UNIVERSIDADE FEDERAL DE SÃO CARLOS
CENTRO DE CIÊNCIAS BIOLÓGICAS E DA SAÚDE
PROGRAMA DE PÓS-GRADUAÇÃO EM ECOLOGIA E RECURSOS NATURAIS

EDNA VIVIANA CALPA ANAGUANO

EFEITOS ECOTOXICOLÓGICOS DE METAIS NA SOBREVIVÊNCIA, DESENVOLVIMENTO E COMPORTAMENTO DE DUAS ESPÉCIES ENDÊMICAS DE ANFÍBIOS ANUROS NEOTROPICAIS

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Tese apresentada ao Programa de Pós-graduação em Ecologia e Recursos Naturais do Centro de Ciências Biológicas e da Saúde da Universidade Federal de São Carlos, como parte dos requisitos para obtenção do título de Doutor em Ciências, área de concentração em Ecologia e Recursos Naturais.

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UNIVERSIDADE FEDERAL DE SÃO CARLOS

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Folha de Aprovação

Defesa de Tese de Doutorado da candidata Edna Viviana Calpa Anaguano, realizada em 10/09/2024.

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O ser humano mal reconhece os demônios de sua criação

Albert Schweitzer

Resumo

A contaminação dos ecossistemas aquáticos por metais é um dos principais fatores responsáveis pela diminuição e perda de populações de anfíbios em nível mundial. Este trabalho teve como objetivo avaliar os efeitos ecotoxicológicos dos sais dos metais, Sulfato de Cobre ($CuSO_4$) e Cloreto de Cádmio ($CdCl_2$) durante a fase larval de duas espécies endêmicas da Região Neotropical *Physalaemus nattereri e Rhinella diptycha*, analisando seus impactos na sobrevivência, morfologia, desenvolvimento e comportamento desses anfíbios. Para *P. nattereri*, os valores de LC50 obtidos nos testes de toxicidade aguda, 24; 48; 72 e 96 horas foram de 0,33; 0,25; 0,25 e 0,22 mg L^{-1} para Cu, e 4,6; 4,33; 4,8, e 4,03 mg L^{-1} para o Cd, respectivamente. Nos testes de exposição, a toxicidade aguda, os girinos tiveram aumento na mortalidade à medida que as concentrações e o tempo de exposição aos metais aumentavam. Durante a exposição crônica, os girinos de *P. nattereri* foram resistentes ao Cu, sem ocorrência de mortalidade, enquanto o Cd provocou mortalidade nas concentrações de 0,04 e 0,4 mg L^{-1}. Não foram observados efeitos dos metais no crescimento e desenvolvimento. Os resultados indicaram que *P. nattereri* apresenta sensibilidade moderada em comparação com outras espécies de anfíbios. A análise de risco mostrou a existência de risco para os anfíbios. Foram obtidos os valores de quocientes de risco (RQ) máximos de 75 para Cu e 972,2 para Cd, respectivamente. No caso da mistura de $CuSO_4$ e $CdCl_2$, o modelo que melhor se ajustou aos dados foi o de Adição de Concentração (CA). A combinação dos sais metálicos gerou uma mistura altamente tóxica, causando sinergismo e representando um risco significativo para a sobrevivência dos girinos de *P. nattereri*. Para os girinos de *R. diptych*a, o valor de LC50 nos testes de toxicidade aguda em 96 horas de exposição foi de 0,49 mg L^{-1} para $CuSO_4$ e 1,09 mg L^{-1} para $CdCl_2$. Analisou-se a distribuição da probabilidade das distâncias percorridas em cada marca de tempo e as mudanças de velocidade para cada grupo. Essa análise revelou que a exposição dos girinos ao $CuSO_4$ e $CdCl_2$ reduziu sua capacidade de mobilidade. O presente estudo revelou a importância de se conhecer as diferenças na sensibilidade das espécies, bem como os efeitos subletais de ambos metais no desenvolvimento e morfologia de *P. nattereri* e de respostas comportamentais de *R. diptycha*. Estes resultados contribuem para o estabelecimento de uma base para a avaliação das consequências da contaminação ambiental pelos metais cobre e cadmio.

Palavras-chave: Avaliação de risco, Contaminação aquática, Ecotoxicidade, Espécies nativas, Metais.

Abstract

The contamination of aquatic ecosystems by metals is one of the main factors responsible for the decline and loss of amphibian populations worldwide. This study aimed to evaluate the ecotoxicological effects of the metal salts Copper Sulfate ($CuSO_4$) and Cadmium Chloride ($CdCl_2$) during the larval stage of *Physalaemus nattereri* and *Rhinella diptycha*, analyzing their impact on survival, morphology, development, and behavior. For *P. nattereri*, the LC50 values obtained in acute toxicity tests at 24, 48, 72, and 96 hours were 0.33, 0.25, 0.25, and 0.22 mg L^{-1} for Cu, and 4.6, 4.33, 4.28, and 4.03 mg L^{-1} for Cd, respectively. Acute toxicity tests showed an increase in mortality as metal concentrations and exposure time increased. During chronic exposure, *P. nattereri* tadpoles were resistant to Cu, with no mortality occurring, while Cd induced mortality at concentrations of 0.04 and 0.4 mg L^{-1}. No significant effects on growth and development were observed due to metal exposure. The results indicated that *P. nattereri* exhibits moderate sensitivity when compared to other amphibian species. Risk assessment showed a risk to amphibians, with maximum risk quotient (RQ) values of 75 for Cu and 972.2 for Cd, respectively. For the mixture of $CuSO_4$ and $CdCl_2$, the model that best fit the data was Concentration Addition (CA). The combination of the metal salts created a highly toxic mixture, causing synergism and representing a significant risk to the survival of *P. nattereri* tadpoles. For *R. diptycha* tadpoles, the LC50 value in acute toxicity tests at 96 hours of exposure was 0.49 mg L^{-1} for $CuSO_4$ and 1.09 mg L^{-1} for $CdCl_2$. We analyzed the probability distribution of distances traveled at each timestamp and the speed changes for each group. This analysis revealed that exposure of the tadpoles to $CuSO_4$ and $CdCl_2$ reduced their mobility capacity. This study highlighted the importance of understanding species-specific sensitivity as well as the sub-lethal effects of both metals on the development and morphology of *P. nattereri* and behavioral responses of *R. diptycha*. These results contribute to establishing a solid foundation for assessing the consequences of environmental contamination by these metals.

Keywords: Risk assessment,, Aquatic contamination, Ecotoxicity, Native species, Metals.

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SUMÁRIO

Esta tese foi elaborada e estruturada inicialmente com uma contextualização geral da pesquisa desenvolvida, incluindo justificativa, objetivos e hipótese, como parte introdutória, seguida de três capítulos, no formato de artigos, a fim de facilitar a apresentação dos resultados. Cada capítulo é subdividido em: resumo, introdução, material e métodos, resultados, discussão e conclusões. O formato dos capítulos segue as normas dos periódicos científicos onde os artigos serão submetidos e as normas propostas para apresentação de teses da Universidade Federal de São Carlos-UFSCar. A seguir, apresentamos uma breve descrição do conteúdo de cada capítulo:

Capítulo 1 - Acute and chronic toxicity of cadmium and copper on tadpoles of *Physalaemus nattereri* (Steindachner, 1893), a Neotropical native amphibian

Neste capítulo, foram avaliados os efeitos dos metais cobre e cádmio em girinos de *Physalaemus nattereri* com o objetivo de avaliar o efeito da toxicidade desses metais na sobrevivência, crescimento e desenvolvimento desta espécie. Foram feitos testes de toxicidade aguda durante quatro dias (96 horas) e testes de toxicidade crônica durante 54 dias, utilizando concentrações semelhantes àquelas já registradas no ambiente. Além disso, foram coletados da literatura dados de valores de LC50 de larvas de diferentes espécies de anfíbios para comparar a sensibilidade das espécies e, finalmente, foi feita uma avaliação preliminar de risco ecológico dos metais em corpos de água do Cerrado.

Capítulo 2 - Effects of cadmium and copper metals isolated and in combined action over *Physalaemus nattereri* (Steindachner, 1893) tadpoles

Neste capítulo, avaliou-se o efeito do cobre, cádmio e a mistura deles sobre girinos de *Physalaemus nattereri.* Foram realizados testes de toxicidade aguda durante 96 horas com os metais individuais e em mistura, sendo o parâmetro analisada a sobrevivência dos girinos.

Capítulo 3 - Effects of cadmium and copper on the swimming behavior of *Rhinella diptycha* (Cope, 1862) tadpoles

Neste capítulo, analisaram-se os efeitos do cobre e do cádmio no comportamento natatório dos girinos de *Rhinella diptycha* quando expostos a esses metais. Além disso, foram feitos ensaios de toxicidade aguda com duração de 96 horas.

Com base nesses três capítulos, as conclusões gerais da tese e as considerações finais foram elaboradas.

1. Introdução e justificativa

1.1. Metais

Os metais são considerados como agentes tóxicos que afetam tanto os ecossistemas aquáticos quanto os terrestres. Suas fontes podem ser naturais, como erupções vulcânicas e o intemperismo químico e físico de solos e rochas (Jeong et al. 2023; Manahan, 2011), ou antropogênicas, provenientes de atividades industriais, mineração, práticas agrícolas e a rápida transformação dos sistemas naturais em sistemas urbanizados. No entanto, o aumento das concentrações dos metais nos corpos d'água causado pelas atividades humanas, torna esses metais tóxicos para os seres vivos (Dirzo e Raven, 2003; Dunson et al., 1992; Fernandes et al., 2021; Ojha et al., 2021). Como os metais são persistentes no ambiente e não se degradam biologicamente, podem ser bioacumulados em diversos tecidos e órgãos dos organismos na biota aquática em diferentes níveis tróficos (Yuan et al., 2017) (Figura 1).

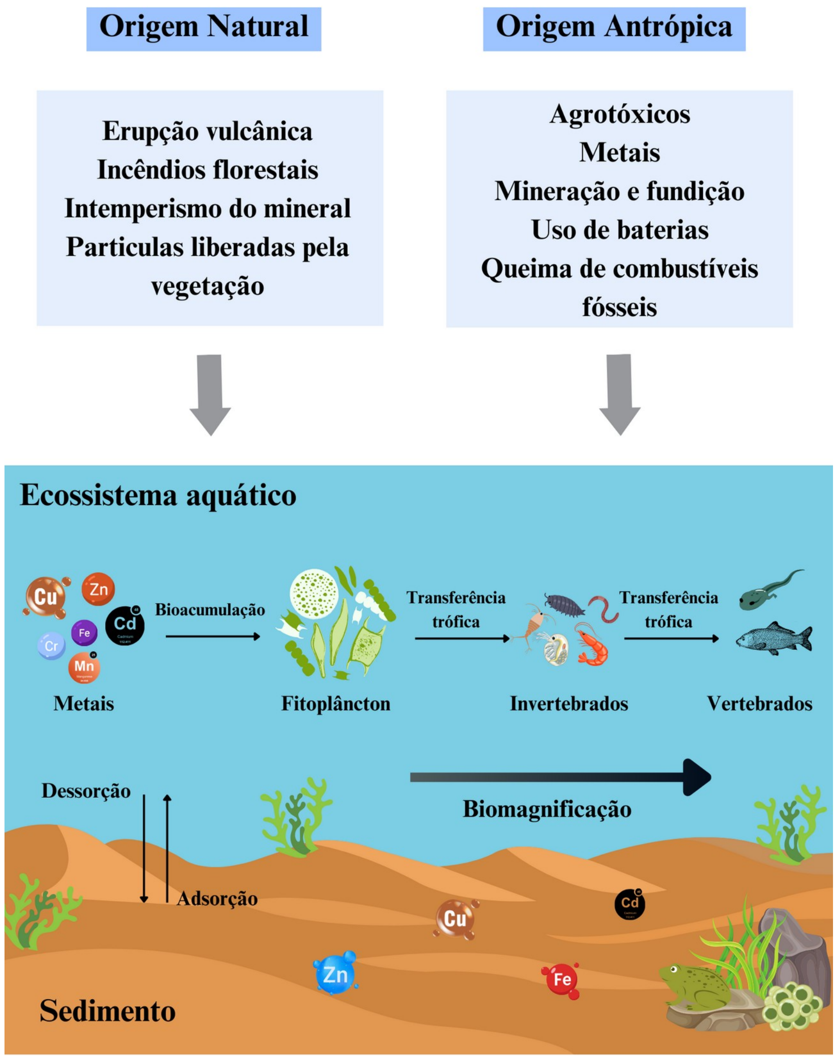

Figura 1. Vias de introdução de metais nos ecossistemas aquáticos: Processos naturais e antropogênicos. Fonte: Adaptado de Jeong et al. (2023).

Metais como o cobre (Cu), zinco (Zn), ferro (Fe), manganês (Mn), boro (B), em concentrações-traço, são considerados micronutrientes, cumprindo funções fisiológicas e bioquímicas nos organismos, já que podem formar parte de biomoléculas como ácidos nucleicos e carboidratos (Ali et al., 2019; C.S Carvalho et al., 2017). Por exemplo, o Cu é um metal essencial em plantas e animais, indispensável para funciones metabólicas como a produção de energia, o transporte de oxigênio e a síntese de hormônios (Jeong et al., 2023; Tsang et al., 2021).

No entanto, o aumento da concentração, frequentemente associado à produção de esgoto doméstico e efluentes industriais e agrícolas (Cabral et al., 2021; Manahan, 2011), pode causar múltiplos efeitos adversos, como inibição do crescimento e da reprodução (Pinto et al., 2021), alterações no sistema endócrino e aumento na produção de espécies reativas de oxigênio (ROS) (Brix et al., 2022). Esse aumento promove o estresse oxidativo causando disfunções celulares e inativação de enzimas como a superóxido dismutase, catalase e glutationa peroxidase, que são essenciais na defesa antioxidante (Jeong et al., 2023). Além disso, o Cu pode se acumular em diferentes órgãos. C.S Carvalho et al. (2017) e Carvalho et al. (2020), expuseram girinos de *Lithobates catesbeianus* ao Cu e observaram concentrações elevadas desse metal no fígado e nos rins, o que resultou em alterações nas funções hepática e renal.

Por outro lado, metais como o cádmio (Cd) não possuem uma função biológica conhecida. Sua concentração nos ecossistemas aquáticos tem aumentado nos últimos anos devido a atividades antrópicas, como a queima de carvão mineral, o recobrimento de aço e ferro, a disposição inadequada de pilhas e baterias, além do descarte de resíduos industriais, domésticos e agrícolas (Lu et al., 2021; Manahan, 2011). O Cd pode substituir metais essenciais, como magnésio (Mg), cálcio (Ca) e zinco (Zn), em algumas enzimas, devido à sua semelhança química e à capacidade de interagir com os sítios ativos ou cofatores metálicos de proteínas, como metaloproteínas (Moiseenko e Gashkina, 2020). Essa substituição pode causar alterações estruturais e prejudicar sua atividade catalítica. Além disso, as ARN e o ADN polimerases podem ser afetadas pelo Cd, interferindo nos processos de replicação e transcrição (Fernandes et al., 2021).

A dinâmica dos metais nos ecossistemas aquáticos é altamente influenciada pelas condições ambientais e pelas propriedades físicas e químicas da água. A temperatura, por exemplo, afeta os ciclos biogeoquímicos dos elementos, propiciando impactos significativos na bioacumulação de metais nos peixes. Além disso, as variações climáticas, de um modo geral possibilitam alterações na bioacumulação e na toxicidade ecológica dos metais, fazendo com que os organismos se tornem mais suscetíveis a intoxicações crônicas (Moiseenko e Gashkina, 2020).

O pH dos ecossistemas aquáticos também influencia no comportamento dos metais (Gorny et al., 2016; Moiseenko e Gashkina, 2020), já que pode modificar a concentração de íons livres desses metais na água ou interagir com eles de forma sinérgica ou antagônica. Essas interações afetam a toxicidade dos metais sobre diferentes organismos aquáticos (Lu et al., 2021). Por exemplo, Lu et al. (2021), avaliaram os efeitos tóxicos combinados de diferentes concentrações de Cd e níveis de pH no desenvolvimento larval de girinos da rã marrom de Zhenhai (*Rana zhenhaiensis*). Eles observaram que o acúmulo de Cd no fígado foi reduzido em ambientes ácidos (pH baixo), enquanto aumentou em ambientes alcalinos (pH alto). Além disso, o pH elevado intensificou a toxicidade do Cd aos girinos, ao passo que o pH baixo não causou o mesmo. Esses resultados indicam que ambientes alcalinos podem potencializar os danos causados pelo Cd.

De modo geral, fatores ambientais como a temperatura, salinidade e oxigênio afetam diretamente a permeabilidade e as propriedades dos metais nos ecossistemas aquáticos, além de influenciar a toxicidade nos organismos desses ambientes (Al Naggar et al., 2018).

1.2. Contaminação química por metais no Brasil

Na atualidade, uma das maiores preocupações em relação ao meio ambiente é a contaminação dos corpos de água causada pela presença de contaminantes químicos (Morin-Crini et al., 2022). A contaminação por metais nos ecossistemas brasileiros deve-se a diversas atividades antropogênicas como a urbanização, mineração, despejos inadequados de efluentes industriais, uso de pesticidas e incêndios florestais (Ellwanger e Chies, 2023).

No Brasil, o Conselho Nacional do Meio Ambiente (CONAMA) estabelece diretrizes e padrões da qualidade ambiental, incluindo limites de concentração de metais na água, conforme as diferentes classes. As águas de Classe 1 destinam-se ao abastecimento humano potável, após tratamento, e à preservação de ecossistemas aquáticos. Já as de Classe 2 são utilizadas para navegação, recreação de contato direto e também para abastecimento humano, desde que tratadas, e as águas de Classe 3 são utilizadas para irrigação, abastecimento animal e atividades de recreação com menor contato direto (CONAMA, 2005)(Tabela 1).

Tabela 1. Resumo dos limites recomendados para Classe 1 de qualidade ambiental de acordo com a Comissão Nacional de Meio Ambiente, Resolução CONAMA nº 357/2005.

Metal	Classe 1 (mg L^{-1})	Classe2 (mg L^{-1})	Classe 3 (mg L^{-1})
Alumínio	0,01	0,2	0,2
Arsênio	0,01	0,01	0,1
Cádmio	**0,001**	**0,001**	**0,01**
Chumbo	0,01	0,01	0,05
Cobre	**0,009**	**0,013**	**0,2**
Cromo total	0,05	0,05	0,05
Mercúrio	0,0002	0,0002	0,025
Níquel	0,025	0,025	0,025
Zinco	0,18	0,18	0,18

No entanto, no Brasil frequentemente são registradas concentrações de metais nos corpos d'água superiores aos limites estabelecidos pelo CONAMA. Uma das principais problemáticas está associada ao uso crescente de fertilizantes nas monoculturas de cana-de-açúcar (Corbi et al., 2006), que acabam sendo transportados para os corpos hídricos, contribuindo a poluição de ecossistemas aquáticos. Um dos exemplos a serem destacados, relaciona-se à região central do estado de São Paulo, onde se tem observado uma crescente contaminação por 2,4-D e o fipronil nos cultivos de cana-de-açúcar (Ogura et al., 2022).

Além disso, a substituição de áreas nativas para a implantação da monocultura e consequentemente o uso de pesticidas, tem influenciado na perda gradual da biodiversidade do solo (Franco et al., 2016). Além disso, o avanço dessas plantações por muitas vezes ocorre de forma rápida e pouco planejada devido às pressões e competitividades comerciais, gera impactos ambientais significativos em diferentes escalas (Caldarelli e Gilio, 2018).

Além dos avanços da cana-de-açúcar, diferentes catástrofes ambientais ocorridas nos últimos anos, como por exemplo o rompimento da barragem do Fundão em Mariana (Minas Gerais) no ano de 2015, têm favorecido a contaminação por metais. No exemplo de Mariana, os rejeitos contaminaram as águas e o solo da bacia do Rio Doce, incorporaram metais como Chumbo (Pb: 0,013-0,097 mg L^{-1}), Arsênio (As: 0.012-0.911 mg L^{-1}), Cobre (Cu: 0,062-1,427 mg L^{-1}), Níquel (Ni: 0,051-1,078 mg L^{-1}), Alumínio (Al: 0,465-9,432 mg L^{-1}) e Manganês (Mn: 0,041-1,638 mg L^{-1}) (M.S Carvalho et al., 2017) em concentrações maiores do que as estabelecidas pelo CONAMA para Classe 3 de qualidade de águas, conforme apresentado na Tabela 1.

Uma análise química dos rejeitos realizada por Girotto et al. (2020) confirmou a presença de metais como Cd, Pb, Fe, Mn, Zn e Al, o estudo avaliou os efeitos agudos e crônicos desses rejeitos em girinos de *Lithobates catesbeianus*, demonstrando que a exposição a diferentes concentrações reduziu a velocidade de nado, o consumo de oxigênio e causou danos morfofisiológicos e comportamentais. Além disso, os altos níveis de metais liberados em Mariana resultaram na contaminação e morte de diversas espécies de peixes na foz do Rio Doce (Gabriel et al., 2020, Vieira et al., 2022). Esses impactos afetaram drasticamente os ecossistemas de água doce e marinhos, comprometendo processos ecológicos essenciais para a subsistência (Bevitório et al., 2022; Foesch et al., 2020) e gerando efeitos diretos e indiretos na saúde humana a médio e longo prazo (Vidal et al., 2024).

Quase quatro anos após o desastre de Mariana, mais uma barragem se rompeu causando novos danos ambientais. Em 2019, a barragem em Brumadinho (Minas Gerais) lançou aproximadamente 13 milhões de m^3 de rejeitos acarretando danos ao meio ambiente e à saúde humana (Guimarães et al., 2024).

Com a chegada do rejeito ao Rio Paraopeba (Minas Gerais), rapidamente foi constatada turbidez 30 vezes maior do que a recomendada (Thompson et al., 2020). Ao se encaminhar para a Bacia do Rio São Francisco foram identificadas quantidades muito acima daquelas recomendadas pelo CONAMA em diversos trechos da bacia, para o Ferro (Fe: 8,412 mg L^{-1}), Alumínio (Al: 1,817 mg L^{-1}), Manganês (Mg: 116 mg L^{-1}) e Titânio, além de outros metais também com toxicidades conhecidas (Martins e Takahashi, 2022). Quando avaliada a qualidade da água observou-se que as concentrações de Fe, Al, Mn, Zn, Cu, Pb, Cd e U estavam acima dos limites permitidos (Martins e Takahashi, 2022) e também nos níveis limites dos metais nos sedimentos para Cr, Ni, Cu e Cd (Vergilio et al., 2020). Quando observados os danos ambientais, os impactos gerados foram muito similares aos citados para Mariana, as amostras de água e sedimento, com seus altos níveis de toxicidade, impactaram drasticamente os organismos de diferentes níveis tróficos como: algas (*Raphidocelis subcapitata*), microcrustáceos (*Daphnia similis*) e peixes (*Danio rerio*) (Martins e Takahashi, 2022).

Além das catástrofes observadas, que inserem uma grande quantidade de metais nos ambientes aquáticos é importante destacar que existem rios que, mesmo sem esses eventos, apresentam altos níveis de toxicidade. Negrão et al. (2021) ao monitorarem o rio Cascavel, na cidade de Guarapuava, Paraná, identificaram concentrações elevadas de metais na macrófita aquática *Egeria densa* em um trecho urbano. Os valores médios encontrados foram: Ni (135,33 mg kg^{-1}), Zn (168,83 mg kg^{-1}), Mg (191 mg kg^{-1}), Cr (34,38 mg kg^{-1}), Mn (138,71 mg kg^{-1}) e Pb (55,02 mg kg^{-1}), evidenciando uma contaminação significativa.

Da mesma forma, altos níveis de Cd (<0,075 mg L^{-1}) e Cr (<0,06 mg L^{-1}) foram verificados na bacia hidrográfica do rio Capibaribe, em Pernambuco (Silva et al., 2023). Esses níveis, segundo os autores, decorrem da contaminação das águas através de resíduos de fábricas de confecção de jeans, que são encontrados em grandes proporções em determinado trecho da bacia e que sem tratamento adequado são direcionados ao rio.

Na região amazônica são encontrados elevados níveis de metais, principalmente decorrentes da mineração ilegal, industrialização e da urbanização. Na Avaliação de Risco Ecológico (ERA) realizada por Gomes et al. (2023) na bacia do Amazonas, foram registradas altas concentrações de metais no sedimento, incluindo Mn (1856,8 mg Kg^{-1}), Cu (537,06 mg Kg^{-1}), Ba (309 mg Kg^{-1}), Pb (221,6 mg Kg^{-1}), Co (160,3 mg Kg^{-1}), Ni (152,25 mg Kg^{-1}), Cr (143,3 mg Kg^{-1}), Zn (129,1 mg Kg^{-1}), Cd (5,4 mg Kg^{-1}) e As (2,8 mg Kg^{-1}) e na água as concentrações registradas foram Co (9,53mg L^{-1}), Pb (3,81 mg L^{-1}), Cr (2,69 mg L^{-1}), Cu (0,62 mg L^{-1}) e Ni (2,55 mg L^{-1}). Contudo, em diversas áreas avaliadas, os limites estabelecidos pelo CONAMA foram ultrapassados. Além disso, 56% das áreas de estudo relacionadas a água e 66 % dos sedimentos apresentaram valores dos quocientes de risco superiores a 1 (RQ > 1) o que indica um alto risco ambiental. Além disso, Araújo et al. (2021) verificaram que afluentes do rio Solimões, no Amazonas, apresentavam níveis elevados de Hg (<0,04 mg L^{-1}) e Al (<0,02 mg L^{-1}) ao avaliarem a toxicidade das águas.

1.3. Misturas químicas

Os modelos conceituais de referência para misturas químicas propostos por Jonker et al. (2005) fundamentam-se em duas abordagens principais para os ecossistemas aquáticos: Adição de Concentração (CA) e Ação Independente (IA).

