FUDMA Journal of Sciences (FJS)
ISSN online: 2616-1370
ISSN print: 2645 - 2944
Vol. 10 No. 24, December, 2026, pp 227 – 231
DOI: https://doi.org/10.33003/fjs-2026-1024-6295

Toxicity and Developmental Biomarker Profiles of Lead, Chromium, Cadmium and Ternary Mixture in *Xenopus Tropicalis*

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Received: 21 Oct. 2026
Accepted: 10 Nov. 2026
Published: 14 Nov. 2026

ABSTRACT
Lead (Pb), chromium (Cr), and cadmium (Cd) are metal contaminants of concern in amphibian habitats, yet developmental evidence for their combined effects in *Xenopus tropicalis* remains limited. This study compared 96h acute toxicity and developmental biomarker profiles following exposure to Pb, Cr, Cd, and Pb-Cr-Cd mixture. Tadpole, late larval, and froglet stages were assessed for mass, survival, malformation, antioxidant enzyme activity, malondialdehyde (MDA), and micronucleus frequency. The 96h LC50 estimates were 18.4 mg $L^{-1}$for Pb, 12.7 mg $L^{-1}$for Cr, 2.9 mg L^{-1} for Cd, and 1.8 mg L^{-1} for the mixture. At the froglet stage, the mixture group had lower final mass (1.927 ± 0.028 g) and survival (92.34 ± 0.36%) than the controls (2.21 ± 0.03 g and 97.44 ± 0.36% respectively), together with higher malformation (11.80 ± 0.25% and 3.50 ± 0.25%), MDA (3.410 ± 0.047 and 2.02 ± 0.05 nmol mg $^{-1}$ protein), and micronucleus frequency (4.88 ± 0.10% and 1.56 ± 0.10%). Across stages, exposure was associated with reduced developmental performance and elevated oxidative stress and genotoxicity endpoints. The mixture showed the highest overall deviation from the control hence, no interaction model was tested, and the findings did not establish synergism. The results support the value of integrating acute toxicity, developmental, and biomarker endpoints when assessing metal exposure in *X. tropicalis*.

Keywords: Amphibian ecotoxicology, Biomarker, Heavy metals, Metal mixture, Oxidative stress, *Xenopus Tropicalis*

INTRODUCTION

Metal contamination remains a concern for amphibian early life stages because eggs and larvae develop in water and can respond to exposure through altered survival, growth, morphology, and physiological condition (Yang et al., 2020). Studies using amphibian models have shown that lead and cadmium can affect developmental outcomes, while chromium exposure requires careful interpretation because toxicity depends strongly on its oxidation state and chemical form (Haywood et al., 2004; Bosisio et al., 2009). The genus *Xenopus* is well suited to developmental toxicology because embryos develop externally and can be assessed for mortality, growth inhibition, and malformations. Modified FETAX work has shown that *Xenopus tropicalis* is an effective test species/ model organism, although responses must be interpreted in relation to species, developmental stage, exposure duration, and test chemistry (Fort et al., 2004). A 48h FETAX study demonstrated cadmium associated lethality and malformations in *X. tropicalis*, providing direct support for the sensitivity of this species to developmental toxicants (Shi, 2009). The three metals considered here represent distinct but environmentally relevant exposure concerns. Cadmium chloride is embryotoxic and teratogenic in *Xenopus laevis* FETAX assays (Sunderman et al., 1991), whereas cadmium exposure in *X. laevis* larvae has also been associated with stress and genotoxicity related responses (Mouchet et al., 2006). Sodium chromate has produced developmental toxicity, uptake, and distribution effects in Xenopus FETAX experiments (Bosisio et al., 2009), and Pb, Cd, and other metals have been evaluated in *X. laevis* embryos and tadpoles as pollutants of ecological concern (Haywood et al., 2004). Comparable studies in other amphibians strengthen the rationale for reporting chromium as Cr (VI) and for assessing developmental growth and morphology. Cr (VI) exposure altered growth, developmental stage, and oxidative stress related transcription in *Bufo gargarizans* embryos, while longer term Pb or Cd exposures in *X. laevis* influenced malformation, growth, and developmental progression (Sharma & Patiño, 2008; Li et al., 2018; Yang et al., 2020).

