UNIVERSIDADE FEDERAL DO PAMPA

MARIA VITÓRIA TAKEMURA MARIANO

TOXICIDADE DO COBRE EM LARVAS DE *Danio rerio:* IMPLICAÇÕES ALARMANTES DE CONCENTRAÇÕES PERMITIDAS NO BRASIL

São Gabriel

2024

MARIA VITÓRIA TAKEMURA MARIANO

TOXICIDADE DO COBRE EM LARVAS DE *Danio rerio:* IMPLICAÇÕES ALARMANTES DE CONCENTRAÇÕES PERMITIDAS NO BRASIL

Dissertação apresentada ao Programa de Pós-graduação *Stricto sensu* em Ciências Biológicas da Universidade Federal do Pampa, como requisito parcial para obtenção do Título de Mestre em Ciências Biológicas.

Orientador: Prof. Dr. Jeferson Luis Franco
Co-Orientadora: Prof. Dra. Thais Posser

São Gabriel

2024

Ficha catalográfica elaborada automaticamente com os dados fornecidos
pelo(a) autor(a) através do Módulo de Biblioteca do
Sistema GURI (Gestão Unificada de Recursos Institucionais) .

M333 Mariano, Maria Vitória Takemura
TOXICIDADE DO COBRE EM LARVAS DE Danio rerio: IMPLICAÇÕES ALARMANTES DE CONCENTRAÇÕES PERMITIDAS NO BRASIL / Maria Vitória Takemura Mariano.
60 p.

Dissertação(Mestrado)-- Universidade Federal do Pampa, MESTRADO EM CIÊNCIAS BIOLÓGICAS, 2024.
"Orientação: Jeferson Luis Franco".

1. peixe-zebra. 2. ecotoxicologia. 3. contaminação. 4. estresse oxidativo. 5. sulfato de cobre. I. Título.

MARIA VITÓRIA TAKEMURA MARIANO

TOXICIDADE DO COBRE EM LARVAS DE *Danio rerio:* IMPLICAÇÕES ALARMANTES DE CONCENTRAÇÕES PERMITIDAS NO BRASIL

Dissertação apresentada ao Programa de Pós-graduação *Stricto sensu* em Ciências Biológicas da Universidade Federal do Pampa, como requisito parcial para obtenção do Título de Mestre em Ciências Biológicas.

Área de concentração: Qualidade Ambiental

Dissertação de Mestrado defendida e aprovada em: 23 de Fevereiro de 2024.

Banca examinadora:

_____

Prof. Dr. Jeferson Luis Franco
Orientador
UNIPAMPA

_____

Prof. Dr. Alcir Dafré
UFSC

_____

Prof. Dr. Marcelo Farina
UFSC

AGRADECIMENTOS

À minha família, por permanecer ao meu lado desde o início desta jornada. Obrigada mamãe, por todo o apoio independente das circunstâncias. Em especial, agradeço ao meu pai. Reconheço e valorizo suas palavras de orgulho, mas tenha certeza que meu orgulho por ser sua filha é ainda maior, por toda sua dedicação e esforço em compreender minha profissão e, não medir esforços para meu desenvolvimento pessoal e profissional.

Ao Vitor, meu grande amor e companheiro de vida, por todos as risadas, por nossas discussões científicas e claro, por toda sua disposição em me auxiliar nos experimentos deste trabalho. Tantos momentos bons compartilhados contigo tornou a vida acadêmica mais leve, tu é essencial para mim.

À minha segunda família, que me acolheu de maneira incomparável. À minha sogra Ivete, ao meu sogro João Luiz, à minha cunhada Joana e a todos os membros da família Oliveira de Rosso! Vocês não apenas me receberam de braços abertos, mas também encheram meu coração com amor e carinho. O frio do sul se torna quase imperceptível diante do acalanto que sinto ao estar com vocês.

Às minhas amigas, não apenas companheiras de laboratório, mas verdadeiras amigas de coração: Kellyn, Jacy, Luana, Giany e especialmente a Karen, por toda parceria, conselhos e discussões instigantes, além das nossas aventuras no congresso!

Aos integrantes do grupo de pesquisa de Diversidade Genética Animal, à pesquisadora Victoria Tura, e aos professores Ricardo José Gunski e Analía Del Valle Garnero. A colaboração de vocês foi essencial para o aprimoramento deste trabalho.

Agradeço aos meus estimados professores, Juliano Tomazzini e Paulo Pinto, pelo esforço dedicado em proporcionar meu aprendizado de novas técnicas, além dos valiosos conselhos e conhecimentos que compartilharam.

Expresso minha gratidão, em especial, ao meu orientador, Prof. Dr. Jeferson Luis Franco, e à Profª Drª Thaís Posser, por me acolherem no GPEOSCEL. Agradeço por inspirarem meu caminho científico e por compartilharem generosamente todo o conhecimento. Estendo meu agradecimento a cada professor que fez parte da minha trajetória acadêmica.

A Universidade Federal do Pampa pela educação pública de extrema qualidade.

A Coordenação de Aperfeiçoamento Pessoal de Nível Superior pela bolsa concedida.

E por todos que contribuíram direta ou indiretamente para realização deste trabalho.

RESUMO

O cobre é um metal de ocorrência natural e desempenha um papel vital no metabolismo dos organismos. No entanto, atividades antrópicas intensificam a concentração deste metal no ambiente, elevando o risco de contaminação. No Brasil, o limite máximo permitido de cobre na água potável é de 2 mg/L, contudo, há lacunas quanto à segurança dos organismos aquáticos. O presente trabalho investigou a toxicidade do cobre em larvas de *Danio rerio* em concentrações ambientalmente relevantes. Larvas de *D. rerio* em 72 horas pós-fertilização foram expostas a concentrações nominais de cobre (0,16 a 48 mg/L) por 24 horas. A toxicidade do cobre foi avaliada utilizando concentrações subletais (0,16, 0,32 e 1,6 mg/L) com as larvas de *D. rerio* em 96 horas pós-fertilização. Como resultado, a CL50 foi estabelecida em 8,4 mg/L. Notavelmente, a concentração mais elevada de cobre (1,6 mg/L) resultou na redução do comprimento corporal (3,31 mm ± 0,10) e aumento na área do saco vitelínico (0,192 mm^2 ± 0,01) que, quando comparado com o grupo controle (3,62 mm ± 0,09, 0,136 mm^2 ±), sugerem possível associação com a interferência na nutrição desses organismos, resultando em prejuízos no desenvolvimento. Ao mesmo tempo que a diminuição da área da bexiga natatória (0,01 mm^2 ± 0,05) em comparação com o grupo controle (0,30 mm^2 ± 0,06), suscita implicações cruciais na flutuabilidade desses organismos, afetando também o desenvolvimento do nado ativo. A exposição ao cobre diminuiu a resposta de escape e capacidade natatória das larvas e, em um ambiente natural, essas alterações podem resultar em maior suscetibilidade à predação, especialmente durante as fases iniciais de desenvolvimento. Alterações em parâmetros oxidativos foram observados, evidenciado pelo aumento nos níveis de espécies reativas de oxigênio peroxidação lipídica, indicando potenciais danos celulares. Além disso, a redução dos níveis de tióis não proteicos e viabilidade celular sugere impactos, em parte, na capacidade antioxidante e, principalmente, na integridade celular, prejudicando a homeostase e comprometendo o desenvolvimento larval. Os resultados destacam que a exposição ao cobre, mesmo em concentrações próximas e inferiores às permitidas na legislação para água potável, provoca impactos adversos em parâmetros morfológicos, comportamentais e bioquímicos. Essas constatações sublinham a urgência de reavaliação dessas concentrações, as quais, apesar de não serem consideradas tóxicas para os humanos, podem comprometer a biota aquática.

Palavras-Chave: peixe-zebra; ecotoxicologia; contaminação; estresse oxidativo; sulfato de cobre

ABSTRACT

Copper, a naturally occurring metal, plays a crucial role in organism metabolism. However, anthropogenic activities contribute to an increase in its concentration in the environment, heightening the potential for contamination. In Brazil, the maximum allowed limit for copper concentration in drinking water is 2 mg/L; nevertheless, safety for aquatic organisms still presents noteworthy limitations. This study investigated the toxicity of copper in *Danio rerio* larvae at environmentally relevant concentrations. *D. rerio* larvae at 72 hours post-fertilization were exposed to nominal copper concentrations (0.16 to 48 mg/L) for 24 hours. Copper toxicity was assessed using sublethal concentrations (0.16, 0.32, and 1.6 mg/L) with *D. rerio* larvae at 96 hours post-fertilization. As a result, the LC50 was established at 8.4 mg/L. Notably, the highest copper concentration (1.6 mg/L) resulted in a reduction in body length (3.31 mm ± 0.10) and an increase in yolk sac area (0.192 mm^2 ± 0.01), which, when compared to the control group (3.62 mm ± 0.09, 0.136 mm^2 ± 0.13), suggests a possible association with interference in the nutrition of these organisms, resulting in developmental impairments. Additionally, the decrease in the swim bladder area (0.01 mm^2 ± 0.05) compared to the control group (0.30 mm^2 ± 0.06) raises crucial implications for the buoyancy of these organisms, also affecting the development of active swimming. Copper exposure reduced the escape response and swimming capacity of the larvae, and in a natural environment, these changes may result in increased susceptibility to predation, especially during the early developmental stages. Changes in oxidative parameters were observed, evidenced by an increase in reactive oxygen species and lipid peroxidation, indicating potential cellular damage. Concurrently, the reduction in non-protein thiol levels and cellular viability suggests impacts, partly on antioxidant capacity and mainly on cellular integrity, disrupting homeostasis and compromising larval development. The results highlight that copper exposure, even at concentrations close to or below those allowed in drinking water legislation, induces adverse effects on morphological, behavioral, and biochemical parameters. These findings underscore the urgent need for a reassessment of these concentrations, which, despite not being considered toxic to humans, may compromise aquatic biota.

Keywords: zebrafish; ecotoxicology; contamination; oxidative stress; copper sulphate

LISTA DE FIGURAS

LISTA DE ABREVIATURAS E SIGLAS

Cu^{+}– cobre reduzido, cuproso

Cu^{+2} – cobre oxidado, cúprico

CTR1 – transportador de cobre 1

CCS – chaperona de cobre para superóxido dismutase

SOD – superóxido dismutase

H_2O_2 – peróxido de hidrogênio

CAT – catalase

Cox17 – chaperona de cobre para citocromo c oxidase

Sco1/Sco2 – Proteína de montagem do citocromo c oxidase

Cco – citocromo c oxidase

ATP – adenosina trifosfato

ATOX1 – proteína antioxidante 1

ATP7A – ATPase Transportador de Cobre α

ATP7B – ATPase Transportador de Cobre β

TGN – rede trans-Golgi

CP – ceruloplasmina

ERO – espécies reativas de oxigênio

MTs – metalotioneinas

GSH – glutationa

mg/L – miligramas por litro

$OH^{\bullet}$ – radical hidroxila

DNA – ácido desoxirribonucleico

$O_2^{-\bullet}$ – Radical ânion superóxido

GPx – glutationa peroxidase

GSSG – glutationa oxidada

GST – glutationa-s-transferase

SH – sulfidrila

hpf – horas pós fertilização

APRESENTAÇÃO

A seção de **INTRODUÇÃO** fornece uma visão geral do tema, enquanto o segmento **REVISÃO BIBLIOGRÁFICA** explora a literatura relacionada aos temas abordados nesta dissertação. Os métodos realizados e os resultados obtidos, são apresentados sob a forma de **ARTIGO,** onde constam as seções: de Materiais e Métodos, Resultados, Discussão e Referências Bibliográficas. Na seção final da dissertação, intitulada **CONSIDERAÇÕES FINAIS** e **PERSPECTIVAS FUTURAS**, são apresentadas interpretações e comentários abrangentes acerca dos resultados. As **REFERÊNCIAS** referem-se somente as citações que aparecem seção **INTRODUÇÃO**.

SUMÁRIO

1. INTRODUÇÃO

O cobre é um metal de transição, reconhecido por ser um elemento traço essencial, desempenhando papel fundamental em processos bioquímicos críticos. Contudo, em razão de suas características redox, íons de cobre estão sujeitos a uma rígida regulação por sistemas de captação, absorção e efluxo, a fim de garantir a homeostase do organismo. Entretanto, é crucial salientar que metais essenciais podem desencadear toxicidade quando concentrações celulares ultrapassam as exigências metabólicas (Chen et al., 2020; Tsang et al., 2021).

Embora o cobre seja naturalmente encontrado no ambiente, emissões antrópicas contribuem para a elevação das concentrações de cobre no ambiente. Esse cenário desperta preocupações sob uma perspectiva ecotoxicológica, uma vez que os metais, incluindo o cobre, são reconhecidos como poluentes ambientais severos (Campana et al., 2012; Poggere et al., 2023; Tao et al., 2022).

Um dos principais mecanismos de toxicidade do cobre a indução de estresse oxidativo, através do aumento na geração de espécies reativas de oxigênio (ERO) (Halliwell and Gutteridge, 2015; Pereira et al., 2016). O impacto das ERO nos organismos é ainda mais pronunciado naqueles em fase de desenvolvimento, devido à associação com a disfunção de biomoléculas fundamentais. Este impacto reflete em alterações significativas em espécies aquáticas em desenvolvimento, como evidenciado em espécies de peixes, que também resultam nas alterações em padrões comportamentais e bioquímicos (Xu et al., 2017; Zhao et al., 2020). Diante deste cenário, torna-se imperativo a avaliação da toxicidade do cobre sobre estes organismos, antes que alcancem proporções maiores como efeitos letais, assim como impactos na saúde humana, uma vez que o excesso de cobre também está relacionado ao desenvolvimento de doenças (Chen et al., 2020; Choo et al., 2013).

Neste contexto, o peixe-zebra (*Danio rerio*) é um organismo modelo eficiente, especialmente na sua fase embrio-larval em estudos de ecotoxicologia. Em virtude da ampla diversidade de fenótipos comportamentais e bioquímicos organismo se configura como um modelo valioso para a investigação da toxicidade de contaminantes ambientais. Adicionalmente, ao considerar o dinâmico processo de desenvolvimento, destaca-se como parâmetro essencial a análise de alterações morfológicas, ressaltando não apenas a sensibilidade deste organismo, mas também seu papel como indicador na avaliação de contaminantes (Martins et al., 2023; Paganotto Leandro et al., 2023, 2021).

2. REVISÃO DE LITERATURA

2.1 Essencialidade do cobre e riscos de toxicidade

O cobre é um metal de transição localizado na família 4 e grupo IB da Tabela Periódica, podendo ocorrer sob a forma elementar, sais orgânicos na forma de íons ou como compostos orgânicos (Barceloux and Barceloux, 1999; Tsang et al., 2021). Este metal apresenta dois estados de oxidação, cuproso (Cu^{+1}; oxidado) e cúprico (Cu^{+2}; reduzido). Em sistemas biológicos, o cobre é encontrado principalmente na forma de $Cu^{+2,}$ uma vez que na presença de oxigênio o Cu^{+1} é facilmente oxidado (Arredondo and Núñez, 2005; Tsang et al., 2021).

Ao participar de processos bioquímicos críticos, o cobre é um micronutriente essencial para os organismos, devido ao seu envolvimento na respiração mitocondrial, defesa antioxidante e componente estrutural de macromoléculas (Apresova et al., 2014; Bhuvanasundar et al., 2014; Boyd et al., 2020; Garcia et al., 2019; Li, 2020; Swaminathan and Gohil, 2022).

Em humanos, a obtenção de cobre ocorre primariamente por meio da dieta, com o Ministério da Saúde preconizando uma ingestão recomendada de 900 µg para adultos, enquanto cerca de 1 mg de cobre é excretado diariamente (Ministério da Saúde, 2005; National Institutes of Health, 2022). Para assegurar as funções fisiológicas essenciais desse metal, a homeostase é estritamente regulada por sistemas intricados de captação, distribuição e excreção, a fim de prevenir o excesso no organismo (Chen et al., 2020).

No trato digestivo de mamíferos, íons de cobre estão predominantemente na forma de Cu^{+2}, o qual pode ser transportado diretamente pelo transportador de metal divalente 1 (DMT1) mas que não pode ser diretamente utilizado pelas células (Shawki et al., 2015). Dessa forma, reductases presentes nos enterócitos são responsáveis por reduzir Cu^{+2} a Cu^{+} e posteriormente, absorvido pelo transportador de cobre 1 (CTR1) (Bossak et al., 2018; Skopp et al., 2019a; Wyman et al., 2008).

No meio intracelular, íons cuprosos são direcionados para diferentes vias de utilização e desintoxicação através de proteínas, denominadas chaperonas, para alvos celulares **(Fig. 1).** No citoplasma, a chaperona de cobre para superóxido dismutase (CCS) medeia o transporte de Cu^{+} e ativação da superóxido dismutase (Wong et al., 2000). O íon de cobre presente nesta enzima atua como centro catalítico, alternando entre seus estados de oxidação, facilitando a transferência de elétrons, crucial para conversão do ânion superóxido (O_2^{-}) em peróxido de

hidrogênio (H_2O_2) (Boyd et al., 2020).

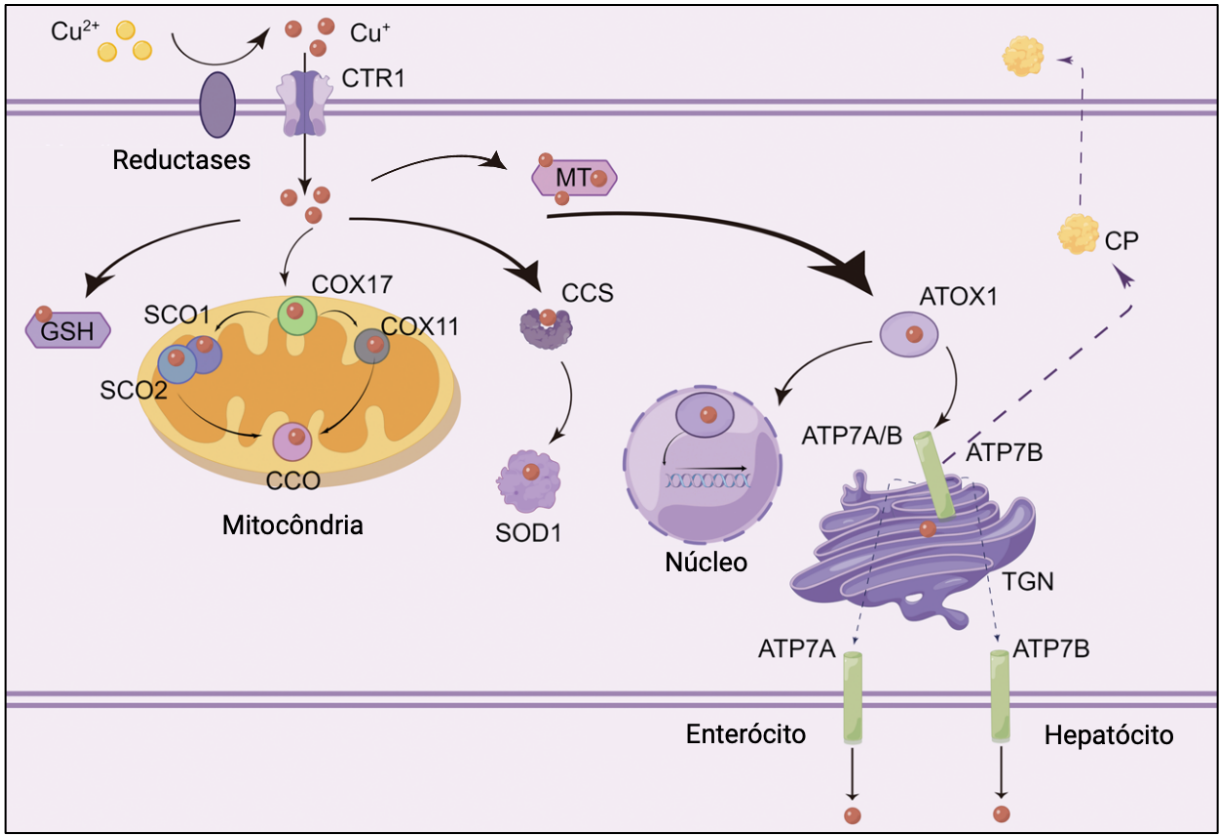


Figura 1 Ilustração dos mecanismos envolvidos na homeostase do cobre em mamíferos. Íons cúpricos (Cu^{+2}) são reduzidos a íons cuprosos (Cu^{+}) por reductase para a incorporação via CTR1. Chaperonas de cobre direcionam íons cuprosos para diferentes destinos intracelulares. No interior da mitocôndria, o COX17 é responsável por entregar Cu^{+} para Sco1/Sco2 e Cox11 para ativar citocromo c oxidase. Simultaneamente, CCS transporta Cu^{+} para ativar a superóxido dismutase (SOD), enzima antioxidante. Nos enterócitos ou hepatócitos, as ATPases se translocam para exportação de Cu^{+} para o sangue ou bile, respectivamente. O transporte intracelular é mediado via ATP7B-TGN para formar a ceruloplasmina (CP), transportado para diversos sistemas. O excesso Cu^{+} induz a expressão de metalotioneínas que sequestram e diminuem a concentração do metal no citoplasma. A glutationa também exerce papel na diminuição de Cu^{+} circundantes, formando complexos estáveis com o íon. Adaptado (Chen et al., 2023).

A chaperona de cobre Cox17, é responsável pelo transporte de Cu^{+} até a mitocôndria, direcionando-os para a Sco1/Sco2 e Cox11, os quais doam Cu^{+} para as subunidades da citoctomo c oxidase (CcO) (Chen et al., 2020; Chojnacka et al., 2015; Skopp et al., 2019b). Também conhecida como complexo IV da cadeia respiratória, essa enzima utiliza dois centros metálicos de cobre para catalisar a etapa final de respiração celular, no qual o citocromo c é oxidado e o oxigênio é reduzido para formar água. Como resultado, prótons são liberados e bombeados através da membrana mitocondrial, contribuindo para a síntese de ATP

(Swaminathan and Gohil, 2022).

O mecanismo pelo qual íons de cobre são incorporados na distribuição sistêmica ocorre por meio da interação entre a chaperona antioxidante 1 (ATOX1), o qual transfere íons de Cu+ para ATPases do tipo P, como ATP7A e ATP7B, essenciais no transporte de cobre para via secretora e excreção de cobre na célula (Chen et al., 2023). A importância fisiológica das ATPases é evidenciada pelas consequências da inativação da ATP7A, associado à doença de Menkes em humanos, caracterizada pelo comprometimento no desenvolvimento neurológico devido à interrupção na entrega de cobre ao cérebro (Horn and Wittung-Stafshede, 2021).

Ao mesmo tempo que, mutações no gene que expressa a ATP7B em humanos resultam em um distúrbio metabólico severo conhecido como doença de Wilson, associado à acumulação de cobre em vários tecidos, especialmente no cérebro e no fígado, pela falha na excreção adequada do metal (Lucena-Valera et al., 2023). Como um exemplo de acumulação de cobre no cérebro, a desregulação da homeostase desse metal emerge como um componente associado à patogênese de doenças neurodegenerativas, como o Alzheimer. O excesso de cobre forma ligações diretas e de alta afinidade com os peptídeos β-amiloide (Aβ), intensificando a agregação e neurotoxicidade (Cheignon et al., 2018; Squitti et al., 2013).