A CA (Loewe e Muischnek, 1926) é empregada para compostos que compartilham mecanismo de ação comum, ou seja, que apresentam interação com um mesmo receptor ou via metabólica. Esse modelo parte do princípio que os compostos da mistura têm modos de ação similares e afetam os mesmos sistemas biológicos. Assim, os efeitos tóxicos individuais podem ser somados para prever o efeito total da mistura. Para tanto é fundamental que cada composto da mistura seja calculado como a fração da dose que corresponde à concentração equivalente do composto puro que causaria o mesmo efeito. Após isso as frações são somadas. Caso a soma exceda o limiar presume-se um efeito tóxico combinado. Assim, o modelo da CA pode ser representado por:

$$\sum_{i=1}^{n} \frac{Ci}{ECxi} = 1$$

Em que, C_i é a concentração do composto químico i na mistura e ECx_i é a concentração de efeito do composto químico i que produz o mesmo efeito (x%) da mistura total (efeito máximo).

A IA (Bliss, 1939), por sua vez, é empregada para compostos que têm diferentes mecanismos de ação, os quais afetam diferentes alvos biológicos ou vias metabólicas. Esse modelo parte do princípio de que os compostos na mistura têm modos de ação diferentes e também independentes. Com isso, não ocorre influência mútua. Assim, o efeito combinado é calculado com a probabilidade de ocorrência independente dos efeitos dos compostos individuais. A probabilidade é fundamental para estimar a resposta combinada, presumindo-se que a ocorrência de um efeito por um dado composto, não afeta a ocorrência de efeitos pelos outros.

$$Y = Umax \prod_{i=1}^{n} q1(Ci)$$

Em que, Y é a resposta biológica, C_i é a concentração dos compostos químicos na mistura, q_i (C_i) é a probabilidade da não-resposta, u_{max} é a resposta do controle para *endpoints* e Π é a função de multiplicação.

Os desvios dos modelos CA e IA, como sinergismo/antagonismo (S/A), proporção da dose (DR) e nível da dose (DL) são obtidos por meio dos parâmetros "a" e "b". O parâmetro "a" é negativo para os desvios sinérgicos e positivo para os antagônicos (a>0=A; a<0=S). Para exemplificar o desvio dependente do DL inclui-se o parâmetro "a", que se relaciona à variação em doses altas e baixas, e o parâmetro "bDL", que remete a qual nível ocorre a mudança na variação. Ressalta-se ainda que em nível DR, os valores dos parâmetros "bDR" e "a" indicam que a variação esta contida na composição da mistura.

Para desvios que são determinados a partir de modelos de referências de misturas, a caracterização pode ser realizada por meio da ferramenta MIXTOX de Jonker et al. (2005). Essa ferramenta propicia a avaliação, além de prever a toxicidade de misturas de substâncias químicas. O MIXTOX permite a incorporação de desvios dos modelos CA e IA para modelar interações sinérgicas e antagônicas mais precisamente. Na Figura 2 é apresentado o isobolograma da relação entre o antagonismo e o sinergismo.

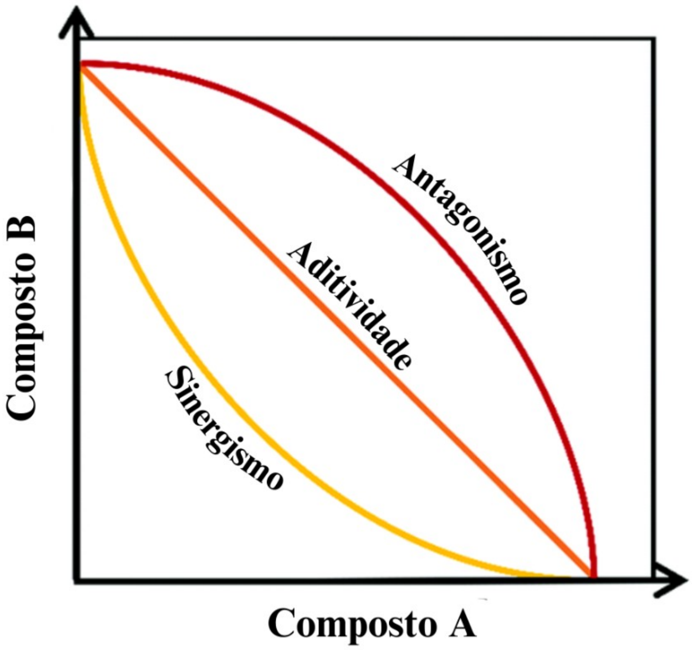

Figura 2. Isobolograma para componente antagônico, aditivo e sinérgico, em que os eixos representam as doses de agentes individuais e as linhas representam a combinação de concentrações dos dois agentes necessárias para atingir um determinado efeito fixo. Fonte: Adaptado de Caesar e Cech (2019).

1.4. Anfíbios e sua importância como bioindicadores ambientais

Os anfíbios constituem um grupo taxonômico diversificado e de importância vital nos ecossistemas, desempenhando várias funções na cadeia trófica, agindo como predadores e presas (Menin et al., 2015), controladores de pragas (Syazali et al., 2021), dispersores de sementes (Gould e Valdez, 2024) e polinizadores de plantas (Oliveira-Nogueira et al., 2023). Sua estreita relação com ambientes aquáticos, pele permeável, mobilidade reduzida e ciclo de vida bifásico (larvas aquáticas e adultos terrestres) fazem com que sejam altamente sensíveis às mudanças ambientais tornando-os valiosos bioindicadores tanto em ecossistemas aquáticos quanto terrestres (Blaustein e Kiesecker, 2002; Blaustein et al., 2003; Moriarty et al., 2013). No entanto, nas últimas décadas, têm-se observado em nível global, uma rápida perda de suas populações, o que tem gerado grandes preocupações quanto à conservação das espécies deste grupo e ao impacto na biodiversidade e no funcionamento dos ecossistemas que habitam (Blaustein e Kiesecker, 2002; Blaustein et al., 2003; Collins e Storfer, 2003;; Luedtke et al., 2023; Stuart et al., 2004).

De acordo com a Lista Vermelha de Espécies Ameaçadas da União Internacional para a Conservação da Natureza (UICN), os anfíbios são o grupo de vertebrados mais ameaçados no mundo, com 40,7% das espécies em perigo de extinção (Luedtke et al., 2023). Com base em diversos estudos, alguns fatores têm sido sugeridos como desencadeadores da perda de espécies. Dentre esses fatores destacam-se as mudanças climáticas (Corn, 2005; He et al., 2023), a exposição à radiação ultravioleta-B (Alton e Franklin, 2017; Blaustein et al., 2003), doenças infecciosas emergentes (Blaustein e Kiesecker, 2002), espécies invasoras (Falaschi et al., 2020; Nunes et al., 2019) e as transformações e perdas de habitat devido principalmente à expansão urbana e desmatamento (Becker et al., 2007). Além destes, a presença de contaminantes químicos no ambiente, como metais, herbicidas e fungicidas, representa uma das principais ameaças, representando elevado risco para os anfíbios (Costa et al., 2023).

Estudos sobre os efeitos ecotoxicológicos em diferentes etapas de desenvolvimento dos girinos relatam as graves consequências desses estressores químicos. Por exemplo, Xia et al. (2012) e Wang et al. (2016) testaram a influência do cobre no desenvolvimento embrionário e nos estágios iniciais das larvas até a metamorfose do sapo asiático *Bufo gargarizans*. Durante os experimentos observaram atrasos no desenvolvimento dos embriões e malformações na cauda (nadadeira dorsal ondulada, cauda flexível e eixo corporal curvado), presença de edemas e pigmentação reduzida do saco vitelino (Xia et al., 2012). Além disso, as larvas de *B. gargarizans* testadas em laboratório desde os estágios iniciais, apresentaram um aumento na mortalidade, inibição do crescimento, redução no tamanho das larvas e atraso no tempo da metamorfose (Wang et al., 2016). Peluso et al. (2023) detectaram que a espécie *Rhinella arenarum* (Hensel, 1867), endêmica do Rio Paraná na Argentina, é afetada gradativamente com a perda da qualidade da água devido à presença de metais, pesticidas e contaminantes emergentes. Com uma exposição crônica, mais intensa, as consequências chegaram a ser letais nessa espécie.

Ao investigar o comportamento de girinos de *Bufo raddei* na China, Zhang et al. (2020) constataram que metais (Cu, Zn, Cd, Pb) conseguem afetar, a longo prazo, o desempenho de movimento e a adaptação ao meio da espécie investigada. Os girinos apresentaram altos níveis de enzimas antioxidantes e irregularidades na natação conforme as alterações da temperatura no ambiente. Por outro lado, os autores destacam que alguns indivíduos, mesmo expostos a essa adversidade, apresentavam adaptações de resistência à natação, o que favorecia o escape em situações de perigo, mesmo que parcialmente.

Zhelev et al. (2020) verificaram a influência de metais e metaloides (Pb, Cd, Cu, Zn, As, Se) em indivíduos adultos de *Pelophylax ridibundus*. Os animais expostos a esses contaminantes apresentavam em sua maioria anemia, hematopoiese suprimida e imunidade baixa. Os autores destacaram ainda que as maiores concentrações desses metais nos anuros encontravam-se no fígado, em vez dos músculos.

Conforme citado, dependendo da exposição e sua duração em relação aos metais, os anfíbios poderão sofrer diversos efeitos letais e não letais. Halliday (2008) destaca que mesmo existindo efeitos que não acarretam a letalidade, eles irão influenciar na redução da aptidão e contribuirão para a diminuição significativa de suas respectivas populações.

1.5. Anfíbios como organismos-teste

1.5.1. *Physalaemus nattereri* (Steindachner, 1893)

Conhecida popularmente como rã de quatro olhos esta é uma espécie nativa do Brasil, Paraguai e Bolívia (Frost, 2024) (Figura 3). Pode ser encontrada em tanques de pastoreio, fragmentos de bosque, pântanos e corpos d'água temporários ou permanentes no bioma Cerrado (Menin et al., 2015; Silva e Rossa-Feres, 2010). Geralmente se reproduz na época de maior precipitação pluviométrica entre outubro e janeiro, formando ninhos de espuma na beira dos corpos d'água (Rodrigues et al., 2004). É uma espécie considerada generalista, com ampla distribuição geográfica. Embora a União Internacional para a Conservação da Natureza (UICN) a classifique como pouco preocupante, esta especie está em risco de extinção devido ao aumento da agricultura intensiva, pastagens e plantações de cana de açúcar (Aquino et al., 2004; Araújo et al., 2009; Boscolo et al., 2017; IUCN, 2024a).

Figura 3. Mapa de distribuição de *Physalaemus nattereri* (Steindachner, 1893). Fonte: IUCN (2024a).

1.5.2. *Rhinella diptycha* (Cope, 1862)

A especie *Rhinella diptycha* conhecida popularmente no Brasil como sapo cururu ou sapo boi, se distribui em alguns países da América do Sul. É nativo do Brasil, Paraguai, Argentina, Bolívia e Uruguai (Frost, 2024). É uma espécie comum, generalista e oportunista, encontrada em áreas abertas e urbanas (Carvalho Batista et al., 2011). Sua reprodução ocorre entre julho e outubro, durante a estação seca e o início da chuvosa. As fêmeas depositam os ovos em cordões gelatinosos em corpos d'água lênticos permanentes ou temporários. A UICN classifica a perda da espécie *R. diptycha* como pouco preocupante (IUCN, 2024b).

Figura 4. Mapa de distribuição de *Rhinella diptycha* (Cope, 1862). Fonte: IUCN (2024b).

1.6. Distribuição de sensibilidade das espécies

A Distribuição de Sensibilidade das Espécies - SSD (do inglês *Species Sensitivity Distribution*), é uma abordagem estatística utilizada para estimar e comparar a sensibilidade de espécies em função da toxicidade das substâncias químicas (Fox et al., 2021). A SSD possibilita quantificar o risco ecológico dos poluentes em uma abordagem ecossistêmica, permitindo assim reduzir as incertezas associadas às peculiaridades das espécies nas respostas à toxicidade e também ao estimar o risco ecológico (Bandeira et al., 2021).

Para uma melhor compreensão em relação à SSD, os dados de toxicidade são avaliados para diversas espécies expostas ao mesmo contaminante. Assim, pode se estimar a concentração do contaminante que causa algum efeito adverso em cada espécie, seja a morte ou inibição do crescimento. Por exemplo, os dados de toxicidade são organizados em distribuição cumulativa, a qual possibilita um ajuste na função estatística (distribuição normal ou log-normal) (Fox, 2016). Assim, a distribuição irá representar a variação na sensibilidade das diferentes espécies ao contaminante (Fox et al., 2021).

Com a SSD, é possível estimar a concentração do contaminante, tanto a mais perigosa para determinada quantidade de espécies, como a menos. Assim, é obtida a estimativa de HCx (Hazardous Concentration for x% of Species), para que sejam delimitados os limites de segurança ambiental (Fox et al., 2021), e que a grande parte das espécies estejam protegidas abaixo desse limiar (Hennig et al., 2023).

Quando aplicada a sistemas aquáticos, a SSD é amplamente utilizada em avaliações de qualidade de água e de risco ecológico por ser uma abordagem simples, computacional, com a geração de dados estatísticos confiáveis (Wang et al., 2024). Ressalta-se que para obtenção de dados de toxicidade com SSD são necessários no mínimo oito táxons diversos, sendo treze ou mais táxons o ideal para gerar um modelo SSD (Wang et al., 2020).

1.7. Justificativa

A destruição de habitat pelas transformações no uso da terra, a propagação de doenças como a quitridiomicose produzida pelo fungo *Batrachochytrium dendrobatidis*, mudanças climáticas, presença de espécies invasoras, além da contaminação química são atualmente considerados os principais fatores que contribuem para o declínio das populações de anfíbios no mundo (IUCN, 2021; Sparling et al., 2010). Os anfíbios são um grupo de organismos altamente vulneráveis às alterações ambientais, devido ao ciclo de vida bifásico, com uma fase larval-aquática e outra pós-metamórfica-terrestre (Blaustein, 1990; Blaustein e Kiesecker, 2002; Blaustein et al., 2003). Além disso, muitos dos ecossistemas aquáticos onde eles se reproduzem e desenvolvem estão sendo contaminados por metais por causa da lixiviação oriunda de processos industriais, agrotóxicos, mineração e a urbanização, o que gera efeitos adversos no comportamento, na morfologia e fisiologia destes organismos. A combinação de contaminantes e toxicidade dos agentes químicos assim como a história de vida dos organismos tem se convertido num desafio para os ecotoxicologistas (Freitas e Almeida, 2016; Freitas et al., 2017; Hallman e Brooks, 2015; Moe et al., 2013).

A toxicidade de muitos compostos químicos como os metais é muito pouco conhecida para os anfíbios nativos do Brasil devido aos poucos estudos de sensibilidade para o grupo. Inclusive, a maior parte dos limites de contaminação estabelecidos para diferentes compostos químicos para os anfíbios, durante a fase aquática são geralmente extrapolados a partir de dados existentes para espécies de peixes (Fryday e Thompson, 2012; Vasconcelos, 2014). Além disso, a susceptibilidade de girinos nativos é muitas vezes diferente daquela dos girinos de espécies modelo, como *Lithobates catesbeianus*, o que limita a compreensão dos efeitos nas populações e comunidades presentes na região. Neste sentido, a importância desta pesquisa se justifica pela urgente necessidade de analisar a sensibilidade dos girinos de espécies de anuros que podem indicar ameaças e consequências que os anfíbios nativos enfrentam na atualidade e assim compreender melhor até que ponto os metais contribuem para o declínio das espécies, além de proporcionar dados da sensibilidade de espécies nativas neotropicais.

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2. Objetivos e Hipóteses

2.1. Objetivo geral

Avaliar os efeitos ecotoxicológicos dos metais cobre e cádmio sob diferentes concentrações durante a fase de vida larval de *Physalaemus nattereri* e *Rhinella diptycha,* espécies nativas neotropicais, a partir de efeitos sobre a sobrevivência, morfologia, desenvolvimento e comportamento.

2.2. Objetivos específicos

2.2.1. Avaliar os efeitos na sobrevivência causados pelos metais cobre e cádmio em girinos de *Physalaemus nattereri* e determinar possíveis efeitos no crescimento e desenvolvimento dos girinos sob concentrações ambientais.

2.2.2. Avaliar os efeitos agudos do cobre e cádmio individuais e em mistura em girinos de *Physalaemus nattereri*.

2.2.3. Avaliar os efeitos agudos e de comportamento natatório causados pelos metais cobre e cádmio em girinos de *Rhinella diptycha*

2.3. Hipóteses

2.3.1. A contaminação por cobre e cádmio causam toxicidade aos girinos de *Physalaemus nattereri,* causando efeitos nocivos na sobrevivência, e de forma crônica no desenvolvimento dos girinos.

2.3.2. Os metais cobre e cádmio, tanto isolado como em mistura, causam alta mortalidade e efeitos sinérgicos aos girinos de *Physalaemus nattereri*.

2.3.3. O cobre e cádmio causam alterações na velocidade e distância do nado e na taxa de sobrevivência dos girinos de *Rhinella diptycha.*

Capítulo 1. Acute and chronic toxicity of cadmium and copper on tadpoles of *Physalaemus nattereri* (Steindachner, 1893), a Neotropical native species

Abstract

The loss of amphibian biodiversity is currently a global concern. In Brazil, there is evidence of population size reduction and apparent local extinctions of native species. A possible cause is the contamination of aquatic ecosystems by metals. We exposed tadpoles of *P. nattereri* to cadmium (Cd) and copper (Cu) to evaluate the effects of these metals on the survival, growth, and development of this species. Acute and chronic toxicity tests were conducted using representative concentrations of environmental conditions in non-eutrophic aquatic ecosystems. Besides, we compared the sensitivity of amphibian species and conducted a preliminary assessment of the ecological risk of these metals in the water bodies of the Brazilian Cerrado. The median lethal concentration (LC50) values obtained in acute toxicity tests at 24, 48, 72, and 96 hours were 0.33, 0.25, 0.25, and 0.22 mg L^{-1} for Cu, and 4.6, 4.33, 4.28, and 4.03 mg L^{-1} for Cd, respectively. Regarding chronic exposure, *P. nattereri* tadpoles showed resistance to Cu, with no mortality, while mortality occurred at concentrations of 0.04 and 0.4 mg L^{-1} for Cd. Furthermore, no effects of the metals on growth and development were observed. The sensitivity values of amphibians revealed that *P. nattereri* exhibits moderate sensitivity compared to non-tropical amphibian species. Concerning the risk analysis, significantly elevated values indicate a potential risk for amphibians inhabiting these aquatic ecosystems. These results highlight the importance of understanding variations in sensitivity and the effects of metal pollution on Neotropical species.

Keywords: Amphibians, Aquatic contamination, Native species, Metals, Risk quotients

1. Introduction

The global concern regarding the loss of amphibian diversity has increased in recent years. One of the main factors contributing to this issue is environmental pollution (Azizishirazi et al., 2021; Egea-Serrano et al., 2012), stemming from human activities such as intensive agriculture, mining, and the discharge of urban and industrial effluents that introduce various toxic substances into aquatic habitats where amphibians reproduce and complete their development (Girotto et al., 2020, 2023; E. Santana et al., 2021). This contamination, characterized by the presence of metals such as copper (Cu), an essential co-factor in proteins and DNA, and cadmium (Cd), an element with unknown biological function in organisms (Santos-Carvalho et al., 2020), can reach highly toxic concentrations, as documented by F.C Santana et al. (2021) in the waters of the Gualaxo do Norte and Carmo rivers after the collapse of the Fundão waste dam in the municipality of Mariana, Minas Gerais, Brazil. In these rivers, concentrations of Cu and Cd have reached values of 0.359 mg L^{-1} and 0.109 mg L^{-1} (F. C Santana et al., 2021) surpassing the limits established by the National Environmental Council-CONAMA 357/2005 (Cu 0.001, Cd 0.009). This poses a potential risk to the health and survival of amphibians due to their sensitivity to these substances, given their morphophysiological characteristics (Blaustein and Wake, 1990; Collins and Storfer, 2023).

Previous studies have documented the sensitivity of amphibians to these contaminants. Ojha et al. (2021) demonstrated that tadpoles of *Polypedates maculatus* exposed to 0.5 and 0.7 mg L^{-1} of Cd exhibited tail deformities such as tumors, pale skin, reduced mobility, delayed metamorphosis, and high mortality. Similarly, Chen et al. (2007) showed that ecologically relevant concentrations of Cu in the United States had adverse effects on *Rana pipiens* larvae, affecting embryonic development, growth rates, survival, behavior, and metamorphosis. Besides, other researchers have documented effects such as changes in body size and histological alterations (Gürkan et al., 2014), decreased larval and embryonic survival (Flynn et al., 2015), delayed metamorphosis, reduced body size, and endocrine-disrupting effects (Azizishirazi et al., 2021; Wang et al., 2016), decreased sensory system related to swimming (Krupa et al., 2021), and high larval mortality at environmental concentrations (Calfee and Little, 2017; Srivastav et al., 2016; Weir et al., 2019). However, the majority of these studies have focused on native species from North America, Asia, Europe, and Africa, leaving a notable gap in our understanding of the sensitivity of Neotropical amphibians (Daam et al., 2020). This is especially relevant since Brazil hosts the greatest diversity of amphibian species in the world, many of which are endemic and endangered (Frost, 2023).

An example of a species at risk is the Cuyaba Dwarf Frog, *P. nattereri* (Anura: Leptodactylidae), whose local populations are declining due to intensive agriculture, livestock farming, and urban expansion (Aquino et al., 2004; Eterovick et al., 2005; IUCN, 2022). Given this scenario, this study aimed to assess the toxicity of Cu and Cd on the survival, growth, and development of *P. nattereri* tadpoles through acute (4 days) and chronic (54 days) toxicity tests to understand their sensitivity and evaluate the risk these contaminants pose to this species, by calculating risk quotients (Rqs).

2. Materials and Methods

2.1. Test and Cultivation Conditions

Spawns of *Physalaemus nattereri* were collected from the temporary Mayaca Lake, located in the municipality of São Carlos, São Paulo state (21°54'18.0"S, 47°52'17"W) (Figure 1). The egg masses were transported in 10-liter containers filled with lake water. These containers were taken to the Limnology and Ecotoxicology Laboratory of the Department of Ecology and Evolutionary Biology at the Federal University of São Carlos, where all experiments were conducted. The collection of egg masses was carried out under a permit from the Chico Mendes Institute for Biodiversity Conservation (ICMBio n°86058-1), and the maintenance and experimentation procedures were approved by the Ethics and Animal Experimentation Committee of the Federal University of São Carlos (CEUA UFSCAR n° 9592171022/ ID 001757).

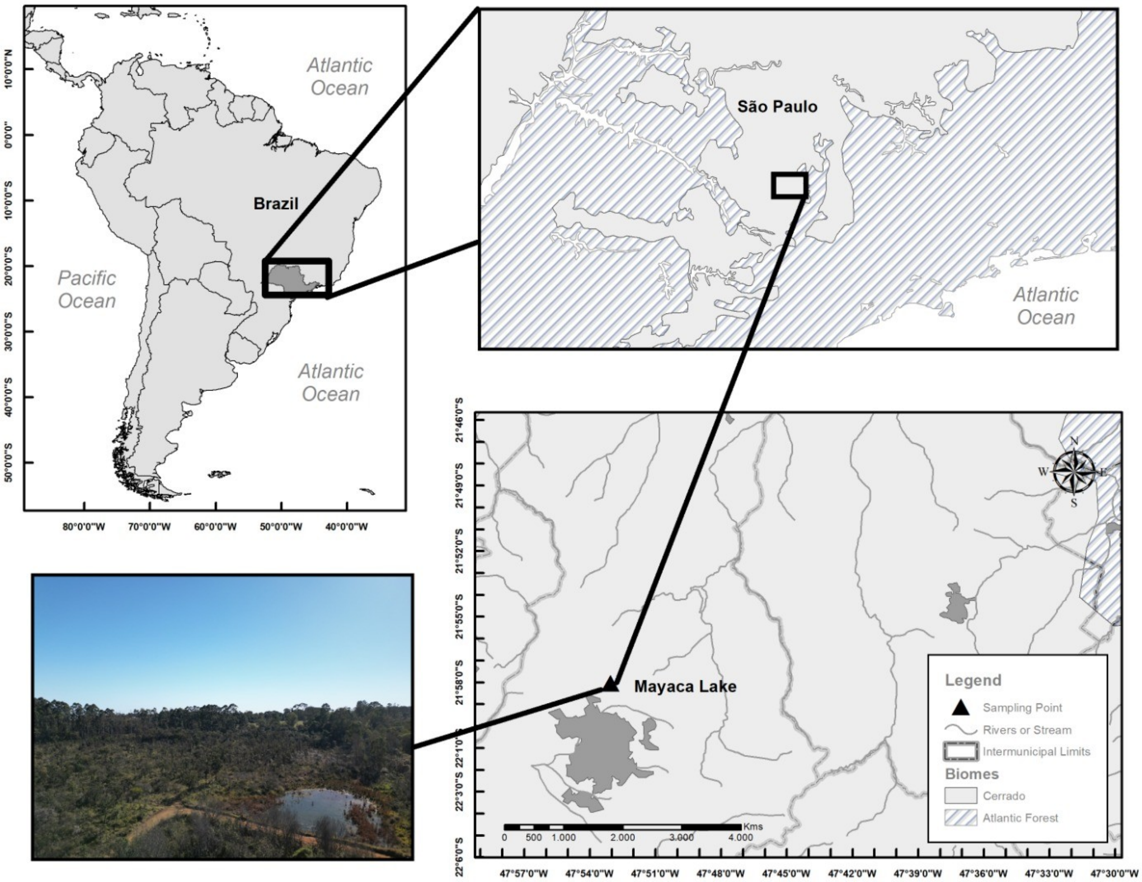

Figure 1. Lake Mayaca location and Collection point of *Physalaemus nattereri* (Steindachner, 1893) egg masses and general view of temporary Mayaca Lake, São Carlos-SP.

