UNIVERSIDADE FEDERAL DO PARANÁ

LUIS PHELIPE DE SOUZA MIRANDA

AVALIAÇÃO ECOGENOTOXICOLÓGICA DE NANOPARTÍCULAS DE ÓXIDO DE ZINCO (NPS-ZNO) NÃO DOPADAS E DOPADAS COM NÍQUEL (NI) NO PEIXE NATIVO RHAMDIA QUELEN

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Dissertação apresentada ao Programa de Pós-Graduação em Genética, Departamento de Genética, Setor de Ciências Biológicas, Universidade Federal do Paraná, como parte das exigências para a obtenção do grau de Mestre em Ciências Biológicas, área de concentração Genética.

Orientador(a): Prof(a). Dr(a). Marta Margarete Cestari

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TERMO DE APROVAÇÃO

Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação GENÉTICA da Universidade Federal do Paraná foram convocados para realizar a arguição da Dissertação de Mestrado de **LUIS PHELIPE DE SOUZA MIRANDA** intitulada: **Avaliação Ecogenotoxicológica de Nanopartículas de Óxido de Zinco (NPs-ZnO) não dopadas e dopadas com níquel (Ni) no peixe nativo *Rhamdia quelen***
, sob orientação da Profa. Dra. MARTA MARGARETE CESTARI, que após terem inquirido o aluno e realizada a avaliação do trabalho, são de parecer pela sua APROVAÇÃO no rito de defesa.
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A minha mãe:

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(in memoriam)

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Remember when you had a dream…

(Angelo, S.)

RESUMO

As nanopartículas de óxido de zinco (NPs-ZnO) são bons semicondutores utilizados na fabricação de sensores eletrônicos, painéis fotovoltaicos e transdutores, tendo sido empregadas, mais recentemente também, na remediação ambiental e na medicina. As NPs-ZnO têm sido testadas *in natura* e com diferentes agentes dopantes, visando o combate a microrganismos, e oferecendo uma possibilidade de substituição aos antibióticos atuais. Desta maneira, essas nanopartículas em diferentes estados teriam aplicações futuras para o tratamento de infecções de origem bacterianas. Porém em consequência da alta empregabilidade e produção deste nanomaterial devemos estar alertas quanto a sua periculosidade a sistemas naturais. Estudos já apontam que as NPs-ZnO podem desencadear danos a diferentes organismos, sendo esta toxicidade altamente relacionada com os meios de produção, mecanismo de contaminação e a natureza dos sistemas-teste. Portanto, se fazem necessários estudos ecotoxicologicos que avaliem os reais impactos destas nanopartículas. A realização de estudos de ecogenotoxicidade com espécies nativas é importante pois são elas que estão presentes em nossos ambientes naturais. Este trabalho teve como objetivo avaliar os efeitos da toxicidade aguda de NPs-ZnO não dopadas e dopadas com níquel em diferentes tecidos de um peixe neotropical. Para tanto, foi utilizada a espécie *Rhamdia quelen* (jundiá) que é um peixe endêmico da América do Sul e muito utilizado na piscicultura. As concentrações das NPs-ZnO não dopadas e dopadas com níquel (0.4, 4, e 40 mg. L^{-1}), utilizadas para os testes ecogenotoxicológicos apresentam ação antimicrobiana contra bactérias gram-negativas e gram-positivas. Desta forma foram testadas em bioensaio hídrico de 96h, semi-estático, para avaliação da toxicidade foram utilizados biomarcadores genéticos como ensaio cometa e teste do Microcúcleo Pisceo, e bioquímicos como lipoperoxidação (LPO), superóxido dismutase (SOD), catalase (CAT), glutationa-S-transferase (GST) e acetilcolinesterase (AChE). Os resultados dos biomarcadores genéticos mostram que nenhuma das nanopartículas gerou efeitos mutagênicos. No entanto, ZnO-NPs (Ni) produziram efeitos genotóxicos no sangue, cérebro, fígado e brânquias de *R. quelen*. Além disso, a atividade das enzimas SOD e GST no fígado e brânquias apresentou alteração para as concentrações de NPs-ZnO 0.4 e 4, mg. L^{-1}. Não houve alterações na atividade de CAT, AChE e LPO.