Apesar da expressão predominante do ATP7A em diversos tecidos e órgãos (exceto o fígado) e a expressão majoritária do ATP7B no fígado, a localização intracelular destes transportadores é destacada pela plasticidade dos mesmos em respostas específicas aos níveis de cobre no microambiente celular (Chen et al., 2020, 2023; La Fontaine et al., 2010).

Em níveis fisiológicos de Cu, as ATPases se localizam na rede trans-Golgi (TGN) onde bombeiam Cu^{+} do citoplasma para o lúmen do TGN (Yi and Kaler, 2014). Com o aumento dos níveis de Cu+, a ATP7A migra para a membrana basolateral dos enterócitos, promovendo a exportação de cobre para a corrente sanguínea, onde alcançam o fígado, principal órgão de armazenamento deste metal (Horn and Wittung-Stafshede, 2021). A ATP7B transloca para a membrana apical dos hepatócitos, contribuindo para a excreção deste metal pela bile e ambos atuam para prevenir efeitos tóxicos do excesso do acúmulo intracelular de Cobre(Chen et al., 2020). Além disso, através da via secretora, a ATP7B é crucial para o fornecimento de cobre à ceruloplasmina, a qual é subsequentemente secretada na circulação sanguínea (Bartee and Lutsenko, 2007; La Fontaine et al., 2010). Posteriormente, quando os níveis de cobre retornam aos níveis fisiológicos, a ATP7A e ATP7B são reciclados de volta ao TGN (Chen et al., 2020).

O potencial tóxico dos íons de cobre não complexados com proteínas está associado à sua natureza redox, capaz de gerar espécies reativas de oxigênio (ERO) (Halliwell and

Gutteridge, 2015). Portanto, proteínas envolvidas na desintoxicação do Cu, como as metalotioneínas (MTs), desempenham papel essencial na homeostase deste metal (Calvo et al., 2017).

As MTs, pertencentes a uma família de proteínas de baixo peso molecular ricas em cisteína, têm sua expressão induzida pelo aumento de íons metálicos como o cobre e zinco (Ziller and Fraissinet-Tachet, 2018). Os grupos tióis presentes nas cisteínas conferem às MTs a capacidade de quelar íons de cobre, evitando assim a toxicidade celular (Calvo et al., 2017)

Além das proteínas como MTs, o tripeptídeo glutationa (GSH), tiol não proteico mais abundante na célula, é reconhecido não apenas por sua função antioxidante, mas também pela capacidade de quelar metais (Jozefczak et al., 2012). Os grupamentos tióis presentes demonstram papel direto na complexação com íons de cobre livre, impedindo a formação excessiva de ERO e contribuindo para manutenção do equilíbrio redox intracelular (Jozefczak et al., 2012; Maryon et al., 2013). Assim, a ação coordenada tanto da MTs e GSH, assim como as demais proteínas envolvidas na homeostase do cobre, contribui para preservar a integridade celular e para regular com precisão os níveis intracelulares de cobre, assegurando uma resposta adaptativa eficiente frente às variações no ambiente celular (Chen et al., 2020; Jozefczak et al., 2012; Morris et al., 2014)

2.2 Contaminação ambiental por metais traço

Os metais traço constituem um grupo de elementos caracterizados por sua densidade maior que 5 g/cm^3 e massas atômicas superiores a 20, portanto, incluem elementos como mercúrio, cobre, arsênio entre outros (Halliwell and Gutteridge, 2015; Zamora-Ledezma et al., 2021).

Embora presentes naturalmente no ambiente, a crescente expansão populacional, aliada ao desenvolvimento industrial acentua a pressão sobre recursos naturais, intensificando a liberação dos metais pesados no ambiente, o qual desperta preocupações do ponto de vista ecotoxicológico, conforme evidenciado por estudos recentes (Azimi et al., 2017; Castelhano Gebara et al., 2021; Kopittke et al., 2019; Liu et al., 2022; Naeem et al., 2021; Taslima et al., 2022; Vellingiri et al., 2022a; Yang et al., 2021). Neste cenário, esses elementos, classificados como severos poluentes ambientais, destacam-se por sua natureza não biodegradável, potencial de bioacumulação e capacidade de causar efeitos deletérios ao ecossistema (Li et al., 2019; Rehman et al., 2019; Vellingiri et al., 2022b).

A principal ênfase em estudos toxicológicos reside na investigação da toxicidade

relacionada a metais pesados não essenciais, ou seja, elementos cuja presença no organismo não é vital para funções biológicas (Fasae and Abolaji, 2022; Fu and Xi, 2020; Sharma and Agrawal, 2005; Tchounwou et al., 2012). Porém, é fundamental destacar que metais essenciais como ferro, cobre, manganês e zinco, além de desempenharem funções vitais nas células, também podem exibir efeitos tóxicos quando a concentração celular excede níveis fisiológicos (Chasapis et al., 2020; Galaris et al., 2019; Santamaria and Sulsky, 2010; Zhang et al., 2015).

2.3 Implicações ambientais da contaminação por cobre

Diversas fontes exercem influência sobre a presença do cobre no ambiente, sendo possível encontrá-lo em ambientes naturais como resultado do intemperismo e atividades vulcânicas (Liu et al., 2022; Mason et al., 2021). No entanto, emissões antrópicas provenientes de setores industriais agrícolas e de mineração se destacam como fatores preponderantes que influenciam diretamente a concentração do cobre no ambiente (Karimi et al., 2021; Li et al., 2019; Poggere et al., 2023; Tao et al., 2022).

Desde o início da década de 1880, compostos de cobre têm desempenhado papel fundamental na proteção de cultivos devido às suas propriedades biocidas, atuando como fungicida de contato (Ayres, 2004). A trajetória que remonta à descoberta acidental do sulfato de cobre ($CuSO_4$), conhecido como calda bordalesa quando misturado com hidróxido de cálcio, evidenciou a eficácia desse composto no controle do míldio da videira (*Plasmopora vitícola*), sendo decisivo para o início do manejo químico de doenças de plantas (Ayres, 2004; Koledenkova et al., 2022; Mondello et al., 2022).

Apesar da aprovação e o uso do cobre na proteção das culturas estarem principalmente ligadas às práticas da agricultura orgânica, os desafios relacionados à sua ação não seletiva e à necessidade de aplicações repetidas em virtude de sua natureza de contato surgem como aspectos críticos (Alves et al., 2023; Karimi et al., 2021; Tamm et al., 2021). Estudos demonstram que o cobre pode levar à contaminação do solo, atingir corpos d’agua e prejudicar organismos aquáticos (Ballabio et al., 2018; Brix et al., 2022; Karimi et al., 2021; Poggere et al., 2023). Diante disso, é evidente que esses compostos, longe de estarem isentos de potenciais impactos ambientais, despertaram a atenção da Comissão Europeia, ao classificá-los como candidatos a substituição (Alves et al., 2023; Brix et al., 2022; Burandt et al., 2024; Tamm et al., 2021).

No Brasil, a qualidade da água potável e o tratamento de efluentes são regulamentados por normas distintas que, embora tenham objetivos complementares, destacam uma disparidade importante em relação à presença de cobre. A Portaria GM/MS nº 888, emitida em 4 de maio de 2021, estabelece que a concentração máxima de cobre na água potável deve ser de 2 mg/L, normativa que se mantém desde dezembro de 2011 (Ministério da Saúde, 2021, 2011). Por outro lado, a resolução CONAMA nº 430, de junho de 2011, define o limite máximo permitido de cobre em efluentes lançados em corpos d'água em 1 mg/L (Conselho Nacional do Meio Ambiente, 2011). Contudo, estudos têm demonstrado que, mesmo concentrações de cobre inferiores aos limites estabelecidos, podem afetar negativamente a biota aquática, perturbando o desenvolvimento de espécies de peixes e a reprodução de anfípodes (Campana et al., 2012; Dornelles Zebral et al., 2019; Johnson et al., 2007; Pompermaier et al., 2021; Wang et al., 2020a; Xu et al., 2017). Dessa forma, embora as concentrações de cobre permitida não são consideradas nocivas para humanos, esses limites podem comprometer a biota aquática, uma vez que a presença do cobre no ambiente aquático pode exceder as concentrações estipuladas (Assis et al., 2019; Leal et al., 2018; Liu et al., 2023; Poggere et al., 2023).

Este cenário normativo destaca a necessidade de avaliação contínua, sobretudo diante das significativas implicações ambientais associadas à presença do cobre (Kopittke et al., 2019; Poggere et al., 2023; Pompermaier et al., 2021). A escassez de estudos que investiguem a segurança da concentração permitida para organismos aquáticos acentua a necessidade de análises aprofundadas, uma vez que a contaminação pelo cobre emerge como potencial risco não apenas para ecossistemas aquáticos, mas também para a saúde humana (Choo et al., 2013; Scheiber et al., 2013; Tao et al., 2022; Xu et al., 2017)

2.3.1 Comportamento do cobre em ambientes aquáticos

A dinâmica do cobre em ambientes aquáticos é fortemente influenciada por propriedades físico-químicas, tais como pH, teor de matéria orgânica, potencial redox e concentração de oxigênio dissolvido. Portanto, a interação entre esses elementos desempenha papel crucial na especiação e biodisponilidade dos íons de cobre na água (Babcsányi et al., 2014; Rader et al., 2019).

Pesquisas indicam que uma parcela significativa do cobre presente na coluna d'água é transportada para o sedimento, especialmente em condições redutoras. Nessas circunstâncias, a alta afinidade do cobre em se ligar a sulfetos e matéria orgânica presente nos sedimentos

resulta na menor disponibilidade de íons na coluna d'água (Rader et al., 2019). Entretanto, essa condição pode ter implicações direta sobre organismos bentônicos que habitam sedimentos ou próximo a ele como evidenciado pelos efeitos tóxicos do cobre nos sedimentos, prejudicando reprodução do anfípode *Melita plumosa* (Campana et al., 2012).

Uma vez presente no ambiente aquático, a dissociação do cobre de compostos orgânicos transforma esse metal em uma forma mais biodisponivel (Cu^{2+}), o que pode impactar negativamente a exposição e toxicidade do cobre para organismos aquáticos, como peixes, altamente sensíveis a poluição por metais pesados (Kadim and Risjani, 2022; Wang et al., 2020b; Zhang et al., 2022).

Em resposta à contaminação, peixes acumulam quantidades significativas de cobre, sendo esses contaminantes absorvidos tanto maneira direta pelas brânquias quanto pela ingestão de presas ou fontes alimentares contaminadas ao longo do tempo (Brix et al., 2022; de Mendonça Francisco et al., 2023; Johnson et al., 2007; Mirzaei VandKhanghah et al., 2022; Zhen et al., 2022). Através da biomagnificação, o cobre percorre diferentes níveis tróficos até chegar aos seres humanos, levantando preocupações quanto ao seu potencial de toxicidade (Kadim and Risjani, 2022; Mirzaei VandKhanghah et al., 2022; Yang et al., 2021; Zhen et al., 2022).

2.3.2 Estresse oxidativo como mecanismo de toxicidade

O estresse oxidativo é definido em escalas de intensidade, variando desde o estresse oxidativo fisiológico (Euestresse) até desequilíbrios em moléculas oxidantes que comprometem a capacidade antioxidante do organismo (Distresse). Este processo, associado à carga tóxica dos oxidantes pode estar vinculado a condições fisiopatológicas (Lushchak, 2014; Sies, 2019). Sob condições fisiológicas, as ERO atuam na sinalização redox modulando fatores de transcrição. Um exemplo notável é o H_2O_2, metabólito redox ativo cuja influência se estende à morfogênese e diferenciação celular (Gauron et al., 2016; Sies, 2017; Wilson et al., 2018).

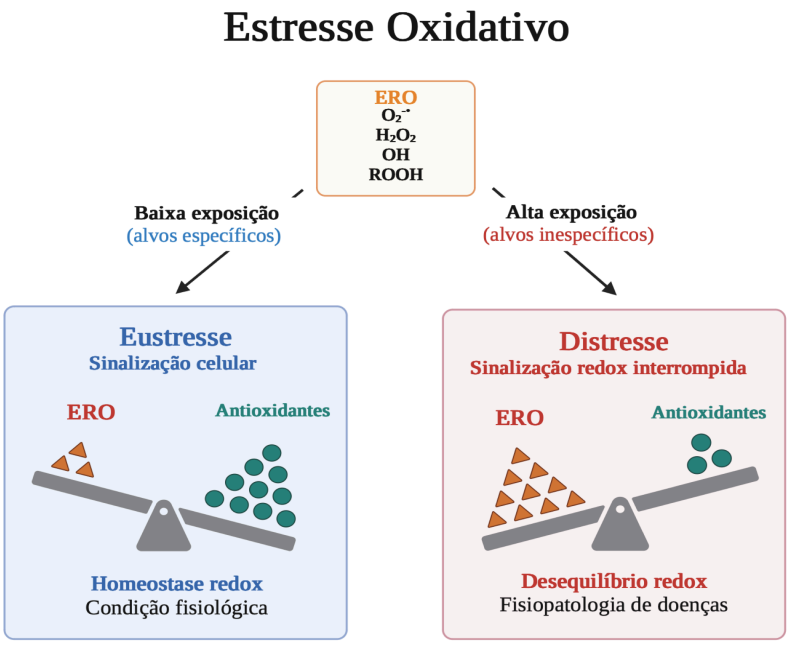


Figura 2 Estresse Oxidativo. Durante a homeostase redox, a manutenção dos níveis de oxidantes e antioxidantes mantêm a estabilidade celular. A exposição a baixos níveis de oxidantes contribui na sinalização redox (Eustresse). Em contrapartida, o aumento na produção de ERO e subsequente exposição elevada a essas oxidantes prejudicam a sinalização celular. O incremento nos níveis de ERO ultrapassa a capacidade antioxidante do organismo, resultando em um desequilíbrio redox (Distresse). Adaptado (Sies, 2019).

Estudos têm demonstrado que o principal mecanismo de toxicidade exercida pelo cobre a indução do estresse oxidativo (Fitzgerald et al., 2017; Halliwell and Gutteridge, 2015; Olivari et al., 2008; Pereira et al., 2016; Zhang et al., 2022). A capacidade do cobre em gerar espécies reativas é associada as suas propriedades redox, ao existir em dois estados de oxidação (Halliwell and Gutteridge, 2015).

Olivari; Hernández; Allende, (2008) constataram que a exposição do peixe-zebra (*Danio rerio*) ao cobre resultou na indução de estresse oxidativo, levando à morte de células ciliadas em neuromastos, além de danos significativos no DNA. A interrupção dessas células compromete o comportamento padrão, conforme descrito em espécies de peixes e anfíbios. Em um ambiente natural, essa perturbação poderia ter impactos significativos na sobrevivência desses organismos, aumentando sua vulnerabilidade à predação. Tal aumento na suscetibilidade é atribuído ao papel crucial dessas células na resposta a estímulos ambientais. (Brix et al., 2022; Hardy et al., 2021; Hernández et al., 2006; Krupa et al., 2021; Linbo et al., 2006)

Além disso, comprometimentos morfológicos, bioquímicos e fisiológicos resultantes da exposição ao cobre, associados a efeitos negativos no desenvolvimento e reprodução de peixes, destacam a importância da investigação abrangente sobre o potencial tóxico do cobre, considerando o comprometimento direto a biota aquática (Acosta et al., 2016; Campana et al.,

2012; Dornelles Zebral et al., 2019; Johnson et al., 2007; Poggere et al., 2023; Wang et al., 2020b; Witeska et al., 2014)

2.4 Mecanismos de defesa antioxidante

Sistema de defesas antioxidantes capacitaram a coexistência em relação à exposição as espécies reativas, ao neutralizar e detoxificar as mesmas, diminuindo os danos causados pelo estresse oxidativo. Esses sistemas incluem enzimas antioxidantes como a superóxido dismutase (SOD), responsável pela conversão do ânion superóxido ($O_2^{-\cdot}$) em peróxido de hidrogênio (H_2O_2), posteriormente convertido em água pela ação da catalase (CAT) ou removido pela glutationa peroxidase (GPx) (Halliwell and Gutteridge, 2015).

O ciclo metabólico da glutationa, caracterizado por reações redox entre sua forma oxidada (GSSG) e reduzida (GSH), desempenha um papel fundamental na neutralização de oxidantes (Halliwell; Gutteridge, 2015). Nesse processo, a glutationa peroxidase (GPx) utiliza a GSH como doador de elétrons, com o grupamento sulfidrila (SH) em seu sítio ativo, constantemente oxidado e reduzido durante o ciclo catalítico. A forma oxidada da glutationa (GSSG) é reciclada para a forma reduzida pela ação da glutationa redutase (GR), regenerando, assim, o ciclo da GSH (Morris et al., 2014) (**Figura 2)**.

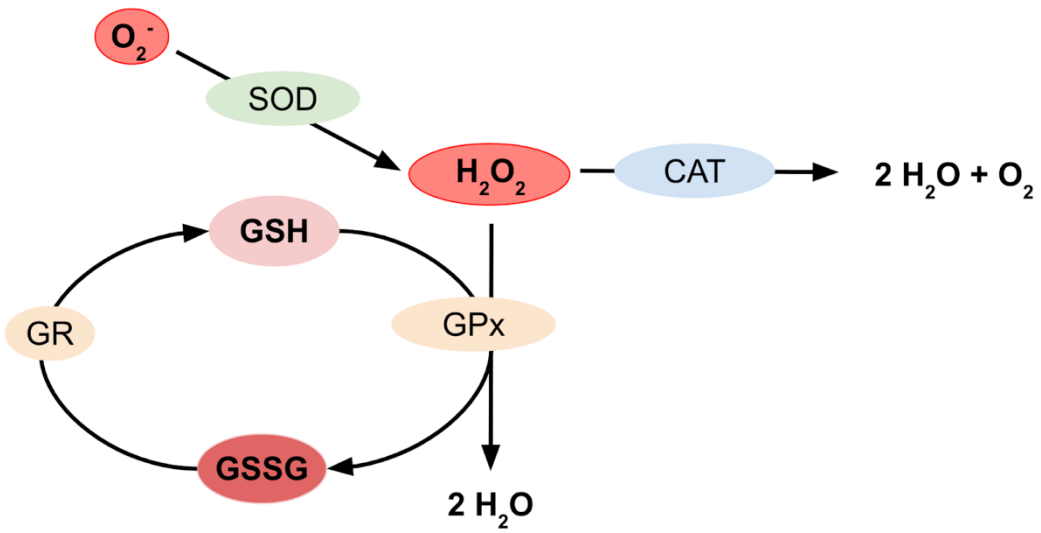


Figura 3 Mecanismos de defesa antioxidante. A SOD catalisa a dismutação do $O_2^{-\cdot}$ em H_2O_2, sendo este convertido pela CAT ou GPx, caracterizados no sistema de defesa antioxidante enzimático. No ciclo metabólico da GSH, a GPx utiliza a GSH como doador de elétrons, permanecendo em sua forma oxidada (GSSG) que pode ser regenerado pela GR. Adaptado de (PENG, C. et al., 2014).

Em estudos de ecotoxicologia, a avaliação da atividade enzimática da CAT, SOD, GPx, assim como dos níveis de GSH, são ferramentas valiosas na análise dos efeitos da exposição a contaminantes ambientais, especialmente em organismos modelo, contribuindo na compreensão do impacto de contaminantes na homeostase redox e efeitos subsequentes (Capriello et al., 2021; Costa-Silva et al., 2018; Glisic et al., 2015; Martins et al., 2023; Paganotto Leandro et al., 2023).

2.5 *Danio rerio* como organismo de modelo em estudos ecotoxicológicos

Com o propósito de avaliar impactos resultante da exposição a diversos contaminantes ambientais, torna-se crucial a utilização de organismos modelo. Nesse contexto, o peixe-zebra (*Danio rerio*), inicialmente introduzido em estudos genéticos, despertou interesse devido ao seu potencial na investigação científica (Streisinger et al., 1981).

Figura 4 Peixe zebra adulto. Fonte: https://l1nk.dev/LbQ7U

O sequenciamento genômico demonstrou que aproximadamente 70% dos genes humanos possui um ortólogo no peixe-zebra, sendo que 84% destes genes, são conhecidos por estarem associados a doenças humanas (Howe et al., 2013). Fato este que contribui com a utilização deste organismo em estudos toxicológicos e comportamentais, permitindo a compreensão em nível genético do respostas biológicas frente a contaminantes ambientais, além possibilitar a inferências pertinentes desses processos em humanos (Capriello et al., 2021; Howe et al., 2013; Zabegalov et al., 2019; Zhang et al., 2015; Zhao et al., 2020).

Entre as particularidades relevantes que o tornam organismo emergente na ciência, destaca-se o tamanho pequeno, medindo de 4 a 5 centímetros na vida adulta (DAMMSKI, *et* al., 2011) alta taxa reprodutiva, desenvolvimento embrionário e fertilização externa (Teame et al., 2019). A transparência do embrião permite o estudo de diferentes estágios de desenvolvi-

mento desde as primeiras horas pós fertilização (hpf), enquanto a transparência da larva possibilita a aplicação de diversas metodologias in vivo com sondas fluorescentes (He et al., 2022; Johnson et al., 2007; Rastogi and Timme-Laragy, 2021).

A morfogênese do peixe-zebra é categorizada com base nas principais mudanças que ocorrem durante cada estágio. Conforme definido pela Organização para a Cooperação e Desenvolvimento Econômico (OCDE), cada fase do desenvolvimento embrionário proporciona pontos chave de interesse toxicológico, contribuindo para a compreensão da toxicidade do composto em estudo (OECD, 2013).

No estágio de Segmentação (10 - 24 hpf), ocorre o início do desenvolvimento dos órgãos, diferenciação celular e desprendimento da cauda, acompanhados pelo aumento do tamanho corporal. Durante o período de Faríngula (24 - 48 hpf), observa-se a organização bilateral, diferenciação das células ciliadas, desenvolvimento da notocorda, faixa dorsal dos melanóforos bem definida, e formação do cérebro, sistema nervoso e circulatório. No estágio de Eclosão (48-72 hpf), destaca-se a abertura parcial da boca indicando nado ativo, e a conclusão da morfogênese, incluindo o desenvolvimento das nadadeiras peitorais, brânquias e mandíbulas. A partir desse momento, a larva manifesta características comportamentais independentes, como atividade natatória, resposta a estímulos sensoriais e comportamento (Kimmel et al., 1995).

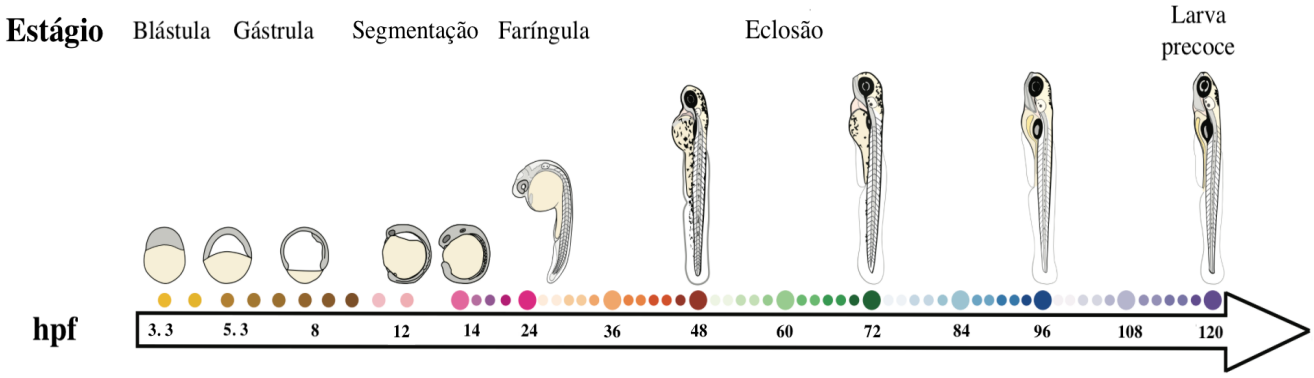


Figura 5 Estágios de desenvolvimento do *D. rerio*. Cronologia dos estágios de desenvolvimento do *D. rerio*, desde blástula até larva precoce, correlacionando cada estágio com horas pós-fertilização.

