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Cadmium and lead: genotoxic effects on the broodstock of the fish *Prochilodus magdalenae* (Characiformes: Prochilodontidae)

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ABSTRACT

Introduction: Toxic metals such as cadmium (Cd) and lead (Pb) pose a significant threat to aquatic ecosystems due to their persistence, capacity for bioaccumulation, and genotoxic potential. These contaminants are of particular concern in tropical freshwater systems, where they may impact native species of ecological, economic, and nutritional importance, such as *Prochilodus magdalenae*, a migratory fish endemic to the Magdalena–Cauca River basin in Colombia.

Objective: To evaluate the acute effects of individual and combined exposure to Cd and Pb on behavioral parameters, mortality, and genetic damage in *P. magdalenae*.

Methods: Three individual doses of each metal and five binary combinations were administered via intraperitoneal injection in adult fish, followed by analysis using genotoxicity biomarkers (micronuclei, nuclear abnormalities, and DNA integrity damage).

Results: Evident behavioral changes, increased mortality, and significant genotoxic damage were demonstrated, particularly in treatments involving combined exposures. The highest mortality rate (75 %) occurred in the 0.8 µg Cd/kg + 14 µg Pb/kg treatment. Pb exposure was associated with a greater frequency of micronucleus formation, while Cd exposure induced an increase in nuclear abnormalities, without a clear dose–response trend. Cell-level analysis of the comet assay suggested primary DNA damage particularly in the 0.2 µg Cd/kg + 8.0 µg Pb/kg and 0.8 µg Cd/kg + 14 µg Pb/kg treatments; however, a reanalysis using the individual fish as the unit of analysis did not confirm statistically significant differences among treatments, underscoring the need for larger per-individual sample sizes in future studies. Nevertheless, non-linear responses were detected in some treatments, possibly due to adaptive cellular responses or apoptosis. A sex-dependent susceptibility was also identified: males exhibited higher mortality rates but lower micronucleus frequencies than females.

Conclusions: The toxic potential of Cd and Pb is highlighted, and the suitability of *P. magdalenae* as a sentinel species for environmental monitoring supported. The study emphasizes the need to consider co-exposure scenarios in ecotoxicological risk assessments, due to their relevance to ecosystem health, human consumption, and food security in regions where this species represents a vital resource.

Key words: co-exposure, micronuclei, comet assay, biomarkers, sentinel species.



RESUMEN

**El cadmio y el plomo: efectos genotóxicos en reproductores
del pez *Prochilodus magdalenae* (Characiformes: Prochilodontidae)**

Introducción: Los metales tóxicos como el cadmio (Cd) y el plomo (Pb) representan una amenaza significativa para los ecosistemas acuáticos debido a su persistencia, capacidad de bioacumulación y potencial genotóxico. Estos contaminantes son de particular preocupación en los sistemas de agua dulce tropicales, donde pueden impactar especies nativas de importancia ecológica, económica y nutricional, como *Prochilodus magdalenae*, un pez migratorio endémico de la cuenca del río Magdalena-Cauca en Colombia.

Objetivo: Evaluar los efectos agudos de la exposición individual y combinada al Cd y Pb sobre los parámetros comportamentales, mortalidad y daño genético en *P. magdalenae*.

Métodos: Se administraron tres dosis individuales de cada metal y cinco combinaciones binarias mediante inyección intramuscular en peces adultos, seguido de análisis utilizando biomarcadores de genotoxicidad (micronúcleos, anormalidades nucleares y daño a la integridad del ADN).

Resultados: Se demostraron cambios comportamentales evidentes, aumento de la mortalidad y daño genotóxico significativo, particularmente en tratamientos que involucraban exposiciones combinadas. La tasa de mortalidad más alta (75 %) ocurrió en el tratamiento 0.8 µg Cd/kg + 14 µg Pb/kg. La exposición al Pb se asoció con una mayor frecuencia de formación de micronúcleos, mientras que la exposición al Cd indujo un aumento en anormalidades nucleares sin una tendencia clara de dosis-respuesta. El análisis a nivel de célula del ensayo cometa sugirió daño primario del ADN, particularmente en los tratamientos 0.2 µg Cd/kg + 8.0 µg Pb/kg y 0.8 µg Cd/kg + 14 µg Pb/kg; sin embargo, un reanálisis usando el pez individual como unidad de análisis no confirmó diferencias estadísticamente significativas, lo que resalta la necesidad de mayores tamaños muestrales por individuo en estudios futuros. Sin embargo, se detectaron respuestas no lineales en algunos tratamientos, posiblemente debido a respuestas celulares adaptativas o apoptosis. También se identificó una susceptibilidad dependiente del sexo: los machos exhibieron tasas de mortalidad más altas, pero frecuencias de micronúcleos más bajas que las hembras.

Conclusiones: Se resalta el potencial tóxico del Cd y Pb y se apoya la idoneidad de *P. magdalenae* como especie centinela para el monitoreo ambiental. El estudio enfatiza la necesidad de considerar escenarios de co-exposición en las evaluaciones de riesgo ecotoxicológico, debido a su relevancia para la salud del ecosistema, el consumo humano y la seguridad alimentaria en regiones donde esta especie representa un recurso vital.

Palabras clave: co-exposición, micronúcleos, ensayo cometa, biomarcadores, especies centinela.

INTRODUCTION

Prochilodus magdalenae (Steindachner, 1879), bocachico, is a migratory fish endemic to the Magdalena–Cauca River basin in Colombia, of considerable ecological, economic, and nutritional importance (Jiménez-Segura et al., 2022; Jiménez-Segura et al., 2010). This species was selected as the model organism to evaluate acute genotoxic effects induced by toxic metal exposure, given that its populations face habitat fragmentation (García-Castro & Márquez, 2024; Jiménez-Segura et al., 2010) and accumulate cadmium (up to 0.0256 mg/L in muscle) and lead (up to 0.676 µg/g in muscle) from environmental contamination (Mancera-Rodríguez & Álvarez-León, 2006; Pájaro-Meza & Sánchez-Rincón, 2016; Mojica et al., 2012; Vivas et al., 2015; Zapata et al., 2018; Zapata et al., 2016).

Toxic metals, traditionally classified by densities exceeding 5 g/cm³ (historically termed ‘heavy metals’), are more accurately grouped according to their chemical properties, biological role, and toxicological behavior rather than density alone (Duffus, 2002). These can be divided into essential elements, which perform physiological functions at trace concentrations (e.g., iron [Fe], copper [Cu], zinc [Zn], cobalt [Co]), and non-essential elements, which have no known biological role and are toxic even at low concentrations (e.g., cadmium [Cd], lead [Pb], mercury [Hg], selenium [Se]). While essential metals perform vital biological roles in organisms (Subotić et al., 2013), non-essential metals have no known physiological function and their accumulation in tissues leads to cellular and systemic damage (Paschoalini et al., 2019). Cadmium and lead have particular concern due to their environmental persistence,

bioaccumulation potential, and interference with key cellular processes. Exposure, even at low concentrations, induces oxidative stress, DNA damage and enzymatic dysfunction, posing significant risks to human and ecological health (Liu et al., 2022). In fish models, cadmium exposure has been linked to neurosensory defects, oxidative stress, immunotoxicity, and disruption of key genes such as *mt1* and *cyp19a1b* (Chouchene et al., 2016; Green & Planchart, 2018; Ruiter et al., 2016; Sonnack et al., 2015; Zhang et al., 2015), while lead exposure alters neuroectodermal gene expression, disrupts synaptogenesis and neurogenesis, and provokes neuromuscular disturbances such as erratic swimming and hyperventilation, with effects that can persist across multiple generations via epigenetic modifications (Bault et al., 2015; Green & Planchart, 2018; Lee & Freeman, 2014; Mager, 2011; Senut et al., 2012; Tchounwou et al., 2012; Xu et al., 2016; Zhu et al., 2016).