Palavras-chave: NPs-ZnO; genotoxicidade; nanopartículas metálicas; nanotoxicologia; toxicologia aquática;

ABSTRACT

Zinc oxide nanoparticles (ZnO-NPs) are good semiconductors used to manufacture electronic sensors, photovoltaic panels, and transducers. They have been used, more recently, in environmental remediation and medicine. ZnO-NPs have been tested in nature and with different doping agents, aiming to combat microorganisms and offering the possibility of replacing current antibiotics. In this way, these nanoparticles in different states would have future applications for treating infections of bacterial origin. However, due to this nanomaterial's high employability and production, we must be alert to its danger to natural systems. Studies already indicate that ZnO-NPs can cause damage to different organisms, with this toxicity being highly related to the means of production, contamination mechanism, and nature of the test systems. Therefore, ecotoxicological studies are necessary to evaluate the real impacts of these nanoparticles. Carrying out ecogenotoxicity studies with native species is important because they are present in our natural environments. This work aimed to evaluate the effects of the acute toxicity of undoped and nickel-doped ZnO-NPs in different tissues of neotropical fish. To this end, the species *Rhamdia quelen* (jundiá), a fish endemic to South America, was widely used in fish farming. The concentrations of ZnO-NPs (0.4, 4, and 40 mg. L-1), used for ecogenotoxicological tests, show antimicrobial action against gram-negative and gram-positive bacteria. In this way, they were tested in a 96-hour, semi-static water bioassay. To evaluate the toxicity of ZnO-NPs, genetic biomarkers were used, such as the comet assay and the Piscean Micronucleus test, and biochemicals such as lipoperoxidation (LPO), superoxide dismutase (SOD), catalase (CAT), glutathione-S-transferase (GST), and acetylcholinesterase (AChE). The genetic biomarker results show that none of the nanoparticles generated mutagenic effects. However, ZnO-NPs (Ni) produced genotoxic effects in the blood, brain, liver, and gills of *R. quelen*. Furthermore, the activity of SOD and GST enzymes in the liver and gills showed changes for ZnO-NPs concentrations of 0.4 and 4 mg. L-1. There were no changes in CAT, AChE, and LPO activity.

Keywords**:** ZnO-NPs; genotoxicity; metallic nanoparticles; nanotoxicology; aquatic toxicology;

LISTA DE SIGLAS

AChE – Acetilcolinesterase
CAT – Catalase
DNA - Ácido desoxirribonucleico
EDS - Espectroscopia de raios X por dispersão em energia (do inglês Energy-dispersive X-ray spectroscopy)
EDTA -Ácido etileno diamino tetracético (do inglês Ethylenidiaminetetracetic Acid)
FAO - Organização para Alimentação e Agricultura (do ingês Food and Agricultura Organization)
GSH - Reduce Glutatione
L -Litro
LPO -peroxidação lipídica (do inglês *lipid peroxidation*)
LMP - Low Melting Point
MET -microscopia eletrônica de transmissão
MN – Micronúleos
Ni -Níquel
NP(s) -nanopartícula (s)
PBS -Tampão fosfato salino (do inglês phosphate buffer saline)
pH- Potencial de Hidrogênio Iônico
ROS - Reactive Oxygen Species
SAED - difração de elétrons de área selecionada (do inglês selected area electron diffraction)
SOD - Superóxide Dismutase
TiO2 -Dióxido de titânio
ZnO-NPs – Nanopartículas de óxido de zinco
NPs-ZnO - Nanopartículas de óxido de zinco

LISTA DE SÍMBOLOS

º C- Graus Celsius

cm – Centímetro

H_2O_2 – Peróxido de Hidrogênio

Kg - Quilograma

L – Litro

mg. L^{-1} – Miligrama por Litro

mA- Miliampere

min- Minuto

ng- Nanograma

Ni – Níquel

O_2^- - Superóxido

OH – Hidroxila

% - Percentual

µg – Micrograma

µm – Micrometro

V- Volt

Zn – Zinco

Zn^{2+} – Ion Zinco

SUMÁRIO

1 INTRODUÇÃO GERAL

A ecotoxicologia se apresenta como campo da ciência responsável pelos estudos dos efeitos toxicológicos que substâncias químicas podem provocar nos diferentes níveis de organização dos sistemas biológicos (Newman; Zhao, 2008; Hellou, 2011; Zhou *et al.*, 2018). Esta ciência analisa o movimento das substâncias no ambiente, incluindo a transformação, migração e degradação (Zhou *et al.*, 2018). Diversos fatores culminaram no desenvolvimento desta ciência, os mais relevantes foram os impactos causados por metais pesados, pesticidas e materiais orgânicos (Chapman, 1995).