The egg masses were kept in the laboratory during the hatching, larval development, and experimental stages. For this purpose, 10-liter aquariums were employed, containing reconstituted water with a pH between 7.0 and 8.0, hardness of 40 ± 2 mg $CaCO_3$ L^{-1}, and conductivity of 140 ± 10 µS cm^{-1} (ABNT, 2016). The temperature was maintained at 25 ± 1°C, and a photoperiod of 12 hours of light/12 hours of darkness was established. The water in the aquariums was constantly aerated with a 50% weekly partial water change. The tadpoles were fed daily with commercial tropical fish food (Tetramin Flakes Tetra), and feeding was suspended 24 hours before the start of the experiments.

2.2. Quantification of Metals

The metallic salts used for the tests were Copper Sulfate ($CuSO_4$-$5H_2O$, CAS 7758-99-8, Dinâmica) and Cadmium Chloride ($CdCl_2$ 2.5 H_2O, CAS 10108-64-2, Carlo Erba); it is noteworthy that they will be referred to as copper (Cu) and cadmium (Cd), respectively. These salts were used to prepare a stock solution with 1 g L^{-1} by adding distilled water. A subsequent solution with nominal 10 mg L^{-1} was used to determine the real concentration and used to check the real concentrations and to prepare all working solutions to be used in all experimental assays. The quantification was performed using a Perkin Elmer PinAAcle 900 T flame and a Zeeman longitudinal atomic absorption spectrophotometer, previously calibrated with cadmium and copper standards (Traces Metal Grade, Sigma-Aldrich) (Table S1). The real concentrations did not show variations exceeding 10% of the predicted concentrations. Therefore, following the guidelines established in ISO (2000), the nominal concentrations were kept.

2.3. Acute Toxicity Test

Tadpoles at stage 26 of the Gosner Scale (Gosner, 1960) were used as test-organisms in the assays. At this stage, tadpoles grow, actively search for food and develop limb buds (McDiarmid and Altig, 1999). We determined the initial sensitivity range based on concentrations used in previous studies with other amphibian species and on preliminary tests conducted with this native species to determine the concentration gradient to be used for each compound: 0.05, 0.20, 0.25, 0.50, 1.0, 2.0 mg L^{-1} of Cu and 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 10.0 mg L^{-1} of Cd. Additionally, a control group with reconstituted water was included.

The assays were conducted in triplicate with 1-liter plastic containers with 500 mL of the test solution and 3 tadpoles per replicate. During the experiment, the animals exposed to the test solution did not receive food. Mortality of the animals was recorded daily over four days (96 hours), the physical and chemical variables pH (QUIMIS Q400AS), dissolved oxygen (HANNA HI9146), hardness (complex metric titration with EDTA), and electrical conductivity (DIGIMED DM3) were measured at the beginning and end of the toxicity tests.

2.4. Species Sensitivity Distribution

We generate the Species Sensitivity Distribution (SSD) curves by comparing the acute toxicity values (LC50) obtained for *Physalaemus nattereri* exposed to Cu and Cd with corresponding values for amphibian species with toxicity data obtained from the US-EPA ECOTOX database (Olker et al., 2022), and literature records, detailed in Table S3 (Supplementary Material). To compare with the US-EPA database and literature records, we selected data related to the developing stage (larvae and tadpoles), endpoint LC50-mortality, test duration of four days (96 hours), species distribution, and conservation status. When multiple toxicity values were available, the geometric mean was calculated for each species.

2.5. Ecological Risk Analysis

The risk quotient (RQ) for amphibians was calculated using the following equation:

$$RQ = \frac{PNEC}{MEC}$$

MEC represents the measured ambient concentration, and PNEC is the predicted No-Effect Concentration. MEC values were obtained from the Web of Science database and information for water bodies in the Brazilian Cerrado and are detailed in Table S4.

Besides, PNEC values were calculated by dividing the HC5 value obtained from the SSD curve by an assessment factor (AF). The AF ranges from 1 to 5, depending on the uncertainty degree in ecotoxicological data for organisms (TGD, 2003). In this case, an AF of 5 was selected due to the availability of data on median lethal concentration (LC50) values used in the construction of SSD curves. RQ values were classified into three levels: Low risk (RQ < 0.1), Medium Risk (RQ = 0.1 - 1), and High risk (RQ > 1) (Gomes et al., 2023; Yan et al., 2022).

2.6. Chronic Toxicity Test

Tadpoles at stage 26 (Gosner, 1960) were exposed to four sublethal concentrations, determined after obtaining the mean lethal concentration for the test organisms (LC50-96h) in acute toxicity tests. One-tenth of the LC50-96h values were calculated, and based on these values, four metal solution concentrations were prepared: 0.025, 0.0025, 0.00025, and 0.000025 mg L^{-1} and 0.4, 0.04, 0.004, 0.0004 mg L^{-1} for Cu and Cd, respectively. The assays were conducted in triplicate, using three tadpoles per replicate. Every four days, the test solution was renewed, and the tadpoles were fed. Throughout the test, continuous aeration of the solution was maintained, and the temperature was kept constant. Physical and chemical variables, such as pH, conductivity, dissolved oxygen, and hardness, were measured at the beginning and end of each solution exchange.

To assess the impact of metals on the growth and development of tadpoles, morphological measurements of snout-vent length (SVL) and total length were conducted. Additionally, body weight was recorded, and the developmental stage was classified according to Gosner (1960). These measurements were taken after a 54-day exposure to the metals. Morphological measurements were performed using a manual pachymeter (0.001 mm precision) and an analytical balance (0.0001 g precision).

2.7. Data Analysis

We determined the LC50-96h values by performing non-linear regression with a three-parameter logistic curve with a 95% confidence interval. We used the scientific data analysis and graphing software tools Sigmaplot v. 12.0 (Systat, 2011), Statistica v. 11.0, and the programming language for statistical computing and graphics R version 4.2.3. Besides, we conducted a normality test to check if the assay results followed a normal distribution using the Shapiro-Wilk test and the Levene test for variance homogeneity. To compare the morphological measurements between each sublethal concentration and the control group, we analyzed variance (ANOVA) and a Kruskal-Wallis test ($p<0.05$) (non-parametric data).

We generated the SSD using the ETX software, version 2.0 (Van Vlaardingen et al., 2004). The resulting curves show the percentage of species threatened by toxic substances, in which the most affected fraction can be observed: HC5 and HC50, proposed by Aldenberg and Jaworska (2000). We also verified the log-normality of the data using the Anderson-Darling test at a significance level of 5%.

3. Results

3.1. Acute Exposure: Determination of LC50

The mortality of Neotropical frog tadpoles *Physalaemus nattereri* significantly increased in response to the rise in concentrations of Cu and Cd, as well as exposure time up to 96 hours, as shown in Figure 2. Tadpole survival was 100% in the control groups for both metals. Regarding Cu acute toxicity, 100% mortality was observed at concentrations of 1.0

and 2.0 mg L^{-1} and also at the concentration of 0.5 mg L^{-1} after 48 hours (Figure 2A). As a result of exposing tadpoles for 24, 48, 72, and 96 hours, the lethal concentrations (LC50) were 0.33, 0.25, 0.25, and 0.22 mg L^{-1} of Cu, respectively (Table 1). On the other hand, acute Cd toxicity resulted in a 100% mortality at concentrations of 5.0 and 10.0 mg L^{-1} after 24 hours of exposure and at a concentration of 4.5 mg L^{-1} after 96 hours of exposure (Figure 2B). For *P. nattereri*, the LC50 values for 24, 48, 72, and 96 hours were 4.60, 4.33, 4.28, and 4.03 mg L^{-1} of Cd, respectively (Table 1).

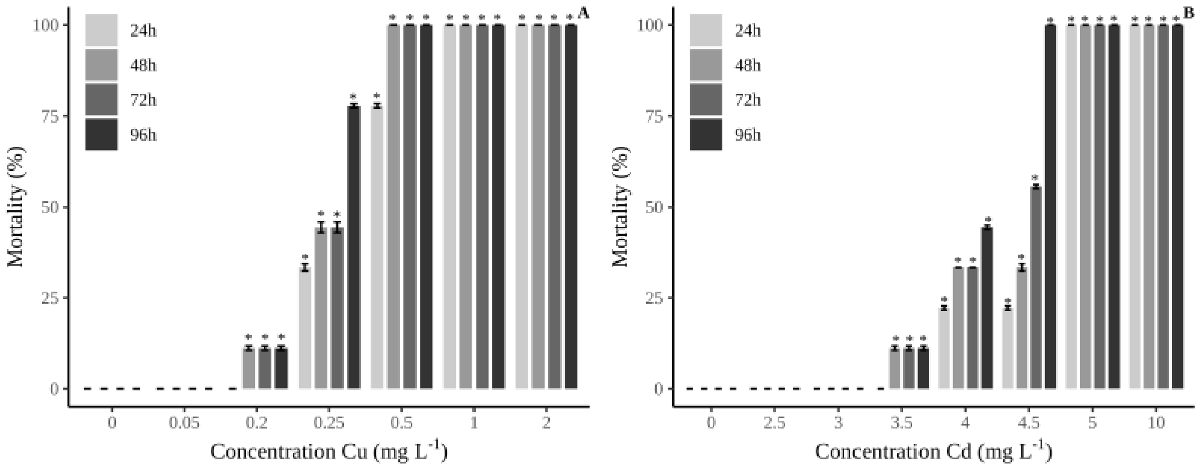

Figure 2. Mortality percentages of *Physalaemus nattereri* (Steindachner, 1893) tadpoles exposed to different concentrations of the metals: (A) Copper (Cu) and (B) Cadmium (Cd) at various time intervals. Standard Deviation (SD), with a significant difference compared to the control groups, is indicated by *p<0.05.

Table 1. Lethal concentrations (LC50) and respective confidence intervals (CI:95%) of the metals Cu and Cd for *Physalaemus nattereri.*

Copper		Cadmium	
Duration (h)	LC50 (mg L–1, CI:95%)	Duration (h)	LC50 (mg L-1, CI:95%)
24h	0.33 (0.26 – 0.40)	24h	4.60 (4,15 – 5,05)
48h	0.25 (0.22 – 0.29)	48h	4.33 (4.03 – 4.73)
72h	0.25 (0.22 – 0.29)	72h	4.28 (4.14 – 4.43)
96h	0.22 (0.21 – 0.23)	96h	4.03 (3.96 – 4.09)

3.2. Species Sensitivity Distribution and Ecological Risk Analysis

To the variations in amphibian species sensitivity to Cu (Figure 3A), it was observed that the most sensitive species were the Eastern Tiger Salamander *Ambystoma tigrinum* (LC50 = 0.035 mg L^{-1}), the Cope's Gray Treefrog *Hyla chrysoscelis* (LC50 = 0.045 mg L^{-1}), and the Asian Common Toad *Duttaphrynus melanostictus* (LC50 = 0.088 mg L^{-1}). The Cuyaba Dwarf Frog *Physalaemus nattereri* (LC50 = 0.22 mg L^{-1}) showed a lower sensitivity to Cu compared to the American Bullfrog *Lithobates catesbeianus* (LC50 = 3.96 mg L^{-1}).

Regarding Cd, the most sensitive species were the Green Toad *Pseudopiladea variabilis*, with an LC50 of 0.035 mg L^{-1}, followed by the Asian Common Toad *D. melanostictus* (LC50 = 0.35 mg L^{-1}), and the Northwestern Salamander *Ambystoma gracile* (LC50 = 0.47 mg L^{-1}). Besides, Neotropical frogs *P. nattereri* (LC50 = 4.03 mg L^{-1}) and the Argentine frog *R. arenarum* (geometric mean of six LC50 values = 2.55 mg L^{-1}) showed moderate sensitivity to Cd, although to a lesser extent in comparison with Chiricahua Leopard frog *Lithobates chiricahuensis* (LC50 = 13.80 mg L^{-1}) and the Marsh Frog *Rana ridibunda* (LC50 = 71.80 mg L^{-1}), which exhibited significantly higher LC50 values, indicating lower sensitivity of Neotropical Frog *P. nattereri* to Cd (Figure 3B).

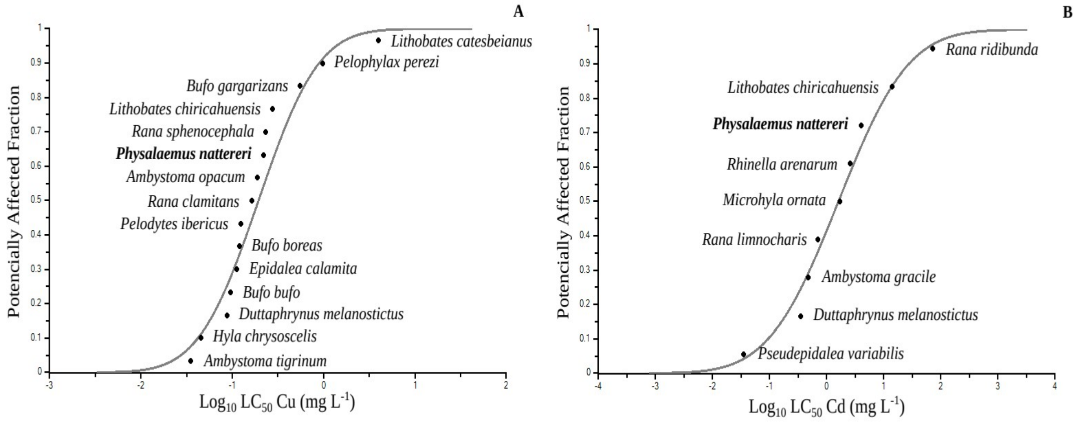

Figure. 3. Sensitivity distribution of species (SSD) constructed based on the LC50 values (mg L^{-1}) *Physalaemus nattereri* (Steindachner, 1893) and data on other amphibians exposed to Cu (A) and Cd (B) from the literature and the ECOTOX database (Table S2 Supplementary Material).

The hazardous concentration (HC – 95% CI) for Cu and Cd was determined using the Sensitivity Distribution Species (SSD). Table 2 details the hazardous concentration values affecting 5% (HC5) and 50% (HC50) of the amphibians evaluated in the SSD. The LC50 for Cu (0.22 mg L^{-1}) and Cd (4.03 mg L^{-1}) are within the range of HC50 values, suggesting that both metals pose a significant risk to the survival of *P. nattereri* and other amphibian species with even lower LC50 values.

Table 2. Mean values of the hazardous concentrations for 5% (HC5) and 50% (HC50) of the species included in the SSD and their respective 95% confidence intervals for the metals Copper (Cu) and cadmium (Cd).

Metal	HC5		HC50	
	HC_5 (mg L^{-1})	CI 95% (mg L^{-1})	HC_{50} (mg L^{-1})	CI 95% (mg L^{-1})
Copper	0.030	0.009 – 0.051	0.190	0.11 – 0.33
Cadmium	0.040	0.002 – 0.178	0.600	0.40 – 6.32

The risk assessment conducted for Cu and Cd in some Brazilian Cerrado water bodies indicated that at minimum, median, or maximum concentrations, Cu and Cd threaten amphibians inhabiting these aquatic ecosystems. Cu, at its minimum concentration, presented a moderate risk (RQ = 0.1), while the risk escalated to high levels at its median (RQ = 1.9) and maximum concentrations (RQ = 75). Regarding Cd, its risk varied, being low at the minimum concentration (RQ = 0.01), moderate at the median concentration (RQ = 0.7), and reaching high levels at the maximum concentration (RQ = 972.2) (Table 3).

Table 3. Minimum, median, and maximum values of MEC, RQ for Cu and Cd, and PNEC calculated from the hazardous concentration values for 5% (HC5) of the amphibians assessed in the SSD

Metal	PNEC	MEC minimum	RQ minimum	MEC median	RQ median	MEC maximum	RQ maximum
Cooper	0.0052	0.00046	0.1	0.00965	1.9	0.39	75
Cadmium	0.0072	0.00006	0.01	5	0.7	7	972.2

3.3. Assessment of the Effects of Chronic Metal Exposure

After 54 days of chronic exposure, a mortality rate of 33.33% was observed in the second week of exposure to Cd at a concentration of 0.4 mg L^{-1}. In the third week, the mortality rate increased to 77.77%; in the fourth week, it reached 88.88%. Besides, at a concentration of 0.04 mg L^{-1}, a mortality rate of 22.22% was recorded in the second week, and 33.33% in the third week. It is important to note that there was no mortality in the tadpoles exposed to Cu during the eight weeks of testing (Table S5). Statistical analyses conducted on morphological measurements (Snout-Vent Length - SVL, Total length, and Weight) of tadpoles after 54 days of exposure to Cu and Cd indicate no significant differences between the treatments and the control group (P > 0.05) (Figure 4, 5; Table S6). Regarding the developmental stage of tadpoles exposed to Cu, the control group exhibited stages 39G, 40G, and 41G, unlike the treatments that showed stages between 39 and 40G (Figure 4D; Table S4). Contrastingly, tadpoles exposed to Cd exhibited different developmental stages. The control group recorded stages 39G, 40G, and 41G, unlike 0.0004 mg L^{-1}, where only stage 40G was observed. At 0.004 and 0.04 mg L^{-1}, stages 39G and 40G were observed. At 0.4 mg L^{-1}, the sole surviving tadpole after Cd exposure was in stage 40G. When comparing Cu and Cd treatments with the control group for both metals, no significant differences were found (P > 0.05) (Figure 5D, Table S6).

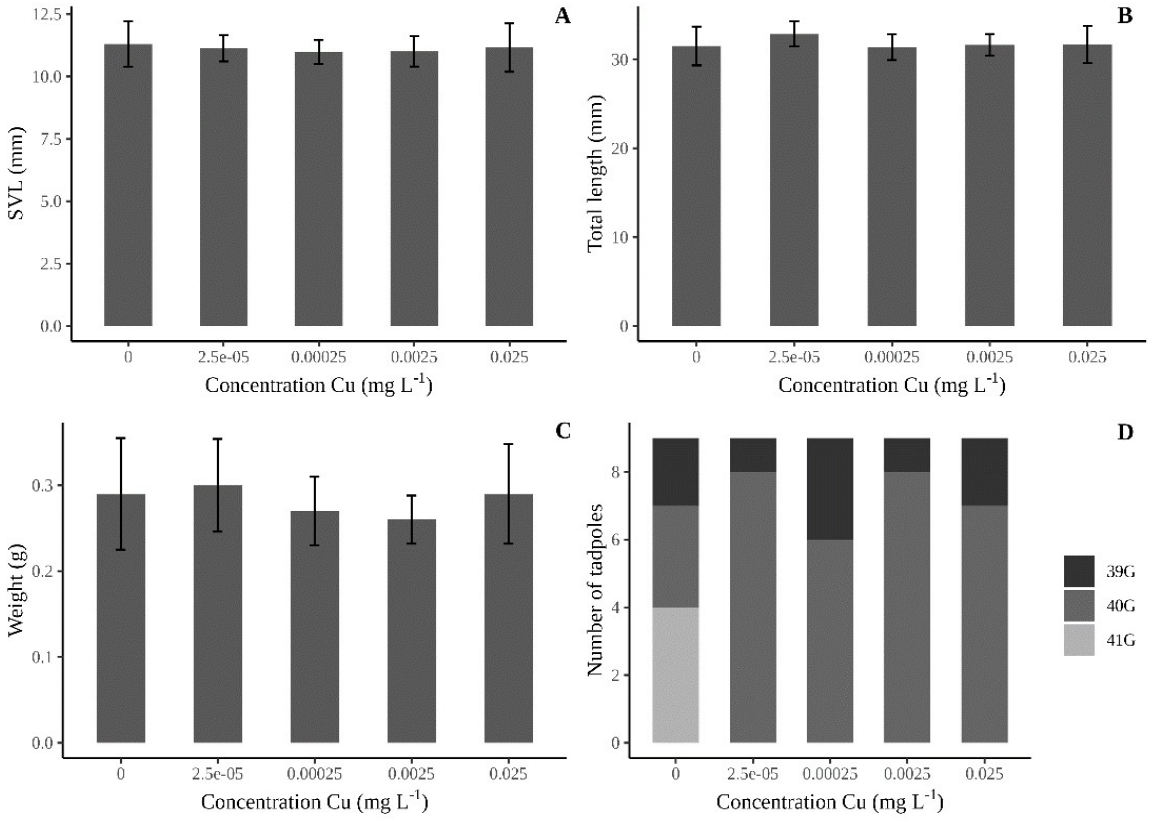

Figure 4. Morphological measurements A) Snout-vent length (SVL), B) Total length, C) Weight, and D) Developmental stage of *Physalaemus nattereri* (Steindachner, 1893) tadpoles after a 54-day exposure to Cu. The bars in graph represent the means ± standard deviation.

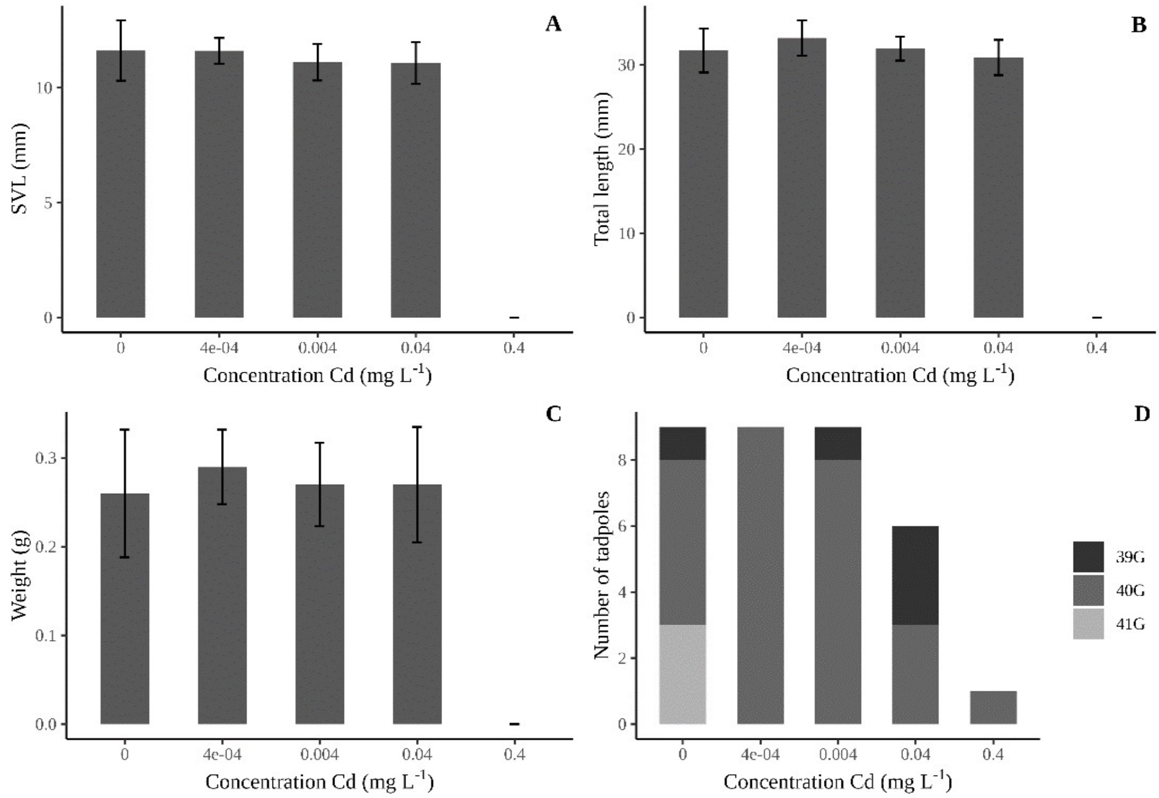

Figure 5. Morphological measurements A) Snout-vent length (SVL), B) Total length, C) Weight, and D) Developmental stage of *Physalaemus nattereri* (Steindachner, 1893) tadpoles after a 54-day exposure to Cd. The bars in the graph represent the means ± standard deviation.

4. Discussion

The results obtained in this study showed a gradual increase in mortality as the exposure time to metals increased, indicating a decrease in LC50 values (Table 1). These results are in agreement with those reported by Srivastav et al. (2016), who exposed tadpoles of the Indian frog *Rana cyanophlyetis* for 24, 48, 72, and 96 hours and found LC50 values of 32.586, 29.994, 27.219, and 23.048 mg L^{-1} of Cd, respectively. Similarly, Patar et al. (2016) reported LC50 values for the alpine frog *Rana limnocharis* of 1.85, 1.62, 1.12, and 0.69 mg L^{-1} of Cd for the mentioned exposure periods. Ojha et al. (2021) also recorded LC50 values for Cd at 24 hours of 5.603 mg L^{-1} and LC_{50} at 48 hours of 4.8 mg L^{-1} when exposing tree frog tadpoles of Chunam *Polypedates maculatus*. On the other hand, Ossana et al. (2010) subjected bullfrog tadpoles of *L. catesbeianus* to Cu and obtained LC50 values of 9.49, 6.89, and 3.96 mg L^{-1} for exposure periods of 48, 72, and 96 hours, respectively.

Furthermore, when comparing the LC50 values for Cu and Cd obtained in our study, it is notable that the Cu LC50 value at 96 hours (0.22 mg L^{-1}) is approximately 18 times lower than the value recorded for Cd (4.03 mg L^{-1}), indicating that Cu is potentially more toxic compared to Cd. This result is in line with the findings of Calfee and Little (2017), who reported LC50 values at 96 hours of 0.27 mg L^{-1} (geometric mean of two LC50) for Cu and 13.8 mg L^{-1} for Cd in Chiricahua leopard frogs *Lithobates chiricahuensis* tadpoles, where the Cu LC50 value is 51 times lower than the Cd LC50 value.