The micronucleus test (MN) and comet assay (CA) are sensitive, rapid biomarkers for detecting DNA damage in erythrocytes, reflecting recent or chronic toxic metal exposure (Al-Sabti & Metcalfe, 1995; Bolognesi et al., 2006; Da Rocha et al., 2009; D'Agostini & La Maestra, 2021; Dalzochio et al., 2019; Dhawan et al., 2009; Frenzilli et al., 2009; Lee & Steinert, 2003; Schaumburg et al., 2012; Strunjak-Perovic et al., 2010; Udroui, 2006; Zapata et al., 2016). Consequently, acute genotoxic assessment using MN and CA in erythrocytes of *Prochilodus magdalenae* is essential to detect sublethal effects, establish sensitivity thresholds, and guide conservation and food security strategies in regions where this species represents a vital resource (Bonassi et al., 2011; Gu et al., 2023; Jiménez-Segura et al., 2022; Monteiro et al., 2011; Sokolova & Lannig, 2008).

MATERIALS AND METHODS

Chemical products: Contaminant solutions at exposure dosages were prepared through appropriate dilutions with physiological saline

of Cd (50 mg/l) and Pb (50 mg/l) stock solutions, which were prepared from corresponding standard substances: 8.18 mg of CdCl₂ (99.7 %, AK Scientific Inc, Union City, CA, USA) in 0.1 l of deionized water, and 6.79 mg PbCl₂ (98.9 %, AK Scientific Inc, Union City, CA, USA) in 0.1 l of deionized water.

Sample collection: This study was conducted at the Fish Research Institute of the Universidad de Córdoba (CINPIC; Montería, Colombia), with approval from the bioethics committee of the Universidad de Antioquia, with environmental license for fishing from the National Authority of Aquaculture and Fisheries-AUNAP of Colombia (Permit Number: 00071 of 2025), complying with animal responsibility and welfare. Male (n=48) and female (n= 48) bocachico maintained in captivity at two years of age were selected, at a density of 0.3 kg of fish/m³. Selected animals were distributed in twelve tanks (8 fish/tank: 4 males and 4 females) of 100 % sterile polyethylene, with dimensions of 55 × 107 cm, volumetric capacity of 250 l in a closed system with water recirculation. Physical and chemical parameters of experimental water, such as dissolved oxygen (6.48 ± 0.97 mg/l), oxygen saturation (80.88 ± 11.17 %), temperature (27.04 ± 1.32 °C), and pH (7.38 ± 0.24), were found within the appropriate range for the species.

The selection of exposure doses was based on three criteria. First, an ethical consideration related to the administration route: immersion exposure would have required maintaining precise and sustained metal concentrations in large water volumes throughout the experimental period, generating substantial volumes of toxic metal-contaminated wastewater that would be technically difficult and environmentally problematic to dispose of safely. Second, an additional ethical consideration related to the biological nature of the model species: *Prochilodus magdalenae* is a native, non-domesticated migratory species, which limits the availability of homogeneous individuals and restricts the use of large sample sizes, reinforcing the need for a controlled parenteral



administration approach. Third, a mechanistic criterion based on sublethal dose determination: before the experiment, tissue samples from a subset of individuals were analyzed at the Toxicology and Environmental Management Laboratory of the Universidad de Córdoba using the PLTX-058 method (graphite furnace atomic absorption spectroscopy, GF-AAS), yielding no quantifiable concentrations of either metal in tissues with detection limits of 8.23 µg/kg and quantification limits of 25.00 µg/kg for Pb, and detection limits of 0.83 µg/kg and quantification limits of 2.50 µg/kg for Cd. Based on these analytical thresholds, doses were intentionally selected within and around these detection and quantification limits to ensure sublethal exposure levels, minimizing the risk of acute lethality while enabling the detection of genotoxic effects at ecotoxicologically relevant concentrations.

For solution administration, an injection volume adjusted proportionally to the body weight of each fish was used, equivalent to 3 µL per gram of body weight (3 µL/g). Fish presented an average weight of 181.13 ± 49.14 g, an average standard length of 22.10 ± 2.05 cm, and an average total length of 26.89 ± 2.39 cm.

To evaluate the genotoxic effects of acute exposure to Cd and Pb in *P. magdalenae*, 48 sexually mature females and 48 males were randomly selected. Fish were exposed for 72 hours to 11 treatments, which included individual doses of Cd and Pb, as well as binary combinations of both metals. Cd was administered at three concentrations: 0.2 µg/kg (Cd1), 0.8 µg/kg (Cd2), and 1.4 µg/kg (Cd3); and Pb at three concentrations: 2.0 µg/kg (Pb1), 8.0 µg/kg (Pb2), and 14.0 µg/kg (Pb3). Although the combination of all possible doses of Cd and Pb would have resulted in nine binary treatments, five representative combinations were selected: Cd1 + Pb1 (0.2–2.0 µg/kg), Cd1 + Pb2 (0.2–8.0 µg/kg), Cd2 + Pb1 (0.8–2.0 µg/kg), Cd2 + Pb3 (0.8–14.0 µg/kg), and Cd3 + Pb2 (1.4–8.0 µg/kg). These combinations were strategically chosen to cover a wide range of low, intermediate, and high concentrations for both Cd and Pb, allowing exploration of possible synergistic

or additive effects without compromising the experimental design or animal welfare. A negative control treatment (C-) was also included, in which fish received only an intraperitoneal injection of 0.9 % NaCl isotonic saline solution. The specific doses used for each treatment are presented in Table 1.

During the 72-hour experimental period, mortality and behavioral observations were recorded in fish from each treatment. This monitoring included continuous surveillance of general behavioral manifestations in each exposure group, as well as an analysis of behavioral alterations in fish that did not survive before death. Recorded behaviors included changes in swimming activity, loss of equilibrium, hyperventilation, lethargy, and abnormal responses to external stimuli. These data were collected and compared across different treatments, which included individual and combined concentrations of Cd and Pb, as well as the negative control group.

Table 1
Experimental design showing the doses of cadmium (Cd), lead (Pb), and their binary combinations administered to *Prochilodus magdalenae*.

Treatments	(µg/kg)
Cd1	0.2
Cd2	0.8
Cd3	1.4
Pb1	2.0
Pb2	8.0
Pb3	14.0
(DP5) Cd1 + Pb1	0.2 + 2.0
(DP1) Cd1 + Pb2	0.2 + 8.0
(DP2) Cd2 + Pb1	0.8 + 2.0
(DP3) Cd2 + Pb3	0.8 + 14.0
(DP4) Cd3 + Pb2	1.4 + 8.0
C (control)	0.0

Concentrations are expressed in µg/kg body weight. DP: binary combination treatments of cadmium and lead. C: control group (no exposure). The notation Cd1–Cd3 and Pb1–Pb3 represents increasing concentration levels for each metal.

After the exposure time, blood extraction was performed to obtain samples necessary for the micronucleus test and comet assay through puncture of the caudal peduncle vein of each fish (Hesser, 1960; Nabi et al., 2022), and then, the fish were euthanized.

Micronucleus (MN) assay and nuclear abnormalities in erythrocytes: Peripheral blood samples were taken from *P. magdalenae* individuals, and smears were made on previously cleaned slides. Blood smears were dried at room temperature (25 °C) for 2 hours and then fixed cold first in Carnoy solution for 5 minutes and subsequently in absolute methanol for 10 minutes. Slides were air-dried at 25 °C for 1 hour before being stained with a 10 % aqueous Giemsa solution for 30 minutes. After staining, slides were washed with distilled water and dried again at room temperature (Al-Sabti & Metcalfe, 1995; Bolognesi et al., 2006; Udriou, 2006).

A total of 270 blood smear slides were analyzed, corresponding to three slides per sampled fish. Of the 96 fish used in the experiment, 16 died during the 72-hour exposure period; blood samples could still be obtained from 10 of these individuals, as they were collected shortly after death, while the remaining 6 were excluded due to sample degradation. Combined with the 80 surviving fish, this resulted in a total of 90 individuals sampled (270 slides). Each slide was examined for 2 000 erythrocytes, yielding a total of 540 000 cells evaluated across all treatment groups. MN in erythrocytes were identified and counted using a microscope at 100× magnification (oil immersion), following criteria described by Al-Sabti and Metcalfe (1995): structures smaller than one-third of the main nucleus, clearly separated from it, non-refrigent, and within intact cytoplasm. Photographs of the most characteristic erythrocyte abnormalities were recorded according to Caramello et al. (2022) criteria: microcyte; notched (nuclear notches); nuclear bud; lobed (lobulated nuclei); eight-shaped nucleus; blebbed (evaginations); nuclear

vacuole, nucleus with plasmatic bridge; binucleated; and normal erythrocytes (NE).