Estudos recentes utilizam embriões e larvas de peixe-zebra para avaliar a toxicidade de metais pesados, destacando que a toxicidade é notoriamente mais pronunciada durante as fases iniciais de desenvolvimento (Briñez-Gallego et al., 2023; Gouva et al., 2020; Horzmann and Freeman, 2018; Lai et al., 2021; Michiels et al., 2017; Mohammadbakir, 2016; Wang et

al., 2020b). A exposição de estágios embrionários e larvais de peixe-zebra a metais como cobre, Zn, Fe e Mn tem mostrado impactos em parâmetros críticos de sobrevivência, incluindo taxas de eclosão, edema pericárdico e malformações (Gouva et al., 2020; Mohammadbakir, 2016).

Além disso, alterações comportamentais servem como indicadores diretos na presença de contaminantes ambientais e, quando consideradas em conjunto com parâmetros bioquímicos, podem funcionar como ferramentas eficazes de monitoramento para ecossistemas aquáticos (Paganotto Leandro et al., 2021; Wang et al., 2023). Dessa forma, a utilização desse modelo na toxicologia de contaminantes é altamente relevante, considerando seu rápido desenvolvimento, repertório comportamental complexo e respostas bioquímicas robustas (Costa-Silva et al., 2018; Martins et al., 2023; Paganotto Leandro et al., 2021; Taslima et al., 2022; Zhao et al., 2014).

3. JUSTIFICATIVA

Considerando as implicações ambientais decorrentes da contaminação por metais pesados no ambiente, torna-se imperativo avaliar os alvos celulares e bioquímicos afetados diante da exposição a esses elementos A escassez de estudos dedicados à segurança da concentração atual para organismos aquáticos ressalta a urgência de análises mais aprofundadas. A contaminação por cobre não apenas representa um potencial risco para os ecossistemas aquáticos, mas também suscita preocupações relacionadas à saúde humana. Diante desse cenário, é essencial investigar e ampliar os conhecimentos, especialmente no que diz respeito à toxicidade do cobre em concentrações subletais e ambientalmente relevantes.

4. OBJETIVOS

4.1 Objetivos Gerais

Avaliar a toxicidade do cobre em larvas de peixe-zebra (*Danio rerio*).

4.2 Objetivos Específicos

Avaliar a influência de diferentes concentrações de cobre na sobrevivência de larvas de peixe-zebra;

Investigar a toxicidade do cobre parâmetros morfológicos, comportamentais e bioquímicos em larvas de peixe-zebra, submetidas a concentrações inferiores e próximas do limite permitido na água potável pela legislação;

Avaliar marcadores de estresse oxidativo em larvas de peixes-zebra expostos a concentrações inferiores e próximas do limite permitido na água potável pela legislação.

5. ARTIGO

Os resultados que fazem parte desta dissertação estão apresentados sob a forma de artigo. Os itens: Material e Métodos, Resultados, Discussão e Referências Bibliográficas encontram-se no artigo, os quais estão dispostos do mesmo modo o qual foi publicado no periódico Journal of Toxicology and Environmental Health, Part A.

JOURNAL OF TOXICOLOGY AND ENVIRONMENTAL HEALTH, PART A
https://doi.org/10.1080/15287394.2023.2290630

Assessing the disparity: comparative toxicity of Copper in zebrafish larvae exposes alarming consequences of permissible concentrations in Brazil

Maria Vitória Takemura Mariano[a], Luana Paganotto Leandro[a,b], Karen Kich Gomes[a], Ana Beatriz dos Santos[a], Vitor Oliveira de Rosso[a], Alcir Luiz Dafre[c], Marcelo Farina[c], Thaís Posser[a], and Jeferson Luis Franco[a]

[a]Oxidative Stress and Cell Signaling Research Group. Interdisciplinary Center for Biotechnology Research – CIPBIOTEC, Federal University of Pampa, São Gabriel, Brazil; [b]Department of Molecular Biology and Biochemistry, Federal University of Santa Maria, Santa Maria, Brazil; [c]Department of Biochemistry, Center for Biological Sciences, Federal University of Santa Catarina, Santa Catarina, Brazil

ABSTRACT

Copper (Cu) is a naturally occurring metal with essential micronutrient properties. However, this metal might also pose increased adverse environmental and health risks due to industrial and agricultural activities. In Brazil, the maximum allowable concentration of Cu in drinking water is 2 mg/L. Despite this standard, the impact of such concentrations on aquatic organisms remains unexplored. This study aimed to evaluate the toxicity of $CuSO_4$ using larval zebrafish at environmentally relevant concentrations. Zebrafish (*Danio rerio*) larvae at 72 hr post-fertilization (hpf) were exposed to nominal $CuSO_4$ concentrations ranging from 0.16 to 48 mg/L to determine the median lethal concentration (LC_{50}), established at 8.4 mg/L. Subsequently, non-lethal concentrations of 0.16, 0.32, or 1.6 mg/L were selected for assessing $CuSO_4$ -induced toxicity. Morphological parameters, including body length, yolk sac area, and swim bladder area, were adversely affected by $CuSO_4$ exposure, particularly at 1.6 mg/L (3.31 mm ±0.1, 0.192 mm^2 ±0.01, and 0.01 mm^2 ±0.05, respectively). In contrast, the control group exhibited values of 3.62 mm ±0.09, 0.136 mm^2 ±0.013, and 0.3 mm^2 ±0.06, respectively. Behavioral assays demonstrated impairments in escape response and swimming capacity, accompanied by increased levels of reactive oxygen species (ROS) and lipid peroxidation. In addition, decreased levels of non-protein thiols and reduced cellular viability were noted. Data demonstrated that exposure to $CuSO_4$ at similar concentrations as those permitted in Brazil for Cu adversely altered morphological, biochemical, and behavioral endpoints in zebrafish larvae. This study suggests that the permissible Cu concentrations in Brazil need to be reevaluated, given the potential enhanced adverse health risks of exposure to environmental metal contamination.

KEYWORDS

Heavy metals; oxidative stress; development; zebrafish; ecotoxicology; copper

Introduction

Anthropogenic activities release significant amounts of heavy metals into the environment, raising concerns from an ecotoxicological perspective (De Oliveira et al. 2021; Francisco et al. 2023; Naeem et al. 2021; Shahjahan et al. 2022). Heavy metals are considered hazardous environmental pollutants, as these elements exert adverse effects on both aquatic and terrestrial ecosystems when released in high concentrations (Rehman et al. 2018; Štrbac et al. 2015; Vellingiri et al. 2022).

Most ecotoxicological studies focus on the toxicity of non-essential heavy metals such as arsenic (As), cadmium (Cd), and mercury (Hg), which are explicitly toxic at low concentrations (Fasae and Abolaji 2022; Fu and Xi 2020; Yang et al. 2021). However, essential metals including iron (Fe), copper (Cu), manganese (Mn), and zinc (Zn) might also exert adverse effects on biological systems (Balachandran et al. 2020; Chasapis et al. 2020; Galaris, Barbouti, and Pantopoulos 2019; Jomova et al. 2022; Santamaria and Sulsky 2010; Stern et al. 2007; Tsang et al. 2020).

Copper (Cu) is a chemical element classified as a transition metal which occurs in elemental form, in inorganic salts as ions or as organic compounds (Barceloux and Barceloux 1999; Brix, Esbaugh and Grosell 2011). Due to its biocidal properties, Cu compounds are frequently used for water treatment to prevent algae growth (Shen et al. 2019) and in agriculture as a fungicide (Gobbi et al. 2020). For instance, copper sulfate ($CuSO_4$), the

CONTACT Jeferson Luis Franco jefersonfranco@unipampa.edu.br Oxidative Stress and Cell Signaling Research Group. Interdisciplinary Center for Biotechnology Research – CIPBIOTEC, Federal University of Pampa, Campus São Gabriel, São Gabriel, Brazil
Supplemental data for this article can be accessed online at https://doi.org/10.1080/15287394.2023.2290630

first fungicide developed by humans, is known as Bordeaux mixture when mixed with calcium hydroxide (Vazqyez-Blanco et al. 2020). The accidental discovery of $CuSO_4$ effectiveness in controlling grapevine downy mildew (*Plasmopora viticola*) was decisive for initiation of chemical management of plant diseases (Koledenkova et al. 2022; Michereff 2001; Mondello et al. 2022).

Research efforts have been directed toward elucidating the mechanisms underlying $CuSO_4$ - initiated toxicity, particularly in the development of aquatic species. As the primary mechanism of toxicity is oxidative stress, the reactive species generated due to excess Cu is known to result in cellular damage in exposed species, inducing apoptosis, hypoxia, developmental impairments, and damage to the mechanosensory system (Fitzgerald et al. 2019; Olivari, Hernández, and Allende 2008; Zhao et al. 2020). Further, excess $CuSO_4$ affects osmoregulation by acting on sodium (Na) uptake and efflux pathways, potentially related to accumulation of the metal in the gills, as observed in *Oncorhynchus mykiss* by Chowdhury et al. (2016). Implications of $CuSO_4$-induced effects on swim bladder development in *Danio rerio* embryos were linked to downregulated genes in the Wnt signaling pathway, indicating crucial role of this pathway in normal embryo development and emphasizing the intricate molecular mechanisms through which $CuSO_4$ compromises fundamental developmental processes (Xu et al. 2017).

In Brazil, the maximum allowable Cu concentration in drinking water is regulated by Ordinance GM/MS No. 888, issued on May 4, 2021, which has remained unchanged since December 2011 (Ministério da Saúde 2011, 2021). However, environmental contamination by Cu still remains a potential enhanced adverse risk to different ecosystems as well as human health (Choo et al. 2013; Scheiber, Dringen, and Mercer 2013; Stern et al. 2007; Tao et al. 2022).

In aquatic environments, factors such as pH, organic matter, redox potential and dissolved oxygen influence the speciation and bioavailability of Cu ions (Babcsányi et al. 2014; Rader et al. 2019; Tao et al. 2001, 2002). Studies indicate that the majority of Cu in the water column is transported to the sediment, especially under reducing conditions, this metal tends to strongly bind to sulfides and organic matter in sediments. Under these conditions, bioavailability of Cu in the water column is relatively low, which might affect benthic organisms living in or near sediments (Cao et al. 2018; Lewis et al. 2016; Rader et al. 2019; Simpson et al. 2012).

Environments with an acidic pH promote the dissociation of Cu from organic compounds. This dissociation transforms Cu into a more bioavailable form (Cu^{2+}), which might adversely impact aquatic organisms to Cu exposure and subsequent toxicity (Carvalho, Bernusso, and Fernandes 2015; Wang et al. 2015). Once present in the aquatic environment, Cu is incorporated into organisms, including fish, which are highly sensitive to environmental pollution by heavy metals (Kadim and An Risjani 2022; Zhang et al. 2022). These organisms are primarily exposed to contaminants through waterborne exposure and dietary intake, although another potential source of exposure might result from interactions with sediment (Barjhoux et al. 2012; Kumar et al. 2023; Taslima et al. 2022).

In response to contamination, fish accumulate significant amounts of Cu, with these metallic contaminants being absorbed directly through the gills in the case of waterborne exposure. Conversely, dietary exposure may result from ingestion of prey or food sources that are contaminated, often involving aquatic organisms that gradually accumulate contaminants over time (Brix et al. 2022; Francisco et al. 2023; Johnson, Carew, and Sloman 2007; VandKhanghah et al. 2022). Through biomagnification, this metal attains different trophic levels until it reaches humans, raising concerns regarding potential toxicity (Kadim and An Risjani 2022; VandKhanghah et al. 2022; Yang et al. 2021; Zhen et al. 2022)

The literature includes a broad spectrum of studies that utilize zebrafish (*Danio rerio)* embryos and larvae to assess toxicity attributed to heavy metals, with the majority emphasizing that the harmful effects of these elements are significantly more pronounced during the early stages of development (Brinez-Gallego et al. 2023; Gouva et al. 2020; Horzmann and Freeman 2018; Lai, Gong, and Tse 2021; Michiels et al. 2017; Mohammadbackir 2016; Wang et al. 2022).

Exposure to zebrafish embryonic and larval stages to metals such as Cu, Zn, Fe, and Mn was reported to impact critical survival parameters,

including hatching rates, pericardial edema, and malformations (Gouva et al. 2020; Mohammadbackir 2016). Further, embryos exposed to environmentally relevant concentrations of dissolved Fe and Mn in an ecotoxicological context demonstrated critical lethal, sublethal, and teratogenic effects, including non-development of the swim bladder and scoliosis (Peixoto et al. 2022).

Behavioral changes serve as direct indicators in the presence of environmental contaminants, and when considered in conjunction with biochemical parameters, might serve as effective monitoring tools for aquatic ecosystems (Leandro et al. 2021; Ma et al. 2022).

Therefore, given its rapid development, complex behavioral repertoire, and robust biochemical responses, this zebrafish (*Danio rerio*) model may be considered pertinent for investigating the effects of toxic substances (Martins et al. 2023; Rivero-Wendt et al. 2023; Taslima et al. 2022). Accordingly, employing this species becomes interesting, given its capacity to encourage diverse research areas, notably within environmental contaminant toxicology (Leandro et al. 2021; Richendrfer et al. 2012).

Based upon published observations using *D. rerio*, it was of interest to examine whether permissible Cu levels in potable water in Brazil, currently set at 2 mg/L may not provide adequate protection against potential toxicity attributed to exposure to this metal in aquatic organisms. Through a comprehensive analysis of morphological, behavioral, and biochemical parameters, this study aimed to assess the toxicological implications of exposure to environmentally relevant Cu concentrations during the early life stages of zebrafish (*D. rerio*).

Material and methods

Chemicals

Copper (II) sulfate ≥ 99% ($CuSO_4$) (7758-96-7), 2,7-dichlorofluorescein diacetate (DCFH2-DA (2044-85-1), 5,5-dithio-bis (2–119 nitrobenzoic) acid (DTNB) (69-78-3), 7-hydroxy-3 H-phenoxazin-3-one-10-oxide sodium salt (Resazurin sodium salt) (62758-13-8) and monochlorobimane (76421-73-3) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). BODIPY™ 581/591 C11 (217075-36-0) (Lipid Peroxidation Sensor) was purchased from Invitrogen (Waltham, MA). All other chemicals were analytical grade and obtained from commercial suppliers.

Zebrafish maintenance

Zebrafish maintenance and reproduction followed approved experimental protocols (CEUA Unipampa: protocol 031/2022). Adult wild-type *D. rerio* specimens were purchased from a local commercial supplier and were housed in a controlled ZebTec® system water with pH 7, conductivity of 400 µS/cm, temperature of 28°C, and dissolved oxygen levels above 95% saturation. Biological and chemical filters as well ultraviolet light, ensured that this water recirculation system upheld water quality under optimal conditions. A 14/10-hr light/dark photoperiod was provided, and zebrafish were fed a combination of commercial flocked fish food and *Artemia sp*. 4 times a day (Westerfield 2000).

To obtain embryos, males and females (aged 6–12 months) were placed in breeding tanks overnight (in a 2:1 ratio, respectively). The following morning, as reproduction is strongly influenced by photoperiod, spawning was induced by light in the early hours of the day (Lawrence 2007). After a three-hr period, the collected embryos were washed with E3 medium (0.17 mM KCl, 0.33 mM $MgCl_2$, 0.5 mM NaCl, and 0.1% methylene blue) (Cerda et al. 2006). This collection conducted after three hr is directly related to hr post-fertilization (hpf) and marks the onset of embryonic development, with the stage determined according to Kimmel et al. (1995). Subsequently, larvae were placed in a Petri dish with E3 medium and incubated until they reached 72 hpf. At this stage, zebrafish larvae were subject to $CuSO_4$ exposure and assessments conducted at 96 hpf. Figure 1a provides a visual representation of the experimental design.

Dose response curve

A stock of $CuSO_4$ with a concentration of 3192 mg/L was prepared using distilled water, and the working solutions were diluted in ZebTec® system water. To establish the dose-response curve, a group of 24 larvae at 72 hpf were exposed to nominal concentrations of $CuSO_4$ ranging from 0.16 to 48 mg/L for 24 h ($n = 3$ replicates *per* concentration) in a 12-well

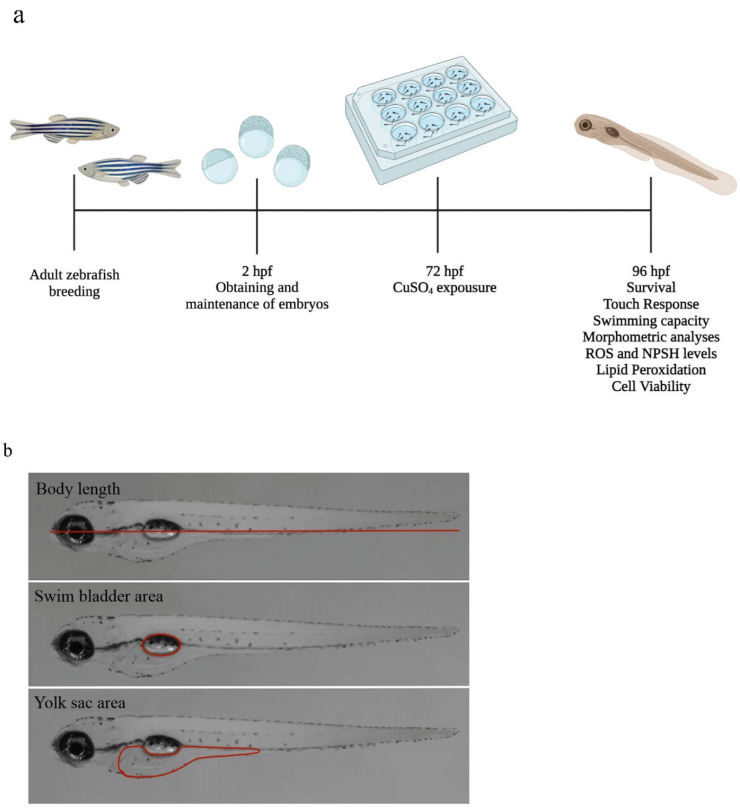


FIGURE 1. Experimental design (a) After the zebrafish larvae reproduced, embryos were collected, sterilized and placed in an incubator until the 72 hpf stage for Cu exposure was reached. To assess Cu toxicity, the larvae were evaluated at 96 hpf. (b) representation of morphologic measurements in zebrafish larvae at 96 hpf. The red line represents the standardized measurement guidelines for each evaluated parameter. The scale bar corresponds to 0.5 mm, serving as a visual reference for the measured parameters. All measurements were performed using image J software.

plate with 6 larvae *per* well in a final volume of 3 mL. As a control group, larvae at the same developmental stage were exposed only to the ZebTec® system water. The LC_{50} was calculated through the dose-response curve to ascertain the concentration at which 50% of the larvae exhibited lethality.

Acute exposure to cu

To ensure low mortality rates and environmentally relevant concentrations that were below the legally permitted limits, the nominal concentrations 0.16, 0.32, and 1.6 mg/L of Cu were selected for further analyses.

Zebrafish larvae 72 hpf were exposed to Cu for 24 hr at nominal concentrations of 0.16, 0.32 or 1.6 mg/L in a 12-well plate (6 larvae *per* well in a final volume of 3 ml) as described by Martins et al (2023) with modifications. The larvae were then kept in an incubator until 96 hpf for further investigations to determine potential metallic effects.

Morphological assessments

Morphological changes were assessed through morphometric measurements in yolk sac area, body length, and swim bladder area in 10 zebrafish larvae at 96 hpf ($n = 3$ replicates per concentration) (Miao et al. 2022). Images of the larvae were captured using a Carl Zeiss Stemi 2000C stereomicroscope equipped with a Moticam 2000 digital camera. To facilitate image capture and limit larval

movement, temporary immobilization was achieved through cold treatment, followed by fixation with 3% methylcellulose on microscope slides. The captured images were then analyzed using Image J software as described by Leandro et al. (2023). As illustrated in Figure 1b, zebrafish larvae total body length was measured from the head tip to tail end. The yolk sac development was measured by demarcating the region encompassing the yolk sac and its extension, while swim bladder assessment involved the delimitation of its complete contour (Miao et al. 2022).

Behavioral responses

Following the exposure period, the touch response and swimming capacity tests were conducted on 15 randomly selected larvae ($n = 3$ replicates *per* concentration) at 96 hpf. Individual larvae were positioned at the center of a Petri dish containing 20 ml ZebTec® system water. After a 2-min acclimatization period, a touch to the larva's tail area was initiated using a 6 mm needle attached to BD Ultrafine™ entomological forceps, acting as the stimulus. The number of stimuli required to elicit the first displacement and the larva's ability to perform an escape response by swimming toward the periphery (swim response) were recorded (Colwill and Creton 2011).

Lipid peroxidation and ROS steady-state levels

The measurement of ROS (reactive oxygen species) levels was conducted using the oxidation of the fluorescent dye 2'-7'-dichlorofluorescein diacetate (DCFH2-DA) (LeBel, Ischiropoulos, and Bondy 1992) through two different methodologies: *ex vivo* homogenate preparation and representative images *in vivo*.

The *ex vivo* method involved homogenizing a total of 15 zebrafish larvae ($n = 4$ replicates *per* concentration) in 100 µl 20 mM HEPES buffer at pH 7 using a manual homogenizer. The homogenate was then centrifuged at 1000 g and 4°C for 10 min, and 10 µl supernatant was separated from each sample for protein measurement. Subsequently, 20 µl supernatant was incubated in a 96-well plate with 274 µl 20 mM HEPES buffer at pH 7 with 6 µl DCFH2-DA (Martins et al. 2023). After a 40-min incubation, fluorescence was read at 488/530 nm excitation/emission wavelengths. The resulting values were normalized using the protein concentration in the samples, and results expressed as a % of control. To obtain representative *in vivo* images of ROS levels, 5 larvae *per* concentration were incubated with 1 ml ZebTec® system water containing 10 µl DCFH2-DA according to Costa-Silva et al. (2018) with modifications. The larvae were incubated in the dark at a temperature of 28°C for 1 hr. To prevent background staining of the fluorescent dye, larvae were temporarily immobilized by cold treatment and washed with ZebTec® system water three times. Subsequently, larvae were removed from the solution and fixed on microscopy slides using 3% methylcellulose. Images were acquired using an Olympus B×63fluorescence microscope with the BW filter at 40× magnification and an exposure of 357 ms.