Aquatic organisms are more commonly exposed to mixtures than to single contaminants. Metal mixture studies in *X. laevis* tadpoles show that low concentrations can alter selected biomarker responses, but the magnitude and direction of mixture effects depend on the constituents, dose, exposure duration, and endpoint (Yologlu and Ozmen, 2015). Accordingly, a mixture group should be interpreted as a treatment condition rather than presumed to be synergistic without an explicit interaction analysis. Complementary studies in other amphibians have documented developmental and tissue level effects after cadmium, Pb-Cd, and related mixed metal exposures; these reports provide biological context but are not species equivalent evidence for *X. tropicalis* (Ya et al., 2019; Ju et al., 2020; Chen et al., 2022). The present study therefore combined acute lethality with stage resolved developmental and biomarker endpoints in *X. tropicalis*. This integrated framework follows the multi endpoint logic of amphibian developmental testing while retaining cautious interpretation of species and mixture specific effects (Fort et al., 2004; Mouchet et al., 2006). Thus, additional evidence of metal retention and tissue related effects supports combining outcome levels in amphibian assessments: Pb accumulated in *X. laevis* tadpoles, Cd accumulated in the liver and kidney of *Rana ridibunda*, and

Pb produced biochemical and physiological changes in adult *Bufo arenarum* (Berzins and Bundy, 2002; Fink and Salibián, 2005). The objectives were to estimate the 96h LC50 of Pb, Cr, Cd, and a Pb-Cr-Cd mixture, compare mass, survival, and malformation across tadpole, late larval, and froglet stages and characterize antioxidant enzyme, lipid peroxidation, and micronucleus responses.

MATERIALS AND METHODS

Test Organisms, Husbandry and Breeding

Healthy adult *X. tropicalis* were collected from local lentic habitats and acclimated for at least two weeks in aerated 20L plastic containers containing 3L of dechlorinated tap water. Containers were arranged to provide both aquatic and dry areas. Water was renewed every two days, uneaten food and faecal material were removed, and adults were fed earthworms twice weekly. Adults of comparable body mass and snout vent length were selected for breeding. Spawning was induced by injecting a male and a female with human chorionic gonadotropin in the dorsal lymph sac. After spawning, adults were removed from the breeding aquarium. Twenty eggs from each clutch were placed in individual plastic exposure containers. Following hatching, larvae were maintained under a 10:14h light: dark photoperiod at 26-27 °C and were fed daily. Five replicates were evaluated for each treatment at each developmental stage.

Test Chemicals and Exposure Design

Lead was administered as lead nitrate [$Pb(NO_3)^2$], cadmium as cadmium chloride ($CdCl_2$), and chromium as potassium dichromate (K_2 Cr_3 O), which served as the Cr(VI) source. Stock solutions were diluted with dechlorinated water to prepare the test media. The developmental study included five groups: control, Pb, Cr, Cd, and a ternary Pb-Cr-Cd mixture. The mixture contained 0.5 mg L^{-1} Pb, 0.5 mg L^{-1} Cr, and 0.1 mg L^{-1} Cd; the corresponding single metal treatments used the same nominal component concentrations (Shah et al., 2024).

Acute Toxicity Assay

A initial 24h range finding assay was conducted at nominal concentrations of 0.01, 0.1, 1, 10, and 100 mg L^{-1} for each test material. Final 96h exposures were then used to estimate the median lethal concentration (LC50) and its 95% confidence interval from mortality data. Acute toxicity estimates were used to rank relative lethality and to inform the choice of sublethal developmental exposures.

Developmental, Biochemical, and Genotoxicity Endpoints

Tadpole, late larval, and froglet stages were monitored for initial and final mass, mass gain, survival, and external malformations. Malformations included oedema-axial-curvature, and abnormal pigmentation. Snout vent length and body mass were measured at two week intervals during development. For biochemical analysis, whole organism homogenates were prepared for early stages, whereas tissue homogenates were prepared for later stages. Superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) activities were determined as antioxidant enzyme endpoints. Malondialdehyde (MDA) was measured as a lipid peroxidation associated endpoint and was normalized to total protein. Micronucleus frequency was assessed in Giemsa stained blood smears by scoring at least 1,500 intact interphase cells per individual.

Statistical Analysis

Results are reported as mean ± standard deviation (SD). Two way analyses of variance were used to evaluate the effects of developmental stage, treatment, and their interaction on final mass, survival, malformation, SOD, CAT, GPx, MDA, and micronucleus frequency. At the froglet stage, exposed groups were compared with the stage matched control using two sided Dunnett tests. Statistical significance was set at $\alpha = 0.05$.