Results were presented as MN and AN frequency using Equation 1. With the MN frequency, the mutagenicity index was calculated according to Equation 2. This index evaluates the genotoxic potential of substances and their possible health impact, indicating the risk of diseases related to genetic instability, such as cancer (Bonassi et al., 2011).

$$\% \text{ MN or NA} = \frac{(\text{Number of cells containing micronuclei})}{\text{total number of cells counted (2000)}} * 100$$

Equation 1

$$MI = \frac{Fq \text{ MN per dose}}{Fq \text{ MN Negative Control}}$$

Equation 2

The evaluated variables included MN frequency and NA.

Comet Assay: The comet assay was performed to detect DNA strand breaks in *P. magdalenae*, according to the method of Singh et al. (1988), with modifications tailored to the organism. Peripheral blood obtained through caudal puncture was used, which was collected in tubes with anticoagulant. From each sample, 5 µL were taken and transferred to Eppendorf tubes containing 1.7 mL of calcium and magnesium-free phosphate buffered saline (PBS). Subsequently, a 10 µL aliquot of this diluted blood was taken and mixed with 90 µL of 0.5 % low-melting-point agarose. The mixture was carefully deposited on a Gelbond® PAG Film slide, taking care to form four drops of 25 µL each, and left to solidify at room temperature.

To ensure adequate cellular dispersion and avoid sample saturation, the dilution was previously verified through observation in a Neubauer chamber. Subsequently, the Gelbond® PAG Film slide was incubated in a lysis solution (containing NaCl, EDTA, Tris, Triton X-100, DMSO, and pH 10) for at least one hour at 4 °C, with the objective of lysing cell membranes and releasing DNA.

After lysis completion, the slide was subjected to electrophoresis under alkaline



conditions (pH 13) for approximately 20 minutes, followed by electrophoretic running for 30 minutes at 25 V and 300 mA, in darkness and at 4 °C. Subsequently, it was neutralized with a neutral pH buffer solution, denatured by quick immersion in methanol, and stained with ethidium bromide at the time of reading.

Finally, the slide was observed under a fluorescence microscope (Nikon Eclipse E200, Nikon) at 40× magnification. The obtained images enabled the evaluation of the degree of DNA damage, analyzing 100 cells per individual using Triton CometScore software version 2.0.0.38, with the tail DNA percentage used as the damage parameter.

Statistical analysis: Statistical analysis: A dual strategy combining a robust non-parametric descriptive approach and a complementary exploratory approach was used for micronuclei (MN), nuclear abnormalities (NA), and comet assay parameters. In the descriptive phase, given that individual cells could not be treated as independent replicates without inflating the effective sample size (pseudoreplication), the Shapiro–Wilk test was used to assess the normality of tail DNA percentage and tail moment distributions at the individual level; both variables deviated significantly from normality (tail DNA percentage: $W = 0.732$, $p < 0.001$; tail moment: $W = 0.260$, $p < 0.001$), supporting the use of non-parametric methods. Levene's test indicated homogeneity of variances across treatments for both variables (tail DNA percentage: $p = 0.807$; tail moment: $p = 0.849$). Accordingly, the individual fish was used as the unit of analysis: median values per fish were compared between treatments using the Kruskal–Wallis test, followed by Dunn's post-hoc test with multiple-comparison adjustment using the Bonferroni method. As a complementary, exploratory analysis, a generalized linear model (GLM) with binomial distribution and logit link function was used to analyze the relative frequency of micronuclei (MN) and nuclear abnormalities (NA), taking treatment (TTO) and sex as explanatory variables. This approach enabled the appropriate modeling

of proportional variables, as MN and NA were expressed as the proportion of affected cells to the total number of cells counted per individual. To correct variability between replicates and ensure homogeneity in observation weights, a constant weight was applied to all observations. Subsequently, multiple comparisons between treatments and doses were performed using the adjusted Tukey method (p -adj) for simultaneous tests. Differences were considered statistically significant when the p -value was less than 0.05. They were considered highly significant when $p < 0.01$, and extremely substantial when $p < 0.001$. Model adequacy was verified through deviance analysis (a generalization of residual variance analysis for GLM), residual inspection, and AIC comparison among models.

For the comet assay, the descriptive Kruskal–Wallis/Dunn analysis described above was applied to the per-individual median tail DNA percentage and tail moment. As a complementary, exploratory analysis reported for comparison with the original cell-level approach, tail DNA percentage and tail moment parameters were used. Initially, data distribution was examined through histograms and residual analysis. Due to asymmetry in tail moment values, a square root transformation was applied to meet the normality assumptions required by ANOVA. One-way analyses of variance (ANOVA) were performed to detect treatment effects both in general groupings by metal type (Cd, Pb, Cd + Pb) and at specific treatment levels (Doses: Cd1, Pb2, Cd1 + Pb1, etc.). Subsequently, Tukey HSD tests were performed for multiple comparisons ($\alpha = 0.05$), and Student's t -tests with the Bonferroni correction were used for contrasts between selected groups. Values of $p < 0.05$ were considered significant, those with $p < 0.01$ highly important, and extremely significant if $p < 0.001$. In all models, assumptions were validated through graphical analysis of residuals and homogeneity of variances. Statistical analyses were performed using R v.4.3.0. For evaluation of micronuclei and nuclear abnormalities, the multcomp package was employed through generalized linear models with Tukey post hoc contrasts. For the

comet assay, the agricolae, stats, and multcomp packages were employed to fit linear models, apply normalization transformations, and perform multiple comparisons adjusted by the Bonferroni method.

Given the exploratory nature of the GLM and cell-level ANOVA analyses described above, and the lack of tank-level replication in the experimental design (each of the twelve treatments was assigned to a single tank), results derived from these complementary models are interpreted with caution and are reported as mechanistic trends rather than confirmatory evidence.

RESULTS

Cd treatments showed a non-monotonic mortality pattern rather than a clear dose-response relationship. In Cd1 (0.2 µg/kg), mortality was 25 %. Treatments Cd2 (0.8 µg/kg) and Cd3 (1.4 µg/kg) resulted in no mortality; however, sublethal signs, such as erratic swimming, lethargy, and diminished response to external stimuli, were observed. In the control group, no mortality was observed, and no behavioral alterations were noted.

In Pb treatments, the Pb1 (2 µg/kg) group showed no mortality or notable alterations. In contrast, in Pb2 (8 µg/kg) the death of one female was recorded, and in Pb3 (14 µg/kg) two males died. Additionally, in the Pb3 group, signs such as ocular opacity, loss of swimming axis, and pallor were observed, with these manifestations being more prominent in male individuals.

The binary combination treatments of cadmium and lead (DP) showed greater toxicity compared to individual metal exposures. Treatments DP1 (0.2 µg Cd/kg + 8.0 µg Pb/kg) and DP5 (0.2 µg Cd/kg + 2.0 µg Pb/kg) presented low mortality of 12.5 % in each treatment. In DP2 (0.8 µg Cd/kg + 2.0 µg Pb/kg), one male died (12.5 %). Treatment DP4 (1.4 µg Cd/kg + 8.0 µg Pb/kg) recorded two deaths (one male and one female) and was associated with severe signs. DP3 (Cd 0.8 + Pb 14 µg/kg) proved to be the most toxic treatment, with

a total mortality of six out of eight fish (75 %), including specifically 100 % mortality in females, accompanied by severe signs indicative of neuromuscular dysfunction and physiological stress (Fig. 1A). These results together suggest toxicity that is dependent on exposure dose and individual sex, as well as the existence of effects exceeding those of individual exposures between cadmium and lead. Notably, sex-based susceptibility appeared to vary depending on the specific treatment: while DP3 was lethal primarily to females, males accounted for a higher overall frequency of mortality across the full set of treatments, suggesting that susceptibility to lethal effects in *P. magdalenae* may be sex- and treatment-specific rather than uniform (Fig. 1B).