Lipid peroxidation, quantified through lipid ROS levels, was assessed using the fluorescent dye C-11 BODIPY (581/591) following the protocol of He et al. (2022) with some modifications. Groups of 5 larvae ($n = 3$ replicate *per* concentration) were incubated with 1 mL ZebTec® system water containing 1 µl 1 mM C-11 BODIPY (581/591). The larvae were incubated in the dark at ambient temperature for 30 min. As a positive control, larvae at the same stage were incubated with 13 µl for 30 min to *tert*-butyl hydroperoxide (t-BOOH) 1 mM. This compound is well recognized for inducing lipid peroxidation (Boix et al. 2020). To prevent background staining, larvae were temporarily immobilized using cold treatment and washed three times with ZebTec® system water. Subsequently, larvae were removed from the solution and fixed on microscopy slides using 3% methylcellulose. Images were captured using an Olympus B×63 fluorescence microscope with the CY3 filter for reduced BODIPY (R-BODIPY) at an exposure of 10 ms, and the BW filter for oxidized BODIPY (O-BODIPY) at an exposure of 200 ms, all at 40× magnification. The relative fluorescence of the images was quantified using ImageJ software by selecting the entire body of the zebrafish larvae as the region of interest. The Corrected Total Cell Fluorescence (CTCF) formula, CTCF = Integrated Density – (Area of selected cell × Mean Fluorescence of background readings), was employed to correct the total fluorescence signal by accounting for the background (El-Sharkawey 2016).

Non-protein thiols levels

The levels of non-protein thiol (NPSH) were determined using a modified version of the assay described by Ellman (1959). A total of 30 zebrafish larvae ($n = 4$ replicates *per* concentration) were homogenized in 200 µl Tris/HCl buffer at pH 8 using a manual homogenizer. From each sample, 10 µl homogenate was separated for protein measurement. Subsequently, perchloric acid 0.5 M was added, and the samples were then centrifuged at 9500 g for 5 min at 4°C. For the assay, 50 µl supernatant was incubated in a 96-well plate containing 190 µl Tris/HCl buffer pH 8 and 10 µl 5 mM DTNB (5,5'-dithiobis-(2-nitrobenzoic acid). The plate was kept in the dark for 15 min at room temperature. Following the incubation period, absorbance was read at 412 nm using a plate reader (Perkin Elmer Enspire 2300). The results were expressed as micromoles of thiol groups *per* milligram of protein (µmol/mg protein).

Glutatione levels in vivo

Glutathione (GSH) levels were assessed using the fluorescent probe monochlorobimane (MCB) described by Rastogi and Timme-Laragy (2021) with modifications. The fluorescence probe interacts with GSH sulfhydryl group altering spectral characteristics that may be visualized through fluorescence microscopy, enabling the representative imaging of GSH levels in the larvae (Glisic et al. 2015). In this assay, 5 larvae *per* concentration were incubated with 1 ml ZebTec® system water containing 1 µl 200 mM MCB. The larvae were incubated for 30 min at 28.5°C in the dark. To prevent background staining, larvae were briefly exposed to cold, washed three times with ZebTec® system water, removed from the solution and then fixed on microscopic slides using 3% methylcellulose. Imaging was performed using an Olympus B×63 fluorescence microscope equipped with a DAPI filter at 40× magnification, with an exposure time of 588 ms.

Cell viability in vivo

Cell viability in zebrafish larvae was assessed using the resazurin assay with modifications (Williams and Renquist 2016). In this assay, 10 larvae *per* well were plated on a 96-well plate containing ZebTec® system water and the CellTiter-Blue® Cell Viability Assay, resulting in a final concentration of 0.08 µM of reagent in the plate ($n = 3$ replicates *per* concentration). The resazurin assay measures the ability of viable mitochondria to reduce resazurin to a fluorescent product called resorufin, which may be quantified measuring fluorescence intensity, as previously described (Reid, D'Aquila, and Biga 2018). Concurrently, the survival of larvae was monitored throughout the entire duration of the assay. The obtained values were normalized based upon protein concentration in the samples, and results expressed as a % relative to control.

Protein quantification

Protein levels in the samples were quantified using the Bradford method (Bradford 1976) which involves the binding of Comassie Brillant Blue G-250 to proteins, leading to a detectable color change measurable through spectrophotometry. Protein standard curves were prepared using known concentrations of bovine serum albumin (BSA). The samples were diluted and pipetted into 96-well plates. Then, a solution containing Coomassie Brilliant Blue G-250 was added to both the standard curve and sample wells. After incubating for 10 min in the dark, absorbance was read at 595 nm using a plate reader (Perkin Elmer Enspire 2300). Protein concentrations in the samples were determined by correlating absorbance values with the standard curve ($R^2 > 0.95$).

Statistical analysis

Normality tests including the Shapiro-Wilk and Bartlett's, were conducted to assess distribution and equality of variances, respectively. For non-parametric data Kruskal-Wallis's test followed by Dunn's posttest was applied and results were expressed as median ± interquartile range. Parametric data were analyzed using One-Way ANOVA followed by Dunnett's posttest, and results expressed as mean ± standard error (SEM). Statistical significance was determined at $p < 0.05$. Statistical analyses were conducted using GraphPad Prism software, version 8.

Results

Acute exposure to cu effect on larval survival, development and locomotor behavior

A 24 hr mortality curve using $CuSO_4$ ranging from 0.16 to 48 mg/L was constructed. Our findings showed that concentrations of 0.16, 0.32, 0.8, or 1.6 mg/L did not significantly affect larval survival compared to controls. However, exposure to concentrations of 3.2 mg/L and higher resulted in a concentration-dependent decrease in the survival rate (Figure 2a). Data obtained from the mortality curve demonstrated 24 hr lethal median concentration (LC_{50}) for larvae to be approximately 8.4 mg/L of $CuSO_4$ (Figure 2b). To explore sublethal effects, further experiments were performed using non-lethal concentrations (0.16–1.6 mg/L $CuSO_4$).

Exposure to $CuSO_4$ exerted a marked adverse effect on larval development that was proportional to the concentration. The larvae displayed a significant decrease in body length and swim bladder area accompanied by a rise in yolk sac area. Quantitative analysis of the images was conducted using ImageJ software and results were presented through graphical representation (Figure 3a-c). The delayed development induced by $CuSO_4$ exposure was illustrated through representative images (Figure 3d).

Exposure to $CuSO_4$ across all tested concentrations, caused the larvae to require additional stimuli to initiate the escape response (Figure 4a). Simultaneously, swimming capacity was impaired as embryos did not exhibit the standard swim toward the periphery compared to the control (Figure 4b).

Impact of acute cu exposure on oxidative parameters and cell viability

Utilizing markers of oxidative stress, including ROS steady-state levels, lipid peroxidation, and decrease in glutathione (GSH), acute $CuSO_4$ exposure was examined as a model of the redox balance in zebrafish larvae. First, larvae exposed to $CuSO_4$ displayed a dose-dependent increase in ROS steady-state levels, as detected in the *ex vivo* assay (Figure 5a). The representative images captured *in vivo* (Figure 5b) further displayed the intensified fluorescence intensity of DCFDA. Quantification of the images using the BODIPY fluorescent probe revealed a significant rise in lipid peroxides, at all concentrations tested (0.16, 0.32, and 1.6 mg/L). This elevation in lipid peroxides was evident from the enhanced shift from red to green fluorescence, indicating the presence of oxidized BODIPY (Figure 5c,d).

Further, treatment with $CuSO_4$ resulted in significant decrease in NPSH levels (Figure 6a). In addition, representative images obtained using the MCB probe for thiol groups confirmed the NPSH results, providing visual evidence of diminished GSH levels *in vivo* (Figure 6b).

The resazurin assay demonstrated a dose-dependent decrease in cell viability in larvae exposed

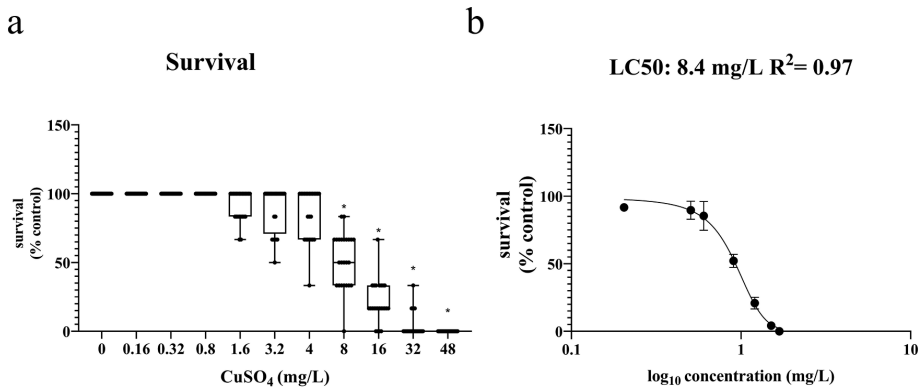


FIGURE 2. Measurement of survival (a) the survival curve of zebrafish larvae at 96 hpf (24 larvae per group, $n = 3$ replicates *per* concentration) (b) the LC_{50} was calculated by the application of a nonlinear fit function after the transformation of values and normalization.

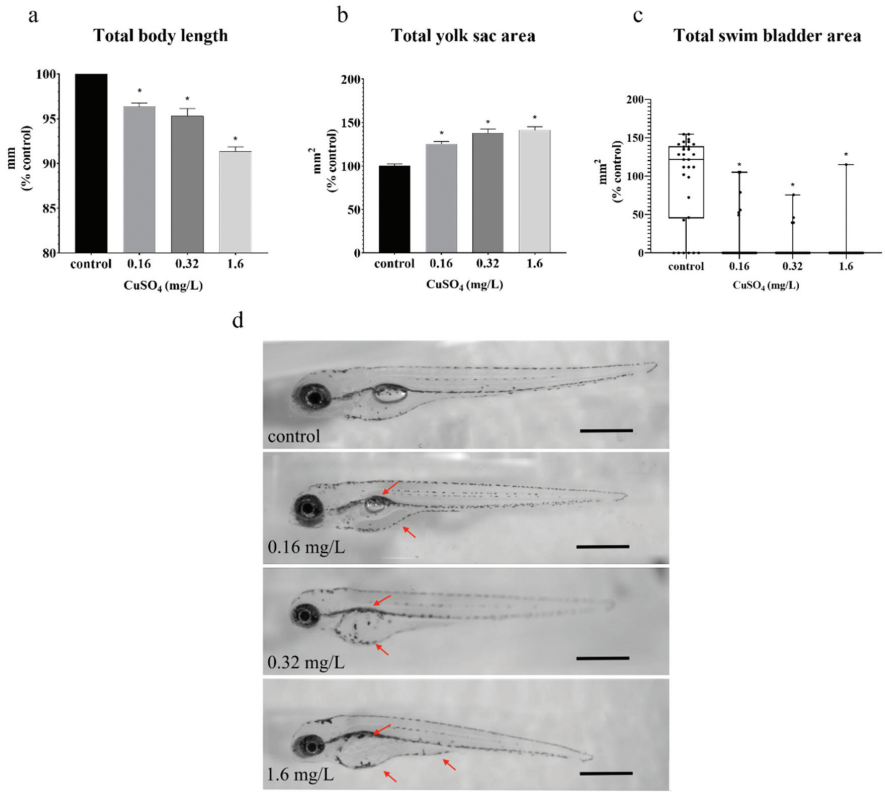


FIGURE 3. Morphological measurements (a) total body length (b) total yolk sac area. Scale bar: 0.5 mm. Parametric data were presented as mean ± standard error (SEM) and analyzed using one-way ANOVA followed by Dunnett's post-hoc test (*$p \leq 0.05$) (c) total swim bladder area. Scale bar: 0.05 mm. Non-parametric data were expressed as median ± interquartile range and analyzed using Kruskal-Wallis and Dunn's multiple comparison test, considered statistically significant at *$p \leq 0.05$.

across all tested concentrations, indicating the detrimental effects of $CuSO_4$ on the overall cell viability of zebrafish larvae (Figure 6c).

Discussion

The current permissible concentration of Cu in Brazilian potable water is set at 2 mg/L (Ministério da Saúde 2021). This study specifically focused on assessing the toxicity of $CuSO_4$ in zebrafish larvae, examining concentrations within the range of the current permissible limits outlined by Brazilian regulations. Given the documented detrimental effects of Cu-induced toxicity on the development of aquatic organisms (Johnson, Carew, and Sloman 2007; Weir et al. 2019), it becomes imperative to establish sensitive tools for effective biomonitoring of this metal in aquatic environments.

Our findings provide compelling evidence regarding the potential toxic effects of exposure to $CuSO_4$ on zebrafish larvae, even below the current permissible concentrations of this metal in potable water. Notably, environmentally relevant concentrations of Cu demonstrated significant adverse effects on various aspects of zebrafish larval development, including survival, morphological abnormalities, behavioral impairments, and biochemical alterations associated with oxidative stress.

The survival of zebrafish larvae exposed to $CuSO_4$ was significantly impaired at concentrations that exceeded environmentally relevant limits (3.2–48 mg/L). However, it is crucial to emphasize that

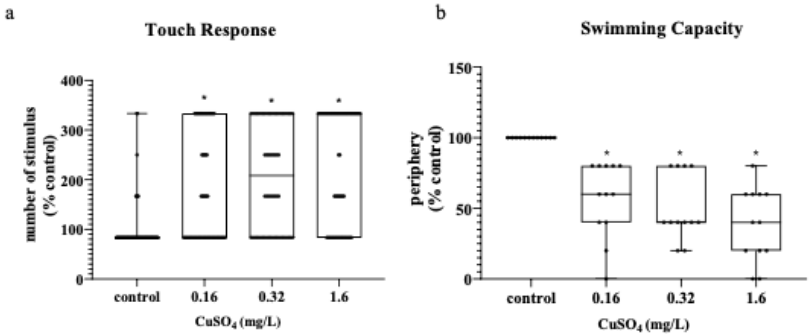


FIGURE 4. Behavioral tests (a) touch stimulus. Zebrafish larvae exposed to Cu exhibited an increased threshold for escape response, requiring a higher number of stimuli (b) swim response. Cu-exposed zebrafish larvae show impaired swimming responses, failing to display the typical movement toward the periphery. Non-parametric data were presented as median ± interquartile range and analyzed using Kruskal-Wallis and Dunn's multiple comparison test, considered statistically significant when $*p \leq 0.05$.

other assessed parameters, though affected at lower levels (0.16, 0.32 and 1.6 mg/L) were not exempt from the sublethal impacts initiated by this metal. Even at lower levels, these sublethal effects might exert significant implications for the health of aquatic ecosystems. Consequently, the consideration of these sublethal impacts is of utmost importance in managing $CuSO_4$ exposure and in establishing more comprehensive and protective environmental regulations, as long-term exposure to sublethal environmental toxicants may pose enhanced ecological risks to aquatic species (Liao et al. 2023)

Our calculations of the LC_{50} of 8.4 mg/L were obtained from a logarithmic dependence. This finding supports our hypothesis that Cu-induced toxicity exhibits dose-dependent responses, in agreement with previous studies that linked exposure duration and $CuSO_4$ levels on zebrafish larvae survival (d'Alençon et al. 2010; Hernandez et al. 2011; Nguyen et al. 2020).

It is widely recognized that heavy metals exert a negative impact on embryonic and larval development of fish, due to their rapid development in these stages, making them more susceptible to these pollutants (Taslima et al. 2022). The morphometric evaluation, centered on body length (mm), yolk sac area (mm^2) and the beginning of swim bladder formation (mm^2), demonstrated that it was impaired at all $CuSO_4$ concentrations tested. Structural deformities of the yolk sac, known as yolk sac edema, are often observed in embryonic and larval fish following exposure to toxicants (Rastogi and Timme-Laragy 2021; Taslima et al. 2022; Witeska et al. 2014). However, such deformities were not found in our current investigation.

Interestingly, our results suggest that the increased yolk sac area accompanied by diminished body size occurring in a dose-dependent manner, might be associated with reduced yolk sac consumption and disrupted adequate nutrition for dynamic growth during this stage (Sant and Timme-Laragy 2018). This may indicate a potential delay in larval development, as the standard length of larvae at 96 hpf is typically approximately 3.5 to 4 mm (Johnson, Carew, and Sloman 2007; Wilson 2012). In our study, the average body length of larvae exposed to 0.16 mg/L and 0.32 mg/L of $CuSO_4$ was 3.49 mm ±0.08 and 3.45 mm ±0.16, respectively. The most notable decrease was observed in the 1.6 mg/L $CuSO_4$, with an average body length of 3.31 mm ±0.1, compared to control group with 3.62 mm ±0.09.

The findings of our study are consistent with Johnson, Carew, and Sloman's 2007, indicating the significance of $CuSO_4$ exposure to detrimental effects on early-life. The reduction in zebrafish larvae length and increase in yolk sac area when exposed to lower $CuSO_4$ concentrations (0.09 mg/L) than those used in this study (0.16, 0.32, and 1.6 mg/L) emphasize the sensitivity of these organisms to Cu-mediated toxicity. Notably, despite the differences in larval age between their research (1 hpf) and our present study

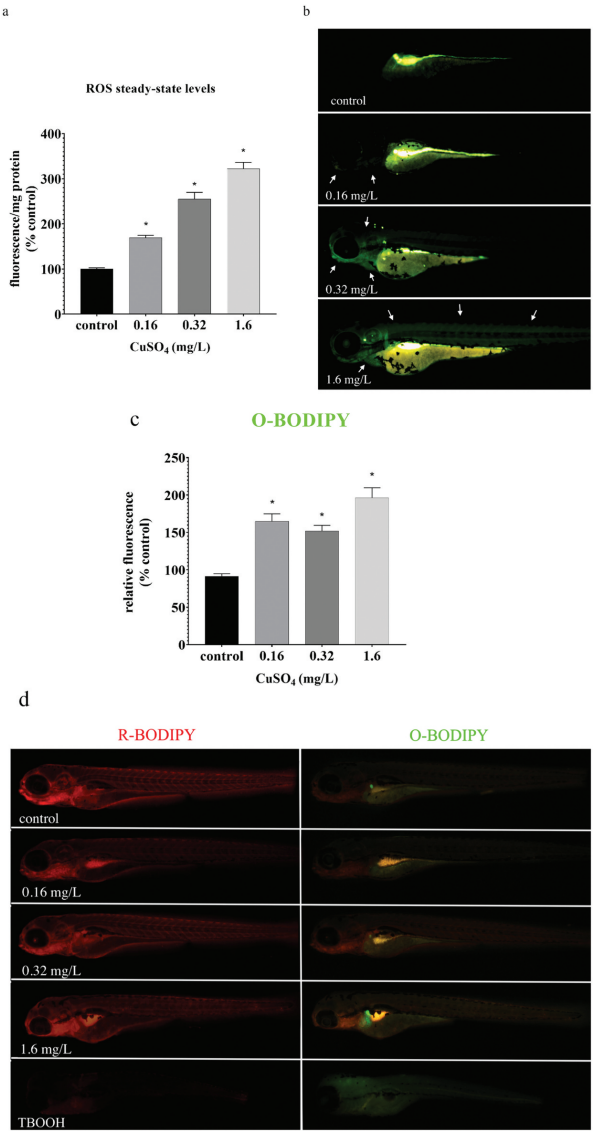


FIGURE 5. ROS production and lipid peroxidation (a) ROS quantification in larval homogenates (*ex vivo*) (b) representative ROS fluorescence images (*in vivo)*, with arrows indicating significant differences. The control group exhibits baseline fluorescence levels, while more pronounced changes in fluorescence are evident at 0.32 mg/L, primarily in the head and along the body. The highest concentration (1.6 mg/L) results in intensified fluorescence, spreading from the head throughout the entire body. Parametric data were presented as mean ± standard error (SEM) and analyzed using one-way ANOVA followed by Dunnett's post-hoc test, considered statistically significant at $*p \leq 0.05$ (c) quantification of green fluorescence intensity (O-BODIPY) indicating lipid peroxidation levels. Parametric data were presented as mean ± standard error (SEM) and analyzed using one-way ANOVA followed by Dunnett's post-hoc test, considered significant at $*p \leq 0.05$ (d) representative images display C11-BODIPY fluorescence, distinguishing between reduced BODIPY (R-BODIPY) and oxidized BODIPY (O-BODIPY). Red fluorescence represents R-BODIPY, with higher intensity in the control group, indicating lipid integrity. The increase in green fluorescence signifies elevated O-BODIPY levels in Cu-exposed larvae, resulting from increased binding of the probe to peroxidized lipids. Tert-butyl hydroperoxide (t-BOOH) was used as a positive control to confirm the assay's sensitivity.

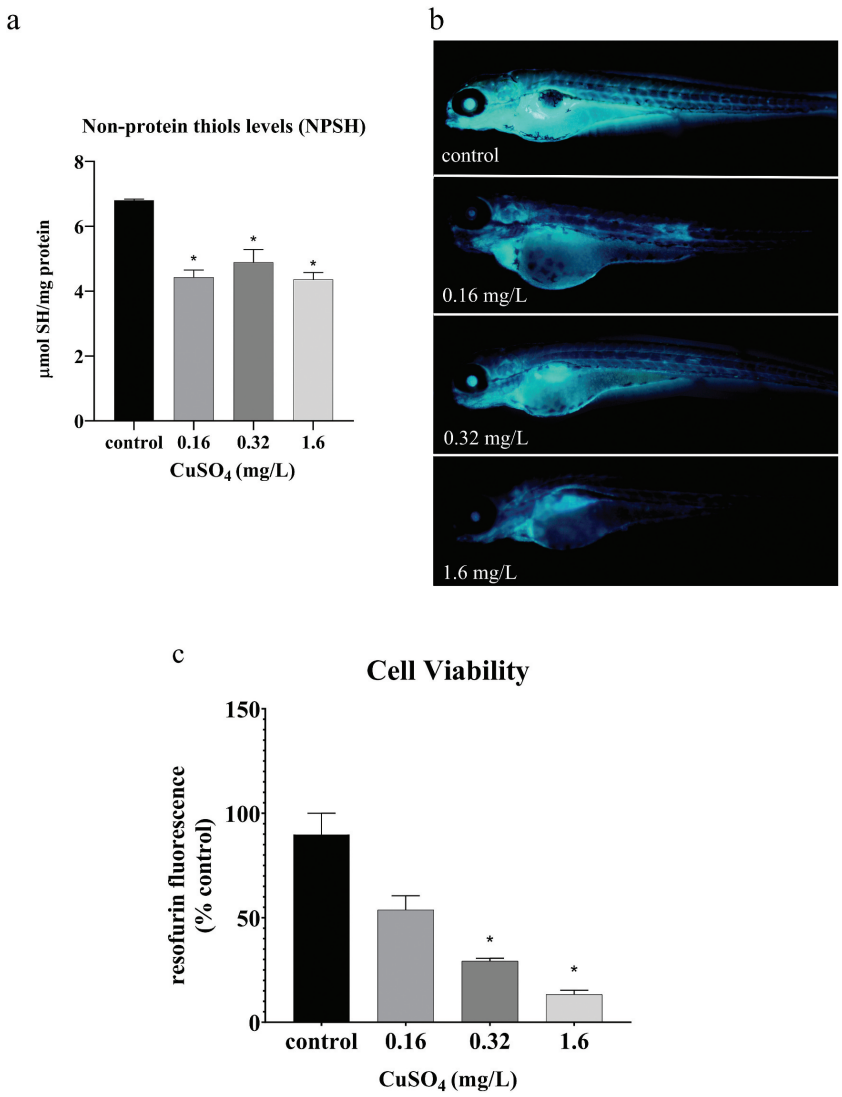


FIGURE 6. Thiols levels and cell viability (a) NPSH quantification of homogenates incubated with DTNB (*ex vivo* assay). Parametric data are expressed as µmol SH/mg protein, presented as mean ± standard error (SEM), and analyzed using one-way ANOVA followed by Dunnett's post-hoc test, considered significant at $*p \leq 0.05$. (b) representative MCB fluorescence image in Cu-exposed larvae, showing distinct fluorescence patterns. Control group larvae exhibit robust and widespread fluorescence, indicating abundant GSH levels. In contrast, Cu-exposed larvae display significantly reduced fluorescence, suggesting lowered GSH levels (c) Cell viability through the resazurin assay. The fluorescence observed in Cu-exposed larvae is reduced, suggesting a decrease in cell viability in comparison to the control group. Parametric data were presented as mean ± standard error (SEM) and analyzed using one-way ANOVA followed by Dunnett's post-hoc test, considered statistically significant at $*p \leq 0.05$.