Os principais objetivos da ecotoxicologia são identificar os primeiros sinais de ação das substâncias químicas, determinar concentrações ambientais aceitáveis e prevenir a degradação ambiental (Hellou, 2011; Philippe *et al.*, 2021). Por isso, este ramo de estudo se dispõe a prever a ação causada por agentes nocivos e identificar as respostas bioquímicas e fisiológicas, além de averiguar a sensibilidade a diferentes classes de químicos e sua toxicidade relativa (Chapman, 2002; Tlili; Mouneyrac, 2021).

Analisando o escopo dos estudos ecotoxicológicos, fica claro que esta área de pesquisa é uma importante ferramenta no controle e no monitoramento de substâncias nocivas aos organismos por meio da identificação das possíveis consequências aos mais diversos compartimentos ambientais (Chaman, 2002; Noventa *et al.*,2018). Sendo assim, a ecotoxicologia pode se inteirar a respeito dos fenômenos de intoxicação ambiental com a finalidade de prevenir, impedir e interromper os efeitos danosos, oferecendo alternativas para a reversão, remediação ou interrupção da degradação ambiental da fonte poluidora (Magalhães; Ferrão, 2008).

Diversas substâncias capazes de promover a intoxicação ambiental são produzidas pela ação antrópica, onde uma grande variedade destas substâncias é denominada de contaminantes emergentes ou *contaminats of emerging concern* (*CECs*), se tratando de substâncias que estão sendo encontradas em concentrações significativamente elevadas do que as esperadas, oferecendo risco a saúde ambiental e humana (Montagner *et al.*, 2019; Pastorino e Ginebreda, 2021). Pesticidas, fármacos, produtos de cuidado pessoal, hormônios, protetores solares, filtros UV, drogas ilícitas, compostos perfluorinatos, desinfetantes, microplásticos e

nanomaterias são as substâncias mais investigadas como contaminantes emergentes (Ziccardi *et al.*, 2016; Montagner, 2019; Khan *et al.*, 2022).

Dentre os contaminantes emergentes atuais, temos diversos nanomateriais, incluindo as nanopartículas (NPs) (EPA, 2010; El-Kalliny *et al.*, 2023). As nanopartículas apresentam pelo menos uma de suas dimensões entre 1-100 nm, o que lhes confere propriedades únicas quando comparadas a mesma substância em escala micrométrica (Freitas, 2018).

A classificação das nanopartículas é diversa e pode se dar de acordo suas dimensões, origem, natureza química e aplicabilidade (Singh, 2016). A diminuição no tamanho das partículas é responsável pela alteração em suas propriedades elétricas (“*gap”* de energia ou *“band gap”)*. Desta forma, as propriedades óticas e elétricas são alteradas, aumentando a aplicabilidade destas substâncias (Arshad *et al.*, 2011, Terna *et al.*, 2021; Yung *et al.*, 2017). Uma de suas aplicações incluem o desenvolvimento de antimicrobianos, para qual uma variedade de substâncias tem sido testada, se apresentando em ordem crescente: as nanopartículas de prata coloidal (Ag), de óxido de zinco (ZnO), de óxido de cobre (CuO), de óxido de ferro III (Fe_2O_3), de óxido de titânio (TiO_2) e dióxido de níquel (NiO) (Freitas, 2018; Raj *et al.*, 2021; Lokapur *et al.*, 2022).

Alguns estudos recentes já apontam o potencial bactericida que as nanopartículas podem apresentar (Hassan *et al.*, 2021; Jimoh *et al.*, 2022; Subha *et al.*,2022). Um estudo realizado por Ansari (2012) evidenciou a ação antimicrobiana das nanopartículas de óxido de Zinco (NPs-ZnO), assinalando que estas NPs são mais indicadas para o uso em substâncias bactericidas quando comparadas as nanopartículas de prata (Ag-NPs) (ROQUE; FREITAS, 2018).

Recentemente, foi desenvolvida no departamento de física da UFPR (Prof. Dr. Ney Mattoso), uma nanopartícula de óxido de zinco pelo método sol-gel, NPs-ZnO dopadas e codopadas com os metais Co, Cu e Ni que apresentam absorção e a emissão de fótons em diferentes regiões do espectro visível, como indicativo de potencialização da atividade bactericida das nanopartículas de ZnO.