Micronucleus assay: A higher frequency of nuclear abnormalities (Fig. 2) and micronuclei was recorded in individuals exposed to metals (Cd and Pb).

Particularly, the binary combination treatments of cadmium and lead, such as DP2 (0.8 µg Cd/kg + 2.0 µg Pb/kg) and DP3 (0.8 µg Cd/kg + 14.0 µg Pb/kg) presented the highest frequencies of nuclear abnormalities (0.0236 and 0.0233, respectively) (Fig. 3A). Regarding micronuclei, the highest frequency was detected in the treatment with the highest lead dose (Pb2: 8.0 µg/kg), reaching a value of 0.0021. This was followed by treatments Pb1 (2.0 µg/kg) and DP3 (0.8 µg Cd/kg + 14.0 µg Pb/kg), with frequencies of 0.0016 and 0.0018, respectively (Fig. 3B).

The mutagenicity index (MI), which expresses the fold-change in micronucleus frequency relative to the negative control, was greater than 1 in all eleven treatments — including both individual and combined exposures. This consistent elevation across every treatment group reinforces the pattern already established through the GLM analysis of micronucleus and nuclear abnormality frequencies described above, in which most treatments showed statistically significant increases relative to the control. While the MI itself is a descriptive ratio rather than a hypothesis-tested

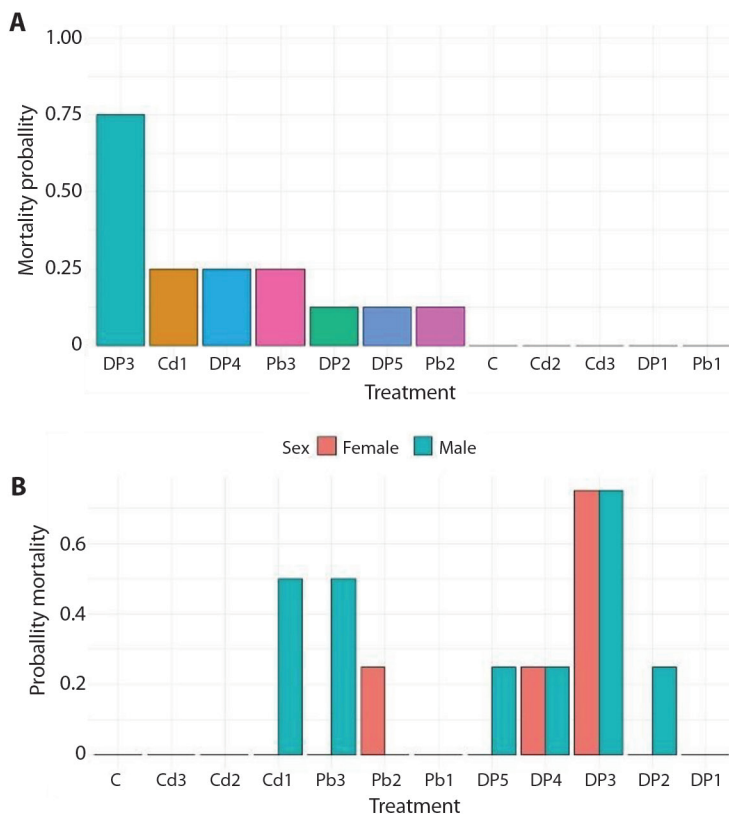


Fig. 1. Mortality probability of *P. magdalenae* exposed to acute doses of cadmium (Cd), lead (Pb), and their binary combinations (DP) for 72 hours. **A.** Overall mortality probability by treatment. **B.** Mortality probability by sex (female and male) across treatments. DP: binary combination of cadmium and lead; C: negative control.

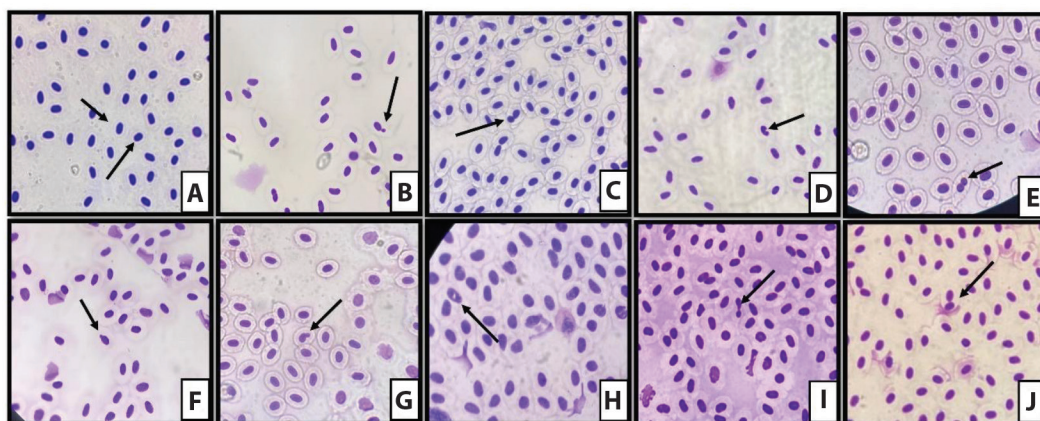


Fig. 2. Blood smears of *P. magdalenae* stained with 10 % Giemsa solution, observed under oil immersion objective (100×). a. Normal erythrocytes. b.-c. Micronuclei of different sizes. d. notched (nuclear notches). e. eight-shaped nucleus. f. nuclear bud. g. blebbed (evaginations). h. nuclear vacuole. i. nucleus with plasmatic bridge. j. binucleated. Images captured at 100× magnification (oil immersion). Note: scale bar could not be added due to the absence of a calibrated reference micrometer at the time of image acquisition.

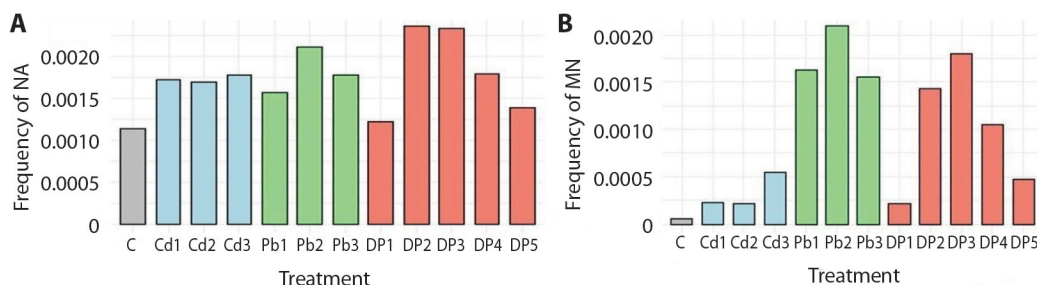


Fig. 3. A. Frequency of nuclear abnormalities in erythrocytes of *P. magdalenae* exposed to doses of Cd and Pb and their binary combinations (DP). **B.** Frequency of micronuclei in erythrocytes of *P. magdalenae* exposed to doses of Cd and Pb and their binary combinations (DP).

statistic in isolation, its uniformity across all treatment groups — regardless of metal type, dose, or combination — adds weight to the biological interpretation that toxic metal exposure consistently increased mutagenic potential in *P. magdalenae* erythrocytes at the doses tested. For nuclear abnormalities, the highest mutagenicity index was detected in the combined treatment DP2 (0.8 μg Cd/kg + 2.0 μg Pb/kg) with 2.08, followed by DP3 (0.8 μg Cd/kg + 14.0 μg Pb/kg) with 2.05 and Pb2 (1.85) (Fig. 4A). Regarding micronucleus formation, lead treatments exhibited the highest values (Pb2: 33.87, Pb1: 26.38 and Pb3: 25.22), along with binary treatments DP3 (29.14) and DP2 (23.05) (Fig. 4B).