RESULTS AND DISCUSSION

Acute Toxicity Classification

The 96h LC50 estimates differed substantially among treatments (Table 1; Fig. 1). Pb had the highest LC50 (18.4 mg L^{-1}), followed by Cr (12.7 mg L^{-1}), Cd (2.9 mg L^{-1}), and the Pb-Cr-Cd mixture (1.8 mg L^{-1}). Thus, within the tested conditions, the acute toxicity ranged from highest to lowest (least) toxicity mixture > Cd > Cr > Pb. The lower LC50 of the mixture indicates greater acute lethality than the individual metal treatments, but does not by itself identify the type of mixture interaction.

Table 1: Lethal Concentration (LC50) Estimates for Pb, Cr, Cd, and the Pb-Cr-Cd Mixture at 96h

Toxicant	96hLC50 (mg L^{-1})	95%CI (mg L^{-1})
Pb	18.4	15.2-22.1
Cr	12.7	10.4-15.5
Cd	2.9	2.3-3.6
Pb-Cr-Cd mixture	1.8	1.4-2.3

Cr was delivered as potassium dichromate. Lower LC50 values indicate greater acute toxicity under the study conditions.

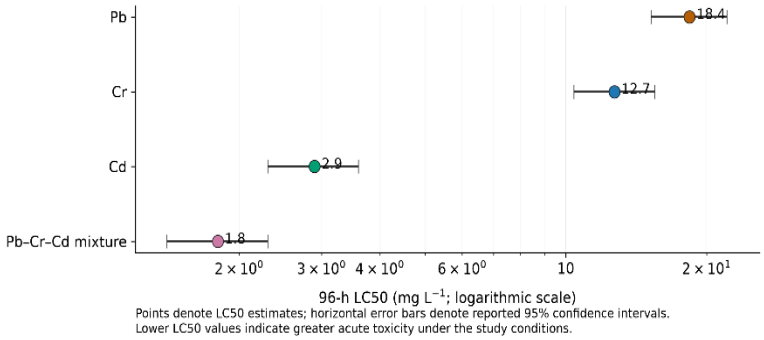


Figure 1: Lethal Concentration (LC50) estimates for individual metals and the ternary mixture

Stage Specific Developmental Outcomes

Final mass increased across developmental stages in all groups, whereas survival declined from the tadpole to froglet stage (Fig. 2). At each stage, exposed groups had lower final mass and survival than the stage matched control. Malformation and MDA increased over development and were consistently highest in the mixture group. At the froglet stage, final mass was 2.21 ± 0.03 g in controls and 2.05 ± 0.03, 2.07 ± 0.03, 2.02 ± 0.03, and 1.93 ± 0.03 g in Pb, Cr, Cd, and mixture groups, respectively. Mixture exposed froglets had a mass gain of 1.42 ± 0.01 g, compared with 1.70 ± 0.01 g in controls, and survival of 92.34 ± 0.36%, compared with 97.44 ± 0.36% in controls. Malformation was 11.80 ± 0.25% in the mixture group with 3.50 ± 0.25% in controls.

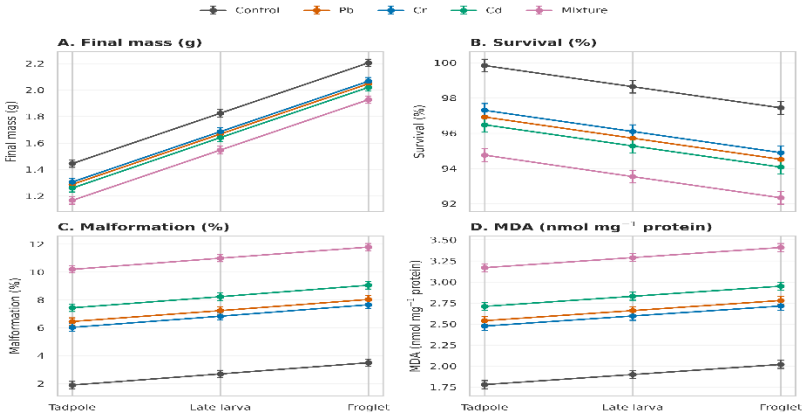


Figure 2: Stage specific lines of final mass, survival, malformation, and MDA with treatment

Oxidative Stress and Genotoxicity Endpoints

At the froglet stage, SOD, CAT, GPx, MDA, and micronucleus frequency were higher in each exposed group than in the control (Table 2). The mixture group had the highest values for all five endpoints: SOD, 11.76 ± 0.10 U mg $^{-1}$ protein; CAT, 7.85 ± 0.06 U mg $^{-1}$ protein; GPx, 4.406 ± 0.036 U mg $^{-1}$ protein; MDA, 3.410 ± 0.047 nmol mg $^{-1}$ protein; and micronuclei, 4.88 ± 0.10%. Relative to the control, the mixture increased MDA by 68.8% and micronucleus frequency by 212.8%.