The SSD curves provide a more accurate representation of how *P. nattereri* and other amphibian species respond to metal exposure. However, it is important to note that the effects of contaminants cannot be generalized by knowing the sensitivity of a single species. For example, *L. catesbeianus* is known to be a reference species in ecotoxicological analysis and highly resistant to Cu (LC50-96h = 3.96 mg L^{-1}) (Ossana et al. 2010; 2013), which does not ensure the protection of *P. nattereri* or the species used in this analysis (Figure 3A). On the other hand, the SSD curve for Cd shows that *P. nattereri* was tolerant to Cd compared to the sensitivity of *R. arenarum* (geometric mean of six LC50-96h = 2.55 mg L^{-1}), a species recorded for South America (Kwet et al., 2004). Without a doubt, when compared to *Pelophylax ridibundus* (LC50-96h = 71.8 mg L^{-1}), a species widely distributed in Europe and Asia (Kuzmin et al., 2009), its high sensitivity to Cd is evident. This shows the importance of conducting toxicity tests with native Neotropical species.

On the other hand, the results obtained from the SSD curves for Cu (HC5 = 0.026 mg L^{-1}) and Cd (0.036 mg L^{-1}) were lower than the concentrations found in some Brazilian water bodies (Table S4). In some cases, they even exceeded the limits established by CONAMA. For instance, Medeiros et al. (2013) recorded a Cu concentration of 0.244 mg L^{-1} in a watershed in Americana, State of São Paulo. Similarly, 0.35 mg L^{-1} in the Carmo and Gualaxo do Norte Rivers (F.C Santana et al., 2021), and 0.28 mg L^{-1} in the Concepción River (Oliveira et al., 2023). These records also surpass the LC50-96h value (0.22 mg L^{-1}) reported in this study for *P. nattereri*. These results support the ecological risk analysis, as both metals underscore the threat to the survival of vulnerable species, such as amphibians inhabiting these ecosystems.

Differences in acute toxicity (LC50) among various amphibian species are not limited to short-term effects but also extend to sublethal consequences after exposure to environmentally relevant concentrations. Previous studies have documented a range of effects on the life history of anurans. For example, Patar et al. (2016) reported acceleration in the metamorphosis time, changes in growth characteristics (both in length and body mass), as well as chromosomal and DNA damage in tadpoles of *R. limnocharis*. Similarly, several authors have reported delays or acceleration in metamorphosis, effects on growth, and high mortality when exposing tadpoles of *P. variabilis* (Gürkan et al., 2014), *Duttaphrynus melanostictus* (Ranatunge et al., 2012), and *Microhyla fissipes* (Hu et al., 2019). Similar to Cd, exposure of tadpoles to sublethal Cu concentrations resulted in changes in individual morphology and development. In a study conducted by Flynn et al. (2015), it was observed that at low Cu levels (10 µg L^{-1}), tadpoles of *Gastrophryne carolinensis* reduced embryo and tadpole survival, and developmental delays. Similarly, Xia et al., (2012) reported developmental delays in embryos and a decrease in tadpole weight and length in *Bufo gargarizans*. In contrast to the previously reported effects, the present study did not identify significant changes in the development or morphology of *P. nattereri* tadpoles (Figura 4, 5; Table S6). However, a decrease in survival was evident at higher sublethal Cd concentrations assessed (0.04 and 0.4 mg L^{-1}). This result shows that, if amphibians extend their exposure to contaminated water bodies, their survival is compromised, even under conditions of low concentrations.

On the other hand, in prolonged exposure to Cu, no mortality of *P. nattereri* tadpoles was observed until development reached stage 40G. Lance et al. (2012) also did not report mortality until the metamorphosis of *Lithobates sphenocephalus*. These variations in sensitivity and sublethal effects among different amphibian species may be intrinsically related to their adaptive capacity to environmental changes (E. Santana et al., 2021). In a recent study conducted by E. Santana et al. (2021), it was demonstrated that *Rhinella ornata* toads found near metal contamination sources exhibited an increase in the size of the liver, kidneys and spleen, organs associated with detoxification and immune functions, and associated chronic stress levels with proximity to contaminated sites, which may be related to possible local adaptation.

Physical and chemical aspects of water, such as hardness and pH, can lead to a reduction or intensification of metal toxicity (Horne and Dunson 1995). The effects of metals on the life history of anurans are complex, and for that reason, it is important to consider the multiple factors that influence the sensitivity of organisms. These factors can explain the discrepancies observed in both LC50 values and sublethal effects induced by contaminants. Understanding these factors, in addition to the consequences of amphibian exposure to metals, provides a comprehensive view of how amphibians are being impacted and how these effects endanger their populations, leading to species loss. Likewise, it is important to address knowledge about the sensitivity of Neotropical amphibians. This group poses a challenge for ecotoxicologists, as it is a highly threatened group, with approximately 41% of its species globally endangered (IUCN, 2022).

5. Conclusion

This study showed that both Cu and Cd caused 100% mortality in *Physalaemus nattereri* tadpoles during short-term exposures to the highest tested concentrations for both metals over 24 and 48 hours. When comparing the median lethal concentration (LC50) values of *P. nattereri* with those of other amphibian species using species sensitivity distribution curves (SSD), it demonstrated a moderate sensitivity of *P. nattereri*. However, high variability was observed among the sensitivity values of the species, emphasizing the necessity of conducting acute toxicity tests on diverse species. This variability highlights the differing responses of amphibians to metal pollution, whether due to local adaptation, evolutionary history, or environmental conditions. Furthermore, given that the range of Cu and Cd concentrations that caused acute toxicity in tadpoles was high, on the order of milligrams, it is concluded that these concentrations are environmentally relevant. As observed in the preliminary ecological risk analysis conducted for Cu and Cd in various rivers and streams within the geographic distribution area of *P. nattereri*, where the RQ values are significantly elevated, indicating that even minimal concentrations pose a threat to their survival in their natural habitat

Regarding the chronic toxicity assessed in this study for both metals, the tested concentrations were very low. After a long exposure of 54 days, it was concluded that there was no chronic effect of morphological deformation or mortality in *P. nattereri* tadpoles during or after exposure to Cu. However, for Cd, there was tadpole mortality after prolonged exposure, allowing the conclusion that this metal is also toxic to tadpoles of this species if the exposure is chronic.

In conclusion, for the conservation of biodiversity and the preservation of various Neotropical amphibian species, including *P. nattereri*, maintaining water quality in their natural habitats, especially regarding metal contamination, is an essential condition.

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Capítulo 2. Effects of cadmium and copper metals isolated and in combined action over *Physalaemus nattereri* (Steindachner, 1893) tadpoles

Abstract

Research on metal toxicity in aquatic organisms has primarily focused on individual exposures, with few studies evaluating the toxicity of metal mixtures in amphibians. This study aimed to assess the effects of copper sulfate ($CuSO_4$) and cadmium chloride ($CdCl_2$) both individually and in combination on the tadpoles of *Physalaemus nattereri*, a native tropical species. *P. nattereri* tadpoles were exposed to the metal salts through acute toxicity tests. For the mixture, a fixed ratio design was used. The median lethal concentration (LC50) values obtained after 96 hours of exposure were 0.22 mg L^{-1} for $CuSO_4$ and 4.02 mg L^{-1} for $CdCl_2$. In the case of the mixture, the model that best fitted data was the Concentration Addition (CA) model. The combination of metal salts produced a highly toxic mixture, causing synergism and posing a significant risk to the survival of *P. nattereri* tadpoles. Therefore, it is essential to conduct risk assessments that consider the toxicity of mixtures, as chemical contaminants in aquatic ecosystems are generally found in combinations.

Keywords: Ecotoxicology; Metal mixtures; Synergism; Tropical amphibians.

1. Introduction

Understanding the effects of stress-inducing metal interactions on organisms from small aquatic ecosystems is a priority for amphibian diversity conservation. Although metals are naturally produced through volcanic rock and soil erosion (Parelho et al., 2014), they serve essential biological roles in the structure and functioning of biomolecules such as DNA and proteins (Altenburger, 2010; Aronzon et al., 2020), However, their bioaccumulation, toxicity, and persistence in environments can trigger many adverse effects (Alnashiri, 2022), such as acting as "metabolic poisons" (Ali et al., 2019). Their toxicity is partly attributed to generating Reactive Oxygen Species (ROS), which may induce oxidative stresses (Carvalho et al., 2017). Furthermore, in contrast to other pollutants, metals tend to accumulate in the environment as they are neither chemically nor biologically degraded, making them particularly hazardous to environmental health (Lance et al., 2013; Wei and Yang, 2015).

Anthropogenic activities such as intensive agricultural and industrial production, mining, and inappropriate discharge of urban effluents, lead to increased metal concentrations in surface and groundwater (Blaustein et al., 2003; Corbi et al., 2006). Essential and non-essential metals that exceed metabolic concentration limits can harm living organisms (Ali et al., 2019). For example, copper is an essential micronutrient for enzymatic processes, different from cadmium, which does not serve a biological function. However, cadmium has an affinity for similar binding sites in organic macromolecules and can act as a substitute for magnesium, zinc, and calcium in some metabolic processes (Carvalho et al., 2017; Sánchez-Bayo, 2011).

Usually, metal risks are assessed individually, but under natural conditions, pollutants interact within the environment. For instance, Santos et al. (2020) evaluated the water quality of the Ivinhema River in the Upper Paraná Basin at Mato Grosso do Sul, Brazil. They recorded concentrations of cadmium (0.008 mg L^{-1}), copper (0.0104 mg L^{-1}), iron (4.000 mg L^{-1}), and nickel (0.050 mg L^{-1}) that exceed the reference values established by the Brazilian National Environment Council (CONAMA, 2005) and the World Health Organization (WHO, 2017). Additionally, they collected fish species, such as *Hemiodus orthonops*, *Leporinus friderici*, *Prochilodus lineatus*, *Pterodoras granulosus*, and *Pimelodus maculatus*, which exhibited genotoxic alterations in erythrocytes and metal bioaccumulation in muscle tissue. Similarly, Girotto et al. (2020) quantified metals in sediment collected after the disaster of the Fundão dam break in Mariana, Brazil. They confirmed the contamination by cadmium (0.007 mg L^{-1}), lead (0.10 mg L^{-1}), iron (29.23 mg L^{-1}), manganese (3.32 mg L^{-1}), zinc (0.08 mg L^{-1}), and aluminum (3.91 mg L^{-1}). Furthermore, they reported morphophysiological damages and behavioral change in *Lithobates catesbeianus* tadpoles exposed to 25, 50, and 100% concentrations of a stock solution containing mining tailings, after 16 days of exposure.

Two reference models, based on the assumption of no interaction between chemicals (Hewlett and Plackett, 1959; Jonker et al., 2005) have been applied to assess environmental risks caused by mixtures of different compounds. These are the Concentration Addition (CA) (Loewe and Muischnek, 1926), and Independent Action (IA) (Bliss, 1939) models. The CA model assumes that chemicals in the mixture have the same or similar mode of action, while the IA model assumes that chemicals have different modes of action (Aronzon et al., 2020). Additionally, both models can experience deviations, such as synergism/antagonism (S/A), dose ratio (DR) dependent, or dose level (DL) dependent (Jonker et al., 2005).

Several studies have applied the CA and IA binary mixture models to metals. For instance, Santos-Lima et al. (2023) analyzed the acute toxicity of metal salts containing copper, cadmium, mercury, and manganese to the ostracod *Strandesia trispinosa*, concluding that depending on the combination of metals, the effects of these metals on mortality can be either synergistic or antagonistic. Besides, Reis et al. (2022) evaluated the effects of a mixture of cadmium and cobalt on the microalga *Raphidocelis subcapitata*. The CA and DR models best-fitted data showing the synergistic effect at high concentrations of cobalt and low concentrations of cadmium. They also evaluated photosynthetic performance, which exhibited antagonistic effects. These model variations and their deviations indicate the importance of understanding the toxicity and the mechanism of action when metal mixtures are tested.

In this scenario, amphibians are the group of animals more directly exposed to a mixture of chemical compounds due to their permeable skin, which makes them more susceptible to the absorption of toxins and contaminants present in water, and due to their high dependence on water bodies for reproduction and larval development. Besides, their low mobility forces them to remain exposed for prolonged periods in contaminated areas, which increases the risk of intoxication and mortality. Nevertheless, for this group, most studies have only focused on exposures to a single metal (Chen et al., 2007; Dobrovoljc et al., 2003; Fernando et al., 2016; Krohn et al., 2020), and few others evaluated the toxicity of metal mixtures for amphibians (Carvalho et al., 2017; Chagas et al., 2020). The aim of this study was to assess the effects of copper sulfate ($CuSO_4$) and cadmium chloride ($CdCl_2$) both individually and in combination on tadpoles of *Physalaemus nattereri*, a native tropical species. We hypothesize that the combination of copper and cadmium results in a highly toxic mixture, causing synergism, which leads to adverse effects and eventually mortality of the tadpoles of *P. nattereri.*

2. Materials and methods

2.1. Test and Cultivation Conditions

Physalaemus nattereri, a native species from Brazil, Paraguay, and Argentina, is commonly found in temporary lakes, natural open areas, and anthropized regions in the Cerrado region (Rossa-Feres and Nomura, 2006). We collected *P. nattereri* eggs from the temporary lake Mayaca in São Carlos, State of São Paulo (21°54', 18"S by 47°52' 17"W). After hatching, the larvae were maintained in the laboratory in reconstituted water with a pH of 7.0-8.0, hardness of 40±2 mg $CaCO_3$/L, dissolved oxygen 6±1 mg L^{-1} and conductivity of 140±10 µS cm (ABNT, 2016). Temperature was controlled at 25±1°C, and we established a photoperiod of 12 hours of light/12 hours of darkness in aquariums with constant aeration. Tadpoles were fed daily with commercial fish food for tropical species (Tetramin Flakes Tetra), and their feeding maintained until 24 hours before the experiments.

We renewed the aquarium water weekly, replacing 50% of total volume. For the experiments we used tadpoles at Gosner developmental stage 27-30 (Gosner, 1960). During this stage, they should grow normally, actively search for food, and develop limb buds (McDiarmid and Altig, 1999). The test organisms were collected under license from the Chico Mendes Institute for Biodiversity Conservation (ICMBio No. 86058-1). Maintenance and experimentation procedures were approved by the Ethics and Animal Experimentation Committee of the Federal University of São Carlos (CEUA UFSCAR No. 9592171022/ID 001757). All experiments were conducted in the Limnology and Ecotoxicology laboratory, Department of Ecology and Evolutionary Biology, Federal University of São Carlos.

2.2. Chemical Analysis

The metallic salts employed for the tests were copper sulfate ($CuSO_4$-$5H_2O$, CAS 7758-99-8, from Dinâmica brand) and cadmium chloride ($CdCl_2$ 2.5 H_2O, CAS 10108-64-2, Carlo Erba). We prepared a stock solution with 1 g L^{-1} by adding distilled water. A nominal solution with 10 mg L^{-1} was applied to determine the real concentration of Cu and Cd. The quantification was performed using a Perkin Elmer PinAAcle 900 T flame, and a Zeeman longitudinal atomic absorption spectrophotometer, calibrated with cadmium and copper standards (Traces Metal Grade, Sigma-Aldrich) (Table S1). The real concentrations did not show variations exceeding 10% of the predicted concentrations. Therefore, the nominal concentrations were kept, according to the guidelines established by the ISO (2000).

2.3. Acute Toxicity Testing of Individual Compounds and Their Mixtures

The sensitivity range of *Physalaemus nattereri* to two reference compounds was determined for the metal salts cadmium chloride ($CdCl_2$) and copper sulfate ($CuSO_4$). The concentration ranges were 2.5, 3, 3.5, 4, 4.5, 5 mg L^{-1} for $CdCl_2$ and 0.05, 0.125, 0.25, 0.5, 1, 2 mg L^{-1} for $CuSO_4$. The control group with only reconstituted water was included.

For the mixture toxicity test, a partial fixed-ratio design (Cassee et al., 1998) was selected, which included 24 combinations of both compounds, one test for each individual compound, and a control (reconstituted water only). We calculated the concentrations of the mixtures based on the expected toxic strengths of 0.125, 0.375, 0.5, 0.75, 1, 1.5, 1.75, and 2 toxic units (TU), where one toxic unit is equal to the LC50-96h value obtained from the individual preliminary test for each metal (LC50-96= 0.22 mg L^{-1} $CuSO_4$; LC50-96 = 4.03 mg L^{-1} $CdCl_2$).

The assays were conducted in triplicate in 1L plastic containers with 500 mL of the test solution and three tadpoles per replicate. Tadpoles exposed to the test solution did not receive food during the experiment. Mortality was recorded daily for four days (96 hours), and the following physical and chemical parameters: pH (Micronal B374), dissolved oxygen (YSI 55-25FT), hardness (complexometric titration with EDTA), electrical conductivity, and temperature (ORION 145 plus) were measured at the beginning and end of toxicity tests.

2.4. Data Analyses

We determined the LC50-96h values by applying a non-linear regression method with a three-parameter logistic curve with a 95% confidence interval. We employed the scientific data analysis and graphing software Sigmaplot v. 12.0 (Systat, 2011) and the statistical programming language R version 4.2.3.

To analyze results of acute toxicity tests with the mixtures, we applied the models described in Jonker et al. (2005) and implemented them with the MIXTOX tool. We examined the Concentration Addition (CA) model (Loewe and Muischnek, 1926) and the Independent Action (IA) model (Bliss 1939). Additionally, we included deviations from the reference models, Dose Ratio (DR) Deviation, Dose Level (DL) Deviation, and Synergistic/Antagonistic (S/A) interactions by adding two parameters (*a* and *b*). After analysis, we chose the best fit using the maximum likelihood method.

3. Results

During the tests, the physical and chemical parameters were recorded at both the beginning and end of the experiment. The measured values were: pH (7.46–7.63), hardness (40–44 mg $CaCO_3$ L^{-1}), electrical conductivity (135.77–150.33 µS cm^{-1}), dissolved oxygen (6.62–7.07 mg L^{-1}), and temperature (25.43–25.75 °C) (Table S2).

3.1. Acute Exposure: Determination of LC50

For the individual acute toxicity tests conducted on *Physalaemus nattereri*, a median lethal concentration (LC50) of 0.27 mg L^{-1} was obtained for $CuSO_4$ and 3.27 mg L^{-1} for $CdCl_2$ after a 96-hour exposure period. No mortality occurred under the tested $CuSO_4$ concentrations of 0.25 and 0.5 mg L^{-1} (mortality=0%). At the concentration of 0.5 mg L^{-1}, mortality was 11.11%. Additionally, for concentrations of 0.5, 1, and 2 mg L^{-1}, the mortality percentage was 100%. For the tested concentrations of 2.5, 3, 3.5, 4, 4.5 and 5 mg L^{-1} of $CdCl_2$, the mortality percentages were 22.22 ± 0.57%, 66.67 ± 0.57%, 44.44%, 44.44 ± 0.57%, 77.78 ± 0.57%, and

100%, respectively (Table S3). It is important to highlight that no mortality occurred in the control group for the tests with both metals.

3.2. Mixtures

Synergistic deviation from the Concentration Addition (CA) was the most suitable model for explaining the effects of the combination of $CuSO_4$ and $CdCl_2$ on the survival of *P. nattereri* tadpoles (i.e. the lowest sum of squared residuals and highest r^2 values) (Table 1). The CA model yielded a sum of squared residuals (SS) of 105.64 ($p=2.26 \times 10^{-42}$) and coefficient of determination $r^2 = 0.66$. The best fit to data by this model showed synergistic deviation; the SS value decreased to 41.36 ($p = 1.08 \times 10^{-15}$) with an r^2 value of 0.87 and "a" of -2.17 (Table 1; Fig 1). On the other hand, the Independent Action (IA) model yielded SS value of 134.50 (p= 3.59 x 10-36; $r^2 = 0.56$), and the deviation that presented the best fit for the model was dose ratio dependency (DR) with SS values of 50.96 ($p=7.24 \times 10^{-19}$) and an r^2 value of 0.83, with "*a*" value of -26.63 and "*b*" of 20.29 (Table 1; Fig 2).

Table 1. Analysis of the acute toxicity tests with tadpoles exposed to mixtures of Cadmium Chloride ($CdCl_2$) and Copper Sulfate ($CuSO_4$) using the concentration addition (CA) and independent action (IA) models. The results were analyzed with the MIXTOX tool (Jonker et al., 2005).

	Concentration Addition				Independent Action			
	CA	**S/A**	DR	DL	IA	S/A	**DR**	DL
max	0.98	**0.98**	0.91	0.91	0.98	0.98	**0.98**	0.98
β Cd	4.19	**3.71**	3.71	3.71	2.66	13.54	**7.80**	3.53
β Cu	27.17	**28.32**	28.32	28.32	2.53	11.30	**10.95**	1.91
LC50 for $CdCl_2$	3.24	**3.46**	3.45	3.45	2.65	3.97	**3.42**	3.87
LC50 for $CuSO_4$	0.14	**0.25**	0.15	0.15	0.10	0.43	**0.28**	0.25
a	-	**-2.17**	-9	-0.0008	-	-22.63	**-26.63**	0.71
bDR / bDL	-	**-**	-4	1.00	-	-	**20.29**	58.77
SS	105.64	**41.36**	NA	NA	134.50	NA	**50.96**	62.29
r2	0.66	**0.87**	NA	NA	0.56	NA	**0.83**	0.80
$\chi 2$ or F test	201.02	**64.28**	NA	NA	172.16	NA	**83.54**	72.21
df	-	**1.00**	NA	NA	-	NA	**2.00**	2.00
p ($\chi 2$/ F)	2.26×10^{-42}	$\mathbf{1.08 \times 10^{-15}}$	NA	NA	3.59×10^{-36}	NA	$\mathbf{7.24 \times 10^{-19}}$	2.09×10^{-16}

where *max* is the maximum response of control (maximum survival in control); β is the slope of the individual dose-response curve; LC50 is the median lethal concentration; *a*, bDR, and bDL are the parameters of the function; L is the objective function used for discrete data; *r2* is the regression coefficient; $\chi 2$ or F test is the test statistic (F test was used for reference model fit and $\chi 2$ test for improvement in fit and the quality of the model fits obtained); df is the degrees of freedom; and p ($\chi 2$ / F) is the significance level of the test statistic. CA is the concentration addition model, and IA is the independent action model, S/A is the synergism or antagonism deviation, DR is the dose-ratio dependent deviation and DL is the dose-level deviation. NA means that the quantity is not applicable.

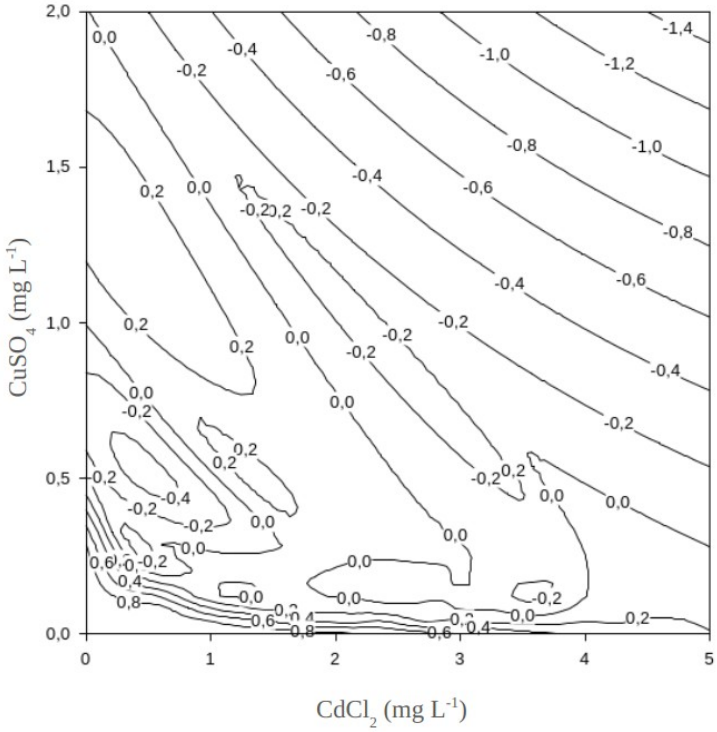

Figure 1. Isobologram of the effect of combining Copper Sulfate ($CuSO_4$) and Cadmium Chloride ($CdCl_2$) on the mortality of *Physalaemus nattereri* (Steindachner, 1893), based on the synergism/antagonism (S/A) of the Concentration Addition (CA) model.

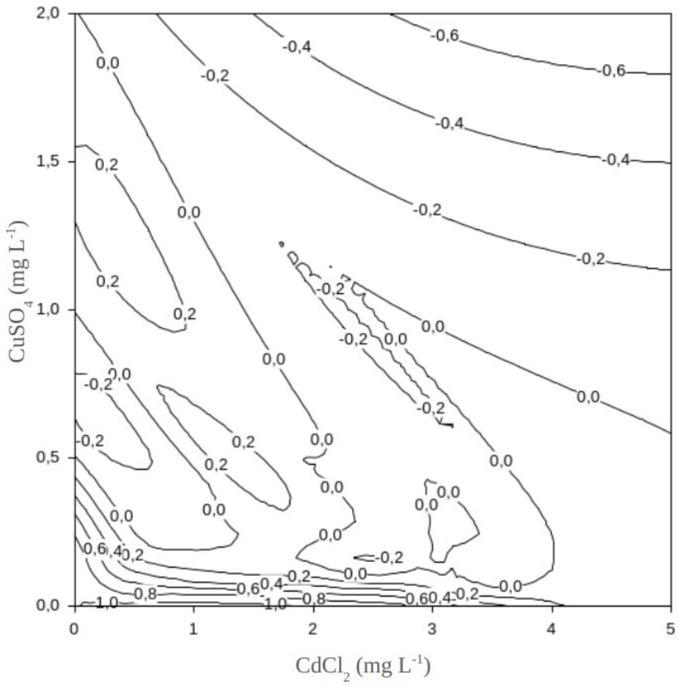

Figure 2. Isobologram of the effect of combining Copper Sulfate ($CuSO_4$) and Cadmium Chloride ($CdCl_2$) on the mortality of *Physalaemus nattereri* (Steindachner, 1893), based on the dose ratio (DR) dependent of the Independent Action (IA) model.