For NA, the model showed that groups exposed to Cd, Pb, and the DP combination presented significantly higher probabilities of NA compared to the control group (Cd: $\beta =$

0.425, $p = 0.0003$; Pb: $\beta = 0.467$, $p < 0.001$; DP: $\beta = 0.445$, $p < 0.001$), with no significant differences among exposed groups ($p > 0.9$). In the case of MN, the model indicated a significant increase in probability in fish exposed to Pb compared to the control group ($\beta = 3.348$, $p = 0.019$), while the DP combination showed a marginal trend ($\beta = 2.665$, $p = 0.063$), and Cd was not statistically significant ($p = 0.2448$). Multiple comparisons revealed that Pb produced a significantly higher frequency of MN than Cd ($p < 0.001$), and that DP presented a lower frequency than Pb ($p = 0.0279$). Regarding sex, no significant differences were found in NA frequency ($p = 0.707$), but there were differences in MN frequency, with males being less prone than females to present this alteration ($\beta = -0.632$, $p = 0.0109$). Both models presented acceptable McFadden's pseudo R^2 values, suggesting adequate statistical fit.

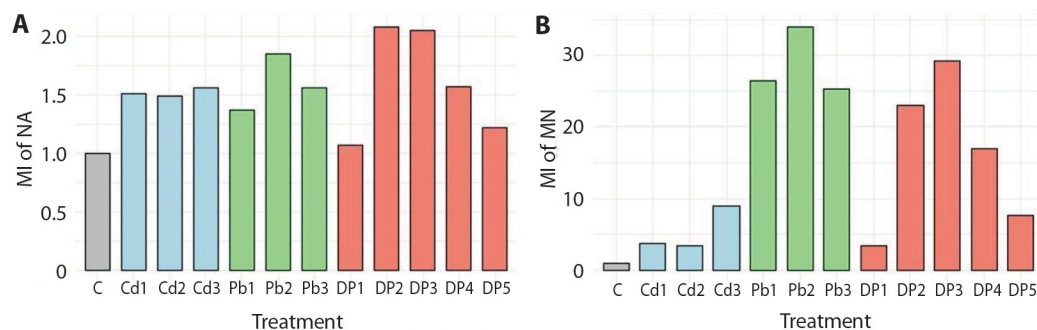


Fig. 4. A. Mutagenicity index of nuclear abnormalities in erythrocytes of *P. magdalenae* exposed to doses of Cd and Pb and their binary combinations (DP). **B.** Mutagenicity index of micronuclei in erythrocytes of *P. magdalenae* exposed to doses of Cd and Pb and their binary combinations (DP).

To explore whether the observed effects on nuclear abnormalities (NA) and micronuclei (MN) presented a dose-dependent pattern, separate logistic models were fitted for each metal (Cd and Pb), considering different concentrations (low, medium, and high). In the case of cadmium, all doses (Cd1, Cd2, and Cd3) generated a significant increase in NA probability compared to the control (Cd1: $\beta = 0.416$, $p = 0.004$; Cd2: $\beta = 0.393$, $p = 0.005$; Cd3: $\beta = 0.452$, $p < 0.001$), although no significant differences were detected between doses ($p > 0.96$), suggesting a sustained effect without a clear dose relationship, as observed in Fig. 5A. For lead, significant increases were also observed at all doses compared to the control (Pb1: $\beta = 0.322$, $p = 0.020$; Pb2: $\beta = 0.648$, $p < 0.001$; Pb3: $\beta = 0.430$, $p = 0.002$), and in this case, Pb2 showed a significantly greater effect than Pb1 ($p = 0.040$), pointing to a possible dose-response relationship, at least in the lower and medium doses (Fig. 5B).

Regarding micronuclei, cadmium did not produce significant increases at any dose ($p > 0.14$), and no differences were observed between different concentrations, indicating an absence of dose-dependent effect on this variable (Fig. 6A). In contrast, lead generated a significant increase in MN probability at all doses compared to the control (Pb1: $\beta = 3.275$, $p = 0.024$; Pb2: $\beta = 3.604$, $p = 0.013$; Pb3: $\beta = 3.157$, $p = 0.030$), although comparisons between doses were not significant ($p > 0.65$), suggesting that the maximum effect could be reached even with the lowest doses, without additional increases at higher concentrations (Fig. 6B). These findings reinforce the results of the general analysis, confirming that lead exhibits greater genotoxic potency, as evidenced by a significant increase in both nuclear abnormalities (NA) and micronuclei (MN) frequency across all tested doses. Cadmium, by contrast, demonstrated a more moderate genotoxic profile, with effects predominantly manifested as

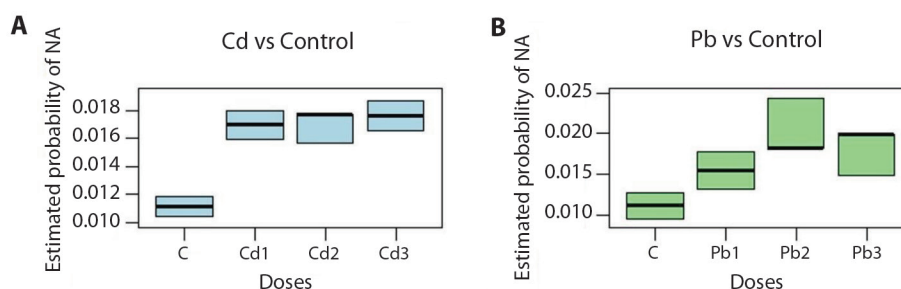


Fig. 5. A. Estimated probability of nuclear abnormalities in erythrocytes of *P. magdalenae* exposed to Cd doses. **B.** Estimated probability of nuclear abnormalities in erythrocytes of *P. magdalenae* exposed to Pb doses.

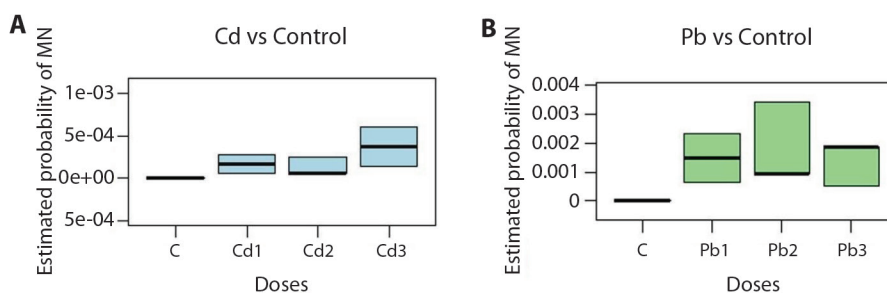


Fig. 6. A. Estimated probability of micronuclei in erythrocytes of *P. magdalenae* exposed to Cd doses. **B.** Estimated probability of micronuclei in erythrocytes of *P. magdalenae* exposed to Pb doses.

NA induction and no statistically significant elevation in MN frequency, suggesting that both metals operate through mechanistically distinct genotoxic pathways.

Finally, with the objective of exploring possible synergistic or antagonistic effects between Cd and Pb, some specific combinations of both metals (DP1–DP5) were evaluated. In the case of nuclear abnormalities (NA), some combinations, such as DP2 (Cd 0.8 µg/kg + Pb 2.0 µg/kg) and DP3 (Cd 0.8 µg/kg + Pb 14 µg/kg), showed significant increases in estimated NA probability compared to the control (DP2: $\beta = 0.766$, $p < 0.001$; DP3: $\beta = 0.680$, $p < 0.001$), with levels similar to or even higher than those observed for the highest individual doses of each metal (Fig. 5A, Fig. 5B). This pattern suggests a possible potentiated combined toxicity, especially in DP2, where both concentrations are intermediate. On the other hand, combinations such as DP1 (Cd 0.2 µg/kg + Pb 8.0 µg/kg) and DP5 (Cd 0.2 µg/kg + Pb 2.0 µg/kg) showed no significant effects compared to the control, nor marked differences with respect to other combinations, suggesting possible antagonism at these specific proportions. Additionally, comparison between combinations evidenced significant differences between DP2 and DP5 ($p < 0.001$) and between DP3 and DP5 ($p = 0.043$), supporting the idea that the interaction between metals strongly depends on their relative concentrations.