(96 hpf), both investigations revealed an impact on body length at similar $CuSO_4$ concentrations. In a similar study, Cu exposure at a concentration of 0.06 mg/L affected zebrafish larvae from 72 to 96 hpf resulting in a detrimental impact on body length, as it was also reduced, emphasizing the harmful effect of Cu on larval growth over an acute exposure (Acosta et al. 2016).

Another important parameter during this development stage is the inflation of the swim bladder, which enables larvae to gradually initiate active swimming (Kimmel et al. 1995). Development and inflation of the swim bladder might be influenced by a combination of environmental and nutritional factors. The canonical Wnt pathway has been identified as a key player in shaping the posterior chamber of the swim bladder (Yin et al. 2011).

A significant decrease in swim bladder development was observed in all larvae exposed to Cu when compared to controls. Xu et al. (2017) observed the downregulated expression of genes associated with the Wnt pathway in zebrafish embryos exposed to Cu, inhibiting the specification and formation of the swim bladder in larvae. This resulted in larvae with small swim bladders and, in some cases, complete absence of swim bladder development in Cu-treated embryos, which might explain the pattern observed in our current study. This effect has broader implications, as it extends to other cyprinid species such as *Leuciscus idus* L. and *Tinca tinca* L (Sikorska and Wolnicki 2010; Witeska et al. 2014),. which Cu exposure during the embryonic-larval phase disrupts development, exemplified by the non-development of the swim bladder.

Previous studies reported that exposure to Cu alters the behavioral profile of larvae (Acosta et al. 2015; Cruz et al. 2017; Johnson, Carew, and Sloman 2007; Santos et al. 2021; Sonnack et al. 2015) It is noteworthy that larvae exposed to Cu required a greater number of stimuli to exhibit an escape response. Further, zebrafish larvae displayed a decrease in swimming capacity, failing to exhibit the typical escape swim toward the periphery seen in controls. This locomotor deficit may be associated with the toxicity attributed to Cu which specifically targets sensory systems.

Although mechanosensory systems were not examined in this study, it is well established that Cu exerts ototoxic effects, specifically targeting neuromasts (Hardy et al. 2021; Newton et al. 2023) Disruption of these cells may exert adverse behavioral consequences, as noted in various fish and amphibian species (Brix et al. 2022; Hardy et al. 2021; Hernández et al. 2006; Krupa et al. 2021; Linbo et al. 2006; Olivari, Hernández, and Allende 2008). Similarly to our study, Hernández et al. (2006) noted the same concentration (0.16 mg/L) $CuSO_4$ utilizing zebrafish larvae at 76 hpf for two hr resulted in complete loss of ciliated cells in neuromasts. Sonnack et al (2015) found that exposure to 0.024 mg/L $CuSO_4$ in zebrafish embryos until larval stage significantly reduced normal escape response associated with damage to motor neurons and neuromasts. These findings indicate an association between the lateral line system and behavioral changes attributed to Cu exposure. Further at lower Cu concentrations, irregular swimming patterns were observed, while the highest metal concentration produced consecutive tail contractions and a reclined posture during the test. This behavior may be associated with Cu's ability to induce locomotor dysfunction in zebrafish during the embryonic-larval phase as documented by Zhang et al. (2015).

Reactive oxygen species (ROS) steady-state levels refer to the balance or equilibrium state of ROS within a biological system (Smith and Murphy 2011). Although ROS are natural by-products of cellular metabolism and play important roles in various physiological processes (Finkel 2011) excessive accumulation or imbalance of ROS leads to oxidative stress, producing damage to cellular components (Halliwell and Gutteridge 2015; Sies, Berndt and Jones 2017). Several investigators demonstrated that exposure to Cu induced accumulation of ROS concentrations in fish, by modifying the levels of biochemical biomarkers, encompassing both enzymatic and non-enzymatic antioxidants (Kumar et al. 2023; Pereira, Campos and Bogo 2016; Taslima et al. 2022). It is established that this observation is attributed to the prooxidant properties of Cu, associated with its capacity to transition between different redox states and engage in redox reactions, resulting in enhanced ROS formation (Halliwell and Gutteridge 2015). Consequently, ROS accumulation leads to oxidation of biomolecules, such as lipids, resulting in lipid peroxidation as demonstrated in *Carassius auratus* (Kong et al. 2013) and *Cyprinus carpio* var. Jian (Jiang et al. 2014).

The pro-oxidant capacity of Cu might also be associated with strong affinity for thiol groups, particularly glutathione (GSH), a vital intracellular peptide in antioxidant defense crucial to maintaining ROS balance, which may be assessed by measuring NPSH levels (Brix et al. 2022; Ellman 1959; Gaetke 2003; Halliwell and Gutteridge 2015). Consistent with our findings, when evaluating biochemical markers of oxidative stress, an increase in steady-state levels of ROS was detected, accompanied by enhanced lipid peroxidation and a reduction in NPSH levels in response to Cu exposure. Interestingly, in our experiments, exposure to 0.32 mg/L increased NPSH levels compared to either 0.16 and 1.6 mg/L $CuSO_4$, but not in controls. This finding suggests an attempt to restore redox balance or involvement of other antioxidant defense components.

The contribution of oxidative stress to the decrease in cell viability becomes evident when an excess of reactive oxygen species (ROS) directly and harmfully damages biological structures, eventually leading to cellular dysfunction (Ayala, Muñoz, and Argüelles 2014; Kowalczyk et al. 2021; Olivari, Hernández, and Allende 2008). Consistent with our findings, exposure to $CuSO_4$ resulted in a dose-dependent decrease in cell viability, as evidenced by the resazurin assay, suggesting a potential correlation between oxidative stress and mitochondrial metabolism. Excessive ROS is known to disrupt mitochondrial function, producing detrimental effects on the organism, ultimately leading to impairment of mitochondrial metabolic activity. This is supported by the assay, which relies on the conversion of resazurin to resorufin via NADH by mitochondrial enzymes (Präbst et al. 2017). Our study confirms the involvement of oxidative stress in Cu-initiated toxicity in zebrafish larvae. It is important to note that Cu, even at concentrations that do not induce high mortality rates, might compromise essential biochemical and behavioral parameters and delay development of exposed organisms (Acosta et al. 2016; Hernández et al. 2006; Johnson, Carew, and Sloman 2007; Olivari, Hernández, and Allende 2008; Wang et al. 2020; Weir et al. 2019). Therefore, environmental monitoring is crucial, as the presence of Cu in the environment severely impairs critical developmental parameters in zebrafish. Consequently, Cu may pose threats to other species in the ecosystem, particularly aquatic organisms, which are also susceptible to heavy metal pollution (Brix et al. 2022; Cremazy et al. 2022; Mason and Parrott 2022).

It is important to acknowledge the limitations of our study. First, our experiments were conducted under controlled lab conditions, and it is necessary to validate our findings in natural aquatic environments to better understand the ecological implications of Cu-initiated toxicity. In addition, this study focused on specific developmental and biochemical parameters, but further investigations need to explore additional endpoints to provide a comprehensive understanding of the effects of Cu on zebrafish larvae. Further, our study examined the influence of Cu exposure at specific concentrations and time points, thus it would be valuable to investigate a broader range of concentrations and exposure durations to establish dose-response relationships and understand potential sublethal effects.

Conclusions

In summary, our study established that exposure to Cu at concentrations below those permitted by legislation, while not producing excessive mortality rates, resulted in significant sublethal effects on zebrafish larvae. Developmental delays, compromised behaviors, and increased oxidative stress, were found emphasizing the vulnerability of early life stages to the adverse effects of Cu-induced toxicity. This finding indicates that the established maximum limits are not deemed safe for aquatic organisms, considering the affected parameters. Data observed underscore the imperative need for rigorous environmental oversight to reassess the maximum allowable concentration, advocating for prudent management strategies to mitigate metallic contamination in aquatic ecosystems. This is particularly crucial given the observed impacts on key physiological and behavioral indicators, emphasizing the potential enhanced ecological risks associated with current Cu concentration standards.

Acknowledgments

We thank professors Analia Del Valle Garnero and Ricardo José Gunski for providing assistance with fluorescence microscopy.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This work was financially supported by CAPES (001); CNPq (405426/2021-6, 406442/2023-3). JLF and TP are CNPq research fellows (314565/2020-5 and 307316/2022-0, respectively).

ORCID

Marcelo Farina http://orcid.org/0000-0001-8255-8515

Data availability statement

The authors report there are no competing interests to declare. Data availability is not applicable for this study.

References

Acosta, D. D. S., N. M. Danielle, S. Altenhofen, M. D. Luzardo, P. G. Costa, A. Bianchini, C. D. Bonan, R. S. da Silva, and A. L. Dafre. 2016. "Copper at Low Levels Impairs Memory of Adult Zebrafish (*Danio rerio*) and Affects Swimming Performance of Larvae." *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology* 185-186:122–130. https://doi.org/10.1016/j.cbpc.2016.03.008.

Ayala, A., M. F. Muñoz, and S. Argüelles. 2014. "Lipid Peroxidation: Production, Metabolism, and Signaling Mechanisms of Malondialdehyde and 4-Hydroxy-2-Nonenal." *Oxidative Medicine and Cellular Longevity* 2014:1–31. https://doi.org/10.1155/2014/360438.

Babcsányi, I., G. Imfeld, M. Granet, and F. Chabaux. 2014. "Copper Stable Isotopes to Trace Copper Behavior in Wetland Systems." *Environmental Science and Technology* 48:5520–5529. https://doi.org/10.1021/es405688v.

Balachandran, R. C., S. Mukhopadhyay, D. McBride, J. Veevers, F. E. Harrison, M. Aschner, E. N. Haynes, and A. B. Bowman. 2020. "Brain Manganese and the Balance Between Essential Roles and Neurotoxicity." *Journal of Biological Chemistry* 295 (19): 6312–6329. https://doi.org/10.1074/jbc.REV119.009453.

Barceloux, D. G. and D. Barceloux. 1999. "Copper." *Journal of Toxicology. Clinical Toxicology* 37 (2): 217–230. https://doi.org/10.1081/CLT-100102421.

Barjhoux, I., M. Baudrimont, B. Morin, L. Landi, P. Gonzalez, and J. Cachot. 2012. "Effects of Copper and Cadmium Spiked-Sediments on Embryonic Development of Japanese Medaka (*Oryzias latipes*)." *Ecotoxicology & Environmental Safety* 79:272–282. https://doi.org/10.1016/j.ecoenv.2012.01.011.

Boix, N., E. Teixido, E. Pique, J. M. Llobet, and J. Gomez-Catalan. 2020. "Modulation and protection effects of antioxidant compounds against oxidant induced developmental toxicity in zebrafish." *Antioxidants (Basel)* 9 (8): 721. Aug 8 https://doi.org/10.3390/antiox9080721.

Bradford, M. 1976. "A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding." *Analytical Biochemistry* 72 (1–2): 248–254. https://doi.org/10.1016/0003-2697(76)90527-3.

Brinez-Gallego, P., D. G. da Costa Silva, M. F. Cordeiro, A. P. Horn, and M. A. Hort. 2023. "Experimental Models of Chemically Induced Parkinson's Disease in Zebrafish at the Embryonic Larval Stage: A Systematic Review." *Journal of Toxicology and Environmental Health, Part B* 26 (4): 201–237. https://doi.org/10.1080/10937404.2023.2182390.

Brix, K. V., G. De Boeck, S. Baken, and D. J. Fort. 2022. "Adverse Outcome Pathways for Chronic Copper Toxicity to Fish and Amphibians." *Environmental Toxicology & Chemistry / SETAC* 41 (12): 2911–2927. https://doi.org/10.1002/etc.5483.

Brix, K. V., A. J. Esbaugh, and M. Grosell. 2011. "The Toxicity and Physiological Effects of Copper on the Freshwater Pulmonate Snail, Lymnaea Stagnalis." *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology* 154 (3): 261–267. https://doi.org/10.1016/j.cbpc.2011.06.004.

Cao, Y., K. Lei, X. Zhang, L. Xu, C. Lin, and Y. Yang. 2018. "Contamination and Ecological Risks of Toxic Metals in the Hai River, China." *Ecotoxicology & Environmental Safety* 164:210–218. https://doi.org/10.1016/j.ecoenv.2018.08.009.

Carneiro, P. T., C. M. Martha, and B. M. Reis. 2016. "Copper Toxicology, Oxidative Stress and Inflammation Using Zebrafish as Experimental Model." *Journal of Applied Toxicology : JAT* 36 (7): 876–885. https://doi.org/10.1002/jat.3303.

Carvalho, C. D. S., V. A. Bernusso, and M. N. Fernandes. 2015. "Copper Levels and Changes in pH induce Oxidative Stress in the Tissue of Curimbata (*Prochilodus lineatus*)." *Aquatic Toxicology (Amsterdam, Netherlands)* 167:220–227. https://doi.org/10.1016/j.aquatox.2015.08.003.

Cerda, G. A., J. E. Thomas, M. L. Allende, R. O. Karlstrom, and V. Palma. 2006. "Electroporation of DNA, RNA, and morpholinos into zebrafish embryos." *Methods* 39 (3): 207–211. https://doi.org/10.1016/j.ymeth.2005.12.009.

Chasapis, C. T., P. A. Ntoupa, C. A. Spiliopoulou, and M. E. Stefanidou. 2020. "Recent aspects of the effects of zinc on human health." *Archives of Toxicology* 94 (5): 1443–1460. https://doi.org/10.1007/s00204-020-02702-9.

Choo, X. Y., L. Alukaidey, A. R. White, and A. Grubman. 2013. "Neuroinflammation and Copper in Alzheimer's Disease." *International Journal of Alzheimer's Disease* 2013:1–12. https://doi.org/10.1155/2013/145345.

Colwill, R. M., and R. Creton. 2011. "Locomotor behaviors in zebrafish (*Danio rerio*) larvae." *Behavioural Processes* 86 (2): 222–229. https://doi.org/10.1016/j.beproc.2010.12.003.

Costa-Silva, D. G., L. P. da Leandro, P. D. B. Vieira, N. R. Carvalho, A. R. Lopes, L. E. Schimith, M. E. M. Nunes, et al. 2018. "N -Acetylcysteine Inhibits Mancozeb-Induced Impairments to the Normal Development of Zebrafish Embryos." *Neurotoxicology and Teratology* 68:1–12. https://doi.org/10.1016/j.ntt.2018.04.003.

Cremazy, A., S. Braz-Mota, K. V. Brix, R. M. Duarte, A. L. Val, and C. M. Wood. 2022. "Investigating the Mechanisms of Dissolved Organic Matter Protection Against Copper Toxicity in Fish of Amazon's Black Waters." *Science of the Total Environment* 843:157032. https://doi.org/10.1016/j.scitotenv.2022.157032.

Cruz, F. F., C. E. Leite, L. W. Kist, G. M. de Oliveira, M. R. Bogo, C. D. Bonan, M. M. Campos, and F. B. Morrone. 2017. "Effects of Caffeine on Behavioral and Inflammatory Changes Elicited by Copper in Zebrafish Larvae: Role of Adenosine Receptors." *Comparative Biochemistry & Physiology Toxicology & Pharmacology: CBP* 194:28–36. https://doi.org/10.1016/j.cbpc.2017.01.007.

d'Alençon, C. A., O. A. Peña, C. Wittmann, V. E. Gallardo, R. A. Jones, F. Loosli, U. Liebel, C. Grabher, and M. L. Allende. 2010. "A High-Throughput Chemically Induced Inflammation Assay in Zebrafish." *BMC Biology* 8 (1): 151. https://doi.org/10.1186/1741-7007-8-151.

De Oliveira, E. C. M., E. S. Caixeta, V. S. V. Santos, and B. B. Pereira. 2021. "Arsenic Exposure from Groundwater: Environmental Contamination, Human Health Effects, and Sustainable Solutions." *Journal of Toxicology and Environmental Health, Part B* 24 (3): 119–135. https://doi.org/10.1080/10937404.2021.1898504.

Ellman, G. L. 1959. "Tissue sulfhydryl groups." *Archives of Biochemistry and Biophysics* 82 (1): 70–77. https://doi.org/10.1016/0003-9861(59)90090-6.

El-Sharkawey, A. E. 2016. *Calculate the Corrected Total Cell Fluorescence (CTCF)*. https://doi.org/10.13140/RG.2.1.1307.8008.

Fasae, K. D., and A. O. Abolaji. 2022. "Interactions and Toxicity of Non-Essential Heavy Metals (Cd, Pb and Hg): Lessons from *Drosophila Melanogaster*." *Current Opinion in Insect Science* 51:100900. https://doi.org/10.1016/j.cois.2022.100900.

Finkel, T. 2011. "Signal Transduction by Reactive Oxygen Species." *The Journal of Cell Biology* 194 (1): 7–15. https://doi.org/10.1083/jcb.201102095.

Fitzgerald, J. A., M. G. Urbina, N. J. Rogers, N. R. Bury, I. Katsiadaki, R. W. Wilson, and E. M. Santos. 2019. "Sublethal Exposure to Copper Supresses the Ability to Acclimate to Hypoxia in a Model Fish Species." *Aquatic Toxicology (Amsterdam, Netherlands)* 217: 105325. http://doi.org/10.1016/j.aquatox.2019.105325 .

Francisco, C. M., L. A. Pavanin, S. Morelli, J. V. M. Bravo, and B. B. Pereira. 2023. "Using Native Fish in Eco-Genotoxic Assessment of Heavy Metal Contamination Pollution Arising from Nearby Large Brazilian Rivers." *Journal of Toxicology and Environmental Health Part A* 86 (2–3): 74–85. https://doi.org/10.1080/15287394.2022.2164754.

Fu, Z., and S. Xi. 2020. "The Effects of Heavy Metals on Human Metabolism." *Toxicology Mechanisms and Methods* 30 (3): 167–176. https://doi.org/10.1080/15376516.2019.1701594.

Gaetke, L. 2003. "Copper toxicity, oxidative stress, and antioxidant nutrients." *Toxicology* 189 (1–2): 147–163. https://doi.org/10.1016/S0300-483X(03)00159-8.

Galaris, D., A. Barbouti, and K. Pantopoulos. 2019. "Iron Homeostasis and Oxidative Stress: An Intimate Relationship." *Biochimica Et Biophysica Acta (BBA) - Molecular Cell Research* 1866 (12): 118535. https://doi.org/10.1016/j.bbamcr.2019.118535.

Glisic, B., I. Mihaljevic, M. Popovic, R. Zaja, J. Loncar, K. Fent, R. Kovacevic, and T. Smital. 2015. "Characterization of Glutathione-S-Transferases in Zebrafish (*Danio rerio*)." *Aquatic Toxicology (Amsterdam, Netherlands)* 158:50–62. https://doi.org/10.1016/j.aquatox.2014.10.013.

Gobbi, A., I. Kyrkou, E. Filippi, L. Ellegaard-Jensen, L. Hansen, and Hestbjerg. 2020. "Seasonal Epiphytic Microbial Dynamics on Grapevine Leaves Under Biocontrol and Copper Fungicide Treatments." *Scientific Reports* 10 (1): 681. https://doi.org/10.1038/s41598-019-56741-z.

Gouva, E., C. Nathanailides, I. Skoufos, I. Paschos, F. Athanassopoulou, and I. S. Pappas. 2020. "Comparative Study of the Effects of Heavy Metals on Embryonic Development of Zebrafish." *Aquaculture Research* 51 (8): 3255–3267 . https://doi.org/10.1111/are.14660.

Halliwell, B., and J. M. C. Gutteridge. 2015. *Free Radicals in Biology and Medicine*. 5th ed. New York: Oxford University Press.

Hardy, K., A. E. Amariutei, F. De Faveri, A. Hendry, W. Marcotti, and F. Ceriani. 2021. "Functional Development and Regeneration of Hair Cells in the Zebrafish Lateral Line." *The Journal of Physiology* 599 (16): 3913–3936. https://doi.org/10.1113/JP281522.

He, F., X. Huang, G. Wei, X. Lin, W. Zhang, W. Zhuang, W. He, T. Zhan, H. Hu, and H. Yang. 2022. "Regulation of ACSL4-Catalyzed Lipid Peroxidation Process Resists Cisplatin Ototoxicity." *Oxidative Medicine and Cellular Longevity* 2022:1–15. https://doi.org/10.1155/2022/3080263.

Hernández, P. P., V. Moreno, F. A. Olivari, and M. L. Allende. 2006. "Sub-Lethal Concentrations of Waterborne Copper are Toxic to Lateral Line Neuromasts in Zebrafish (*Danio rerio*)." *Hearing Research* 213 (1–2): 1–10. https://doi.org/10.1016/j.heares.2005.10.015.

Hernandez, P. P., C. Undurraga, V. E. Gallardo, N. Mackenzie, M. L. Allende, and A. E. Reyes. 2011. "Sublethal Concentrations of Waterborne Copper Induce Cellular Stress and Cell Death in Zebrafish Embryos and Larvae." *Biological Research* 44 (1): 7–15. https://doi.org/10.4067/s0716-97602011000100002.

Horzmann, K. A., and J. L. Freeman. 2018. "Making Waves: New Developments in Toxicology with the Zebrafish." *Toxicological Sciences: An Official Journal of the Society of Toxicology* 163 (1): 5–12. https://doi.org/10.1093/toxsci/kfy044.

Jasim, C. M., M. Girgis, and W. C. M. 2016. "Revisiting the Mechanisms of Copper Toxicity to Rainbow Trout: Time Course, Influence of Calcium, Unidirectional Na+ Fluxes, and Branchial Na+, K+ ATPase and V-Type H+ ATPase Activities." *Aquatic Toxicology (Amsterdam, Netherlands)* 177: 51–62. https://doi.org/10.1016/j.aquatox.2016.05.009.

Jiang, W., Y. Liu, K. Hu, J. Jiang, S. Li, L. Feng, and X. Zhou. 2014. "Copper Exposure Induces Oxidative Injury, Disturbs the Antioxidant System and Changes the Nrf2/ARE (CuZnsod) Signaling in the Fish Brain: Protective Effects of Myo-Inositol." *Aquatic Toxicology (Amsterdam, Netherlands)* 155: 301–313. http://doi.org/10.1016/j.aquatox.2014.07.003 .

Johnson, A., E. Carew, and K. A. Sloman. 2007. "The Effects of Copper on the Morphological and Functional Development of Zebrafish Embryos." *Aquatic Toxicology (Amsterdam, Netherlands)* 84 (4): 431–438. https://doi.org/10.1016/J.AQUATOX.2007.07.003.