4. Discussion

Our results indicated that the Concentration Addition (CA) model with synergistic deviation provides the best fit for the acute toxicity data in *Physalaemus nattereri* tadpoles ($SS = 41.36$, $p < 0.05$, $r^2 = 0.87$, $a = -2.17$) (Fig. 1). This suggests that there is a synergy that increases the toxicity of copper sulfate ($CuSO_4$) and cadmium chloride ($CdCl_2$) when both metals are present. However, the Independent Action (IA) model also fit the data, although the deviation that provided the best fit was dependent on the dose ratio (DR) ($SS = 50.96$, $p < 0.05$, $r^2 = 0.83$, $a = -26.63$, $b = 20.29$) (Fig. 2). This model suggests that metal interactions are not uniformly predictable and depend on the relative concentration of each metal.

We selected the Concentration Addition (CA) model with synergistic deviation as the most appropriate for predicting the toxicity of the $CuSO_4$ and $CdCl_2$ mixture in *P. nattereri* tadpoles. This model showed that the combination of these metal salts was more toxic than the sum of their individual toxicities. Similarly, Santos Lima et al. (2023) determined that the CA model was also the most suitable for explaining the effects of the $CuSO_4$ + $CdCl_2$ mixture on the survival of the tropical ostracod *Strandesia trispinosa*. However, their study found that the deviation of the mixture exhibited an antagonistic effect on ostracod survival.

The mixture of chemical compounds can result in additive, synergistic and antagonistic interactions (Ali et al., 2019). In our study, we observed a synergistic effect on the survival of *P. nattereri* tadpoles when combining $CuSO_4$ and $CdCl_2$. Similarly, Li et al. (2024) demonstrated that the combination of these metals resulted in a mortality rate of *Oryzias latipes* larvae that was two to four times higher than that caused by the metals individually. Additionally, the effects of the metals were evident in the timing and success of hatching and in the swim bladder of the larvae. The synergistic toxic effect of Cu + Cd mixtures can also impact first trophic level populations, suppressing algal growth as observed in *Chlorella* sp. by Franklin et al. (2002). Likewise, Rebolledo et al. (2024) found that binary mixtures of As, Cd, Cu, Fe, Pb, and Zn have the potential to induce synergistic effects in the rotifer *Proales similis*.

The difference between synergistic, antagonistic, or absent effects in organisms may be due to the concentrations of chemical compounds and the duration of exposure (Carvalho et al., 2017; Rebolledo et al., 2024). For example, Carvalho et al. (2017) found that, at low concentrations of Cu, Cd, and Zn (1 $\mu g\ L^{-1}$ for each metal), in binary combination, short-term exposure (2 days) of *Lithobates catesbeianus* tadpoles resulted in a synergistic effect in the liver and muscles. However, they observed an antagonistic effect in the kidney after prolonged exposure of 16 days. Similarly, the chemical interaction of metals can increase or decrease their bioavailability and toxicity (Altenburger, 2010). Furthermore, metal interactions in aquatic ecosystems not only depend on their concentration and exposure time but also on the simultaneous presence of other contaminants, the physical and chemical properties of the water, the biological responses of organisms, and environmental factors such as temperature, pH, and dissolved organic matter (Adams et al., 2020).

On the other hand, this study demonstrated that although the concentrations of the mixture that affected the survival of *P. nattereri* are high compared to the limit values established in Resolution 357/2005 of the National Environmental Council (CONAMA) (Cu: 0.009 mg/L; Cd: 0.001 mg/L), the concentrations recorded in some water bodies also exceed these limits and form part of a "contaminant cocktail."

For example, in the Gualaxo do Norte and Carmo rivers (MG), which were contaminated after the collapse of the Fundão dam in 2015, copper reached 0.35 mg L^{-1} and cadmium 0.109 mg L^{-1}. Additionally, other metals such as chromium (0.137 mg L^{-1}), manganese (0.989 mg L^{-1}), iron (3.013 mg L^{-1}), zinc (0.710 mg L^{-1}), and aluminum (1.388 mg L^{-1}) were also recorded (Santana et al., 2021), impacting aquatic biodiversity and local communities. Considering our results, it is crucial to conduct ecotoxicological tests not only on individual contaminants but also to provide evidence of the potential risks of metal combinations in aquatic ecosystems. Furthermore, investigating the effects of mixtures on different organisms, particularly amphibians, is needed as this information remains limited.

Considering these results, it is crucial to conduct ecotoxicological tests not only on individual contaminants but also to assess the potential risks posed by metal combinations in aquatic ecosystems. Furthermore, given the limited available information, investigating the effects of these mixtures on different organisms, particularly amphibians, is essential to better understand the broader ecological consequences of metal pollution.

5. Conclusion

The mixture of metallic salts, copper sulfate ($CuSO_4$) and cadmium chloride ($CdCl_2$) has a synergistic effect on the survival of *Physalaemus nattereri* tadpoles. The concentration addition (CA) reference model with synergistic deviation was the best fit for the data. This study showed the importance of evaluating the mixture of toxic effects of metals mixtures to risk assessments in more realistic scenarios. The evident synergistic effects of these metals on the survival of aquatic organisms highlight the need for environmental policies adjusting guidelines for more effective protection of aquatic biota.

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Capítulo 3. Effects of cadmium and copper on the swimming behavior of *Rhinella diptycha* (Cope, 1862) tadpoles

Abstract

Amphibians, as part of the freshwater biota, are particularly sensitive to environmental pollution. The global decline in their diversity in recent decades is closely linked to the degradation of water bodies, primarily caused by chemical pollution. In the present work the toxicity of two common metals, copper and cadmium, was evaluated for an endemic native amphibian species. *Rhinella diptycha* tadpoles of variable stages between 25 to 30 stages. They were acclimated to controlled laboratory conditions and then used as test-organisms in acute toxicity tests with copper sulfate ($CuSO_4$) and cadmium chloride ($CdCl_2$), separately, to determine the effect of the metals on their survival. The results of the acute toxicity tests showed that copper sulfate is more toxic compared to cadmium chloride, with median lethal concentration (LC50) values of 0.49 mg L^{-1} for $CuSO_4$ and 1.09 mg L^{-1} for $CdCl_2$ after 96 hours of exposure. Subsequently, a behavioral analysis was conducted to evaluate swimming speed and distance with the tadpoles that survived the acute toxicity test. To analyze the distance traveled by the tadpoles during the essays and their respective speed, we analyzed the probability distribution of the distances traveled at each timestamp and the speed changes for each group. This analysis revealed that the exposition of tadpoles to the salt metals reduced their mobility capacity.

Keywords: Cadmium Chloride, Copper Sulfate, Swimming Behavior, Tropical Tadpoles.

1. Introduction

The responses of amphibian tadpoles to stressors, such as chemical pollution in aquatic ecosystems, are essential for their development and survival. Many pollutants can alter their behavior, affecting their ability to feed, reproduce, or avoid predators (Araújo et al., 2014; Moreira et al., 2019). These changes often have negative effects not only on individuals but also on their populations and communities (Sievers et al., 2019).

Amphibians are among the vulnerable taxa to environmental pollutants being one of the most threatened groups, worldwide (Luedtke et al., 2023; Stuart et al., 2004). Their permeable skin facilitates the exchange of gases, ions, and water directly from the environment, also allowing the entrance of chemical substances, found in water bodies and sediments (Girotto et al., 2020; Simoncelli et al., 2015). As a results in a range of adverse effects can be observed, since from mortality to sublethal effects such as morphophysiological and behavioral alterations (Egea-Serrano et al., 2012; Wang et al., 2022).

Metals are among the environmental pollutants that most affect amphibians due to their high toxicity, bioaccumulation capacity, and environmental persistence (Ali et al., 2019; Patar et al., 2016). Metal contamination, intensified by anthropogenic activities has increased significantly, making amphibians particularly vulnerable. For example, exposure to cadmium, a highly toxic metal, has been shown to cause high mortality and morphological deformities in *Polypedates maculatus* tadpoles (Ojha et al., 2021), alterations in gonad development in *Lithobates catesbeianus* (Abdalla et al., 2013), and negative effects on development, metamorphosis, erythrocyte abnormalities, and survival in *Microhyla fissipes* larvae (Hu et al., 2019). Besides, the presence of copper and other metals in aquatic ecosystems often has similar adverse effects, potentially compromising the health of animals, as well as their survival and swimming activity (Chen et al., 2007; Krupa et al., 2021; Wang et al., 2016).

Changes in swimming patterns, such as reduced speed, or the appearance of erratic movements, can indicate neurotoxicity or a general deterioration of tadpole health (Araújo et al., 2014; Chen et al., 2007), which affects their fitness and may increase mortality, contributing to population decline (Sievers et al., 2019). For example, exposure to environmental pollutants with neurotoxic potentials, such as agrochemicals (Faria et al., 2020; Giacomet et al., 2021), has been shown to cause alterations in swimming frequency (Almeida et al., 2019) and a decrease in swimming speed (Freitas et al., 2019; Moreira et al., 2019). Similar effects have also been observed with metal exposure (Motta et al., 2023). Chen et al. (2007) found that when exposing *Rana pipiens* tadpoles to environmentally relevant concentrations of copper, there were negative effects on larval survival, development, and swimming performance. This suggests that copper can disrupt the neuromuscular functions of tadpoles, as reflected in reduced swimming speed, increasing their susceptibility to predation (Faria et al., 2020).

In this context, the present study aimed to evaluate the effects of exposure to copper and cadmium on the survival and swimming behavior of *R. diptycha* tadpoles, a species widely distributed in South America. We hypothesized that exposure to these metals, increase mortality, but also induce significant decrease in swimming speed and distance, having direct or indirect impact on the survival and development of them.

2. Materials and methods

2.1. Test and Cultivation Conditions

Rhinella diptycha is a South American endemic species native to Brazil, Uruguay, Bolivia, Paraguay, and Argentina, commonly found in open and urban areas (Frost, 2024). For this study the tadpoles of *R. diptycha* were collected at the Experimental Aquaculture Station of Hydrobiology Department at the Federal University of São Carlos (UFSCar), São Paulo state, Brazil (21°58'54.95"S, 47°52'33.04"W). They were transported in 5-liter containers with water from the Station's tanks to the Limnology and Ecotoxicology Laboratory of the Department of Ecology and Evolutionary Biology at the UFSCar.

In the laboratory, tadpoles were acclimated and maintained in reconstituted water with a pH between 7.0 and 8.0, hardness of 40±2 mg $CaCO_3$ L^{-1}, and conductivity of 140±10 µS cm^{-1} (ABNT, 2016). We kept temperature at 25±1°C and established a 12-hour light and 12-hour dark cycle in aquariums under continuous aeration. The tadpoles were daily fed with commercial tropical fish food (Tetramin Flakes Tetra) and suspended feeding was interruped 24 hours before the experiments began. We collected the tadpoles under a permit from the Chico Mendes Institute for Biodiversity Conservation (ICMBio n°86058-1) and ensured that the maintenance and experimental procedures were previously approved by the Animal Ethics and Experimentation Committee of the Federal University of São Carlos (CEUA UFSCAR n°9592171022/ID 001757).

2.2. Acute Toxicity Tests

The metal salts used in the experiments were Copper Sulfate ($CuSO_4{\cdot}5H_2O$, CAS 7758-99-8, Dinâmica brand) and Cadmium Chloride ($CdCl_2{\cdot}2.5H_2O$, CAS 10108-64-2, Carlo Erba). These salts were used to prepare a stock solution with 1 g L^{-1} with distilled water. A subsequent solution with nominal concentration of 10 mg L^{-1} was employed to quantify the real concentration and to prepare all working solutions. The quantification was performed with a Perkin Elmer PinAAcle 900 T flame and a Zeeman longitudinal atomic absorption spectrophotometer, calibrated with cadmium and copper standards (Traces Metal Grade, Sigma-Aldrich) (Table S1). The real concentrations did not show variations exceeding 10% of the predicted concentrations. Therefore, following the guidelines established in ISO (2000), the nominal concentrations were kept.

Concentrations of 0.025, 0.05, 0.25, 0.50, 0.75, and 1 mg L^{-1} of $CuSO_4$ and 0.25, 0.50, 0.75, 1.00, 1.50, and 2,00 mg L^{-1} of $CdCl_2$ were applied, including a control group with reconstituted water only. The experiments were conducted with tadpoles in developmental stages 27-30 according to Gosner (1960). One-liter plastic containers, each with 500 mL of test solution and 3 tadpoles per replicate were used. Animal mortality was recorded daily for 96 hours, and the tadpoles exposed to test solutions were not fed during the experiments. The physical and chemical variables—pH (QUIMIS Q400AS), dissolved oxygen (HANNA HI9146), hardness (complexometric titration with EDTA), and electrical conductivity (DIGIMED DM3) of the test solutions were measured at the beginning and end of each toxicity test.

2.3. Effects on swimming behavior

To evaluate the swimming mobility of the tadpoles after acute exposure periods to the metals, the tadpoles were transferred and acclimated for 1 minute in a plastic tray with 400 mL of reconstituted water. Then, a stimulation was performed to activate movement (3 taps), and swimming activity was recorded for 1 minute using a digital camera placed above the plastic tray (210 x 140 x 55 mm). The data obtained included maximum speed (m/s) and distance traveled (cm) (Freitas et al., 2019; Girotto et al., 2020; Moreira et al., 2019).

2.4. Data Analysis

We calculated LC50 values using a non-linear regression based on a three-parameter logistic curve with a 95% confidence interval. Additionally, swimming activity data, such as distance traveled and swimming speed, were analyzed using Kinovea software v. 0.8.26 (Freitas et al., 2019; Girotto et al., 2020; Moreira et al., 2019). The distribution analysis was performed through histogram visualization of distance-travelings and velocity changes. We also conducted a data distribution analysis by computing and visualizing the histograms (50 bins) of the distances traveled at each timestamp (mm) and the respective velocity changes, for each replica. We apply the first-order derivative of the P cumulative recordings to compute the distance traveling and velocity changes. To assess the similarity between histograms, we performed Kolmogorov-Smirnov tests for each pair of histograms, evaluating their statistical significance through the corresponding p-values. Data analyses were performed using the Sigmaplot scientific software v. 12.0 (Systat, 2011) and the high-level and general-purpose programming language Python v 3.13.

3. Results

During the tests, the physical and chemical variables (pH, hardness, conductivity, dissolved oxygen, and temperature) of the reconstituted water of the tadpoles laboratory maintenance and the test solutions remained within the established range under the culture conditions (Table S2).

3.1. Acute Exposure: Determination of LC50

The median lethal concentration (LC50) values determined for *Rhinella diptycha* tadpoles were 0.49 mg L^{-1} for copper sulfate ($CuSO_4$) and 1.09 mg L^{-1} for cadmium chloride ($CdCl_2$) after a 96-hour exposure period. No mortality was observed at $CuSO_4$ concentrations of 0.25 and 0.5 mg L^{-1}. At a concentration of 0.75 mg L^{-1}, mortality was 11.11%, increasing to 44.44% at 1 mg L^{-1}, 77.77% at 1.5 mg L^{-1}, and reaching 100% at 2 mg L^{-1} (Figure 1A). For $CdCl_2$, no mortality was observed at concentrations of 0.025, 0.05, and 0.25 mg L^{-1}. At 0.5 mg L^{-1}, mortality was 66.66%, and 100% mortality was registered at concentrations of 0.75 and 1 mg L^{-1} (Figure 1B). No mortality was observed in the control group.

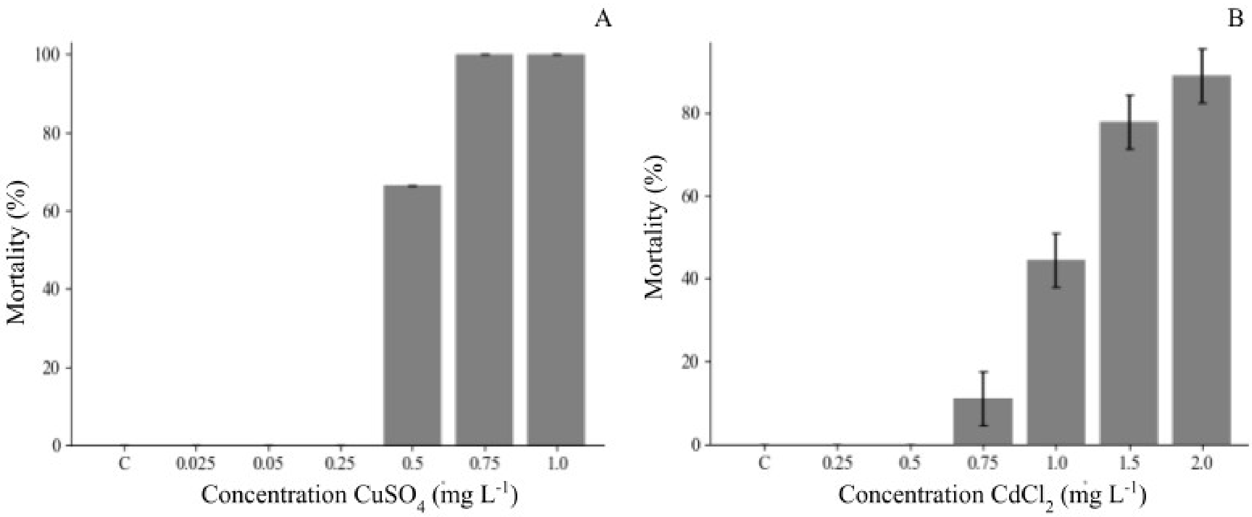

Figure 1. Mortality percentages of *Rhinella diptycha* (Cope, 1862) tadpoles exposed to different concentrations of the metals: (A) Copper Sulfate ($CuSO_4$) and (B) Cadmium Chloride ($CdCl_2$) at 96h. Standard Deviation (SD).

3.2. Effects on swimming behavior

The tadpoles in the control group traveled an average distance of 62.010 ± 31.69 cm, while those exposed to concentrations of 0.025, 0.5, 0.25, and 0.5 mg L^{-1} showed average distances of 31.268 ± 13.430 cm, 79.559 ± 39.750 cm, 43.572 ± 18.652 cm, and 33.061 ± 22.58 cm, respectively (Figure 2A; Table S3). The tadpoles in the control group presented an average speed of 0.254 ± 0.071 m/s. In comparison, the average speeds for the concentrations of 0.025, 0.5, 0.25, and 0.5 mg L^{-1} were 0.222 ± 0.134 m/s, 0.341 ± 0.195 m/s, 0.180 ± 0.065 m/s, and 0.292 ± 0.207 m/s, respectively (Figure 2B; Table S3).

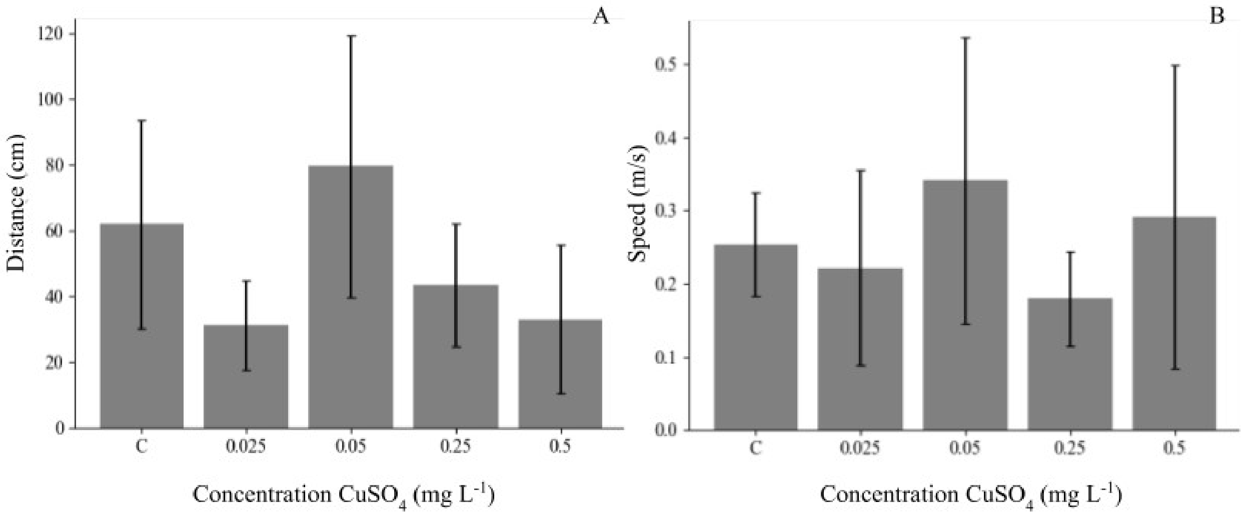

Figure 2. Swimming behavior of *Rhinella diptycha* (Cope, 1832) tadpoles exposed to different concentrations of Cooper Sulfate ($CuSO_4$): (A) Distance (cm) and (B) Speed (m/s). Standard Deviation (SD).

Tadpoles in the control group traveled an average distance of 58.888 ± 1.933 cm, while those exposed to concentrations of 0.25, 0.75, and 1 mg L^{-1} traveled average distances of 55.859 ± 33.766 cm, 34.456 ± 16.143 cm, 62.342 ± 38.687 cm, and 26.944 ± 2.401 cm, respectively. However, at concentrations of 1.5 and 2 mg L^{-1}, the distance traveled was recorded for only one individual, with values of 17.042 and 33.329 cm, respectively (Figure 3A; Table S3). The tadpoles in the control group had an average swimming speed of 0.407 ± 0.039 m/s. In comparison, the average speeds for concentrations of 0.25, 0.5, 0.75, and 1 mg L^{-1} were 0.505 ± 0.45 m/s, 0.297 ± 0.168 m/s, 0.415 ± 0.289 m/s, and 0.541 ± 0.505 m/s, respectively. The speed values for a single individual at concentrations of 1.5 and 2 mg L^{-1} were 0.513 and 0.395 m/s, respectively (Figure 3B).

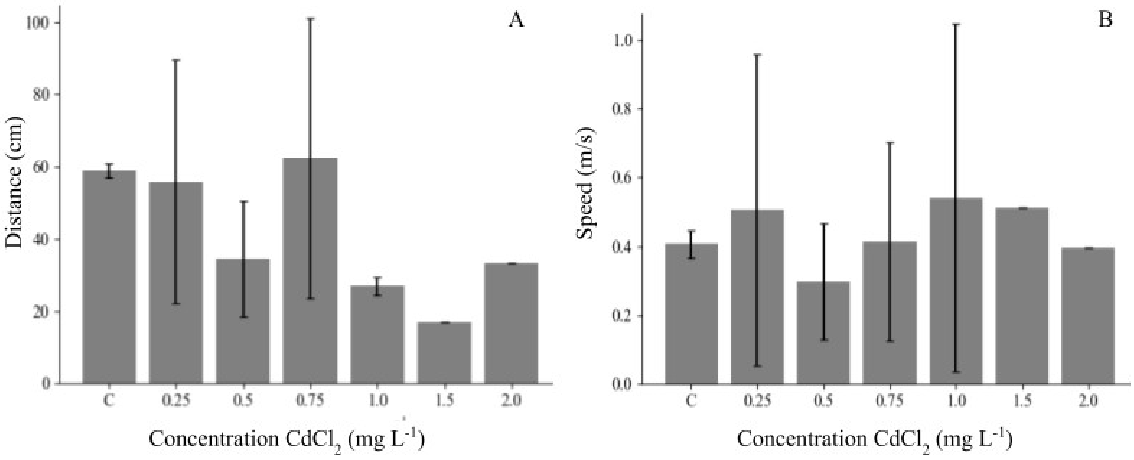

Figure 3. Swimming behavior of *Rhinella diptycha* (Cope, 1862) tadpoles exposed to different concentrations of Cadmium Chloride ($CdCl_2$): (A) Distance (cm) and (B) Speed (m/s). Standard Deviation (SD).

Through the histogram analysis, we aimed to discover the central tendency of the tadpoles' behavior during the essays. The histogram analysis of the distance traveled by the control groups revealed that most of the time the tadpoles moved very short distances (almost still), but they also were more active (traveled longer distances) when compared with the treatment groups, see Figures 4 and 6. This tendency can be observed in the histograms of control groups in which most of the counted distance-travelings are less than 0.1cm (close to zero) and a few greater than 0.1 cm. However, histograms of the treatment groups revealed that tadpoles presented a significant distance-traveling reduction indicating that the exposition to the metal salts affected their normal (group control) activity, one can see this tendency in the respective histograms where almost all the distances traveled by tadpoles were less than 0.1 cm for the treatment groups. We also observed that for both, control and treatment groups, the tadpoles performed some very long distance-travelings (abrupt movements), this is due to the stimulus we applied as part of the essays (three touch), this can be observed on the right tails of the histograms where longer distances are placed (Figure S1 and S2). The histogram analysis also visually revealed that contrary to expected, the tadpoles did not present a normally distributed distance-traveling behavior, in which they were expected to move more actively most of the time and remain still or move longer distances fewer times. Speed distributions were also analyzed (Figures 5 and 7), and the respective histograms confirmed the observations inferred from the distance distributions, in these histograms, we can see that the tadpoles presented few speed changes (positive or negative) and most of the time they remained almost still (Figure S3 and S4).