In the case of MN, combinations DP2 and DP3 also resulted in significant increases compared to the control group (DP2: $\beta = 3.116$,

$p = 0.032$; DP3: $\beta = 3.430$, $p = 0.021$), as observed in Fig. 7A. These combinations reach levels comparable to those observed with individual Pb exposure (Fig. 7B), reinforcing the hypothesis of a relevant joint effect. However, as with NA, other combinations such as DP1, DP4, and DP5 did not present significant effects compared to the control. Unlike the results observed with Pb exposure alone, where all doses generated robust increases in MN, the combined effects did not show consistent improvement, indicating that at specific proportions the mixture may not potentiate the lead impact, and may even attenuate it. Overall, these findings demonstrate that the interaction between cadmium and lead is neither linear nor uniform but rather depends on the balance between specific concentrations of each metal.

Statistical analysis using a generalized linear model (GLM) confirmed that all exposure groups Cd, Pb, and their binary combinations (DP) presented significantly higher probabilities of nuclear abnormalities (NA) compared to the negative control (Cd: $\beta = 0.425$, $p = 0.0003$; Pb: $\beta = 0.467$, $p < 0.001$; DP: $\beta = 0.445$, $p < 0.001$), with no statistically significant differences among the three exposed groups ($p > 0.9$). Dose-specific analysis revealed that all individual Cd doses significantly increased NA probability relative to the control (Cd1: $\beta = 0.416$, $p = 0.004$; Cd2: $\beta = 0.393$, $p = 0.005$; Cd3: $\beta = 0.452$, $p < 0.001$), although no significant differences were detected between doses ($p > 0.96$), suggesting a sustained genotoxic effect without a clear dose-response pattern.

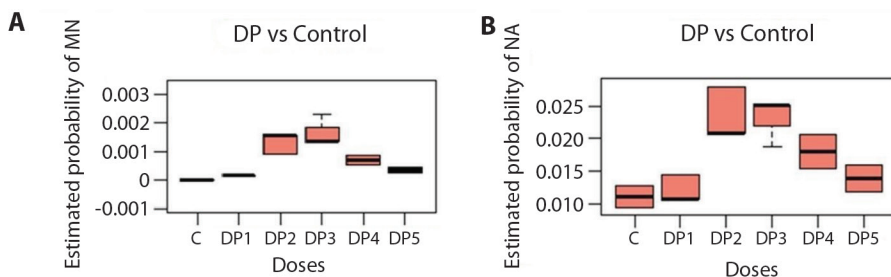


Fig. 7. A. Estimated probability of micronuclei in erythrocytes of *P. magdalenae* exposed to doses of Cd and Pb combinations. B. Estimated probability of nuclear abnormalities in erythrocytes of *P. magdalenae* exposed to doses of combinations of Cd and Pb.



For Pb, all doses also produced significant NA increases (Pb1: $\beta = 0.322$, $p = 0.020$; Pb2: $\beta = 0.648$, $p < 0.001$; Pb3: $\beta = 0.430$, $p = 0.002$), with Pb2 showing a significantly greater effect than Pb1 ($p = 0.040$), indicating a partial dose-response relationship at lower and intermediate concentrations. Regarding micronuclei (MN), only the Pb group showed a statistically significant increase compared to the control ($\beta = 3.348$, $p = 0.019$), while Cd was not statistically significant ($p = 0.2448$), and the DP combination showed only a marginal trend ($\beta = 2.665$, $p = 0.063$). At the dose level, all individual Pb doses significantly increased MN probability (Pb1: $\beta = 3.275$, $p = 0.024$; Pb2: $\beta = 3.604$, $p = 0.013$; Pb3: $\beta = 3.157$, $p = 0.030$), though comparisons between doses were not significant ($p > 0.65$), suggesting that even the lowest Pb dose may elicit a near-maximal MN response. Multiple comparisons further confirmed that Pb produced a significantly higher MN frequency than Cd ($p < 0.001$), and that DP combinations presented a lower MN frequency than Pb alone ($p = 0.0279$). Regarding sex, no significant differences were found in NA frequency ($p = 0.707$), while males were significantly less

prone than females to present MN ($\beta = -0.632$, $p = 0.0109$).

Comet Assay: Genetic damage in *P. magdalenae* was evaluated following acute exposure to Cd, Pb, and their binary combinations (DP). The comet assay was performed using peripheral blood cells, and tail DNA percentage and tail moment were quantified. Both parameters revealed highly significant differences between treatment groups. Specifically, ANOVA for tail moment demonstrated a significant effect ($F_{11,4262} = 23.09$ $p < 2e^{-16}$), indicating that the type and combination of toxic metals significantly altered genetic material integrity.

Post-hoc analysis with Bonferroni correction, applied to treatment medians, allowed the establishment of five statistically distinct groups (a–e) for tail DNA percentage and tail moment (Table 2). This grouping indicates that treatments sharing the same letter (e.g., “a”, “ab”, or “abc”) do not present significant differences among themselves, while those in groups with different letters (e.g., “a” vs “c” or “ab” vs “e”) differ statistically significantly ($p < 0.05$). Combined treatments with Cd and Pb evidenced

Table 2
Median values ($\pm$ SD) between different treatments on tail DNA percentage and tail moment in *Prochilodus magdalenae*.

Treatment	Tail DNA (%)		Tail DNA (SD)	Tail Moment (%)		Tail Moment (SD)
DP3	4.374	a	7.32	0.703	ab	0.34
DP1	4.108	ab	15.25	1.036	a	16.22
Pb3	3.984	ab	7.91	0.572	bc	3.63
Cd2	3.966	ab	6.80	0.386	de	0.31
DP2	3.944	ab	9.24	0.505	bcd	1.83
DP4	3.897	abc	13.72	0.589	abc	5.62
Pb1	3.841	abc	6.12	0.449	cd	0.34
Cd3	3.830	abc	5.75	0.283	e	0.22
Cd1	3.801	abc	8.53	0.444	cd	1.84
Pb2	3.733	bc	7.19	0.335	de	0.50
DP5	3.642	c	8.23	0.370	de	1.46
Control (C)	3.562	c	NA	0.500	bcd	NA

Treatments sharing the same letter do not present statistically significant differences ($p > 0.05$), while treatments assigned different letters differ significantly ($p < 0.05$), as determined by Bonferroni-corrected post-hoc analysis following one-way ANOVA.

the greatest genetic damage. DP3 (Cd 0.8 µg/kg + Pb 14 µg/kg) was placed in group “a” for tail DNA percentage (4.37%), while DP1 (Cd 0.2 µg/kg + Pb 8 µg/kg) was classified in “ab” (4.11 %). However, both treatments shared group “a” for the tail moment, with median values of 0.703 and 1.036, respectively, reflecting a greater degree of DNA fragmentation. These values were significantly higher than those observed in the control group (3.56 % tail DNA; tail moment: 0.500), which was placed in groups “c” and “bcd”, respectively. This confirms that binary combinations of Cd and Pb generate more severe genotoxic damage than individually administered metals, suggesting a potentiated combined toxicity between both contaminants.

As a robustness check, given that the analysis above treated each of the ~100 scored cells per fish as an independent replicate, a sensitivity reanalysis was conducted using the individual fish as the unit of analysis ($n = 56$ fish with usable comet-assay data across the 12 treatments, ranging from 1 to 7 individuals per group; median tail DNA percentage and tail moment calculated per fish). Shapiro–Wilk tests confirmed significant deviations from normality for both variables at the individual level ($p < 0.001$ in both cases), while Levene’s test indicated homogeneous variances across treatments ($p > 0.05$ for both variables), justifying the use of Kruskal–Wallis rather than parametric ANOVA for this reanalysis. Under this more conservative approach, Kruskal–Wallis tests found no statistically significant differences among treatments for either tail DNA percentage ($H = 13.71$, $df = 11$, $p = 0.249$) or tail moment ($H = 9.83$, $df = 11$, $p = 0.546$), and Dunn’s post-hoc pairwise comparisons (Bonferroni-adjusted) yielded $p = 1.000$ for all 66 pairwise combinations for both variables. This discrepancy with the cell-level results in Table 2 illustrates the effect of pseudoreplication — arising from treating multiple cells from the same individual as independent observations — on statistical power, and suggests that the apparent group differences in Table 2 should be interpreted with caution pending

confirmation with larger numbers of individuals per treatment.