Jomova, K., M. Makova, S. Y. Alomar, S. H. Alwasel, E. Nepovimova, K. Kuca, C. J. Rhodes, and M. Valko. 2022. "Essential Metals in Health and Disease." *Chemico-Biological Interactions* 367:110173. https://doi.org/10.1016/j.cbi.2022.110173.

Kadim, M. K., and Y. An Risjani. 2022. "Biomarker for Monitoring Heavy Metal Pollution in Aquatic Environment: An Overview Toward Molecular Perspectives *Emerging Contam*." *Emerging Contaminants* 8:195–205. https://doi.org/10.1016/J.EMCON.2022.02.003.

Kimmel, C. B., W. W. Ballard, S. R. Kimmel, B. Ullmann, and T. F. Schilling. 1995. "Stages of Embryonic Development of the Zebrafish." *Developmental Dynamics* 203 (3): 253–310. https://doi.org/10.1002/AJA.1002030302.

Koledenkova, K., Q. Esmaeel, C. Jacquard, J. Nowak, C. Clément, and E. Ait Barka. 2022. "Plasmopara Viticola the Causal Agent of Downy Mildew of Grapevine: From Its Taxonomy to Disease Management." *Front Microbiology* 13: 889472. https://doi.org/10.3389/fmicb.2022.889472.

Kong, X., H. Jiang, S. Wang, X. Wu, W. Fei, L. Li, G. Nie, and X. Li. 2013. "Effects of Copper Exposure on the Hatching Status and Antioxidant Defense at Different Developmental Stages of Embryos and Larvae of Goldfish *Carassius Auratus*." *Chemosphere* 92 (11): 1458–1464. https://doi.org/10.1016/j.chemosphere.2013.04.004.

Kowalczyk, P., D. Sulejczak, P. Kleczkowska, I. Bukowska-Ośko, M. Kucia, M. Popiel, E. Wietrak, K. Kramkowski, K. Wrzosek, and K. Kaczyńska. 2021. "Mitochondrial Oxidative Stress—A Causative Factor and Therapeutic Target in Many Diseases." *International Journal of Molecular Sciences* 22 (24): 13384. https://doi.org/10.3390/ijms222413384.

Krupa, P. M., S. T. McMurry, M. Minghetti, and J. B. Belden. 2021. "Effects of Copper on the Neuromasts of *Xenopus Laevis*." *Archives of Environmental Contamination and Toxicology* 80 (4): 769–778. https://doi.org/10.1007/s00244-020-00778-z.

Kumar, N., N. K. Chandan, S. Bhushan, D. K. Singh, and S. Kumar. 2023. "Health Risk Assessment and Metal Contamination in Fish, Water and Soil Sediments in the East Kolkata Wetlands, India, Ramsar Site." *Scientific Reports* 13 (1): 1546. https://doi.org/10.1038/s41598-023-28801-y.

Kumar, N., N. K. Chandan, S. Bhushan, D. K. Singh, and S. Kumar. 2023. "Health Risk Assessment and Metal Contamination in Fish, Water and Soil Sediments in the East Kolkata Wetlands, India, Ramsar Site. " *Scientific Reports* 13: 1. https://doi.org/10.1038/s41598-023-28801-y.

Lai, K. P., Z. Gong, and W. K. F. Tse. 2021. "Zebrafish as the Toxicant Screening Model: Transgenic and Omics Approaches." *Aquatic Toxicology (Amsterdam, Netherlands)* 234:105813. https://doi.org/10.1016/j.aquatox.2021.105813.

Lawrence, C. 2007. "The Husbandry of Zebrafish (Danio Rerio): A Review." *Aquaculture* 269 (1–4): 1–20. https://doi.org/10.1016/j.aquaculture.2007.04.077.

Leandro, L. P., R. S. de Mello, D. G. Costa- Silva, M. E. M. Nunes, A. R. Lopes, I. K. Martins, T. Posser, and J. L. Franco. 2021. "Behavioral Changes Occur Earlier Than Redox Alterations in Developing Zebrafish Exposed to Mancozeb." *Environmental Pollution* 268:115783. https://doi.org/10.1016/j.envpol.2020.115783.

Leandro, L. P., M. V. T. Mariano, K. K. Gomes, A. B. Santos, J. S. Anjos, N. R. Carvalho, M. E. M. Nunes, T. Posser, and J. L. Franco. 2023. "Permissible Concentration of Mancozeb in Brazilian Drinking Water Elicits Oxidative Stress and Bioenergetic Impairments in Embryonic Zebrafish." *Environmental Pollution* 333:122013. https://doi.org/10.1016/J.ENVPOL.2023.122013.

LeBel, C. P., H. Ischiropoulos, and S. C. Bondy. 1992. "Evaluation of the Probe 2',7'-Dichlorofluorescin as an Indicator of Reactive Oxygen Species Formation and Oxidative Stress." *Chemical Research in Toxicology* 5 (2): 227–231. https://doi.org/10.1021/tx00026a012.

Lewis, C., R. Ellis, E. Vernon, K. Elliot, S. Newbatt, and R. W. Wilson. 2016. "Ocean Acidification Increases Copper Toxicity Differentially in Two Key Marine Invertebrates with Distinct Acid-Base Responses." *Scientific Reports* 6 (1): 21554. https://doi.org/10.1038/srep21554.

Liao, W., Z. Zhu, C. Feng, Z. Yan, Y. Hong, D. Liu, and X. Jin. 2023. "Toxicity Mechanisms and Bioavailability of Copper to Fish Based on an Adverse Outcome Pathway Analysis." *Journal of Environmental Sciences* 127: 495–507. https://doi.org/10.1016/j.jes.2022.06.002.

Linbo, T. L., C. M. Stehr, J. P. Incardona, and N. L. Scholz. 2006. "Dissolved Copper Triggers Cell Death in the Peripheral Mechanosensory System of Larval Fish." *Environmental Toxicology & Chemistry / SETAC* 25 (2): 597–603. https://doi.org/10.1897/05-241r.1.

Ma, Q., R. K. Poopal, J. Zhang, X. Chen, and Z. Ren. 2022. "Real-Time Determination of Water Status Upon Simultaneous Zebrafish Exposure to Sublethal Concentrations of CuSo4." *Aquatic Toxicology (Amsterdam, Netherlands)* 252:106296. https://doi.org/10.1016/j.aquatox.2022.106296.

Martins, I. K., L. G. Pereira, M. E. M. Nunes, A. R. Lopes, L. P. Leandro, R. S. de Mello, M. V. T. Mariano, J. L. Franco, and T. Posser. 2023. "Exposure to Mancozeb Results in Increased MAPK Phosphorylation and Locomotor Deficits in Zebrafish Larvae." *Comparative Biochemistry & Physiology Toxicology & Pharmacology: CBP* 270:109659. https://doi.org/10.1016/j.cbpc.2023.109659.

Mason, M. W., and B. B. Parrott. 2022. "Acute Copper Toxicity Displays a Nonmonotonic Relationship with Age Across the Medaka (*Oryzias latipes*) Life Span." *Environmental Toxicology & Chemistry / SETAC* 41 (12): 2999–3006. https://doi.org/10.1002/etc.5481.

Miao, W., L. He, T. Zhang, and C. Li. 2022. "Lentinan impairs the early development of zebrafish embryos, possibly by disrupting glucose and lipid metabolism." *Processes* 10 (1): 120. https://doi.org/10.3390/pr10010120.

Michereff, S. J. 2001. *Fundamentos de Fitopatologia*. Recife: Universidade Federal Rural de Pernambuco.

Michiels, E. D., L. Vergauwen, A. Hagenaars, E. Fransen, V. S. Dongen, V. J. S. Cruchten, L. Bervoets, and D. Knapen. 2017. "Evaluating Complex Mixtures in the Zebrafish Embryo by Reconstituting Field Water Samples: A Metal Pollution Case Study." *International Journal of Molecular Sciences* 18 (3): 539. https://doi.org/10.3390/ijms18030539.

Ministério da Saúde. 2011. *PORTARIA GM/MS* Nº 2.914/2011, *DE 12 DE DEZEMBRO DE 2011*.

Ministério da Saúde. 2021. *PORTARIA GM/MS Nº 888, DE 4 DE MAIO DE 2021*. https://www.in.gov.br/en/web/dou/-/portaria-gm/ms-n-888-de-4-de-maio-de-2021-318461562.

Mohammadbackir, S. 2016. Impacts of waterborne copper and silver on the early life stage (ELS) ofzebrafish (*Danio rerio*): physiological, biochemical and molecular responses. University of Plymouth, PhD dissertation.

Mondello, V., C. Lemaître-Guillier, P. Trotel-Aziz, R. Gougeon, A. Acedo, P. Schmitt-Kopplin, M. Adrian, C. Pinto, O. Fernandez, and F. Fontaine. 2022. "Assessment of a New Copper-Based Formulation to Control Esca Disease in Field and Study of Its Impact on the Vine Microbiome." *Vine Physiology and Enological Parameters of the Juice. J Fungi (Basel)* 8: 2. http://doi.org/10.3390/jof8020151.

Naeem, S., M. Ashraf, M. E. Babar, S. Zahoor, and S. Ali. 2021. "The Effects of Some Heavy Metals on Some Fish Species." *Environmental Science and Pollution Research International* 28 (20): 25566–25578. https://doi.org/10.1007/s11356-021-12385-z.

Newton, K. C., D. Kacev, S. R. O. Nilsson, A. L. Saettele, S. A. Golden, and L. Sheets. 2023. "Lateral Line Ablation by Ototoxic Compounds Results in Distinct Rheotaxis Profiles in Larval Zebrafish." *Communications Biology* 6 (1): 84. https://doi.org/10.1038/s42003-023-04449-2.

Nguyen, T. H., H. D. Le, T. N. T. Kim, H. P. The, T. M. Nguyen, V. Cornet, J. Lambert, and P. Kestemont. 2020. "Anti–Inflammatory and Antioxidant Properties of the Ethanol Extract of *Clerodendrum Cyrtophyllum* Turcz in Copper Sulfate-Induced Inflammation in Zebrafish." *Antioxidants (Basel)* 9 (3): 192. https://doi.org/10.3390/antiox9030192.

Olivari, F. A., P. P. Hernández, and M. L. Allende. 2008. "Acute copper exposure induces oxidative stress and cell death in lateral line hair cells of zebrafish larvae." *Brain Research* 1244:1–12. https://doi.org/10.1016/j.brainres.2008.09.050.

Peixoto, P. V. L., Í. B. L. de Andrade, B. C. P. Sales, and L. C. Pereira. 2022. "Rupture of Brumadinho Dam (Minas Gerais, Brazil): Embryotoxicity in Zebrafish Induced by Metal Mixture-Contaminated Water." *Journal of Environmental Science and Health Part B, Pesticides, Food Contaminants, and Agricultural Wastes* 57 (6): 479–488. https://doi.org/10.1080/03601234.2022.2064675.

Präbst, K., H. Engelhardt, S. Ringgeler, and H. Hübner. 2017. "Basic colorimetric proliferation assays: MTT, WST, and resazurin." *Cell Viability Assays* 1–17. https://doi.org/10.1007/978-1-4939-6960-9_1.

Rader, K. J., R. F. Carbonaro, E. D. van Hullebusch, S. Baken, and K. Delbeke. 2019. "The Fate of Copper Added to Surface Water: Field, Laboratory, and Modeling Studies." *Environmental Toxicology & Chemistry / SETAC* 38 (7): 1386–1399. https://doi.org/10.1002/etc.4440.

Rastogi, A., and A. R. Timme-Laragy. 2021. "Using monochlorobimane to visualize glutathione utilization in the developing zebrafish (*Danio rerio*) embryo." *Current Protocols* 1 (2): e552. https://doi.org/10.1002/cpz1.24.

Rehman, K., F. Fatima, I. Waheed, and M. S. H. Akash. 2018. "Prevalence of Exposure of Heavy Metals and Their Impact on Health Consequences." *Journal of Cellular Biochemistry* 119 (1): 157–184. https://doi.org/10.1002/jcb.26234.

Reid, R. M., A. L. D'Aquila, and P. R. Biga. 2018. "The Validation of a Sensitive, Non-Toxic in vivo Metabolic Assay Applicable Across Zebrafish Life Stages." *Comparative Biochemistry & Physiology Toxicology & Pharmacology: CBP* 208:29–37. https://doi.org/10.1016/j.cbpc.2017.11.004.

Richendrfer, H., S. D. Pelkowski, R. M. Colwill, and R. Creton. 2012. "On the Edge: Pharmacological Evidence for Anxiety-Related Behavior in Zebrafish Larvae." *Behavioural Brain Research* 228 (1): 99–106. https://doi.org/10.1016/j.bbr.2011.11.041.

Rivero-Wendt, C. L. G., L. G. Fernandes, A. N. Dos Santos, I. L. Brito, J. A. Dos Santos Jaques, E. Dos Santos Dos Anjos, and C. E. Fernandes. 2023. "Effects of Chloramine on Zebrafish Embryos Malformations Associated with Cardiotoxicity and Neurotoxicity." *Journal of Toxicology and Environmental Health Part A* 86 (11): 372–381. https://doi.org/10.1080/15287394.2023.2205271.

Santamaria, A. B., and S. I. Sulsky. 2010. "Risk assessment of an essential element: manganese." *Journal of Toxicology and Environmental Health Part A* 73 (2–3): 128–155. https://doi.org/10.1080/15287390903337118.

Santos, D., A. Luzio, C. Matos, J. Bellas, S. M. Monteiro, and L. Félix. 2021. "Microplastics Alone or Co-Exposed with Copper Induce Neurotoxicity and Behavioral Alterations on Zebrafish Larvae After a Subchronic Exposure." *Aquatic Toxicology (Amsterdam, Netherlands)* 235:105814. https://doi.org/10.1016/j.aquatox.2021.105814.

Sant, K. E., and A. R. Timme-Laragy. 2018. "Zebrafish as a Model for Toxicological Perturbation of Yolk and Nutrition in the Early Embryo." *Current Environmental Health Reports* 5 (1): 125–133. https://doi.org/10.1007/s40572-018-0183-2.

Scheiber, I., R. Dringen, and J. F. Mercer. 2013. "Copper: Effects of Deficiency and Overload." *Metal Ions in Life Sciences* 13:359–387. https://doi.org/10.1007/978-94-007-7500-8_11.

Shahjahan, M., K. Taslima, M. S. Rahman, M. Al-Emran, S. I. Alam, and C. Faggio. 2022. "Effects of Heavy Metals on Fish Physiology - a Review." *Chemosphere* 300:134519. https://doi.org/10.1016/j.chemosphere.2022.134519.

Shen, X., H., Zhang, X. He, H. Shi, C. Stephan, H. Jiang, C. Wan, and T. Eichholz. 2019. "Evaluating the Treatment Effectiveness of Copper-Based Algaecides on Toxic Algae Microcystis aeruginosa Using Single Cell-Inductively Coupled Plasma-Mass Spectrometry." *Analytical & Bioanalytical Chemistry* 411 (21): 5531–5543. https://doi.org/10.1007/s00216-019-01933-9.

Sies, H., C. Berndt, and D. P. Jones. 2017. "Oxidative stress." *Annual Review of Biochemistry* 86 (1): 715–748. https://doi.org/10.1146/annurev-biochem-061516-045037.

Sikorska, J., and J. Wolnicki. 2010. "Cadmium and Copper Toxicity to Tench *Tinca Tinca* (L.) Larvae After a Short-Term Exposure." *Reviews in Fish Biology and Fisheries* 20 (3): 417–423. https://doi.org/10.1007/s11160-009-9145-y.

Simpson, S. L., H. Yverneau, A. Cremazy, C. V. Jarolimek, H. L. Price, and D. F. Jolley. 2012. "DGT-Induced Copper Flux Predicts Bioaccumulation and Toxicity to Bivalves in Sediments with Varying Properties." *Environmental Science & Technology* 46 (16): 9038–9046. https://doi.org/10.1021/es301225d.

Smith, R. A., and M. P. Murphy. 2011. "Mitochondria-Targeted Antioxidants as Therapies." *Discovery Medicine* 11 :106–114.

Sonnack, L., S. Kampe, E. Muth-Köhne, L. Erdinger, N. Henny, H. Hollert, C. Schäfers, et al. 2015. "Effects of Metal Exposure on Motor Neuron Development, Neuromasts and the Escape Response of Zebrafish Embryos." *Neurotoxicology and Teratology* 50:33–42. https://doi.org/10.1016/j.ntt.2015.05.006.

Stern, B. R., M. Solioz, D. Krewski, P. Aggett, T. C. Aw, S. Baker, K. Crump, et al. 2007. "Copper and Human Health: Biochemistry, Genetics, and Strategies for Modeling Dose-Response Relationships." *Journal of Toxicology and Environmental Health, Part B* 10 (3): 157–222. https://doi.org/10.1080/10937400600755911.

Štrbac, S., M. Kašanin-Grubin, B. Jovančićević, and P. Simonović. 2015. "Bioaccumulation of Heavy Metals and Microelements in Silver Bream (*Brama Brama L.*), Northern Pike (*Esox Lucius L.*), Sterlet (*Acipenser Ruthenus L.*), and Common Carp (*Cyprinus Carpio L.*) from Tisza River, Serbia." *Journal of Toxicology and Environmental Health Part A* 78 (11): 663–665. https://doi.org/10.1080/15287394.2015.1023406.

Tao, S., A. Long, F. Xu, and R. Dawson. 2002. "Copper Speciation in the Gill Microenvironment of Carp (*Cyprinus carpio*) at Various Levels of pH." *Ecotoxicology & Environmental Safety* 52 (3): 221–226. https://doi.org/10.1006/eesa.2002.2193.

Tao, M., K. Nie, R. Zhao, Y. Shi, and W. Cao. 2022. "Environmental Impact of Mining and Beneficiation of Copper Sulphate Mine Based on Life Cycle Assessment." *Environmental Science and Pollution Research International* 29 (58): 87613–87627. https://doi.org/10.1007/s11356-022-21317-4.

Tao, S., Y. Wen, A. Long, R. Dawson, J. Cao, and F. Xu. 2001. "Simulation of Acid–Base Condition and Copper Speciation in the Fish Gill Microenvironment." *Computers & Chemistry* 25 (3): 215–222. https://doi.org/10.1016/s0097-8485(00)00083-8.

Taslima, K., M. Al-Emran, M. S. Rahman, J. Hasan, Z. Ferdous, M. F. Rohani, and M. Shahjahan. 2022. "Impacts of Heavy Metals on Early Development, Growth and Reproduction of Fish - a Review." *Toxicology Reports* 9:858–868. https://doi.org/10.1016/j.toxrep.2022.04.013.

Tsang, T., J. M. Posimo, A. A. Gudiel, M. Cicchini, D. M. Feldser, and D. C. Brady. 2020. "Copper is an essential regulator of the autophagic kinases ULK1/2 to drive lung adenocarcinoma." *Nature Cell Biology* 22 (4): 412–424. https://doi.org/10.1038/s41556-020-0481-4.

VandKhanghah, M. M., A. Hedayati, S. Nazeri, A. H. Mohammadi, and R. Ghorbani. 2022. "Biomagnification of Copper Along the Aquatic Food Chain (*Artemia franciscana*, Danio rerio, and Astronotus ocellatus)." *Biological Trace Element Research* 200 (4): 1854–1860. https://doi.org/10.1007/s12011-021-02781-4.

Vázquez-Blanco, R., M. Arias-Estévez, E. Bååth, and D. Fernández-Calviño. 2020. "Comparison of Cu Salts and Commercial Cu Based Fungicides on Toxicity Towards Microorganisms in Soil." *Environmental Pollution* 257: 113585. https://doi.org/10.1016/j.envpol.2019.113585.

Vellingiri, B., A. Suriyanarayanan, P. Selvaraj, K. S. Abraham, M. Y. Pasha, H. Winster, A. V. G. S. Gopalakrishnan, et al. 2022. "Role of Heavy Metals (Copper (Cu), Arsenic (As), Cadmium (Cd), Iron (Fe) and Lithium (Li)) Induced Neurotoxicity." *Chemosphere* 301:134625. https://doi.org/10.1016/j.chemosphere.2022.134625.

Wang, Z. et al. 2022. "Early-Life Lead Exposure Induces Long-Term Toxicity in the Central Nervous System: From Zebrafish Larvae to Juveniles and Adults." *Science of the Total Environment* 804: 150185 https://doi.org/10.1016/j.scitotenv.2021.150185.

Wang, X., D. Wei, Y. Ma, and M. J. McLaughlin. 2015. "Derivation of soil ecological criteria for copper in Chinese soils." *PLoS One* 10 (10): e0140306. https://doi.org/10.1371/journal.pone.0140306.

Wang, R. F., L. M. Zhu, J. Zhang, X. P. An, Y. P. Yang, M. Song, and L. Zhang. 2020. "Developmental toxicity of copper in marine medaka (*Oryzias melastigma*) embryos and larvae." *Chemosphere* 247:125923. https://doi.org/10.1016/j.chemosphere.2020.125923.

Weir, S. M., S. Yu, D. E. Scott, and S. L. Lance. 2019. "Acute Toxicity of Copper to the Larval Stage of Three Species of Ambystomatid Salamanders." *Ecotoxicology* 28 (9): 1023–1031. https://doi.org/10.1007/s10646-019-02102-5.

Westerfield, M. 2000. *The Zebrafish Book. A Guide for the Laboratory Use of Zebrafish*(Danio rerio). 4 ed. Eugene: University of Oregon Press. https://zfin.org/zf_info/zfbook/cont.html .

Williams, S. Y., and B. J. Renquist. 2016. "High Throughput Danio rerio Energy Expenditure Assay." *Journal of Visualized Experiments: JoVe* 27 (107): e53297. https://doi.org/10.3791/53297.

Wilson, C. 2012. "Aspects of Larval Rearing." *ILAR Journal / National Research Council, Institute of Laboratory Animal Resources* 53 (2): 169–178. https://doi.org/10.1093/ilar.53.2.169.

Witeska, M., P. Sarnowski, K. Ługowska, and E. Kowal. 2014. "The Effects of Cadmium and Copper on Embryonic and Larval Development of Ide *Leuciscus Idus L.*" *Fish Physiology and Biochemistry* 40 (1): 151–163. https://doi.org/10.1007/s10695-013-9832-4.

Xu, J., R. Zhang, T. Zhang, G. Zhao, Y. Huang, H. Wang, and J. X. Liu. 2017. "Copper Impairs Zebrafish Swimbladder Development by Down-Regulating Wnt Signaling." *Aquatic Toxicology (Amsterdam, Netherlands)* 192:155–164. https://doi.org/10.1016/j.aquatox.2017.09.018.

Yang, F., Y. Yun, G. Li, and N. Sang. 2021. "Heavy Metals in Soil from Gangue Stacking Areas Increases Children Health Risk and Causes Developmental Neurotoxicity in Zebrafish Larvae." *The Science of the Total Environment* 794:148629. https://doi.org/10.1016/j.scitotenv.2021.148629.

Yin, A., S. Korzh, C. L. Winata, V. Korzh, and Z. Gong. 2011. "Wnt Signaling is Required for Early Development of Zebrafish Swimbladder." *PLoS One* 6 (3): e18431. https://doi.org/10.1371/journal.pone.0018431.