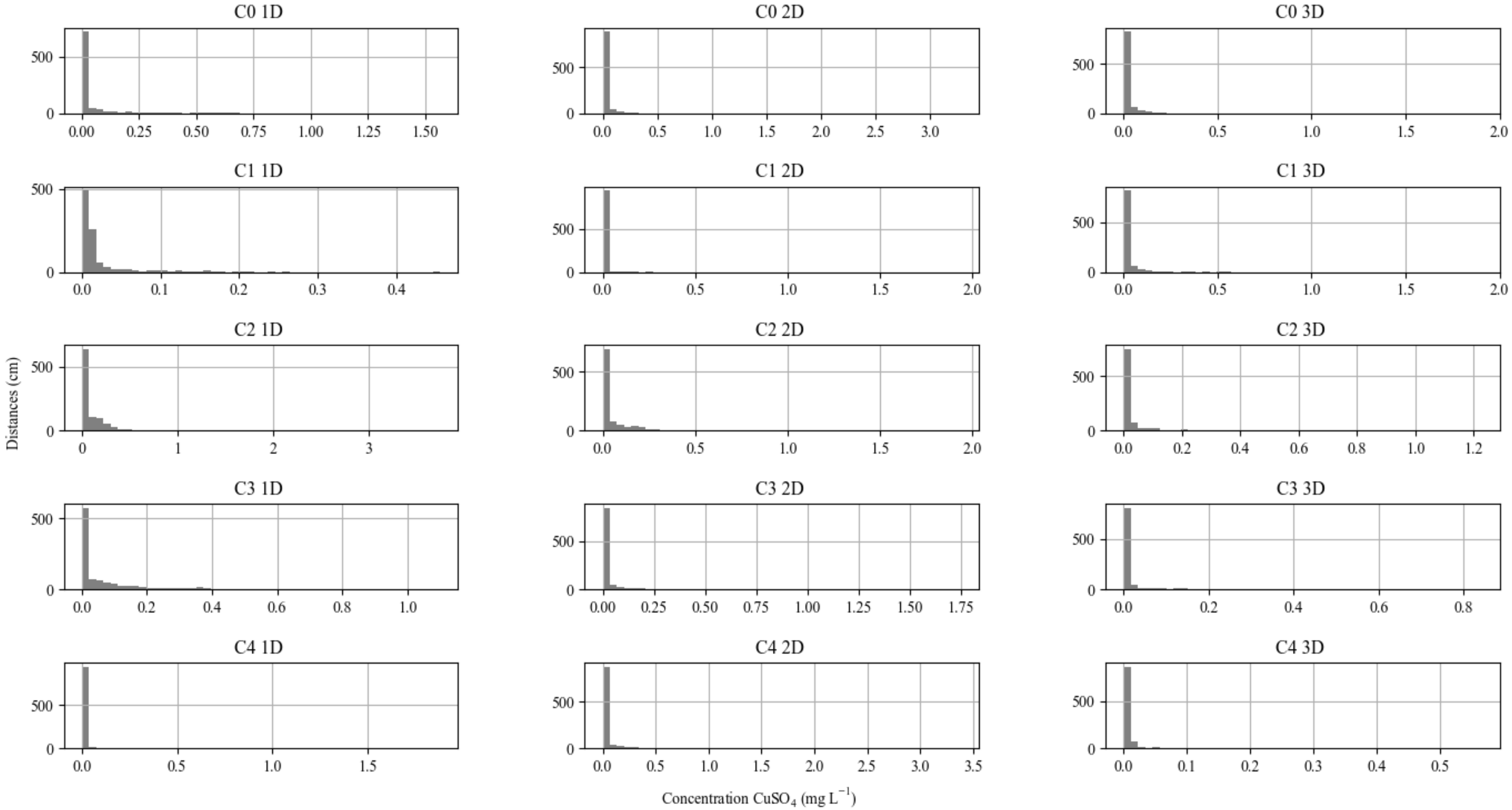

Figure 4. Histograms of Distance traveled at each timestamp (cm) by *Rhinella diptycha* (Cope, 1862) tadpoles previously exposed during 96h to concentration of Copper Sulfate ($CuSO_4$)

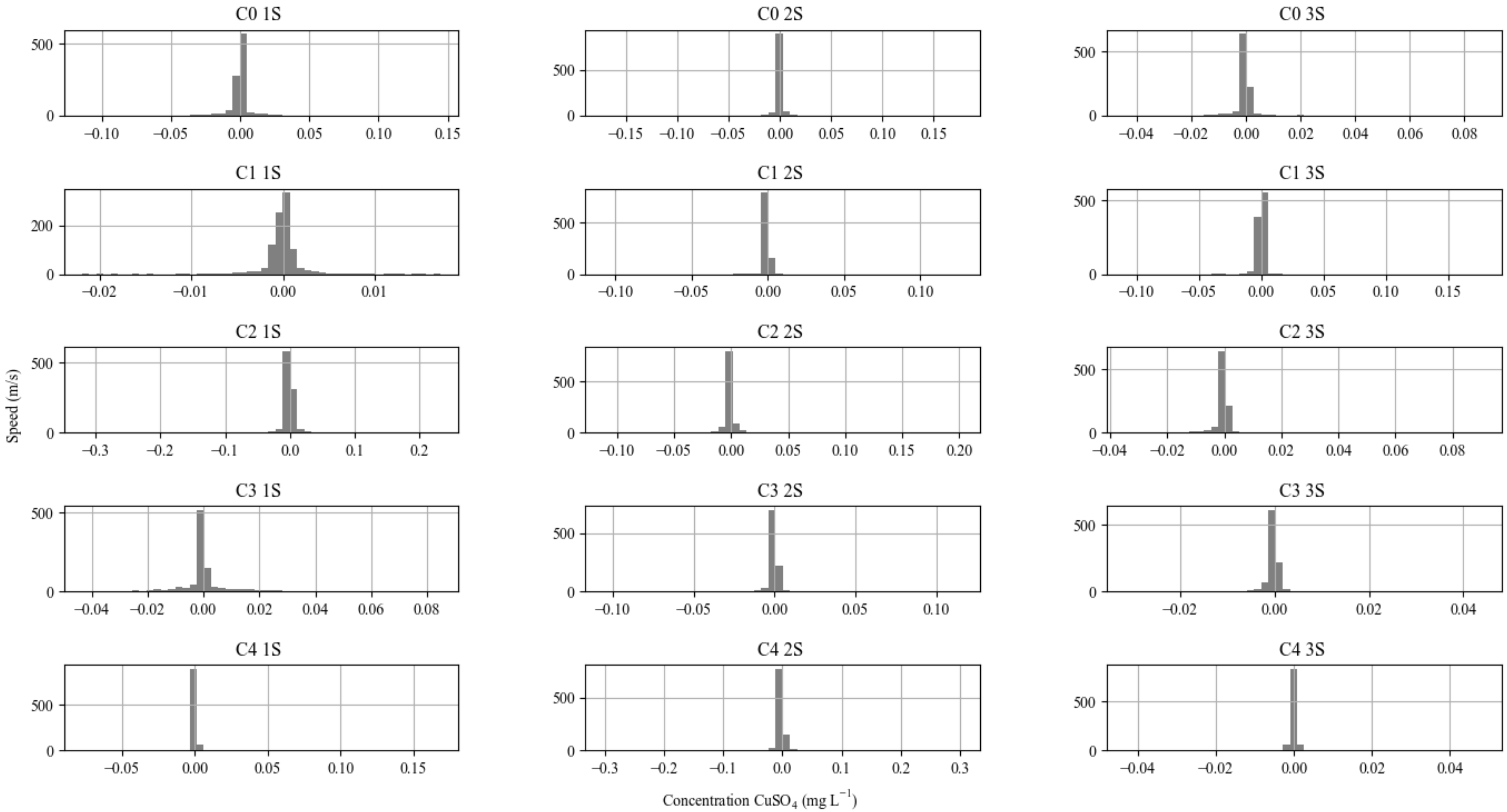

Figure 5. Histograms of Speed changes (s/m) by *Rhinella diptycha* (Cope, 1862) tadpoles previously exposed during 96h to concentration of Copper Sulfate ($CuSO_4$)

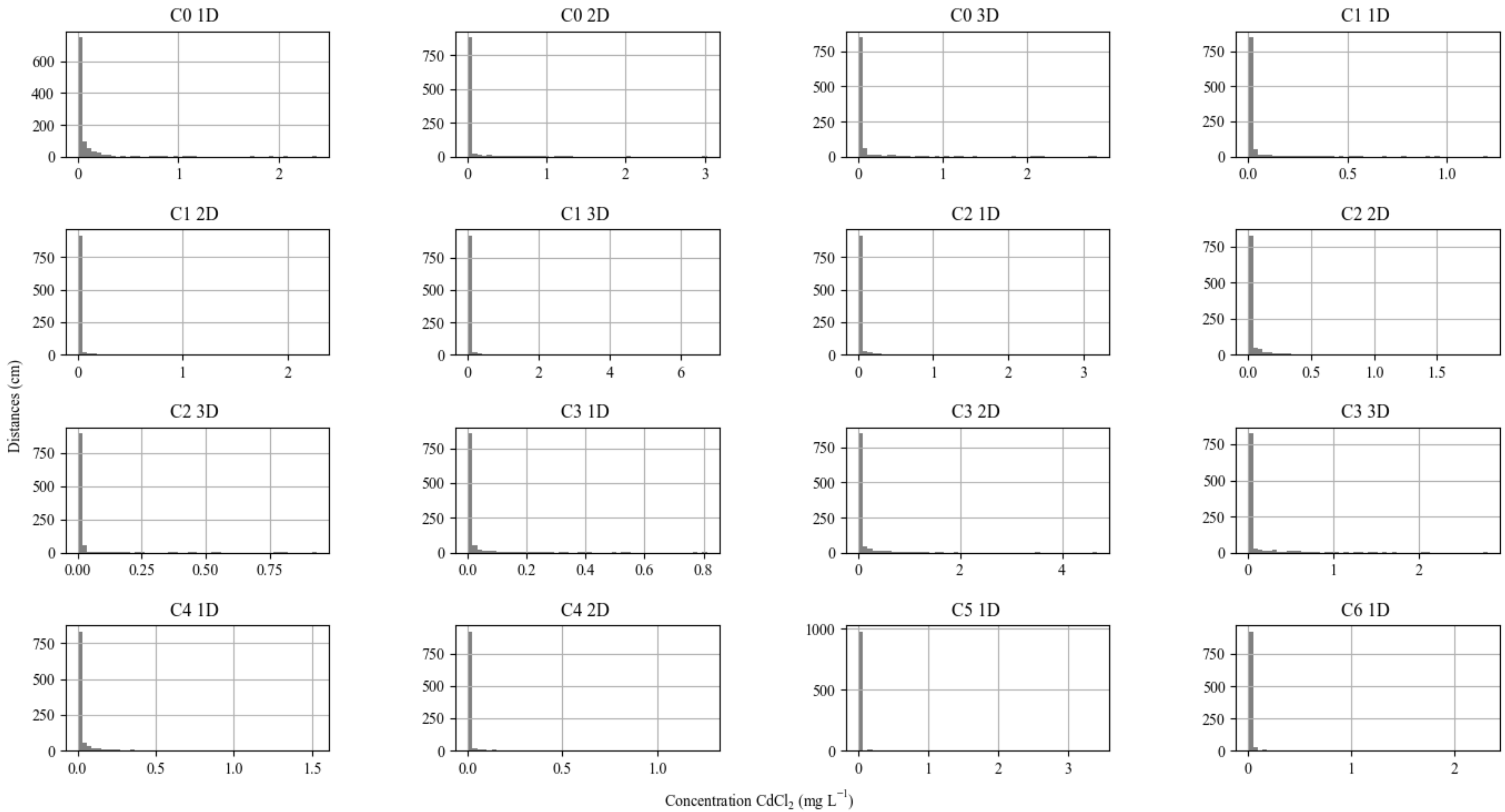

Figure 6. Histograms of Distance traveled at each timestamp (cm) by *Rhinella diptycha* (Cope, 1862) tadpoles previously exposed during 96h to concentration of Cadmium Chloride ($CdCl_2$).

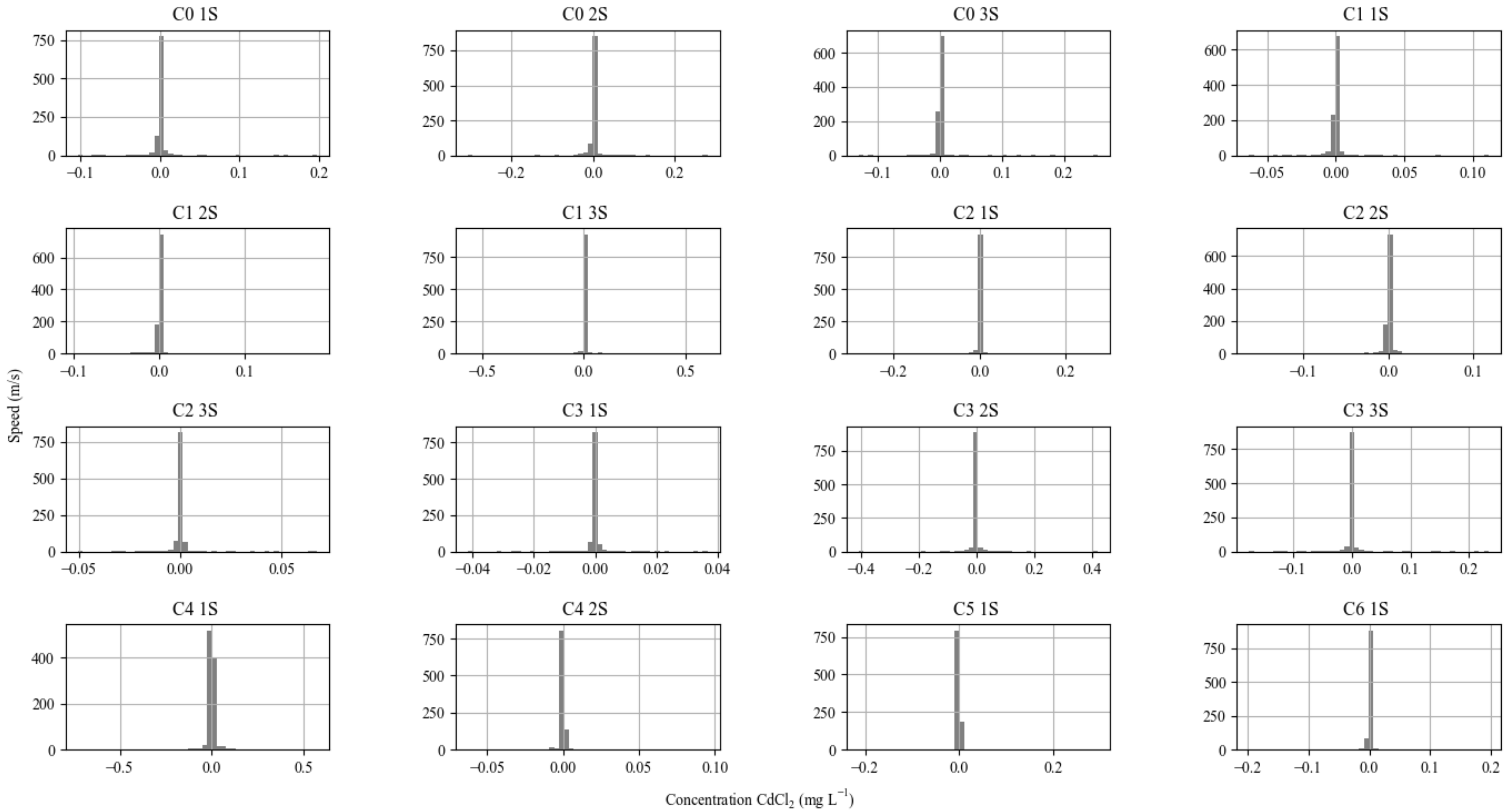

Figure 7. Histograms of Speed changes (s/m) by *Rhinella diptycha* (Cope, 1862) tadpoles previously exposed during 96h to concentration Cadmium Chloride ($CdCl_2$)

We observed that for the higher concentration (cadmium and copper), the tadpoles tended to reduce their mobility and remain more still. Even after the touch stimuli, exposed tadpoles moved slower than the control groups.

4. Discussion

The median lethal concentration values obtained for *Rhinella diptycha* tadpoles indicate that copper sulfate (LC50: 0.49 mg L^{-1} $CuSO_4$) is 2.22 times more toxic than cadmium chloride (LC 50: 1.09 $CdCl_2$) after 96 hours of exposure. For both metal salts, tadpole mortality increased as concentrations increased, reaching 100% at the highest concentrations, suggesting a dose-dependent response to the toxicity of $CuSO_4$ and $CdCl_2$. These results are consistent with a similar study conducted by Andrade (2020) where LC50 values for the native tropical species *Boana faber* and *Scinax fuscovarius* of 0.8 mg L^{-1} and 0.27 mg L^{-1} $CuSO_4$, and LC50 values of 2.46 mg L^{-1} and 2.79 mg L^{-1} $CdCl_2$, were reported respectively. Calfee and Little (2017) reported LC50-96h values of Cu (0.22 mg L^{-1}) and Cd (13.8 mg L^{-1}) for *Lithobates chiricahuensis* tadpoles. According to the LC50 values, copper was more toxic than cadmium to the aforementioned species. In addition, we recorded LC50 values of 0.22 mg L^{-1} $CuSO_4$ and 4.03 mg L^{-1} $CdCl_2$ for *Physalaemus nattereri* tadpoles in a previous study (Calpa-Anaguano et al., 2024).

The tadpoles exposed to metals exhibited a tendency to: remain motionless, erratic swimming, a slower response to tactile stimuli, irregular tail movements and unbalanced body posture indicating that their behavior was significantly affected. To statistically confirm those observations, we conducted a distribution analysis of the distance traveled and speed changes at each timestamp (mm), we visually analyzed the corresponding histograms and noticed that most occurrences were close to zero (distance and speed) which indicates that the tadpoles remain almost still during the experiments for control and treatment groups.

The histograms also revealed that this tendency is more noticeable for higher concentrations, i.e., fewer occurrences distant than zero were recorded as the concentrations increased. This distribution analysis is consistent with our observation that the tadpoles presented a change in their expected behavior. Besides, the histogram analysis showed that copper is the metal that most affected the tadpoles' behavior, as reported in the study conducted by Chen et al. (2007) where they found that exposure to copper led to a reduction in swimming speed in *Rana pipiens* tadpoles, suggesting that copper may have adverse effects on the neuromuscular functions of tadpoles.

In this scenario, several analogous studies were conducted, among the most notable are the work conducted by Simonato et al. (2016) that analyzed the effects of copper exposure on *Prochilodus lineatus*, where they observed changes in swimming parameters (decrease in swimming distance and time) associated with the inhibition of muscle AChE. Lefcort et al. (1998) studied *Rana luteiventris* and the subtle effects of metals such as cadmium, the authors conclude that heavy metals can alter the expected interactions between tadpoles and their predators. In the same line, Lu et al. (2021) demonstrated that cadmium causes a decrease in swimming speed and jumping distance in *Rana zhenhaiensis* tadpoles. However, the interaction between Cd and pH affected the jumping distance only. Girotto et al. (2020) related a reduction in the swimming ability of *Lithobates catesbeianus* tadpoles when exposed to mining waste from the collapse of the Fundão Dam. Through these works and our results, we can suggest that Copper and Cadmium are neurotoxic pollutants that cause behavioral changes and trigger indirect effects that influence the swimming ability of tadpoles.

The direct consequence of our discoveries becomes evident by analyzing the natural aquatic ecosystem in Brazil (Table 1), where the tadpoles subjects of our study are native species. We stress that an important finding in our work is related to the LC50 values in which we computed 0.49 mg L^{-1} for $CuSO_4$ and 1.09 mg L^{-1} for $CdCl_2$, which are values seriously below those registered in some of their natural habitats.

Locations worthy of attention are the Doce river (MG), where a copper concentration of 0.39 mg L^{-1} has been found (Gomes et al., 2018). In the Gualaxo do Norte and Carmo rivers, copper reached 0.35 mg L^{-1}, and cadmium 0.109 mg L^{-1} (Santana et al., 2021), while water bodies in urban and peri-urban areas of the municipality of Uberlândia (MG) the concentrations of copper and cadmium recorded in sediment ranged from 40.33 to 61.67 mg L^{-1} and 0.001 to 0.010 mg L^{-1}, respectively. Additionally, cadmium concentrations in water ranged from 0.042 to 0.110 mg L^{-1} (Caixeta et al., 2022). These recorded concentrations are much higher than those considered environmentally safe by the Brazilian National Environmental Council (CONAMA) — 0.001 mg L^{-1} for copper and 0.009 mg L^{-1} for cadmium.

Therefore, the concentrations found in these locations exceeded the LC50 values for several native amphibians such as *B. faber*, *S. fuscovarius*, *P. nattereri*, and *R. diptycha* (the subject of our study), indicating a strong negative impact on their survival. Besides, based on our findings, we can infer that changes observed in the natural activity of *R. diptycha*, mainly their capacity to avoid predators and to gather food, are also observed in the other native amphibians. Furthermore, we observed that all the mentioned impacts become more evident in early stages of *R. diptycha*, which can significantly reduce their population size by hindering many individuals from reaching metamorphosis and progressing to the reproductive adult stage.

Table 1. Limit concentrations of Copper (Cu) and Cadmium (Cd) established by CONAMA resolution (2005) and literature reported concentrations (mg L^{-1}) for several Brazilian water bodies.

Metal			
Copper (mg L^{-1})	Cadmium (mg L^{-1})	Study area	Citation
0.001	0.009	All Brazilian localities	CONAMA, 2005
0.004 - 0.085	0.001 - 0.005	São Carlos - SP	Chiba et al., 2011
0.016 - 0.244	0.001 - 0.0031	Americana - SP	Medeiros et al., 2013
0.002	0.004 - 0.009	Pirajibú River – SP	Silva, 2022
-	0.004	Sorocaba River - SP	Castro, 2002
-	0.006	Dourado River - SP	Figueiredo, 2018
0.00156	-	Gualaxo do Norte River - MG	Hatje et al., 2017
0.0039	-	Doce River – MG	Gomes et al., 2018
0.0012	0.0003 - 0.0036	Turvo Sujo River - MG	Barros et al., 2009
0.014	0.015	São Francisco River Basin, Petrolina – PE e Juazeiro – BA	Souza et al., 2016
0.359	0.109	Gualaxo do Norte and Carmo River - MG	Santana et al., 2021
0.00046 - 0.00488	0.00041	Wetland in the upper Rio Claro Basin - MG	Luko-Sulato et al. 2021
-	0.042-0.110	High-anthropogenic urban area Uberlândia - MG	Caixeta et al. 2022
0.0146	0.0033	Pardo River -SP	Machado et al. 2016
0.62	7.66	Amazonas River Basin	Gomes et al. 2023
0.007	0.0003	Atibaia River - SP	Rupias et al. 2021
0.0055-0.0121	0.0009	Sinos River -RS	Weber et al. 2013
-	0.001-0.013	Rio Jundiaí River - RN	Guedes et al. 2005
0.0033	0.00003	Monte Alegre River – SP	Alves et al. 2010
0.001-0.014	-	Tapacurá River - PE	Aprile and Bouvy, 2003
0.13 – 0.28	-	Conceição River - MG	Oliveira et al. 2023
0.04	-	Gualaxo do Norte River, Paracatu District - MG	Gimenes et al. 2020
-	0.007	Stock solution containing mining tailings from Fundão - MG	Girotto et al. 2020
0.0103 – 0.0105	0.0075 - 0.0080	Ivinhema River, Upper Paraná River basin - MS	Santos et al. 2020
0.001- 0.009	0.008 – 0.009	Miranda River - MS	Sampaio 2003
0.0005 – 0.0014	0.00006 - 0.00016	João Leite River - GO	Silva et al. 2021

5. Conclusion

The LC50 values obtained for *R. diptycha* tadpoles showed that copper sulfate ($CuSO_4$) was more toxic than cadmium chloride ($CdCl_2$), with mortality increasing in a dose-dependent manner for both metals. The probability distribution analysis revealed that tadpoles exposed to the highest concentrations of $CuSO_4$ and $CdCl_2$ exhibited behavioral alterations. Additionally, some effects of the metals on the tadpoles were observed, including immobility, erratic swimming, and slower responses to tactile stimuli. These changes suggest that exposure to these metals has a severe impact on locomotor behavior and body posture of the tadpoles, although these effects were always evident in all statistical analyses. Furthermore, the metal concentrations found in several Brazilian rivers significantly exceed the LC50 values for *R. diptycha* and other native amphibians. This indicates a severe negative impact on the survival and health of tadpole populations, with potential adverse effects on their behavior and well-being. The concentrations of copper and cadmium in these aquatic ecosystems exceed the limits considered safe by CONAMA, pointing out the urgent need for more stringent monitoring and regulation of metal levels in aquatic habitats in order to protect amphibians and other sensitive groups.

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Conclusões Gerais da Tese

Com este estudo, as seguintes conclusões gerais puderam ser obtidas

- Os metais Cobre e Cádmio causam toxicidade nos girinos de *Physalaemus nattereri* e *Rhinella diptycha* em concentrações ambientalmente relevantes. Os resultados referentes ao cobre, com valores de LC50 menores, em comparação com o cádmio, indicam que para essas especies nativas o cobre é mais tóxico do que o cádmio.
- Os metais cobre e cádmio, após 4 e 54 dias de exposição respectivamente, causam mortalidade aos girinos de *Physalaemus nattereri.* No entanto, não foram observados efeitos no crescimento e desenvolvimento dos mesmos.
- Os resultados evidenciaram que diferentes espécies de anfíbios exibem sensibilidades específicas a diferentes contaminantes, o que indica a importância da inclusão de mais espécies nativas em estudos futuros sobre os contaminantes químicos em regiões neotropicais.
- A análise preliminar de risco ecológico realizada para cobre e cádmio em diversos rios e córregos na área de distribuição de *Physalaemus nattereri* revelou que os valores de RQ são significativamente elevados. Isso sugere que mesmo concentrações baixas desses metais representam uma ameaça substancial à sobrevivência dos girinos em seu habitat natural.
- A combinação dos sais metálicos gerou uma mistura altamente tóxica, causando sinergismo e representando um risco significativo para a sobrevivência dos girinos de *Physalaemus nattereri*. O modelo que melhor ajustou os dados foi o da Adição de Concentração.
- Nas maiores concentrações testadas para os sais metálicos $CuSO_4$ e $CdCl_2$, foram observadas alterações no comportamento dos girinos de *Rhinella diptycha,* como nado errático, imobilidade, e respostas lentas a estímulos táteis na avaliação do desempenho de natação.