Table 2: Froglet Developmental, Biochemical, and Genotoxicity Outcomes with Treatment

Endpoint	Control	Pb	Cr	Cd	Mixture
Final mass (g)	2.205 ± 0.028	2.047 ± 0.028	2.066 ± 0.028	2.020 ± 0.028	1.927 ± 0.028
Mass gain (g)	1.695 ± 0.013	1.537 ± 0.012	1.556 ± 0.013	1.510 ± 0.013	1.417 ± 0.013
Survival (%)	97.44 ± 0.36	94.52 ± 0.40	94.90 ± 0.38	94.08 ± 0.40	92.34 ± 0.36
Malformation (%)	3.50 ± 0.25	8.04 ± 0.27	7.66 ± 0.27	9.06 ± 0.27	11.80 ± 0.25
SOD (U mg $^{-1}$ protein)	9.16 ± 0.10	10.58 ± 0.10	10.46 ± 0.10	10.90 ± 0.10	11.76 ± 0.10
CAT (U mg $^{-1}$ protein)	6.17 ± 0.06	7.09 ± 0.06	7.01 ± 0.06	7.29 ± 0.06	7.85 ± 0.06
GPx (U mg $^{-1}$ protein)	3.386 ± 0.036	3.942 ± 0.040	3.896 ± 0.036	4.068 ± 0.040	4.406 ± 0.036
MDA (nmol mg $^{-1}$ protein)	2.020 ± 0.047	2.780 ± 0.047	2.714 ± 0.052	2.950 ± 0.047	3.410 ± 0.047
Micronuclei (%)	1.56 ± 0.10	3.38 ± 0.10	3.22 ± 0.10	3.78 ± 0.10	4.88 ± 0.10

Values are mean ± SD (n = 5 per treatment). SOD, superoxide dismutase; CAT, catalase; GPx, glutathione peroxidase; MDA, malondialdehyde

Statistical Comparisons

The two-way analysis of variance identified significant main effects of developmental stage and treatment on final mass - survival – malformation - MDA, and micronucleus frequency ($P < 0.001$). Stage × treatment interactions were not significant for these endpoints ($P > 0.05$). At the froglet stage, each metal and mixture group differed from the control for the core developmental, MDA, and micronucleus endpoints after Dunnett adjustment ($P < 0.001$).

Discussion

The study experiment showed Pb-Cr-Cd mixture had the lowest 96h LC50, followed by Cd, Cr, and Pb. This ranking describes relative acute lethality under the study conditions; it does not establish that the same ordering will occur under different exposure durations, water chemistries, or metal species. Following literature, information for *X. tropicalis* is narrow. Shi (2009) reported cadmium associated lethality and malformations in a 48h *X. tropicalis* FETAX assay, whereas Fort et al. (2004) established that *X. tropicalis* can be used in a modified FETAX model. These studies support the use of the species and the endpoint framework, but neither provides direct confirmation of Pb, Cr, or ternary mixture effects as that of the present study. The relatively high Pb LC50 in the present assay should not be interpreted as an absence of developmental hazard. In *X. laevis*, prolonged Pb exposure produced delayed malformations, and cadmium reduced growth or produced concentration related malformations in separate *X. laevis* studies (Sharma and Patiño, 2008). Chromium comparisons require additional restraint because the present chromium source was potassium dichromate and Cr toxicity is dependent on chemical form; chromate specific developmental toxicity, uptake, and distribution have been reported in a *Xenopus* FETAX study (Bosisio et al., 2009). The present developmental stages, showed exposed groups had lower final mass, survival and higher malformation than controls, with the largest deviations in the mixture group. The results in the study did not identify the tissue, molecular, or endocrine processes responsible for the observed developmental profile. But instead, the direction of the Cd

associated developmental findings, which is compatible with prior *Xenopus* work. Cadmium caused mortality, growth inhibition, and/or malformations in *X. tropicalis* or *X. laevis* developmental assays, although the species, exposure periods, and concentration ranges differed from those used in these studies (Sharma and Patiño, 2008; Shi, 2009). Evidence from other amphibians provides contextual, rather than species equivalent, support for considering mixture effects during development. In *Bufo gargarizans*, combined Cd and Pb exposure was associated with lower body mass and altered skeletal or thyroid related outcomes; thus, those findings cannot be extrapolated directly to *X. tropicalis* (Fort et al., 2004; Chen et al., 2022).