Similarly, the combined treatment DP1 (Cd 0.2 µg/kg + Pb 8 µg/kg) resulted in significantly greater genetic damage compared to the negative control (C). Quantification using the comet assay revealed that the median tail DNA percentage in DP4 was 3.89 %, which was higher than that of the control (3.46 %). Similarly, the tail moment in DP4 (median = 0.81) significantly exceeded the control value (median = 0.43) ($p < 0.05$). These findings suggest that joint exposure to lead and a low concentration of cadmium has a more pronounced toxic effect than individual exposure to each metal. The increase in both comet assay parameters evidences greater cellular DNA fragmentation, suggesting a possible synergistic interaction between Cd and Pb in this treatment. Although this comparison did not remain significant in the individual-level sensitivity analysis described above.

In contrast, treatments involving individual exposure to Cd or Pb generated relatively low levels of genetic damage, comparable to each other and the control group. These treatments were statistically grouped within group “b”, indicating the absence of significant differences with the control ($p > 0.05$). For example, Cd1 (0.2 µg/kg Cd), Pb1 (2 µg/kg Pb), and Cd2 (0.8 µg/kg Cd) presented median tail DNA percentages ranging between 3.73 and 3.97 %, while median tail moment values were situated between 0.34 and 0.44.

Likewise, analysis of intragroup variability, evaluated through standard deviation (SD), revealed marked differences between treatments. Regarding tail DNA percentage, combined treatments DP1 (SD = 15.25), DP4 (SD = 13.72), and DP2 (SD = 9.24) presented the greatest dispersions, evidencing high heterogeneity in individual genotoxic responses within these groups. Among individual cadmium treatments, Cd1 (SD = 8.53) showed the greatest variability. Regarding tail moment, DP1 also exhibited the highest standard deviation (SD = 16.22), followed by DP4 (SD = 5.62) and Pb3 (SD = 3.63). In contrast, treatments



such as Cd3 (SD = 0.22) and Cd2 (SD = 0.31) showed the lowest dispersions. These results suggest that combined exposures to Cd and Pb, especially in DP1 and DP4, induce more variable genotoxic responses among individuals, compared to individual metal exposures. This greater variability could reflect differences in individual susceptibility to the potentiated combined toxicity of heavy metals. Notably, in the underlying individual-level data, a single fish in each of the DP1 and DP4 treatments exhibited tail DNA percentages exceeding 40 %, more than double the respective group medians; excluding these two individuals attenuated the Kruskal–Wallis result for tail DNA percentage ($H = 19.19$, $p = 0.058$), while tail moment remained clearly non-significant ($p = 0.283$). This suggests that part of the apparent variability in these two treatments may reflect individual outlier responses rather than a consistent group-level effect.

The previous results suggest patterns consistent with synergistic interactions, though not formally quantified using mixture-toxicity models between cadmium and lead. Treatments DP1 and DP3, in particular, induced significantly greater DNA damage than exposures to the metals separately.

DISCUSSION

The results of this study demonstrate that both Cd and Pb, individually or in combination, are capable of inducing behavioral alterations, increasing mortality, and generating genetic damage in *P. magdalenae* following acute exposure. The observed variability in toxicity among treatments underscores the complexity of the underlying mechanisms, where metal concentration, metal interactions, and individual sex play a fundamental role.

Regarding behavioral responses and mortality, clear dose dependence was evidenced, particularly in the case of Cd. The observed sex-differentiated vulnerability could be linked to differences in endocrine systems or in the expression of detoxifying proteins, such as metallothioneins (Awad et al., 2024). Although

intermediate and high Cd concentrations (Cd2: 0.8 µg/kg and Cd3: 1.4 µg/kg) did not induce mortality, they triggered sublethal signs such as erratic swimming, lethargy, and diminished response to stimuli, which are associated with neurotoxicity and disruption of the locomotor and homeostatic system (Green & Planchart, 2018; Paduraru et al., 2023). Despite the absence of mortality at higher Cd doses, the death of two males at the lowest dose (Cd1, 0.2 µg/kg) suggests potential non-linearity in toxicity under certain individual physiological or sex-specific conditions, possibly reflecting hormetic adaptive responses, differential activation of detoxification mechanisms such as metallothioneins at higher doses, or individual susceptibility factors — a pattern that warrants further investigation.

It should be noted that mortality percentages reported here are based on small group sizes ($n = 8$ fish per treatment), meaning that each individual death corresponds to a 12.5 percentage-point change; these estimates should therefore be interpreted as indicative rather than precise, and larger sample sizes are recommended in future studies.

Pb treatments manifested dose-dependent toxicity, with Pb3 (14 µg/kg) showing mortality in males (50 %) and evident signs such as ocular opacity and loss of swimming axis, consistent with its known neurotoxic impact on neurosensory and muscular structures (Lee & Freeman, 2014; Mondal et al., 2018; Shahjahan et al., 2022).

Cd exposure resulted in a significant increase in nuclear abnormalities (NA) even at low concentrations, although without clearly evidence to defined dose-response relationship, suggesting the existence of a relatively low genomic toxicity threshold. Studies conducted in other fish species under chronic or subchronic conditions have reported persistent cellular and biochemical effects at low Cd concentrations, including alterations in enzymatic metabolism, hepatocyte vacuolization, and intestinal apoptosis (Almeida et al., 2002; Younis et al., 2015). Although these studies involve longer exposure durations and different

species, they provide relevant mechanistic context for understanding the cellular responses observed in the present acute study, particularly the induction of NA without a clear dose-dependent pattern, which may reflect early activation of oxidative stress pathways even at minimal exposure thresholds.

In contrast, Pb showed a more defined pattern of micronuclei (MN) induction, strongly associated with interference in mitotic processes, particularly with microtubule and centrosome function, conferring a well-characterized aneugenic and clastogenic action (Lee & Freeman, 2014). The progressive increase in MN observed can be attributed to both structural and oxidative mechanisms, as Pb not only acts directly on mitotic machinery but also generates reactive oxygen species (ROS) that provoke lipid peroxidation, antioxidant dysfunction, and DNA damage (Zhu et al., 2016). Although the mechanistic evidence for Pb-induced MN has been documented in developmental zebrafish models, which differ in exposure context from the present acute study, these findings provide a useful framework for interpreting the dose-correlated genotoxic responses observed in *P. magdalenae*.

A significant effect of sex on micronuclei frequency was observed, with males presenting a lower incidence of this type of genetic damage, however, given the small subgroup sizes involved (approximately four individuals per sex per treatment), this finding should be interpreted with caution and considered preliminary until confirmed in future studies with larger, sex-balanced samples. This difference may suggest hormonal variations between sexes or higher expression of detoxifying proteins in males, which could contribute to a greater capacity to mitigate genotoxic effects (Awad et al., 2024). However, this interpretation remains speculative in the context of the present study and warrants further investigation through targeted molecular analyses. This pattern contrasts with the higher mortality observed in males, which could be related to sex-specific physiological factors and the synergistic interaction between Cd and Pb, since eight of the ten dead

males were exposed to combined treatments. In *P. magdalenae*, this interaction may have overwhelmed antioxidant defense systems, thereby increasing lethality in this group (Almeida et al., 2002; Chen et al., 2022; Ibrahim et al., 2021).

Co-exposure to cadmium and lead (DP) resulted in substantially more severe effects than individual treatments, indicating synergistic and, in some cases, antagonistic toxic interactions (Shahjahan et al., 2022). These patterns consistent with synergistic interactions, though not formally quantified using mixture-toxicity models can be explained at the molecular level by metallothionein (MT) saturation, competition between metals for detoxification pathways, and potentiation of oxidative stress, which act convergently on genomic integrity (Chen et al., 2022; Duan et al., 2017; Sato & Kondoh, 2002; Shahjahan et al., 2022). These mechanisms are consistent with what has been reported in other aquatic models, where acute co-exposure to toxic metals amplifies toxicity through increased hepatic bioaccumulation, mitochondrial damage, and disruption of essential cellular processes (Abdel-Warith et al., 2011; Ali et al., 2025; Chakraborty, 2021; Mondal et al., 2018; Younis et al., 2015). It is important to note that the term synergistic is used here in a descriptive sense to indicate that combined exposures produced greater effects than those observed with either metal alone. A formal synergy analysis using concentration addition or independent action models was not conducted in the present study and is recommended for future research to quantitatively characterize the nature of Cd–Pb interactions in *P. magdalenae*.

