321213-321213.
497 10.1155/2013/321213
498 Mitchelmore CL, and Chipman JK. 1998. DNA strand breakage in aquatic organisms and the
499 potential value of the comet assay in environmental monitoring. *Mutat Res* 399:135-
500 147. 10.1016/s0027-5107(97)00252-2
501 Nayak YN, Gaonkar SL, Saleh EAM, Dawsari AMAL, Harshitha, Husain K, and Hassan I.
502 2022. Chloramine-T (N-chloro-p-toluenesulfonamide sodium salt), a versatile reagent
503 in organic synthesis and analytical chemistry: An up to date review. *J Saudi Chem Soc*
504 26:101416. https://doi.org/10.1016/j.jscs.2021.101416
505 Powell, MD., Speare DJ, and MacNair N. 1994. Effects of intermittent chloramine-T exposure
506 on growth, serum biochemistry, and fin condition of juvenile rainbow trout
507 (Oncorhynchus mykiss). *Can J Fish Aquat Sci* 51 8:1728–36. 10.1139/f94-174
508 Quezada-Rodriguez PR, Taylor RS, Samsing F, Rigby M, Wood AT, Nowak BF, and Wynne
509 JW. 2022. Effect of a prophylactic treatment with chloramine-T on gill histology and
510 microbiome of Atlantic salmon (Salmo salar) under commercial conditions.
511 *Aquaculture* 546:737319. https://doi.org/10.1016/j.aquaculture.2021.737319
512 Rang HP, Ritter JM, Flower RJ, and G. H. 2016. *Rang & Dale's Pharmacology*. London:
513 Churchill Livingstone Elsevier.
514 Ranzani-Paiva MJT, Pádua SB, Tavares-Dias M, and Egami MI. 2013. *Métodos para análise*
515 *hematológica em peixes.* Maringá/Paraná: Editora da Universidade Estadual de
516 Maringá (EDUEM).
517 Rivero-Wendt CLG, Fernandes LG, Dos Santos AN, Brito IL, Dos Santos Jaques JA, Dos
518 Santos Dos Anjos E, and Fernandes CE. 2023. Effects of Chloramine T on zebrafish
519 embryos malformations associated with cardiotoxicity and neurotoxicity. *J Toxicol*
520 *Environ Health A*:1-10. 10.1080/15287394.2023.2205271
521 Rivero-Wendt CLG, Miranda-Vilela AL, Domingues I, Oliveira R, Monteiro MS, Moura-
522 Mello MAM, Matias R, Soares AMVM, and Grisolia CK. 2020. Steroid androgen 17
523 alpha methyltestosterone used in fish farming induces biochemical alterations in
524 zebrafish adults. *J Environ Sci Health A Tox Hazard Subst Environ Eng* 55:1321-
525 1332. 10.1080/10934529.2020.1790954

Rothmann C, Levinshal T, Timan B, Avtalion RR, and Malik Z. 2000. Spectral imaging of red blood cells in experimental anemia of Cyprinus carpio. *Comp Biochem Physiol A Mol Integr Physiol* 125:75-83. 10.1016/s1095-6433(99)00157-9

Sanchez, JG, Speare DJ, Macnair N, and Johnson G. 1996. Effects of a prophylactic chloramine-T treatment on growth performance and condition indices of rainbow trout. *J Aquat Anim Health* 8: 278–84. 10.1577/1548-8667(1996)008<0278:EOAPCT>2.3.CO;2

Schmidt LJ, Gaikowski MP, Gingerich WH, Stehly GR, Larson WJ, Dawson VK, and Schreier TM. 2007. Environmental assessment of the effects of chloramine-T use in and discharge by freshwater aquaculture. In: Medicine FsCfV, editor: U.S. Food and Drug Administration (FDA). p 136.

Shi Y, Cao XW, Tang F, Du HR, Wang YZ, Qiu XQ, Yu HP, and Lu B. 2009. In vitro toxicity of surface water disinfected by different sequential treatments. *Water Res* 43:218-228. 10.1016/j.watres.2008.10.007

Silva ALN, Rodrigues RA, Siqueira MS, Farias KNN, Kuibida KV, Franco-Belussi L, and Fernandes CE. 2021. Transaminase profile and hepatic histopathological traits in Piaractus mesopotamicus exposed to insecticide Diflubenzuron. *Environ Sci Pollut Res* 28:22002-22010. 10.1007/s11356-020-12013-2

Suvarna SK, Layton C, and Bancroft JD. 2019. *Bancroft's Theory and Practice of histological techniques.* . China: Elsevier.

Tatsumi T, and Fliss H. 1994. Hypochlorous acid mobilizes intracellular zinc in isolated rat heart myocytes. *J Mol Cell Cardiol* 26:471-479. 10.1006/jmcc.1994.1058

Tavares-Dias M. 2021. Toxic, physiological, histomorphological, growth performance and antiparasitic effects of copper sulphate in fish aquaculture. *Aquaculture* 535:736350. https://doi.org/10.1016/j.aquaculture.2021.736350

Teixidó E, Piqué E, Gómez-Catalán J, and Llobet JM. 2013. Assessment of developmental delay in the zebrafish embryo teratogenicity assay. *Toxicol In Vitro* 27:469-478. https://doi.org/10.1016/j.tiv.2012.07.010

Tkachenko H, Kurhaluk N, and Grudniewska J. 2013. Effects of chloramine-T exposure on oxidative stress biomarkers and liver biochemistry of rainbow trout, Oncorhynchus mykiss (Walbaum), brown trout, Salmo trutta (L.), and grayling, Thymallus thymallus (L.). *Fish Aquatic Life* 21:41-51. doi:10.2478/aopf-2013-0005

Vazquez J, Belmont AS, and Sedat JW. 2001. Multiple regimes of constrained chromosome motion are regulated in the interphase Drosophila nucleus. *Curr Biol* 11:1227-1239. 10.1016/s0960-9822(01)00390-6

Yadav S, Dhodpakar R, and Kapley A. 2020. Disinfection by-products and their effect on aquatic and agriculture ecosystem. In: Prasad MNV, ed. *Disinfection By-products in Drinking Water: Detection and Treatment*. UK: Butterworth-Heinemann, 205-233.