Considerações Finais

Os resultados deste estudo evidenciam que os girinos nativos expostos a concentrações de metais, como cobre e cádmio, apresentam alterações que incluem mudanças na sobrevivência e comportamento, especialmente pronunciadas nas primeiras etapas de vida. Estas alterações podem ter efeitos prejudiciais a longo prazo nas populações de anfíbios nativos, dificultando a sobrevivência, o processo de metamorfose e com redução da capacidade de atingir a fase reprodutiva.

Além disso, os resultados evidenciam que em diversos ecossistemas aquáticos do Brasil, são registradas concentrações elevadas de cobre e cádmio representando elevado risco para a fauna nativa, ressaltando a importância de que sejam adotadas medidas para mitigação dos efeitos nocivos causados.

A implementação de regulamentações mais rígidas para o manejo de resíduos industriais e para práticas agrícolas em áreas próximas aos corpos d’água é de extrema urgência. É ainda necessário ampliar o monitoramento ambiental, especialmente em zonas vulneráveis à contaminação por metais, visando prevenir impactos ecológicos graves.

Devem ser implementadas políticas de conservação e restauração de habitats aquáticos, juntamente com processos efetivos de educação ambiental e com desenvolvimento de tecnologias limpas nas indústrias. Somente por meio de uma abordagem integrada que envolva a cooperação entre governos, indústrias e comunidades será possível mitigar os efeitos da poluição por metais e proteger a biodiversidade dos ecossistemas aquáticos.

Acute and chronic toxicity of cadmium and copper on tadpoles of *Physalaemus nattereri* (Steindachner, 1893), a Neotropical native species

Table S1. Quantification of metal concentrations in the stock solutions used in the toxicity assays carried out with the *Physalaemus nattereri* (Steindachner, 1893) tadpoles.

Compounds	Nominal concentration (mg L^{-1})	Measured concentration (mg L^{-1})	Accuracy concentration (mg L^{-1})	Detection limit (mg L^{-1})	Quantification limit (mg L^{-1})
Copper sulfate ($CuSO_4$-$5H_2O$)	10	10.3	103.2±0.9	25	1.3
Cadmium-chloride ($CdCl_2$ 2.5 H_2O)	10	9.1	91.1±0.9	0.01	0.5

Table S2. Initial and final physical and chemical variables of the solutions with copper and cadmium in the assays with *Physalaemus nattereri* (Steindachner, 1893) tadpoles.

Compounds	Variables	Initial	Final
Copper sulfate ($CuSO_4$-$5H_2O$)	pH	7.41 ± 0.45	7.72± 0.40
	Electric conductivity (µs/cm)	137.54 ± 2.20	135.62± 4.58
	Dissolved Oxygen (mg L^{-1})	6.62 ± 1.46	6.85± 1.32
	Temperature (C°)	25.43 ± 0.32	25.55 ± 0.27
	Hardness ($CaCO_3$)	40± 0.26	-
Cadmium-chloride ($CdCl_2$ 2.5 H_2O)	pH	7.41 ± 0.45	7.72± 0.40
	Electric conductivity (µs/cm)	139.26 ± 3.38	138.42± 2.48
	Dissolved Oxygen (mg L^{-1})	6.22 ± 1.56	6.65± 1.42
	Temperature (C°)	25.73 ± 0.82	25.25 ± 0.77
	Hardness ($CaCO_3$)	40± 0.52	-

Table S3. Lethal Concentrations (LC50) data for Copper and Cadmium toxicity used for the construction of SSD curves for different amphibian species.

Compound	Species	Developmental stage	LC50 (mg L^{-1})	Test Duration (days)	Distribution	Conservation Status	References
$CuSO_4$ - $5H_2O$	*Ambystoma opacum*	Larva, Harrison 46	0.187	4	North America	LC	Weir et al. 2019
$CuSO_4$ - $5H_2O$	*Ambystoma tigrinum*	Larva, Harrison 46	0.350	4	North America	LC	Weir et al. 2019
$CuSO_4$	*Bufo boreas syn. Anaxyrus boreas*	NA	0.120	4	North America	LC	Dwyer et al., 2005
$CuSO_4$ - $5H_2O$	*Bufo bufo*	Gosner 25	0.960	4	Europe and Asia	LC	García-Muñoz et al., 2011
$CuSO_4$ - $5H_2O$	*Bufo gargarizans*	Gosner 29	0.552	4	Asia	LC	Chai et al., 2014
$CuSO_4$ - $5H_2O$	*Duttaphrynus melanostictus syn. Bufo melanostictus*	Gosner 25	0.30	4	Asia	LC	Shuhaimi-Othman et al., 2012
$CuSO_4$ - $5H_2O$	*Duttaphrynus melanostictus syn. Bufo melanostictus*	NA	0.28	4	Asia	LC	Shuhaimi-Othman et al., 2013
$CuSO_4$ - $5H_2O$	*Duttaphrynus melanostictus syn. Bufo melanostictus*	Gosner 25	0.72	4	Asia	LC	Arambawatta-Lekamge et al., 2021
$CuSO_4$ - $5H_2O$	*Duttaphrynus melanostictus syn. Bufo melanostictus*	NA	0.320	4	Asia	LC	Khangarot and Pay, 1987
$CuSO_4$ - $5H_2O$	*Epidalea calamita*	Gosner 25	0.113	4	Europe	LC	García-Muñoz et al., 2011
$CuSO_4$ - $5H_2O$	*Epidalea calamita*	Gosner 25	0.110	4	Europe	LC	García-Muñoz et al., 2009
$CuSO_4$ - $5H_2O$	*Epidalea calamita*	Gosner 19	0.80	4	Europe	LC	García-Muñoz et al., 2009
$CuSO_4$ - $5H_2O$	*Hyla chrysoscelis syn. Dryophytes chrysoscelis*	Gosner 25	0.45	4	North America	LC	Brown et al., 2012
$CuSO_4$ - $5H_2O$	*Lithobates catesbeianus syn. Aquarana catesbeiana*	Gosner 25-26	3.960	4	North America	LC	Ossana et al., 2010
$CuSO_4$ - $5H_2O$	*Lithobates chiricahuensis*	Gosner 25	0.220	4	North America	VU	Calfee and Little 2017
$CuSO_4$ - $5H_2O$	*Lithobates chiricahuensis*	Gosner 28-31	0.340	4	North America	VU	Calfee and Little, 2017
$CuSO_4$ - $5H_2O$	*Pelodytes ibericus*	Gosner 25	0.125	4	Europe	LC	García-Muñoz et al., 2011
$CuSO_4$ - $5H_2O$	*Pelophylax perezi*	Gosner 25	0.970	4	Europe	LC	Santos et al., 2013
$CuSO_4$ - $5H_2O$	*Rana clamitans syn. Aquarana clamitans*	Gosner 25	0.163	4	North America	LC	Brown et al., 2012
$CuSO_4$	*Rana sphenocephala syn. Lithobates sphenocephalus*	Gosner 25	0.230	4	North America	LC	Bridges et al., 2002
$CuSO_4$ - $5H_2O$	*Physalaemus nattereri*	Gosner 25-26	0.220	4	South America	LC	This study
$CdCl_2$	*Ambystoma gracile*	Larva-3 months	0.468	4	North America	LC	Nebeker et al., 1995
$CdCl_2 \cdot 2\ 1/2H_2O$	*Duttaphrynus melanostictus*	Gosner 25	0.320	4	Asia	LC	Shuhaimi-Othman et al., 2013
$CdCl_2 \cdot 2\ 1/2H_2O$	*Duttaphrynus melanostictus*	Gosner 25	0.300	4	Asia	LC	Shuhaimi-Othman et al., 2012
$CdCl_2 \cdot 2H_2O$	*Duttaphrynus melanostictus*	Gosner 25	0.431	4	Asia	LC	Arambawatta-Lekamge et al., 2021
$CdCl_2 \cdot 2.5H_2O$	*Lithobates chiricahuensis*	Gosner 25	13.800	4	North America	VU	Calfee and Little, 2017
$CdCl_2$	*Microhyla ornata*	1 week	1.580	4	Asia	LC	Rao et al., 1987
$CdCl_2$	*Microhyla ornata*	4 weeks	1.810	4	Asia	LC	Rao et al., 1987
$CdCl_2$	*Rana ridibunda syn. Pelophylax ridibundus*	NA	71.800	4	Europe and Asia	LC	Loumbourdis et al., 1999

$CdCl_2$	*Pseudepidalea variabilis syn Bufotes viridis*	Gosner 26	0.035	4	Asia	LC	Gürkan et al., 2014
$CdCl_2$	*Rana limnocharis*	Gosner 26-28	0.690	4	Asia	LC	Patar et al., 2016
$CdCl_2$	*Rhinella arenarum*	-	2.080	4	South America	LC	Muino et al.,1990
$CdCl_2 \cdot 2.5H_2O$	*Rhinella arenarum*	Gosner 26	1.090	4	South America	LC	Mastrangelo et al., 2011
$CdCl_2$	*Rhinella arenarum*	Gosner 26	2.190	4	South America	LC	Ferrari et al., 1993
$CdCl_2$	*Rhinella arenarum*	Gosner 26	2.650	4	South America	LC	Ferrari et al., 1993
$CdCl_2$	*Rhinella arenarum*	Gosner 28-29	3.060	4	South America	LC	Ferrari et al., 1993
$CdCl_2$	*Rhinella arenarum*	Gosner 28-29	6.770	4	South America	LC	Ferrari et al., 1993
$CdCl_2 \cdot 2.5H_2O$	*Physalaemus nattereri*	Gosner 25-26	4.030	4	South America	LC	This study

LC - Least Concern

VU - Vulnerable

Table S4. Quantified concentrations of Cu and Cd in some water bodies in Brazil

Metal		Study area	Citation
Copper (mg L^{-1})	**Cadmium (mg L^{-1})**		
0.001	0.009	Brazilian localities	CONAMA, 2005
0.004 - 0.085	0.001 - 0.005	São Carlos - SP	Chiba et al. 2011
0.016 - 0.244	0.001 - 0.0031	Americana - SP	Medeiros et al. 2013
0.002	0.004 - 0.009	Pirajibú River – SP	Silva 2022
-	0.004	Sorocaba River - SP	Castro 2002
-	0.006	Dourado River - SP	Figueiredo 2018
0.00156	-	Gualaxo do Norte River - MG	Hatje et al. 2017
0.0039	-	Doce River - MG	Gomes et al. 2018
0.0012	0.0003 - 0.0036	Turvo Sujo River - MG	Barros et al. 2009
0.359	0.109	Gualaxo do Norte and Carmo River - MG	Santana et al. 2021
0.00046 - 0.00488	0.00041	Wetland in the upper Rio Claro Basin - MG	Luko-Sulato et al. 2021
-	0.042-0.110	High-anthropogenic urban area Uberlândia - MG	Caixeta et al. 2022
0.13 – 0.28	-	Conceição River - MG	Oliveira et al. 2023
0.04	-	Gualaxo do Norte River, Paracatu District - MG	Gimenes et al. 2020
-	0.007	Stock solution containing mining tailings from Fundão - MG	Girotto et al. 2020
0.0103 – 0.0105	0.0075 - 0.0080	Ivinhema River, Upper Paraná River basin - MS	Santos et al. 2020
0.001- 0.009	0.008 – 0.009	Miranda River - MS	Sampaio 2003
0.0005 – 0.0014	0.00006 - 0.00016	João Leite river - GO	Santos-Silva et al. 2021

Table S5. Mean values of cumulative mortality during the 54-days exposure of *P. nattereri* tadpoles to Cu and Cd.

Metal concentration (mg L^{-1})	Percentage of mortality							
	Weeks							
	1	2	3	4	5	6	7	8
Cooper								
Control	0	0	0	0	0	0	0	0
0.000025	0	0	0	0	0	0	0	0
0.00025	0	0	0	0	0	0	0	0
0.0025	0	0	0	0	0	0	0	0
0.025	0	0	0	0	0	0	0	0
Cadmium								
Control	0	0	0	0	0	0	0	0
0.0004	0	0	0	0	0	0	0	0
0.004	0	0	0	0	0	0	0	0
0.04	0	0	22.22	33.33	33.33	33.33	33.33	33.33
0.4	0	33.33	77.77	88.88	88.88	88.88	88.88	88.88

Table S6. Mean values and Standard Deviation (SD) of SVL, total length, weight, and development stage of *Physalaemus nattereri* (Steindachner, 1893) tadpoles at the beginning of the experiment and after 54 days of exposure to Cu and Cd.

Metal concentration (mg L^{-1})	Tadpole growth parameters			
	SVL (mm)	Total Length (mm)	Weight (g)	Gosner Stage
Dados initial	4.91 ± 0.27	13.38 ± 0.68	0.03472 ± 0.0032	26
Cooper				
Control	11.3 ± 0.91	31.52 ± 2.18	0.29 ± 0.065	39 - 41
0.000025	11.13 ± 0.53	32.88 ± 1.41	0.30 ± 0.054	39 - 40
0.00025	10.98 ± 0.48	31.39 ± 1.45	0.27 ± 0.040	39 - 40
0.0025	11.01 ± 0.61	31.64 ± 1.22	0.26 ± 0.028	39 - 40
0.025	11.16 ± 0.97	31.68 ± 2.09	0.29 ± 0.058	39 - 40
Cadmium				
Control	11.61 ± 1.31	31.70 ± 2.60	0.26 ± 0.072	39 - 41
0.0004	11.60 ± 0.56	33.18 ± 2.09	0.29 ± 0.042	40
0.004	11.10 ± 0.79	31.92 ± 1.42	0.27 ± 0.047	39 - 40
0.04	11.07± 0.91	30.87 ± 2.10	0.27 ± 0.065	39 - 40
0.4	11.71	33.66	0.33	40

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Effects of cadmium and copper metals isolated and in combined action over *Physalaemus nattereri* (Steindachner, 1893) tadpoles

Table S1. Quantification of metal concentrations in the stock solutions used in the toxicity assays carried out with the *Physalaemus nattereri* (Steindachner, 1893) tadpoles.

Compounds	Nominal concentration (mg L^{-1})	Measured concentration (mg L^{-1})	Accuracy concentration (mg L^{-1})	Detection limit (mg L^{-1})	Quantification limit (mg L^{-1})
Copper sulfate ($CuSO_4$-$5H_2O$)	10	10.3	103.2±0.9	25	1.3
Cadmium-chloride ($CdCl_2$ 2.5 H_2O)	10	9.1	91.1±0.9	0.01	0.5

Table S2. Initial and final physical and chemical variables of the solutions with copper sulfate and cadmium chloride and mixture in the assays with *Physalaemus nattereri* (Steindachner, 1893) tadpoles.

Metals	Variables	Initial	Final
Copper sulfate ($CuSO_4$-$5H_2O$)	pH	7.46± 0.35	7.55± 0.49
	Electric conductivity (µs/cm)	139.04 ± 5.20	144.94 ± 4.58
	Dissolved Oxygen (mg L^{-1})	6.95 ± 0.58	7.07 ± 2.50
	Temperature (C°)	25.75 ± 0.29	25.47 ± 0.36
	Hardness ($CaCO_3$)	42 ± 0.70	42 ± 0.93
Cadmium-chloride ($CdCl_2$ 2.5 H_2O)	pH	7.50 ± 0.35	7.63 ± 0.51
	Electric conductivity (µs/cm)	138.10 ± 3.42	139.19 ± 2.97
	Dissolved Oxygen (mg L^{-1})	7.07 ± 0.67	7.07 ± 0.67
	Temperature (C°)	25.69 ± 0.17	25.61 ± 0.38
	Hardness ($CaCO_3$)	44 ± 0.86	42 ± 1.09
Copper sulfate ($CuSO_4$-$5H_2O$) + Cadmium-chloride ($CdCl_2$ 2.5 H_2O)	pH	7.57 ± 0.33	7.60± 0.40
	Electric conductivity (µs/cm)	150.33 ± 2.89	135.77 ± 3.43
	Dissolved Oxygen (mg L^{-1})	6.62 ± 1.46	6.85± 1.32
	Temperature (C°)	25.43 ± 0.32	25.55 ± 0.27
	Hardness ($CaCO_3$)	40 ± 1.12	-

Table S3. Mortality in acute toxicity tests with *Physalaemus nattereri* (Steindachner, 1893) tadpoles exposed to cadmium chloride and copper sulfate, both individually and in combination, Toxic strengths andToxic units (TU)

Toxic strengths	Toxic units (TU)		Concentration (mg L^{-1})		Mortality
	Q1 (Cadmium)	Q1 (Copper)	Q1 (Cadmium)	Q1 (Copper)	96-h
Control			0	-	0
Q1			2.5	-	2
Q1			3	-	6
Q1			3.5	-	4
Q1			4	-	4
Q1			4.5	-	7
Q1			5	-	9
Q2			-	0.05	0
Q2			-	0.125	0
Q2			-	0.25	1
Q2			-	0.5	9
Q2			-	1	9
Q2			-	2	9
0.125	0.0625	0.0625	0.25	0.01	0
0.375	0.25	0.125	1.01	0.03	0
0.375	0.125	0.25	0.50	0.06	0
0.5	0.375	0.125	1.51	0.03	0
0.5	0.25	0.25	1.01	0.06	2
0.75	0.125	0.375	0.50	0.08	2
0.75	0.5	0.25	2.02	0.06	7
0.75	0.375	0.375	1.51	0.08	6
0.75	0.25	0.5	1.01	0.11	8
0.75	0.125	0.625	0.50	0.14	7
0.75	0.625	0.125	2.52	0.03	1
1	0.75	0.25	3.02	0.06	9
1	0.5	0.5	2.02	0.11	9
1	0.375	0.625	1.51	0.14	9
1	0.25	0.75	1.01	0.17	9
1	0.125	0.875	0.50	0.20	9
1	0.875	0.125	3.53	0.03	5
1	0.625	0.375	2.52	0.08	9
1.5	0.5	1	2.02	0.22	9
1.5	1	0.5	4.03	0.11	9
1.5	0.75	0.75	3.02	0.17	9
1.75	0.75	1	3.02	0.22	9
1.75	1	0.75	4.03	0.17	9
2	1	1	4.03	0.22	9

Effects of cadmium and copper on the swimming behavior of *Rhinella diptycha* (Cope, 1862) tadpoles

Table S1. Quantification of metal concentrations in the stock solutions used in the toxicity assays carried out with the *Rhinella diptycha* (Cope, 1862) tadpoles.

Compounds	Nominal concentration (mg L^{-1})	Measured concentration (mg L^{-1})	Accuracy concentration (mg L^{-1})	Detection limit (mg L^{-1})	Quantification limit (mg L^{-1})
Copper sulfate ($CuSO_4$-$5H_2O$)	10	10.3	103.2±0.9	25	1.3
Cadmium-chloride ($CdCl_2$ 2.5 H_2O)	10	9.1	91.1±0.9	0.01	0.5

Table S2. Initial and final physical and chemical variables of the solutions with copper sulfate and cadmium chloride in the assays with *Rhinella diptycha (Cope, 1862)* tadpoles.

Compounds	Variables	Initial	Final
Copper sulfate ($CuSO_4$-$5H_2O$)	pH	7.72± 0.40	7.81± 0.45
	Electric conductivity (µs/cm)	149.14 ± 4.58	140.15 ± 4.58
	Dissolved Oxygen (mg L^{-1})	6.95 ± 0.58	7.07 ± 2.20
	Temperature (C°)	25.98 ± 0.36	25.53 ± 0,29
	Hardness ($CaCO_3$)	42 ± 0.35	-
Cadmium-chloride ($CdCl_2$ 2.5 H_2O)	pH	7.46± 0.38	7.81 ± 0.38
	Electric conductivity (µs/cm)	137.54 ± 4,25	139.68 ± 3.20
	Dissolved Oxygen (mg L^{-1})	6.65 ± 0.42	6.80 ± 0.76
	Temperature (C°)	25.36 ± 0.25	25.82 ± 0.42
	Hardness ($CaCO_3$)	44 ± 0.86	-

Table S3. Results of the swimming behavior assays for *Rhinella diptycha* (Cope, 1862) tadpoles. The table presents the replicates, the concentration of copper sulfate (mg L^{-1}) and cadmium chloride (mg L^{-1}), as well as the speed (m/s) and distance (cm), including the mean and standard deviation (SD) for each parameter.

Replicas	Concentration (mg L^{-1})	Speed (m/s)	Mean	SD	Replicas	Distance (cm)	Mean	SD
				Copper Sulfate ($CuSO_4$)				
C0 1S	0	0.240			C0 1D	98.470		
C0 2S	0	0.330	0.250	0.070	C0 2D	40.980	62.010	31.700
C0 3S	0	0.190			C0 3D	46.580		
C1 1S	25	0.070			C1 1D	25.520		
C1 2S	25	0.310	0.220	0.130	C1 2D	21.620	31.240	13.430
C1 3S	25	0.280			C1 3D	46.580		
C2 1S	0.05	0.560			C2 1D	124.660		
C2 2S	0.05	0.270	0.340	0.200	C2 2D	64.380	79.560	39.750
C2 3S	0.05	0.190			C2 3D	49.630		
C3 1S	0.25	0.180			C3 1D	64.910		
C3 2S	0.25	0.250	0.180	0.060	C3 2D	35.460	43.570	18.650
C3 3S	0.25	0.120			C3 3D	30.350		
C4 1S	0.5	0.740			C4 1D	32.220		
C4 2S	0.5	0.510	0.290	0.210	C4 2D	56.060	33.060	22.590
C4 3S	0.5	0.090			C4 3D	10.910		
				Cadmium Chloride ($CdCl_2$)				
C0 1S	0	0.360			C0 1D	58.880		
C0 2S	0	0.440	0.410	0.040	C0 2D	56.960	58.890	1.930
C0 3S	0	0.420			C0 3D	60.820		
C1 1S	0.25	0.170			C1 1D	31.090		
C1 2S	0.25	0.320	0.500	0.450	C1 2D	42.160	55.860	33.770
C1 3S	0.25	1.020			C1 3D	94.320		
C2 1S	0.5	0.470			C2 1D	42.050		
C2 2S	0.5	0.280	0.300	0.170	C2 2D	45.400	34.460	16.140
C2 3S	0.5	0.140			C2 3D	15.920		
C3 1S	0.75	0.130			C3 1D	17.740		
C3 2S	0.75	0.700	0.410	0.290	C3 2D	86.870	62.340	38.690
C3 3S	0.75	0.410			C3 3D	82.410		
C4 1S	1	0.900	0.540	0.500	C4 1D	28.640	26.940	2.400
C4 2S	1	0.180			C4 2D	25.250		
C5 1S	1.5	0.510	-	-	C5 1D	17.040	-	-
C6 1S	2	0.400	-	-	C6 1D	33.330	-	-

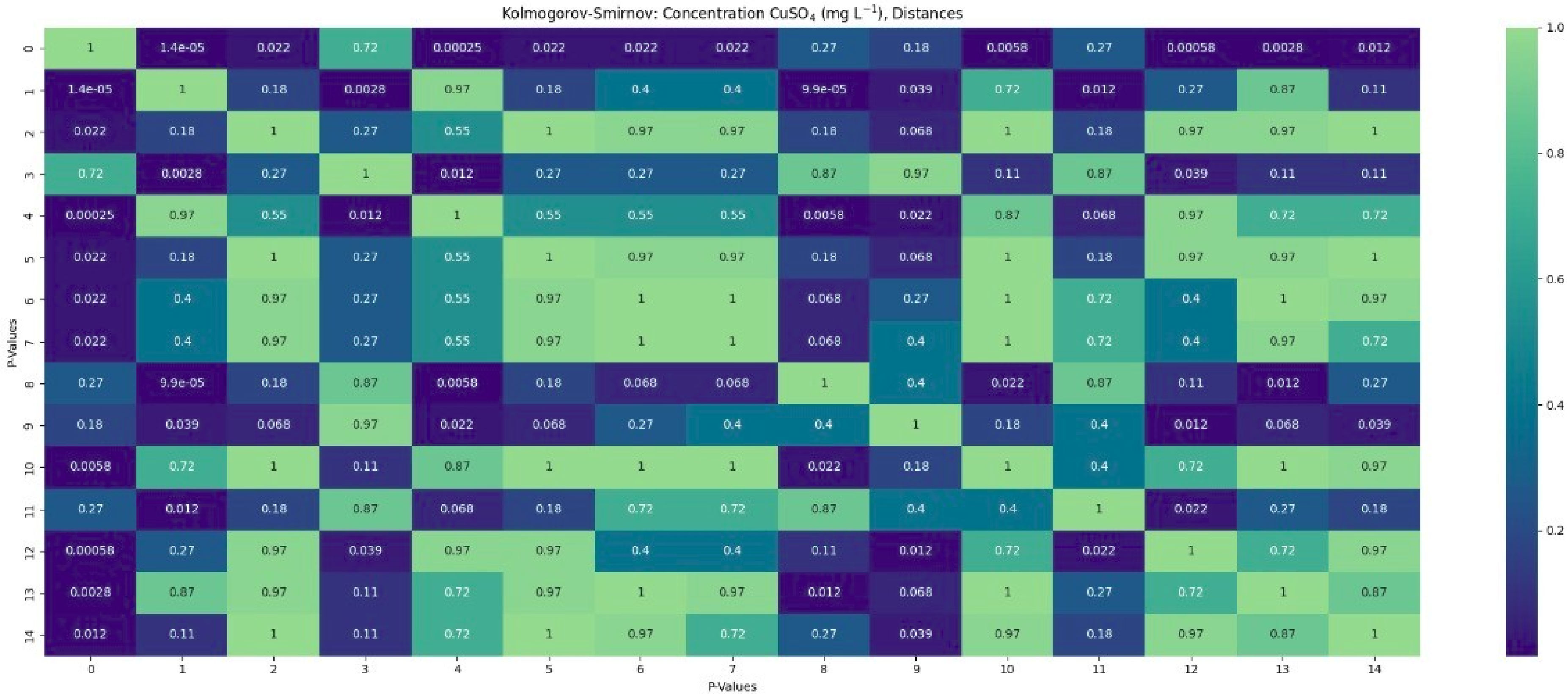


Figure S1. Kolmogorov-Smirnov test results (p-values) assessing the effect of copper sulfate ($CuSO_4$) on the swimming distance of *Rhinella diptycha* (Cope, 1862) tadpoles.