However, not all co-exposure schemes showed an amplified response. In treatments DP1 (Cd 0.2 + Pb 8 µg/kg) and DP5 (Cd 0.2 + Pb 2 µg/kg), genotoxic effects did not exceed those observed in control groups or individual treatments, suggesting possible antagonistic interactions. Such effects may arise from the activation of cross-defense pathways, such as the Nrf2 transcription factor pathway, or from competition between Cd and Pb for cellular transporters, which reduces their intracellular



accumulation and, consequently, their toxicity (Wang et al., 2013; Wang & Gallagher, 2013). Collectively, these findings demonstrate that the interaction between Cd and Pb is neither linear nor uniform but depends on the balance between specific concentrations of each metal and the organism's adaptive capacity.

In the Comet assay, it was demonstrated that both cadmium and lead induced genetic damage in *P. magdalenae* compared to the control group; however, a clear dose-dependent relationship was not evident. In the case of lead, Pb3 (14 µg/kg) caused the highest tail DNA percentage and highest tail moment, reflecting more intense genotoxic damage. However, Pb2 (8 µg/kg) presented values lower than Pb1 (2 µg/kg), which interrupts the expected linearity. This pattern suggests a possible hormetic response, where intermediate exposures induce cellular defense mechanisms that attenuate damage, such as the activation of antioxidant enzymes or the expression of stress proteins (Calabrese & Baldwin, 2001; Gasulla et al., 2016; Tomasello et al., 2012). A similar behavior was observed with cadmium: Cd2 (0.8 µg/kg) recorded the highest tail DNA percentage, while Cd3 (1.4 µg/kg) showed values even lower than the control, particularly in the tail moment. This could indicate a more efficient adaptive response to high doses, such as induction of metallothioneins or antioxidant enzymes that limit DNA damage (Flora et al., 2008; Sato & Kondoh, 2002) or a possible loss of highly damaged cells that are eliminated by apoptosis and, therefore, are not detected in the comet analysis (Collins et al., 2008). Collectively, these results are consistent with a non-linear response, where the intensity of genetic damage does not increase proportionally with dose, but fluctuates as a function of activation or saturation of cellular protection mechanisms, as has been described in toxic metal toxicity models (Calabrese & Baldwin, 2001).

It should be noted that this non-monotonic pattern was identified in the cell-level analysis; an individual-level sensitivity analysis (see Results) did not detect statistically significant differences between doses, so this hypothesis

remains speculative and warrants confirmation with larger per-treatment sample sizes.

Although the absolute increases in tail DNA percentage may appear modest, they represent statistically significant elevations of 15–23 % above the control baseline and are consistent with values reported for other neotropical fish species under acute toxic metal exposure (Da Rocha et al., 2009; Frenzilli et al., 2009; Monteiro et al., 2011); however, as noted above, these differences were not statistically significant once the individual fish was used as the unit of analysis, and should be interpreted as a possible early, subtle signal warranting confirmation with larger individual sample sizes.. Given that the comet assay detects primary and transient DNA strand breaks at early stages after acute exposure, these differences are biologically relevant as indicators of early genotoxic stress that, if maintained under chronic or environmentally realistic exposure conditions, could progress to more stable chromosomal damage detectable by micronucleus endpoints (Collins, 2004; Fenech, 2000).

The combined treatments DP1 (Cd 0.2 + Pb 8 µg/kg) and DP3 (Cd 0.8 + Pb 14 µg/kg) produced the numerically highest comet assay values at the cell level; however, this pattern was not statistically confirmed at the individual level, and therefore cannot currently be interpreted as evidence that these combinations exceed cellular repair capacities beyond what is expected from individual exposures alone. Notably, treatment DP1 presented lower MN and NA frequencies than the negative control yet showed one of the highest DNA damage values in the comet assay. This apparent discrepancy can be attributed to the temporal and functional differences between biomarkers: while the comet assay detects primary and transient DNA strand breaks at very early stages after exposure (Collins, 2004), the micronucleus test reflects more stable chromosomal damage manifested after cell division (Fenech, 2000). It is therefore possible that the genetic damage in DP1 was intense but acute, affecting cells that had not yet divided, or activating

apoptotic pathways prior to micronucleus formation (Frenzilli et al., 2009).

The joint evaluation of morphological damage biomarkers (NA and MN) alongside the Comet assay proved fundamental for a robust and differentiated characterization of genotoxicity. Individual Cd exposure predominantly induced NA, suggesting sublethal nuclear damage such as invaginations or binucleated cell formation (Abass, 2024; Cherednichenko et al., 2024; Razzaq et al., 2021), consistent with single-strand breaks and oxidative stress mechanisms (Da Rocha et al., 2009; Yu et al., 2020; Zhou et al., 2008). Pb, by contrast, induced a greater frequency of MN, pointing toward an aneugenic mechanism linked to alterations in the mitotic machinery. In the Comet assay, Pb caused DNA fragmentation similar to that of Cd but with less severity than some of the combinations, reinforcing its more specific action during the cell cycle, particularly during mitosis (Atli et al., 2015; Lee & Freeman, 2014; Luzhna et al., 2013). These differentiated genotoxic profiles reinforce the complementary value of using multiple biomarkers in ecotoxicological assessments.

Although the genotoxic effects observed in this study do not directly reflect typical environmental exposure scenarios — given that intraperitoneal injection was used rather than natural routes such as oral, dermal, or branchial exposure — this approach guaranteed precise and controlled doses per individual, facilitating the identification of key cellular responses and metal action mechanisms at the systemic level. Analysis in erythrocytes, while it might seem a limitation due to their high turnover rate and absence of bioaccumulation, constitutes a sensitive tool to detect systemic genetic damage, since it reflects active hematopoietic processes during brief but intense exposures. Despite mature erythrocytes in fish having a low division rate, the increase in micronuclei observed suggests alterations during erythropoiesis, probably associated with metal interference with the achromatic spindle and cell cycle dynamics. Additionally, induced toxicity suggests a pattern of sexual dimorphism: while

males showed greater susceptibility to mortality, females presented a higher frequency of micronuclei, which could indicate physiological differentiation in response mechanisms to genotoxic stress induced by toxic metals. This differential response, if maintained under chronic or environmentally relevant exposure conditions, could compromise cellular integrity, negatively affect reproductive function, and consequently put at risk the population stability of *P. magdalenae*, a native species of significant ecological and socioeconomic importance. Furthermore, the response of *P. magdalenae* was comparable to that observed in model species such as *D. rerio*, which not only validates its use in this type of bioassay but also reinforces its potential as a sentinel species for evaluating genotoxicity in neotropical ecosystems. Persistence of NA and MN in somatic cells may serve as early indicators of carcinogenic processes, a risk associated with chronic exposures to toxic metals (Da Rocha et al., 2009; Frenzilli et al., 2009), with direct consequences for development, reproduction, and organism survival (Luzhna et al., 2013; Razzaq et al., 2021), constituting a solid foundation for future research oriented toward the integration of biomarkers in environmental monitoring strategies with an ecotoxicological approach.

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Author contribution statement:

Diana Carolina López-Obando contributed to the conception and design of the study, data collection, data analysis, interpretation of results, and drafting of the manuscript.

Silvana Osorio-Cardona participated in the experimental design, data acquisition, and interpretation of results, and contributed to the manuscript review and editing.

Licet Yurany Montoya-Gaviria was involved in fieldwork coordination, data collection, and interpretation of results, and contributed to the writing and revision of the manuscript.

Luz Yaneth Orozco-Jiménez participated in methodological supervision, data interpretation, and manuscript revision, contributing to the improvement of scientific rigor and structure.

Jonny Andrés Yepes-Blandón supervised the entire research process, contributed to the conceptual design, data analysis, interpretation of results, and critically reviewed and approved the final version of the manuscript.

Ethical statement: The authors declare that they all agree with this publication and made significant contributions; that there is no conflict of interest of any kind; and that we followed all pertinent ethical and legal procedures and requirements. All financial sources are fully and clearly stated in the acknowledgments section. A signed document has been filed in the journal archives.

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