The increases in SOD, CAT, GPx, MDA, and micronucleus frequency in exposed froglets show that the treatments were associated with altered antioxidant enzyme activity, lipid peroxidation associated response, and micronucleus frequency. These endpoints are not, on their own, proof of a single oxidative or genotoxic mechanism. The use of this endpoint panel is supported by adjacent amphibian evidence, but response direction is tissue and time dependent. In *Lithobates catesbeianus*, single and combined metal exposures altered SOD, CAT, GPx, and lipid hydroperoxides differently across liver, kidney, and muscle and between sampling times (Carvalho et al., 2020). Cadmium also produced oxidative stress and histology associated changes in *Bufo gargarizans* and *Rhinella arenarum* (Medina et al., 2016; Wu et al., 2017). Cadmium related stress and genotoxicity findings in *X. laevis* larvae support the inclusion of micronucleus frequency as a genotoxicity endpoint (Mouchet et al., 2006). However, because the present study did not include histology, gene expression, or time course measurements, the observed enzyme, MDA, and micronucleus differences described biological response patterns and its causal pathway.

Ternary Mixture

The mixture group showed the lowest LC50 and the largest deviations from the control for the principal developmental and biomarker endpoints. This result identifies the mixture as the treatment associated with the greatest observed effect in this study. Amphibian studies indicate that combined metal exposure can produce outcomes that differ from single metal exposure. For example, low concentration mixtures altered selected biomarkers in *X. laevis* tadpoles, and Cd-Pb co-exposure in *Bufo gargarizans* was associated with more severe developmental outcomes than those reported for single metal groups (Yoloğlu and Ozmen, 2015; Chen et al., 2022). Differences in species, metal composition, dose, and duration prevent these studies from establishing the interaction type in the present Pb-Cr-Cd exposure. The present design evaluated one mixture composition and did not estimate a concentration addition or independent action model; the findings did not really distinguish synergism, additivity, or antagonism. The conclusion therefore, showed the ternary mixture had the greatest observed toxicity under the tested conditions. Interpretation of the Cr component should remain specific to the dichromate derived Cr (VI) exposure used in the study by Bosisio et al., 2009.

Contributions and Limitations

The contribution of the study is its integration of acute lethality with developmental, biochemical, and genotoxicity associated endpoints across three life stages. This multi-endpoint approach is consistent with the developmental test rationale of FETAX and with amphibian studies that combine organism level and biomarker outcomes (Fort et al., 2004; Mouchet et al., 2006). The limitations of the study reported the experimental unit, water quality conditions, exposure, timing of sampling, and analytical confirmation of Pb, Cd, and Cr concentrations in the test media. These details are especially important for Cr(VI), for which chemical form is central to toxicological interpretation, and for antioxidant endpoints, which can vary among tissues and sampling times (Bosisio et al., 2009; Carvalho et al., 2020). Finally, the available literature provides only limited direct metal evidence in *X. tropicalis*. Most comparison studies concern *X. laevis* or other amphibian taxa; they support endpoint selection and biological plausibility, but should not be treated as species-specific confirmation of the present Pb, Cr, or mixture effects (Fort et al., 2004; Shi, 2009).

Conclusion

The studies present exposure conditions of Pb, Cr, Cd, and the Pb-Cr-Cd mixture produced distinct acute toxicity and developmental response profiles in *X. tropicalis*. The mixture had the lowest 96h LC50 and the largest observed changes in froglet mass, survival, malformation, MDA, and micronucleus frequency. These results support the use of integrated developmental and biomarker endpoints in *X. tropicalis*, consistent with the species' suitability for developmental testing and the direct Cd FETAX evidence available for this model (Fort et al., 2004; Shi, 2009). They do not establish the mechanism of the biomarker pattern or the presence of mixture synergism.

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