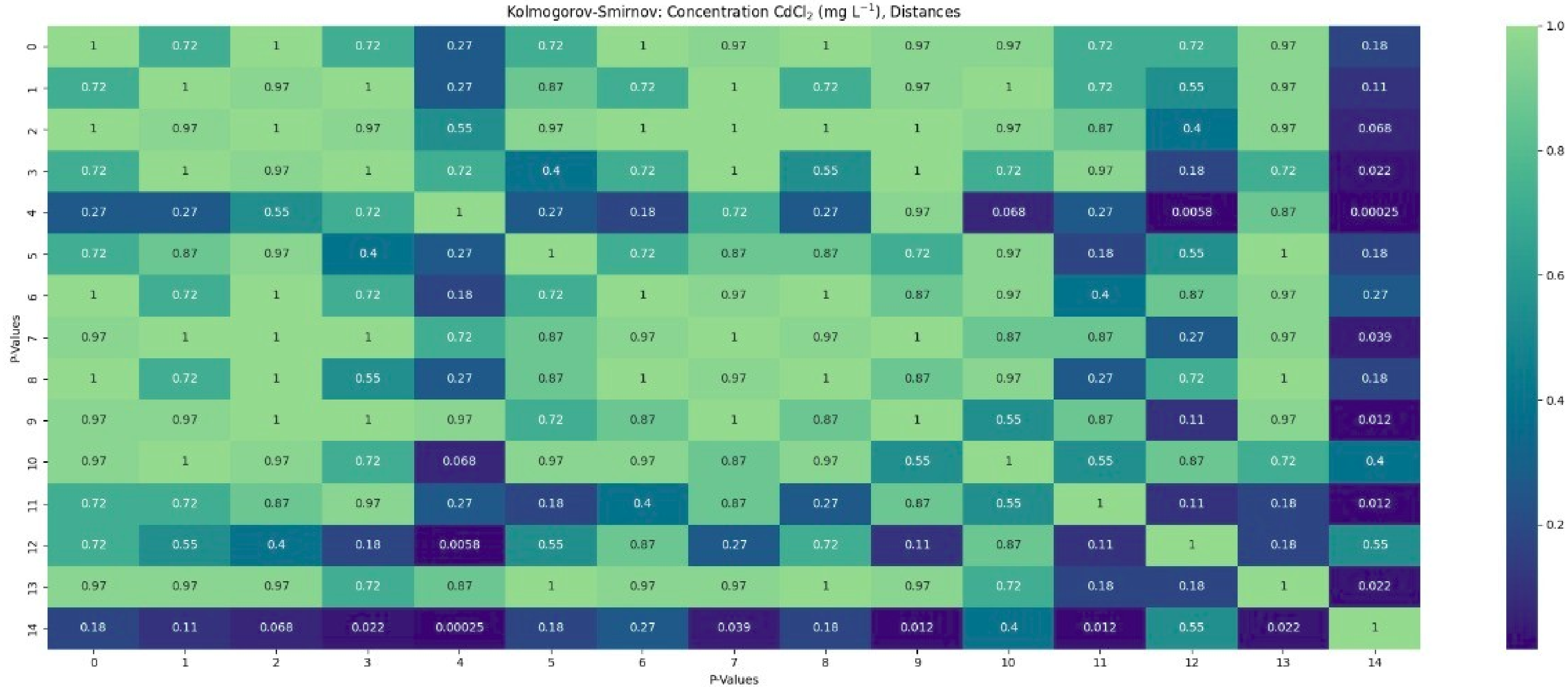


Figure S2. Kolmogorov-Smirnov test results (p-values) assessing the effect of cadmium chloride ($CdCl_2$) on the swimming distance of *Rhinella diptycha* (Cope, 1862) tadpoles.

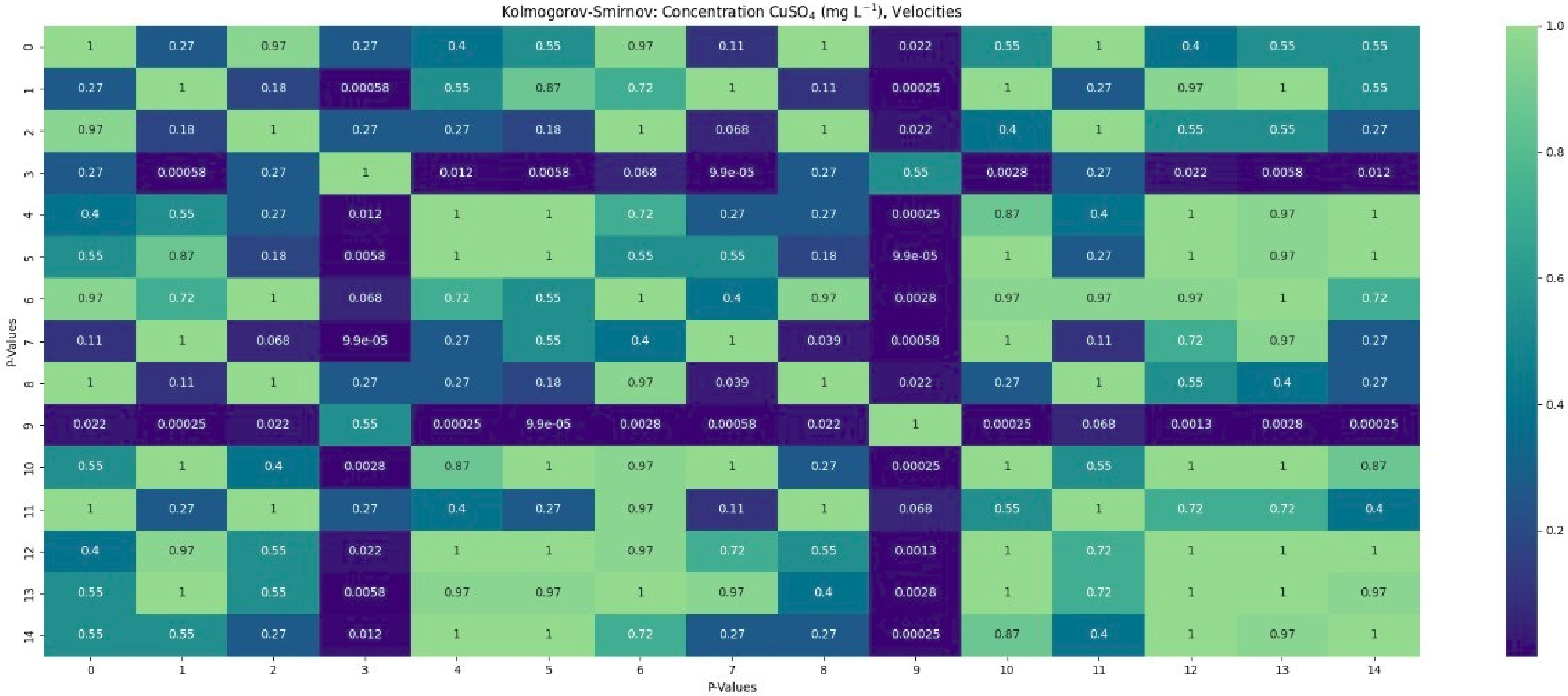


Figure S3. Kolmogorov-Smirnov test results (p-values) assessing the effect of copper sulfate ($CuSO_4$) on the swimming velocities of *Rhinella diptycha* (Cope, 1862) tadpoles.

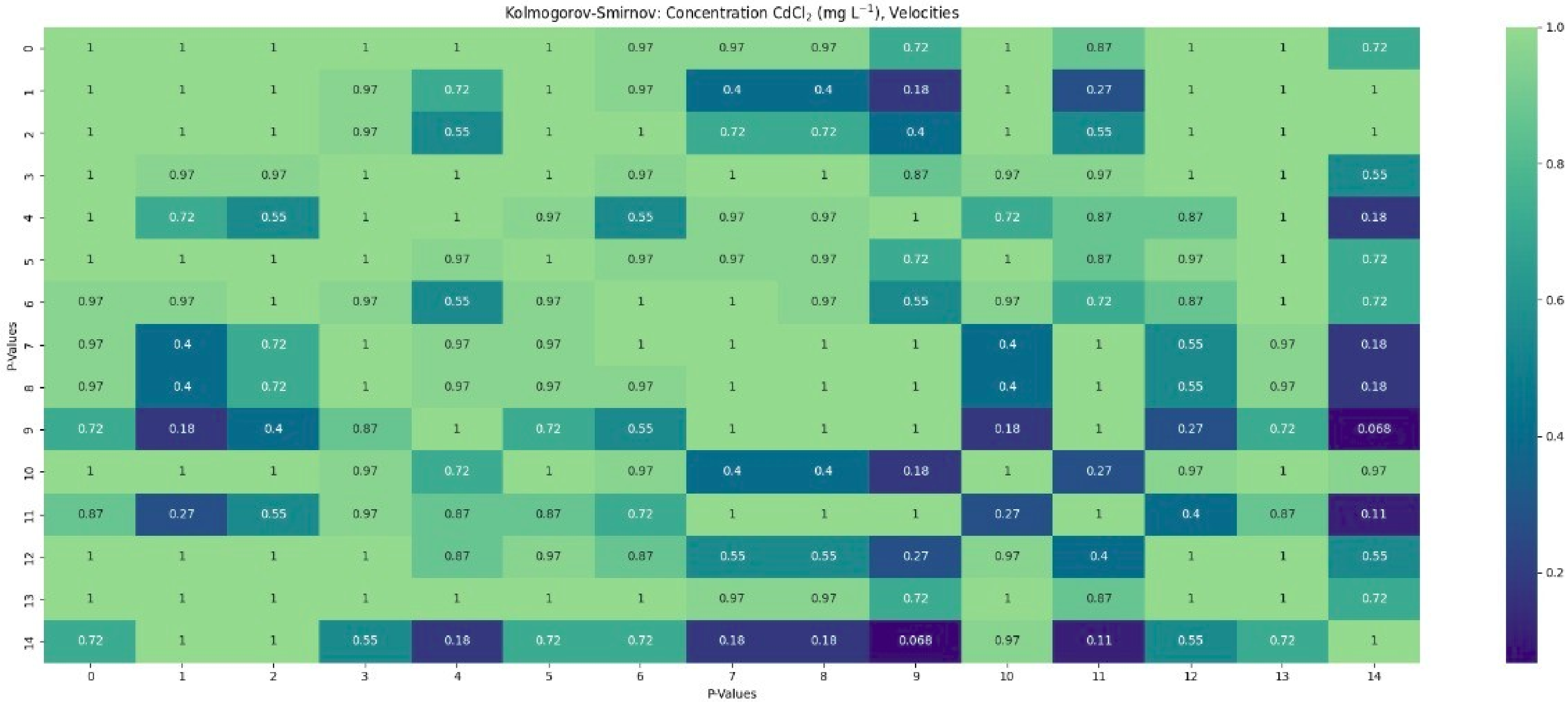


Figure S4. Kolmogorov-Smirnov test results (p-values) assessing the effect of cadmium chloride ($CdCl_2$) on the swimming velocities of *Rhinella diptycha* (Cope, 1862) tadpoles.

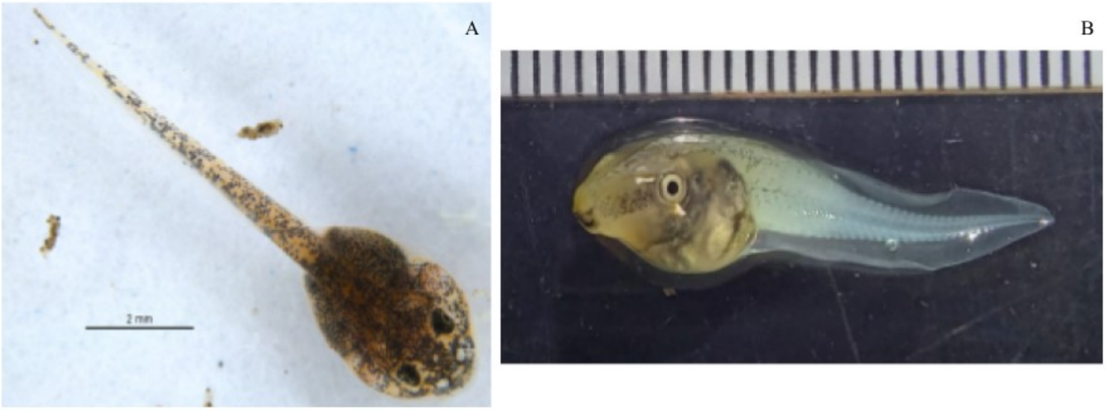


Figura 1. Girinos de (A) *Physalaemus nattereri* (Steindachner, 1893) em estágio 25G (B) *Rhinella diptycha* (Cope, 1862) em estágio 27G.

Figura 2. Sítios de coleta (A) Lagoa temporário Mayaca, localizado no município de São Carlos, estado de São Paulo (21°54'18,0"S, 47°52'17"W) (B) Estação Experimental de Aquicultura do Departamento de Hidrobiologia da Universidade de São Carlos, no estado de São Paulo, Brasil (21°58'54,95"S, 47°52'33,04"W).

Figura 3. (A) Desova de *Physalaemus natteri*, (B) Coleta manual de desova pelo técnico Luiz Aparecido Joaquim, do Departamento de Ecologia e Biologia Evolutiva da Universidade Federal de São Carlos-SP.

Figura 4. Procedimento de manutenção para aclimatação das desovas de *Physalaemus nattereri* (Steindachner, 1893).

Figura 5. Adulto e girinos de *Rhinella diptycha* (Cope, 1862).

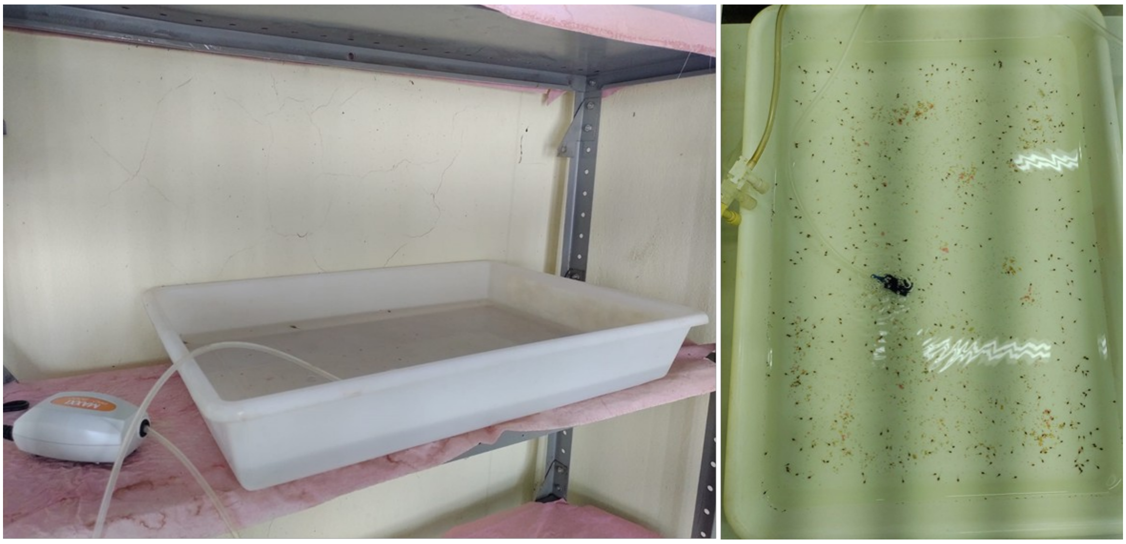


Figura 6. Manutenção na sala de cultivo, bandejas com água reconstituída e aeração constante.

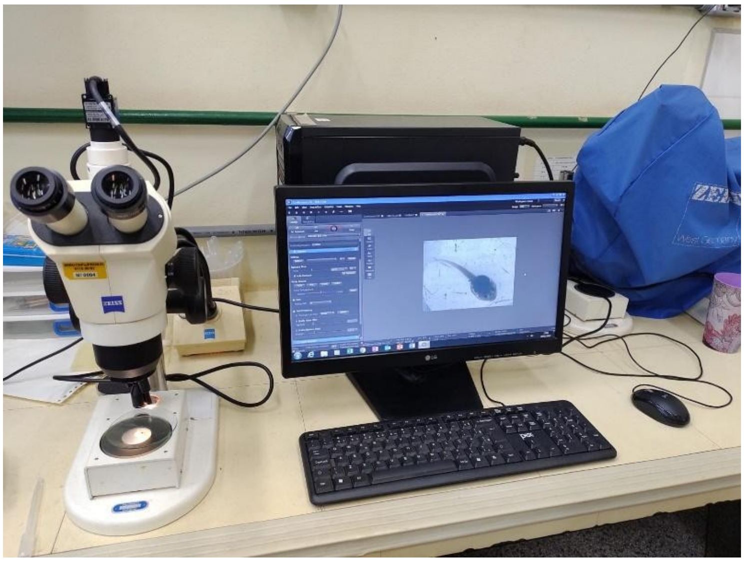
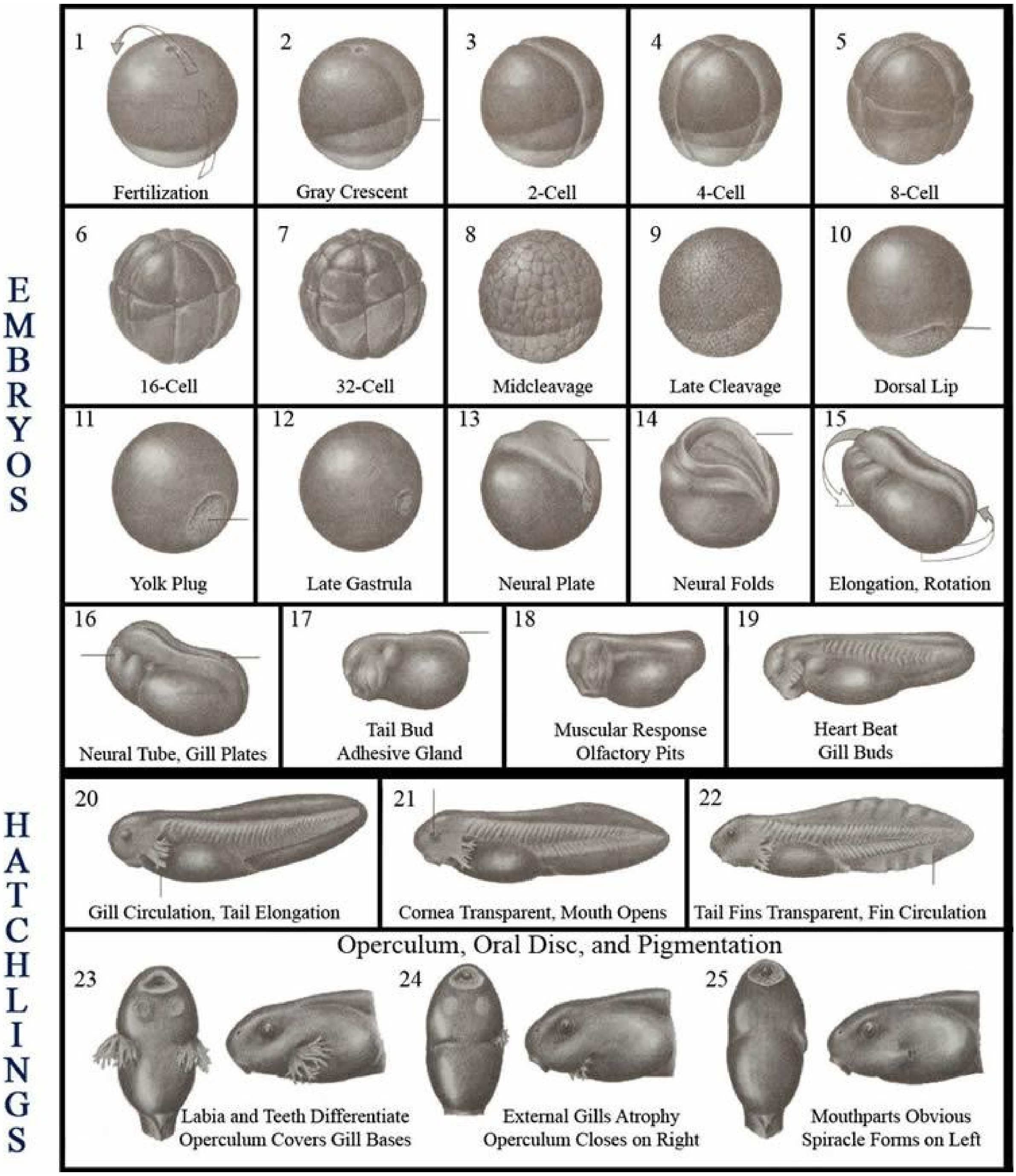


Figura 7. Montagem da câmera em um estereomicroscópio Zeiss Stemi SVI para a identificação e classificação dos girinos.

Figura 8. Montagem para experimentos de toxicidade aguda e crônica


EMBRYOS
HATCHLINGS
1 Fertilization
2 Gray Crescent
3 2-Cell
4 4-Cell
5 8-Cell
6 16-Cell
7 32-Cell
8 Midcleavage
9 Late Cleavage
10 Dorsal Lip
11 Yolk Plug
12 Late Gastrula
13 Neural Plate
14 Neural Folds
15 Elongation, Rotation
16 Neural Tube, Gill Plates
17 Tail Bud
Adhesive Gland
18 Muscular Response
Olfactory Pits
19 Heart Beat
Gill Buds
20 Gill Circulation, Tail Elongation
21 Cornea Transparent, Mouth Opens
22 Tail Fins Transparent, Fin Circulation
Operculum, Oral Disc, and Pigmentation
23 Labia and Teeth Differentiate
Operculum Covers Gill Bases
24 External Gills Atrophy
Operculum Closes on Right
25 Mouthparts Obvious
Spiracle Forms on Left

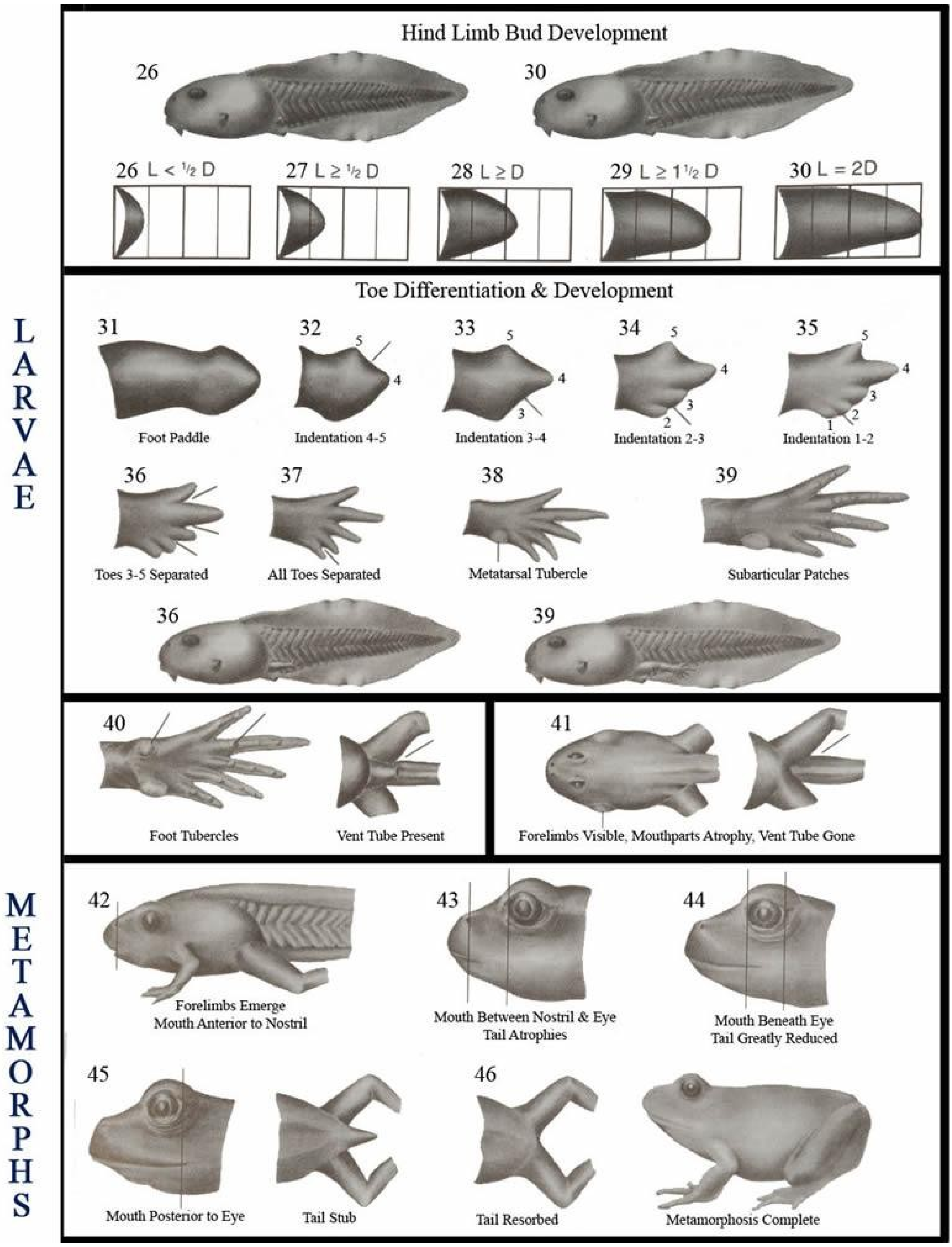


Figura 9. Estádios de desenvolvimento desde embriões até metamorfose, Segundo a classificação de Gosner (1960). Fonte: Adaptado de McDiarmid e Altig (1999).