Zhang, T., L. Xu, J. J. Wu, W. M. Wang, J. Mei, X. F. Ma, and J. X. Liu. 2015. "Transcriptional Responses and Mechanisms of Copper-Induced Dysfunctional Locomotor Behavior in Zebrafish Embryos." *Toxicological Sciences: An Official Journal of the Society of Toxicology* 148 (1): 299–310. https://doi.org/10.1093/toxsci/kfv184.

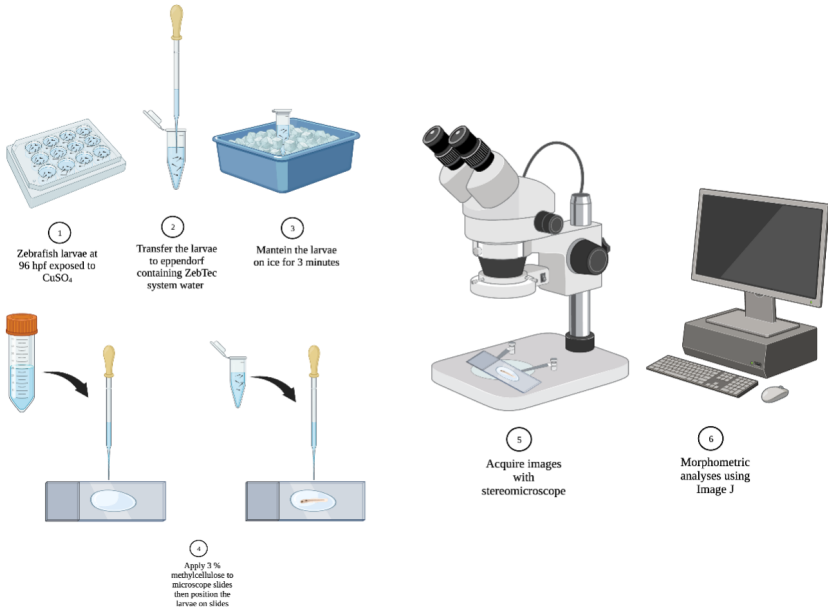
Zhang, L., Z. Yang, M. Yang, F. Yang, G. Wang, D. Liu, X. Li, L. Yang, and Z. Wang. 2022. "Copper-Induced Oxidative Stress, Transcriptome Changes, Intestinal Microbiota, and Histopathology of Common Carp (*Cyprinus carpio*)." *Ecotoxicology & Environmental Safety* 246:114136. https://doi.org/10.1016/j.ecoenv.2022.114136.

Zhao, G., H. Sun, T. Zhang, and J. Liu. 2020. "Copper Induce Zebrafish Retinal Developmental Defects via Triggering Stresses and Apoptosis." *Cell communication and signaling : CCS* 18 (1): 45. https://doi.org/10.1186/s12964-020-00548-3.

Zhen, Y., L. Ge, Q. Chen, J. Xu, Z. Duan, J. J. Loor, and M. Wang. 2022. "Latent Benefits and Toxicity Risks Transmission Chain of High Dietary Copper Along the Livestock–Environment–Plant–Human Health Axis and Microbial Homeostasis: A Review." *Journal of Agricultural and Food Chemistry* 70 (23): 6943–6962. https://doi.org/10.1021/acs.jafc.2c01367.

Supplementary Material

Supplementary Figure 1:



Legend to Figure

Supplementary Figure 1. Illustration of obtaining representative images. After the exposure period, five larvae from each experimental group are transferred to 1.5 ml Eppendorf tubes. Subsequently, these tubes are placed on ice for three minutes, inducing temporary insensibility to cold and restricting larval movements to enhance the acquisition of morphological images. Concurrently, microscopy slides are prepared by adding a 3% methylcellulose solution using a Pasteur pipette. After the three minutes on ice, the larvae are carefully retrieved with the Pasteur pipette and positioned on the microscopy slide. Images are captured through a stereomicroscope equipped with a camera, ensuring precise documentation of morphological features.

6. CONSIDERAÇÕES FINAIS

Demonstramos no presente trabalho que a exposição ao cobre em concentrações inferiores aos limites estabelecidos pela legislação, resultou em efeitos subletais significativos em larvas de *Danio rerio*, como impactos no desenvolvimento, alterações comportamentais e indução de estresse oxidativo. Dessa forma, ressaltam a sensibilidade das fases iniciais de desenvolvimento de organismos aquáticos e sua correlação com os danos oxidativos resultantes da exposição ao cobre. Adicionalmente, nossos resultados efetivamente indicam que os limites máximos atualmente estabelecidos não garantem um ambiente seguro para organismos aquáticos, sublinhando a urgência de avaliações mais rigorosas para reavaliar as concentrações permitidas. Isso se torna crucial para mitigar a contaminação metálica em ecossistemas aquáticos, especialmente considerando a interação potencial entre o cobre outros elementos em um ambiente complexo.

7. PERSPECTIVAS FUTURAS

Os resultados obtidos evidenciam de maneira eficaz a toxicidade do cobre em concentrações inferiores aos limites estabelecidos pela legislação brasileira. Entretanto, é imperativo destacar a necessidade de estudos suplementares para aprofundar a compreensão dos efeitos decorrentes da exposição ao cobre, com foco na avaliação de diferentes períodos de exposição no peixe-zebra. Um caminho promissor para investigação adicional consiste na análise do papel do cobre na modulação da morte ferroptótica, uma forma recentemente identificada de morte celular. A exploração desse aspecto seria de grande interesse, visando compreender se a ferroptose representa outro mecanismo de toxicidade associado ao cobre e quais as vias bioquímicas e gênicas envolvidas. Além disso, considerando a presença do cobre no ambiente e suas complexas interações, torna-se crucial examinar o efeito sinérgico desses fatores na tolerância térmica de organismos aquáticos, considerando as crescentes preocupações relacionadas às mudanças climáticas globais.

8. REFERÊNCIAS

Acosta, D. da S., Danielle, N.M., Altenhofen, S., Luzardo, M.D., Costa, P.G., Bianchini, A., Bonan, C.D., da Silva, R.S., Dafre, A.L., 2016. Copper at low levels impairs memory of adult zebrafish (Danio rerio) and affects swimming performance of larvae. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology 185–186, 122–130. https://doi.org/10.1016/j.cbpc.2016.03.008

Alves, A., Ribeiro, R., Azenha, M., Cunha, M., Teixeira, J., 2023. Effects of Exogenously Applied Copper in Tomato Plants' Oxidative and Nitrogen Metabolisms under Organic Farming Conditions. Horticulturae 9, 323. https://doi.org/10.3390/horticulturae9030323

Apresova, M.A., Efremova, I.E., Babayants, A.A., Cheknev, S.B., 2014. γ-Globulin Fraction Proteins and Their Metal Complexes with Copper Cations in Induction of IL-8 Production. Bull Exp Biol Med 156, 823–825. https://doi.org/10.1007/s10517-014-2460-x

Arredondo, M., Núñez, M.T., 2005. Iron and copper metabolism. Mol Aspects Med 26, 313–327. https://doi.org/10.1016/J.MAM.2005.07.010

Assis, E.M. de, Souza, M.J. dos S., Faria, M.C. da S., Rodrigues, J.L., Garcez, A., Bomfeti, C.A., Barcellos, N.T., 2019. High concentrations of toxic metals in water consumed by the Maxakali indigenous community in Brazil. Ambiente e Agua - An Interdisciplinary Journal of Applied Science 14, 1. https://doi.org/10.4136/ambi-agua.2215

AYRES, P.G., 2004. Alexis Millardet: Frances forgotten mycologist. Mycologist 18, 23–26. https://doi.org/10.1017/S0269915X04001090

Azimi, A., Azari, A., Rezakazemi, M., Ansarpour, M., 2017. Removal of Heavy Metals from Industrial Wastewaters: A Review. ChemBioEng Reviews 4, 37–59. https://doi.org/10.1002/cben.201600010

Babcsányi, I., Imfeld, G., Granet, M., Chabaux, F., 2014. Copper Stable Isotopes To Trace Copper Behavior in Wetland Systems. Environ Sci Technol 48, 5520–5529. https://doi.org/10.1021/es405688v

Ballabio, C., Panagos, P., Lugato, E., Huang, J.-H., Orgiazzi, A., Jones, A., Fernández-Ugalde, O., Borrelli, P., Montanarella, L., 2018. Copper distribution in European topsoils: An assessment based on LUCAS soil survey. Science of The Total Environment 636, 282–298. https://doi.org/10.1016/j.scitotenv.2018.04.268

Barceloux, D.G., Barceloux, D., 1999. Copper. J Toxicol Clin Toxicol 37, 217–230. https://doi.org/10.1081/CLT-100102421

Bartee, M.Y., Lutsenko, S., 2007. Hepatic copper-transporting ATPase ATP7B: function and inactivation at the molecular and cellular level. BioMetals 20, 627. https://doi.org/10.1007/s10534-006-9074-3

Bhuvanasundar, R., John, A., Sulochana, K., Coral, K., Deepa, P.R., Umashankar, V., 2014. A molecular model of human Lysyl Oxidase (LOX) with optimal copper orientation in the catalytic cavity for induced fit docking studies with potential modulators. Bioinformation 10, 406–412. https://doi.org/10.6026/97320630010406

Bossak, K., Drew, S.C., Stefaniak, E., Płonka, D., Bonna, A., Bal, W., 2018. The Cu(II) affinity of the N-terminus of human copper transporter CTR1: Comparison of human and mouse sequences. J Inorg Biochem 182, 230–237. https://doi.org/10.1016/j.jinorgbio.2018.01.011

Boyd, S.D., Ullrich, M.S., Skopp, A., Winkler, D.D., 2020. Copper Sources for Sod1 Activation. Antioxidants 9, 500. https://doi.org/10.3390/antiox9060500

Briñez-Gallego, P., da Costa Silva, D.G., Cordeiro, M.F., Horn, A.P., Hort, M.A., 2023. Experimental models of chemically induced Parkinson's disease in zebrafish at the embryonic larval stage: a systematic review. Journal of Toxicology and Environmental Health, Part B 26, 201–237. https://doi.org/10.1080/10937404.2023.2182390

Brix, K. V., De Boeck, G., Baken, S., Fort, D.J., 2022. Adverse Outcome Pathways for Chronic Copper Toxicity to Fish and Amphibians. Environ Toxicol Chem 41, 2911–2927. https://doi.org/10.1002/etc.5483

Burandt, Q.C., Deising, H.B., von Tiedemann, A., 2024. Further Limitations of Synthetic Fungicide Use and Expansion of Organic Agriculture in Europe Will Increase the Environmental and Health Risks of Chemical Crop Protection Caused by Copper-Containing Fungicides. Environ Toxicol Chem 43, 19–30. https://doi.org/10.1002/etc.5766

Calvo, J., Jung, H., Meloni, G., 2017. Copper metallothioneins. IUBMB Life 69, 236–245. https://doi.org/10.1002/iub.1618

Campana, O., Simpson, S.L., Spadaro, D.A., Blasco, J., 2012. Sub-Lethal Effects of Copper to Benthic Invertebrates Explained by Sediment Properties and Dietary Exposure. Environ Sci Technol 46, 6835–6842. https://doi.org/10.1021/es2045844

Capriello, T., Monteiro, S.M., Félix, L.M., Donizetti, A., Aliperti, V., Ferrandino, I., 2021. Apoptosis, oxidative stress and genotoxicity in developing zebrafish after aluminium exposure. Aquatic Toxicology 236, 105872. https://doi.org/10.1016/J.AQUATOX.2021.105872

Castelhano Gebara, R., de Oliveira Gonçalves Alho, L., Bruno de Abreu, C., da Silva Mansano, A., Moreira, R.A., Swerts Rocha, G., Gama Melão, M. da G., 2021. Toxicity and Risk Assessment of Zinc and Aluminum Mixtures to *Ceriodaphnia silvestrii* (Crustacea: Cladocera). Environ Toxicol Chem 40, 2912–2922. https://doi.org/10.1002/etc.5162

Chasapis, C.T., Ntoupa, P.-S.A., Spiliopoulou, C.A., Stefanidou, M.E., 2020. Recent aspects of the effects of zinc on human health. Arch Toxicol 94, 1443–1460. https://doi.org/10.1007/s00204-020-02702-9

Cheignon, C., Tomas, M., Bonnefont-Rousselot, D., Faller, P., Hureau, C., Collin, F., 2018. Oxidative stress and the amyloid beta peptide in Alzheimer's disease. Redox Biol 14, 450–464. https://doi.org/10.1016/j.redox.2017.10.014

Chen, J., Jiang, Y., Shi, H., Peng, Y., Fan, X., Li, C., 2020. The molecular mechanisms of copper metabolism and its roles in human diseases. Pflügers Archiv - European Journal of Physiology 2020 472:10 472, 1415–1429. https://doi.org/10.1007/S00424-020-02412-2

Chen, X., Cai, Q., Liang, R., Zhang, D., Liu, X., Zhang, M., Xiong, Y., Xu, M., Liu, Q., Li, P., Yu, P., Shi, A., 2023. Copper homeostasis and copper-induced cell death in the pathogenesis of cardiovascular disease and therapeutic strategies. Cell Death Dis 14, 105. https://doi.org/10.1038/s41419-023-05639-w

Chojnacka, M., Gornicka, A., Oeljeklaus, S., Warscheid, B., Chacinska, A., 2015. Cox17 Protein Is an Auxiliary Factor Involved in the Control of the Mitochondrial Contact Site and Cristae Organizing System. Journal of Biological Chemistry 290, 15304–15312. https://doi.org/10.1074/jbc.M115.645069

Choo, X.Y., Alukaidey, L., White, A.R., Grubman, A., 2013. Neuroinflammation and Copper in Alzheimer's Disease. Int J Alzheimers Dis 2013, 1–12. https://doi.org/10.1155/2013/145345

Conselho Nacional do Meio Ambiente, 2011. CONAMA RESOLUÇÃO Nº 430, DE 13 DE MAIO DE 2011 [WWW Document].

Costa-Silva, D.G. da, Leandro, L.P., Vieira, P. de B., de Carvalho, N.R., Lopes, A.R., Schimith, L.E., Nunes, M.E.M., de Mello, R.S., Martins, I.K., de Paula, A.A., Cañedo, A.D., Moreira, J.C.F., Posser, T., Franco, J.L., 2018. N -acetylcysteine inhibits Mancozeb-induced impairments to the normal development of zebrafish embryos. Neurotoxicol Teratol 68, 1–12. https://doi.org/10.1016/j.ntt.2018.04.003

de Mendonça Francisco, C., Pavanin, L.A., Morelli, S., Bravo, J.V.M., Pereira, B.B., 2023. Using native fish in eco-genotoxic assessment of heavy metal contamination pollution arising from nearby large Brazilian rivers. J Toxicol Environ Health A 86, 74–85. https://doi.org/10.1080/15287394.2022.2164754

Dornelles Zebral, Y., Roza, M., da Silva Fonseca, J., Gomes Costa, P., Stürmer de Oliveira, C., Gubert Zocke, T., Lemos Dal Pizzol, J., Berteaux Robaldo, R., Bianchini, A., 2019. Waterborne copper is more toxic to the killifish Poecilia vivipara in elevated temperatures: Linking oxidative stress in the liver with reduced organismal thermal performance. Aquatic Toxicology 209, 142–149. https://doi.org/10.1016/J.AQUATOX.2019.02.005

Fasae, K.D., Abolaji, A.O., 2022. Interactions and toxicity of non-essential heavy metals (Cd, Pb and Hg): lessons from Drosophila melanogaster. Curr Opin Insect Sci 51. https://doi.org/10.1016/J.COIS.2022.100900

Fitzgerald, J.A., Katsiadaki, I., Santos, E.M., 2017. Contrasting effects of hypoxia on copper toxicity during development in the three-spined stickleback (Gasterosteus aculeatus). Environmental Pollution 222, 433–443. https://doi.org/10.1016/j.envpol.2016.12.008

Fu, Z., Xi, S., 2020. The effects of heavy metals on human metabolism. Toxicol Mech Methods 30, 167–176. https://doi.org/10.1080/15376516.2019.1701594

Galaris, D., Barbouti, A., Pantopoulos, K., 2019. Iron homeostasis and oxidative stress: An intimate relationship. Biochimica et Biophysica Acta (BBA) - Molecular Cell Research 1866, 118535. https://doi.org/10.1016/j.bbamcr.2019.118535

Garcia, L., Mansilla, N., Ocampos, N., Pagani, M.A., Welchen, E., Gonzalez, D.H., 2019. The mitochondrial copper chaperone COX19 influences copper and iron homeostasis in arabidopsis. Plant Mol Biol 99, 621–638. https://doi.org/10.1007/s11103-019-00840-y

Gauron, C., Meda, F., Dupont, E., Albadri, S., Quenech'Du, N., Ipendey, E., Volovitch, M., Del Bene, F., Joliot, A., Rampon, C., Vriz, S., 2016. Hydrogen peroxide (H2O2) controls axon pathfinding during zebrafish development. Dev Biol 414, 133–141. https://doi.org/10.1016/J.YDBIO.2016.05.004

Glisic, B., Mihaljevic, I., Popovic, M., Zaja, R., Loncar, J., Fent, K., Kovacevic, R., Smital, T., 2015. Characterization of glutathione-S-transferases in zebrafish (Danio rerio). Aquat Toxicol 158, 50–62. https://doi.org/10.1016/J.AQUATOX.2014.10.013

Gouva, E., Nathanailides, C., Skoufos, I., Paschos, I., Athanassopoulou, F., Pappas, I.S., 2020. Comparative study of the effects of heavy metals on embryonic development of zebrafish. Aquac Res 51, 3255–3267. https://doi.org/10.1111/are.14660

Halliwell, B., Gutteridge, J., 2015. Free radicals in biology and medicine.

Hardy, K., Amariutei, A.E., De Faveri, F., Hendry, A., Marcotti, W., Ceriani, F., 2021. Functional development and regeneration of hair cells in the zebrafish lateral line. J Physiol 599, 3913–3936. https://doi.org/10.1113/JP281522

He, F., Huang, X., Wei, G., Lin, X., Zhang, W., Zhuang, W., He, W., Zhan, T., Hu, H., Yang, H., 2022. Regulation of ACSL4-Catalyzed Lipid Peroxidation Process Resists Cisplatin Ototoxicity. Oxid Med Cell Longev 2022, 1–15. https://doi.org/10.1155/2022/3080263

Hernández, P.P., Moreno, V., Olivari, F.A., Allende, M.L., 2006. Sub-lethal concentrations of waterborne copper are toxic to lateral line neuromasts in zebrafish (Danio rerio). Hear Res 213, 1–10. https://doi.org/10.1016/J.HEARES.2005.10.015

Horn, N., Wittung-Stafshede, P., 2021. ATP7A-Regulated Enzyme Metalation and Trafficking in the Menkes Disease Puzzle. Biomedicines 9, 391. https://doi.org/10.3390/biomedicines9040391

Horzmann, K.A., Freeman, J.L., 2018. Making Waves: New Developments in Toxicology With the Zebrafish. Toxicological Sciences 163, 5–12. https://doi.org/10.1093/toxsci/kfy044

Howe, K., Clark, M.D., Torroja, C.F., Torrance, J., Berthelot, C., Muffato, M., Collins, J.E., Humphray, S., McLaren, K., Matthews, L., McLaren, S., Sealy, I., Caccamo, M., Churcher, C., Scott, C., Barrett, J.C., Koch, R., Rauch, G.-J., White, S., Chow, W., Kilian, B., Quintais, L.T., Guerra-Assunção, J.A., Zhou, Y., Gu, Y., Yen, J., Vogel, J.-H., Eyre, T., Redmond, S., Banerjee, R., Chi, J., Fu, B., Langley, E., Maguire, S.F., Laird, G.K., Lloyd, D., Kenyon, E., Donaldson, S., Sehra, H., Almeida-King, J., Loveland, J., Trevanion, S., Jones, M., Quail, M., Willey, D., Hunt, A., Burton, J., Sims, S., McLay, K., Plumb, B., Davis, J., Clee, C., Oliver, K., Clark, R., Riddle, C., Elliott, D., Threadgold, G., Harden, G., Ware, D., Begum, S., Mortimore, B., Kerry, G., Heath, P., Phillimore, B., Tracey, A., Corby, N., Dunn, M., Johnson, C., Wood, J., Clark, S., Pelan, S., Griffiths, G., Smith, M., Glithero, R., Howden, P., Barker, N., Lloyd, C., Stevens, C., Harley, J., Holt, K., Panagiotidis, G., Lovell, J., Beasley, H., Henderson, C., Gordon, D., Auger, K., Wright, D., Collins, J., Raisen, C., Dyer, L., Leung, K., Robertson, L., Ambridge, K., Leongamornlert, D., McGuire, S., Gilderthorp, R., Griffiths, C., Manthravadi, D., Nichol, S., Barker, G., Whitehead, S., Kay, M., Brown, J., Murnane, C., Gray, E., Humphries, M., Sycamore, N., Barker, D., Saunders, D., Wallis, J., Babbage, A., Hammond, S., Mashreghi-Mohammadi, M., Barr, L., Martin, S., Wray, P., Ellington, A., Matthews, N., Ellwood, M., Woodmansey, R., Clark, G., Cooper, J.D., Tromans, A., Grafham, D., Skuce, C., Pandian, R., Andrews, R., Harrison, E., Kimberley, A., Garnett, J., Fosker, N., Hall, R., Garner, P., Kelly, D., Bird, C., Palmer, S., Gehring, I., Berger, A., Dooley, C.M., Ersan-Ürün, Z., Eser, C., Geiger, H., Geisler, M., Karotki, L., Kirn, A., Konantz, J., Konantz, M., Oberländer, M., Rudolph-Geiger, S., Teucke, M., Lanz, C., Raddatz, G., Osoegawa, K., Zhu, B., Rapp, A., Widaa, S., Langford, C., Yang, F., Schuster, S.C., Carter, N.P., Harrow, J., Ning, Z., Herrero, J., Searle, S.M.J., Enright, A., Geisler, R., Plasterk, R.H.A., Lee, C., Westerfield, M., de Jong, P.J., Zon, L.I., Postlethwait, J.H., Nüsslein-Volhard, C., Hubbard, T.J.P., Crollius, H.R., Rogers, J., Stemple, D.L., 2013. The zebrafish reference genome sequence and its relationship to the human genome. Nature 496, 498–503. https://doi.org/10.1038/nature12111

Johnson, A., Carew, E., Sloman, K.A., 2007. The effects of copper on the morphological and functional development of zebrafish embryos. Aquat Toxicol 84, 431–438. https://doi.org/10.1016/J.AQUATOX.2007.07.003

Jozefczak, M., Remans, T., Vangronsveld, J., Cuypers, A., 2012. Glutathione Is a Key Player in Metal-Induced Oxidative Stress Defenses. Int J Mol Sci 13, 3145–3175. https://doi.org/10.3390/ijms13033145

Kadim, M.K., Risjani, Y., 2022. Biomarker for monitoring heavy metal pollution in aquatic environment: An overview toward molecular perspectives. Emerg Contam 8, 195–205. https://doi.org/10.1016/J.EMCON.2022.02.003

Karimi, B., Masson, V., Guilland, C., Leroy, E., Pellegrinelli, S., Giboulot, E., Maron, P.-A., Ranjard, L., 2021. Ecotoxicity of copper input and accumulation for soil biodiversity in vineyards. Environ Chem Lett 19, 2013–2030. https://doi.org/10.1007/s10311-020-01155-x

Kimmel, C.B., Ballard, W.W., Kimmel, S.R., Ullmann, B., Schilling, T.F., 1995. Stages of embryonic development of the zebrafish. Developmental Dynamics 203, 253–310. https://doi.org/10.1002/AJA.1002030302

Koledenkova, K., Esmaeel, Q., Jacquard, C., Nowak, J., Clément, C., Ait Barka, E., 2022. Plasmopara viticola the Causal Agent of Downy Mildew of Grapevine: From Its Taxonomy to Disease Management. Front Microbiol 13. https://doi.org/10.3389/fmicb.2022.889472

Kopittke, P.M., Menzies, N.W., Wang, P., McKenna, B.A., Lombi, E., 2019. Soil and the intensification of agriculture for global food security. Environ Int 132, 105078. https://doi.org/10.1016/j.envint.2019.105078

Krupa, P.M., McMurry, S.T., Minghetti, M., Belden, J.B., 2021. Effects of Copper on the Neuromasts of Xenopus Laevis. Arch Environ Contam Toxicol 80, 769–778. https://doi.org/10.1007/s00244-020-00778-z

La Fontaine, S., Ackland, M.L., Mercer, J.F.B., 2010. Mammalian copper-transporting P-type ATPases, ATP7A and ATP7B: Emerging roles. Int J Biochem Cell Biol 42, 206–209. https://doi.org/10.1016/j.biocel.2009.11.007

Lai, K.P., Gong, Z., Tse, W.K.F., 2021. Zebrafish as the toxicant screening model: Transgenic and omics approaches. Aquatic Toxicology 234, 105813. https://doi.org/10.1016/j.aquatox.2021.105813

Leal, P.R., Moschini-Carlos, V., López-Doval, J.C., Cintra, J.P., Yamamoto, J.K., Bitencourt, M.D., Santos, R.F., Abreu, G.C., Pompêo, M.L.M., 2018. Impact of copper sulfate application at an urban Brazilian reservoir: A geostatistical and ecotoxicological approach. Science of The Total Environment 618, 621–634. https://doi.org/10.1016/j.scitotenv.2017.07.095

Li, C., Zhou, K., Qin, W., Tian, C., Qi, M., Yan, X., Han, W., 2019. A Review on Heavy Metals Contamination in Soil: Effects, Sources, and Remediation Techniques. Soil and Sediment Contamination: An International Journal 28, 380–394. https://doi.org/10.1080/15320383.2019.1592108

Li, Y., 2020. Copper homeostasis: Emerging target for cancer treatment. IUBMB Life 72, 1900–1908. https://doi.org/10.1002/iub.2341

Linbo, T.L., Stehr, C.M., Incardona, J.P., Scholz, N.L., 2006. DISSOLVED COPPER TRIGGERS CELL DEATH IN THE PERIPHERAL MECHANOSENSORY SYSTEM OF LARVAL FISH. Environ Toxicol Chem 25, 597. https://doi.org/10.1897/05-241R.1

Liu, J.H., Cheng, J., Zhou, L., Feng, L.P., Hu, Y.T., Algeo, T.J., 2022. Copper and gallium isotopic behavior in highly weathered soils. Chem Geol 594, 120757. https://doi.org/10.1016/J.CHEMGEO.2022.120757

Liu, Y., Wang, H., Cui, Y., Chen, N., 2023. Removal of Copper Ions from Wastewater: A Review. Int J Environ Res Public Health 20, 3885. https://doi.org/10.3390/ijerph20053885

Lucena-Valera, A., Ruz-Zafra, P., Ampuero, J., 2023. Wilson's disease: overview. Med Clin (Barc) 160, 261–267. https://doi.org/10.1016/j.medcli.2022.12.016

Lushchak, V.I., 2014. Free radicals, reactive oxygen species, oxidative stress and its classification. Chem Biol Interact 224, 164–175. https://doi.org/10.1016/j.cbi.2014.10.016

Martins, I.K., Pereira, L.G., Nunes, M.E.M., Lopes, A.R., Leandro, L.P., de Mello, R.S., Mariano, M.V.T., Franco, J.L., Posser, T., 2023. Exposure to Mancozeb results in increased MAPK phosphorylation and locomotor deficits in zebrafish larvae. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology 270, 109659. https://doi.org/10.1016/j.cbpc.2023.109659

Maryon, E.B., Molloy, S.A., Kaplan, J.H., 2013. Cellular glutathione plays a key role in copper uptake mediated by human copper transporter 1. American Journal of Physiology-Cell Physiology 304, C768–C779. https://doi.org/10.1152/ajpcell.00417.2012

Mason, E., Wieser, P.E., Liu, E.J., Edmonds, M., Ilyinskaya, E., Whitty, R.C.W., Mather, T.A., Elias, T., Nadeau, P.A., Wilkes, T.C., McGonigle, A.J.S., Pering, T.D., Mims, F.M., Kern, C., Schneider, D.J., Oppenheimer, C., 2021. Volatile metal emissions from volcanic degassing and lava–seawater interactions at Kīlauea Volcano, Hawai'i. Commun Earth Environ 2, 79. https://doi.org/10.1038/s43247-021-00145-3

Michiels, E., Vergauwen, L., Hagenaars, A., Fransen, E., Dongen, S., Van Cruchten, S., Bervoets, L., Knapen, D., 2017. Evaluating Complex Mixtures in the Zebrafish Embryo by Reconstituting Field Water Samples: A Metal Pollution Case Study. Int J Mol Sci 18, 539. https://doi.org/10.3390/ijms18030539

Ministério da Saúde, 2021. PORTARIA GM/MS Nº 888, DE 4 DE MAIO DE 2021.

Ministério da Saúde, 2011. PORTARIA GM/MS Nº 2.941, DE 12 DE DEZEMBRO DE 2011.

Ministério da Saúde, 2005. RESOLUÇÃO-RDC Nº 269, DE 22 DE SETEMBRO DE 2005 [WWW Document]. URL https://bvsms.saude.gov.br/bvs/saudelegis/anvisa/2005/rdc0269_22_09_2005.html

Mirzaei VandKhanghah, M., Hedayati, A., Nazeri, S., Mohammadi Azarm, H., Ghorbani, R., 2022. Biomagnification of Copper Along the Aquatic Food Chain (Artemia franciscana, Danio rerio, and Astronotus ocellatus). Biol Trace Elem Res 200, 1854–1860. https://doi.org/10.1007/s12011-021-02781-4

Mohammadbakir, S., 2016. Impacts of waterborne copper and silver on the early life stage (ELS) of zebrafish (Danio rerio): physiological, biochemical and molecular responses. University of Plymouth. https://doi.org/https://dx.doi.org/10.24382/3520

Mondello, V., Lemaître-Guillier, C., Trotel-Aziz, P., Gougeon, R., Acedo, A., Schmitt-Kopplin, P., Adrian, M., Pinto, C., Fernandez, O., Fontaine, F., 2022. Assessment of a New Copper-Based Formulation to Control Esca Disease in Field and Study of Its Impact on the Vine Microbiome, Vine Physiology and Enological Parameters of the Juice. Journal of Fungi 8, 151. https://doi.org/10.3390/jof8020151

Morris, G., Anderson, G., Dean, O., Berk, M., Galecki, P., Martin-Subero, M., Maes, M., 2014. The Glutathione System: A New Drug Target in Neuroimmune Disorders. Mol Neurobiol 50, 1059–1084. https://doi.org/10.1007/s12035-014-8705-x

Naeem, S., Ashraf, M., Babar, M.E., Zahoor, S., Ali, S., 2021. The effects of some heavy metals on some fish species. Environmental Science and Pollution Research 28, 25566–25578. https://doi.org/10.1007/s11356-021-12385-z

National Institutes of Health, 2022. Copper - Health Professional Fact Sheet [WWW Document]. URL https://ods.od.nih.gov/factsheets/Copper-HealthProfessional/ (accessed 2.29.24).

Olivari, F.A., Hernández, P.P., Allende, M.L., 2008. Acute copper exposure induces oxidative stress and cell death in lateral line hair cells of zebrafish larvae. Brain Res 1244, 1–12. https://doi.org/10.1016/j.brainres.2008.09.050

Paganotto Leandro, L., Siqueira de Mello, R., da Costa- Silva, D.G., Medina Nunes, M.E., Rubin Lopes, A., Kemmerich Martins, I., Posser, T., Franco, J.L., 2021. Behavioral changes occur earlier than redox alterations in developing zebrafish exposed to Mancozeb. Environmental Pollution 268, 115783. https://doi.org/10.1016/j.envpol.2020.115783

Paganotto Leandro, L., Vitória Takemura Mariano, M., Kich Gomes, K., Beatriz dos Santos, A., Sousa dos Anjos, J., Rodrigues de Carvalho, N., Eugênio Medina Nunes, M., Posser, T., Luis Franco, J., 2023. Permissible concentration of mancozeb in Brazilian drinking water elicits oxidative stress and bioenergetic impairments in embryonic zebrafish. Environmental Pollution 333, 122013. https://doi.org/10.1016/J.ENVPOL.2023.122013

Peng, C., Wang, X., Chen, J., Jiao, R., Wang, L., Li, Y.M., Zuo, Y., Liu, Y., Lei, L., Ma, K.Y., Huang, Y., Chen, Z.-Y., 2014. Biology of Ageing and Role of Dietary Antioxidants. Biomed Res Int 2014, 1–13. https://doi.org/10.1155/2014/831841

Pereira, T.C.B., Campos, M.M., Bogo, M.R., 2016. Copper toxicology, oxidative stress and inflammation using zebrafish as experimental model. Journal of Applied Toxicology 36, 876–885. https://doi.org/10.1002/jat.3303

Poggere, G., Gasparin, A., Barbosa, J.Z., Melo, G.W., Corrêa, R.S., Motta, A.C.V., 2023. Soil contamination by copper: Sources, ecological risks, and mitigation strategies in Brazil. Journal of Trace Elements and Minerals 4, 100059. https://doi.org/10.1016/j.jtemin.2023.100059

Pompermaier, A., Varela, A.C.C., Fortuna, M., Mendonça-Soares, S., Koakoski, G., Aguirre, R., Oliveira, T.A., Sordi, E., Moterle, D.F., Pohl, A.R., Rech, V.C., Bortoluzzi, E.C., Barcellos, L.J.G., 2021. Water and suspended sediment runoff from vineyard watersheds affecting the behavior and physiology of zebrafish. Science of The Total Environment 757, 143794. https://doi.org/10.1016/j.scitotenv.2020.143794

Rader, K.J., Carbonaro, R.F., van Hullebusch, E.D., Baken, S., Delbeke, K., 2019. The Fate of Copper Added to Surface Water: Field, Laboratory, and Modeling Studies. Environ Toxicol Chem 38, 1386–1399. https://doi.org/10.1002/etc.4440

Rastogi, A., Timme-Laragy, A.R., 2021. Using Monochlorobimane to Visualize Glutathione Utilization in the Developing Zebrafish (*Danio rerio*) Embryo. Curr Protoc 1. https://doi.org/10.1002/cpz1.24

Rehman, M., Liu, L., Wang, Q., Saleem, M.H., Bashir, S., Ullah, S., Peng, D., 2019. Copper environmental toxicology, recent advances, and future outlook: a review. Environmental Science and Pollution Research 26, 18003–18016. https://doi.org/10.1007/s11356-019-05073-6

Santamaria, A.B., Sulsky, S.I., 2010. Risk Assessment of an Essential Element: Manganese. J Toxicol Environ Health A 73, 128–155. https://doi.org/10.1080/15287390903337118

Scheiber, I., Dringen, R., Mercer, J.F.B., 2013. Copper: Effects of Deficiency and Overload. pp. 359–387. https://doi.org/10.1007/978-94-007-7500-8_11

Sharma, R.K., Agrawal, M., 2005. Biological effects of heavy metals: an overview. J Environ Biol 26, 301–13.

Shawki, A., Anthony, S.R., Nose, Y., Engevik, M.A., Niespodzany, E.J., Barrientos, T., Öhrvik, H., Worrell, R.T., Thiele, D.J., Mackenzie, B., 2015. Intestinal DMT1 is critical for iron absorption in the mouse but is not required for the absorption of copper or manganese. American Journal of Physiology-Gastrointestinal and Liver Physiology 309, G635–G647. https://doi.org/10.1152/ajpgi.00160.2015

Sies, H., 2019. Oxidative Stress, in: Stress: Physiology, Biochemistry, and Pathology. Elsevier, pp. 153–163. https://doi.org/10.1016/B978-0-12-813146-6.00013-8

Sies, H., 2017. Hydrogen peroxide as a central redox signaling molecule in physiological oxidative stress: Oxidative eustress. Redox Biol 11, 613–619. https://doi.org/10.1016/J.REDOX.2016.12.035

Skopp, A., Boyd, S.D., Ullrich, M.S., Liu, L., Winkler, D.D., 2019a. Copper–zinc superoxide dismutase (Sod1) activation terminates interaction between its copper chaperone (Ccs) and the cytosolic metal-binding domain of the copper importer Ctr1. BioMetals 32, 695–705. https://doi.org/10.1007/s10534-019-00206-3

Skopp, A., Boyd, S.D., Ullrich, M.S., Liu, L., Winkler, D.D., 2019b. Copper–zinc superoxide dismutase (Sod1) activation terminates interaction between its copper chaperone (Ccs) and the cytosolic metal-

binding domain of the copper importer Ctr1. BioMetals 32, 695–705. https://doi.org/10.1007/s10534-019-00206-3

Squitti, R., Simonelli, I., Ventriglia, M., Siotto, M., Pasqualetti, P., Rembach, A., Doecke, J., Bush, A.I., 2013. Meta-Analysis of Serum Non-Ceruloplasmin Copper in Alzheimer's Disease. Journal of Alzheimer's Disease 38, 809–822. https://doi.org/10.3233/JAD-131247

Streisinger, G., Walker, C., Dower, N., Knauber, D., Singer, F., 1981. Production of clones of homozygous diploid zebra fish (Brachydanio rerio). Nature 291, 293–296. https://doi.org/10.1038/291293a0

Swaminathan, A.B., Gohil, V.M., 2022. The Role of COA6 in the Mitochondrial Copper Delivery Pathway to Cytochrome c Oxidase. Biomolecules 12, 125. https://doi.org/10.3390/biom12010125

Tamm, L., Thuerig, B., Apostolov, S., Blogg, H., Borgo, E., Corneo, P.E., Fittje, S., de Palma, M., Donko, A., Experton, C., Marín, É.A., Pérez, Á.M., Pertot, I., Rasmussen, A., Steinshamn, H., Vetemaa, A., Willer, H., Herforth-Rahmé, J., 2021. Copper use in organic agriculture in twelve European countries. agriRxiv 2021. https://doi.org/10.31220/agriRxiv.2021.00108

Tao, M., Nie, K., Zhao, R., Shi, Y., Cao, W., 2022. Environmental impact of mining and beneficiation of copper sulphate mine based on life cycle assessment. Environmental Science and Pollution Research 29, 87613–87627. https://doi.org/10.1007/s11356-022-21317-4

Taslima, K., Al-Emran, M., Rahman, M.S., Hasan, J., Ferdous, Z., Rohani, M.F., Shahjahan, M., 2022. Impacts of heavy metals on early development, growth and reproduction of fish – A review. Toxicol Rep 9, 858–868. https://doi.org/10.1016/j.toxrep.2022.04.013

Tchounwou, P.B., Yedjou, C.G., Patlolla, A.K., Sutton, D.J., 2012. Heavy Metal Toxicity and the Environment. pp. 133–164. https://doi.org/10.1007/978-3-7643-8340-4_6

Teame, T., Zhang, Z., Ran, C., Zhang, H., Yang, Y., Ding, Q., Xie, M., Gao, C., Ye, Y., Duan, M., Zhou, Z., 2019. The use of zebrafish (Danio rerio) as biomedical models. Animal Frontiers 9, 68–77. https://doi.org/10.1093/af/vfz020

Test No. 236: Fish Embryo Acute Toxicity (FET) Test, 2013. . OECD. https://doi.org/10.1787/9789264203709-en

Tsang, T., Davis, C.I., Brady, D.C., 2021. Copper biology. Current Biology 31, R421–R427. https://doi.org/10.1016/j.cub.2021.03.054

Vellingiri, B., Suriyanarayanan, A., Selvaraj, P., Abraham, K.S., Pasha, Md.Y., Winster, H., Gopalakrishnan, A.V., G, S., Reddy, J.K., Ayyadurai, N., Kumar, N., Giridharan, B., P, S., Rao, K.R.S.S., Nachimuthu, S.K., Narayanasamy, A., Mahalaxmi, I., Venkatesan, D., 2022a. Role of heavy metals (copper (Cu), arsenic (As), cadmium (Cd), iron (Fe) and lithium (Li)) induced neurotoxicity. Chemosphere 301, 134625. https://doi.org/10.1016/j.chemosphere.2022.134625

Vellingiri, B., Suriyanarayanan, A., Selvaraj, P., Abraham, K.S., Pasha, Md.Y., Winster, H., Gopalakrishnan, A.V., G, S., Reddy, J.K., Ayyadurai, N., Kumar, N., Giridharan, B., P, S., Rao, K.R.S.S., Nachimuthu, S.K., Narayanasamy, A., Mahalaxmi, I., Venkatesan, D., 2022b. Role of heavy metals (copper (Cu), arsenic (As), cadmium (Cd), iron (Fe) and lithium (Li)) induced neurotoxicity. Chemosphere 301, 134625. https://doi.org/10.1016/j.chemosphere.2022.134625

Wang, R.-F., Zhu, L.-M., Zhang, J., An, X.-P., Yang, Y.-P., Song, M., Zhang, L., 2020a. Developmental toxicity of copper in marine medaka (Oryzias melastigma) embryos and larvae. Chemosphere 247, 125923. https://doi.org/10.1016/j.chemosphere.2020.125923

Wang, R.-F., Zhu, L.-M., Zhang, J., An, X.-P., Yang, Y.-P., Song, M., Zhang, L., 2020b. Developmental toxicity of copper in marine medaka (Oryzias melastigma) embryos and larvae. Chemosphere 247, 125923. https://doi.org/10.1016/j.chemosphere.2020.125923

Wang, Y.H., Wang, Y.Q., Yu, X.G., Lin, Y., Liu, J.X., Wang, W.Y., Yan, C.H., 2023. Chronic environmental inorganic arsenic exposure causes social behavioral changes in juvenile zebrafish (Danio rerio). Science of The Total Environment 867, 161296. https://doi.org/10.1016/J.SCITOTENV.2022.161296

Wilson, C., Muñoz-Palma, E., González-Billault, C., 2018. From birth to death: A role for reactive oxygen species in neuronal development. Semin Cell Dev Biol 80, 43–49. https://doi.org/10.1016/J.SEMCDB.2017.09.012

Witeska, M., Sarnowski, P., Ługowska, K., Kowal, E., 2014. The effects of cadmium and copper on embryonic and larval development of ide Leuciscus idus L. Fish Physiol Biochem 40, 151–163. https://doi.org/10.1007/s10695-013-9832-4

Wong, P.C., Waggoner, D., Subramaniam, J.R., Tessarollo, L., Bartnikas, T.B., Culotta, V.C., Price, D.L., Rothstein, J., Gitlin, J.D., 2000. Copper chaperone for superoxide dismutase is essential to activate mammalian Cu/Zn superoxide dismutase. Proceedings of the National Academy of Sciences 97, 2886–2891. https://doi.org/10.1073/pnas.040461197

Wyman, S., Simpson, R.J., McKie, A.T., Sharp, P.A., 2008. Dcytb (Cybrd1) functions as both a ferric and a cupric reductase in vitro. FEBS Lett 582, 1901–1906. https://doi.org/10.1016/j.febslet.2008.05.010

Xu, J.P., Zhang, R.T., Zhang, T., Zhao, G., Huang, Y., Wang, H.L., Liu, J.X., 2017. Copper impairs zebrafish swimbladder development by down-regulating Wnt signaling. Aquatic Toxicology 192, 155–164. https://doi.org/10.1016/J.AQUATOX.2017.09.018

Yang, F., Yun, Y., Li, G., Sang, N., 2021. Heavy metals in soil from gangue stacking areas increases children health risk and causes developmental neurotoxicity in zebrafish larvae. Science of The Total Environment 794, 148629. https://doi.org/10.1016/j.scitotenv.2021.148629

Yi, L., Kaler, S., 2014. ATP7A trafficking and mechanisms underlying the distal motor neuropathy induced by mutations in ATP7A. Ann N Y Acad Sci 1314, 49–54. https://doi.org/10.1111/nyas.12427

Zabegalov, K.N., Kolesnikova, T.O., Khatsko, S.L., Volgin, A.D., Yakovlev, O.A., Amstislavskaya, T.G., Friend, A.J., Bao, W., Alekseeva, P.A., Lakstygal, A.M., Meshalkina, D.A., Demin, K.A., de Abreu, M.S., Rosemberg, D.B., Kalueff, A. V., 2019. Understanding zebrafish aggressive behavior. Behavioural Processes 158, 200–210. https://doi.org/10.1016/j.beproc.2018.11.010

Zamora-Ledezma, C., Negrete-Bolagay, D., Figueroa, F., Zamora-Ledezma, E., Ni, M., Alexis, F., Guerrero, V.H., 2021. Heavy metal water pollution: A fresh look about hazards, novel and conventional remediation methods. Environ Technol Innov 22, 101504. https://doi.org/10.1016/J.ETI.2021.101504

Zhang, L., Yang, Z., Yang, M., Yang, F., Wang, G., Liu, D., Li, X., Yang, L., Wang, Z., 2022. Copper-induced oxidative stress, transcriptome changes, intestinal microbiota, and histopathology of common carp (Cyprinus carpio). Ecotoxicol Environ Saf 246, 114136. https://doi.org/10.1016/j.ecoenv.2022.114136

Zhang, T., Xu, L., Wu, J.-J., Wang, W.-M., Mei, J., Ma, X.-F., Liu, J.-X., 2015. Transcriptional Responses and Mechanisms of Copper-Induced Dysfunctional Locomotor Behavior in Zebrafish Embryos. Toxicological Sciences 148, 299–310. https://doi.org/10.1093/toxsci/kfv184

Zhao, G., Sun, H., Zhang, T., Liu, J.-X., 2020. Copper induce zebrafish retinal developmental defects via triggering stresses and apoptosis. Cell Communication and Signaling 18, 45. https://doi.org/10.1186/s12964-020-00548-3

Zhao, L., Xia, Z., Wang, F., 2014. Zebrafish in the sea of mineral (iron, zinc, and copper) metabolism. Front Pharmacol 5. https://doi.org/10.3389/fphar.2014.00033

Zhen, Y., Ge, L., Chen, Q., Xu, J., Duan, Z., Loor, J.J., Wang, M., 2022. Latent Benefits and Toxicity Risks Transmission Chain of High Dietary Copper along the Livestock–Environment–Plant–Human Health Axis and Microbial Homeostasis: A Review. J Agric Food Chem 70, 6943–6962. https://doi.org/10.1021/acs.jafc.2c01367

Ziller, A., Fraissinet-Tachet, L., 2018. Metallothionein diversity and distribution in the tree of life: a multifunctional protein. Metallomics 10, 1549–1559. https://doi.org/10.1039/C8MT00165K