Considerando os fatores abordados até o momento, este trabalho de pesquisa visou avaliar a ecotoxicidade das nanopartículas de oxido de zinco em dois estados, dopadas e não dopadas com níquel, utilizando o organismo teste nativo *Rhamdia quelen* como bioindicador. Visando contribuir com a literatura acerca das respostas

ecotoxicológicas à materiais nanoparticulados e contribuindo com a elucidação da ação deste contaminante emergente em concentrações bactericidas.

2 REVISÃO BIBLIOGRAFICA

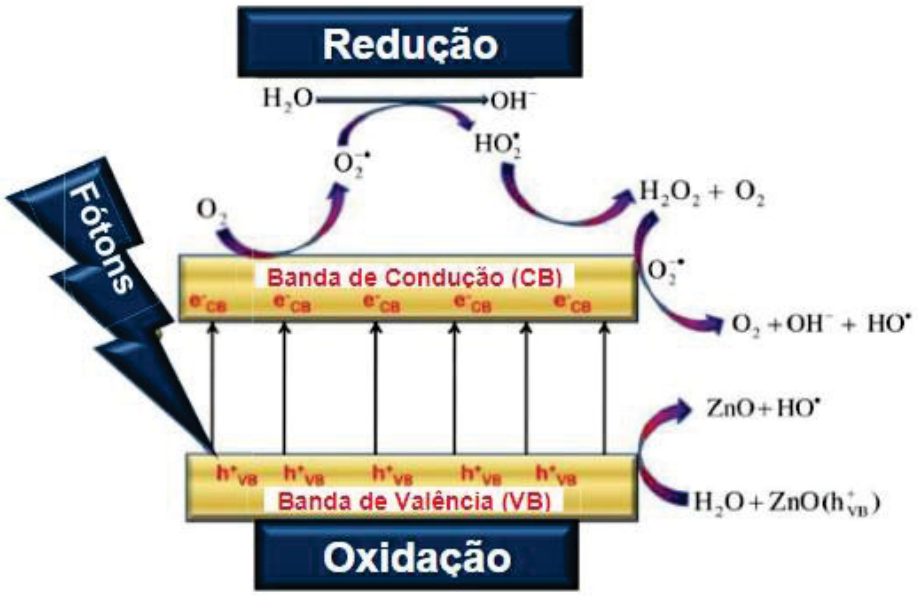
2.1 NANOPARTÍCULAS DE ZnO E ATIVIDADE ANTIBACTERIANA

As nanopartículas de óxido de Zinco já são amplamente utilizadas como sensores, semicondutores, catalizadores, aditivos em embalagens, cosméticos e protetores solares (Ma *et al.*, 2013; Wong *et al.*, 2020). Essas nanopartículas também apresentam utilização na área médica em sondas, tratamentos de câncer, carreadores de medicamentos e antimicrobianos (Bondarenko *et al.*, 2013; Mirzaei; Darroudi, 2017; Smaoui *et al.*, 2023), sendo consideradas ideais para o tratamento de patógenos orais, estando na composição de cremes dentais (Khan *et al.*, 2016).

Basith e colaboradores (2014), apontam que a utilização no campo biomédico está diretamente associada a esta nanopartícula ser não tóxica, biossegura e biocompatível, além de ser quimicamente estável e seus precursores serem naturalmente abundantes. Desta maneira testes antibacterianos com essas nanopartículas têm aumentado, de maneira a se apresentar como uma alternativa a utilização dos antibióticos atuais, especialmente por agirem contra bactérias gram-negativa e gram-positivas (Akhil et al., 2016; Khan et al., 2016).

A ação antibacteriana das NPs-ZnO está diretamente ligada a atividade fotocatalítica, associada a energia luminosa absorvida necessária para a promoção do movimento dos elétrons para fora da banda de valência, ou seja, ao “*gap”* de energia do composto. De maneira concisa, a ação desse movimento de elétrons na superfície das nanopartículas resulta na geração de espécies reativas de oxigênio (ROS) por foto-indução, o que ocasiona estresse oxidativo na célula bacteriana e conseguinte morte celular (Basith et al., 2014; Freitas, 2018).

FIGURA 1 – PRODUÇÃO FOTOINDUZIDA DE ESPÉCIES REATIVAS DE OXIGÊNIO POR COMPOSTOS DE ZNO



FONTE: FREITAS (2018).

O “*gap*” de energia das NPs-ZnO é próximo de 3,37 eV em temperatura ambiente (SHAHZAD *et al.*, 2013; VIJAYAPRASATH *et al.*, 2016). A ação bactericida desta substância depende da absorção de comprimentos de ondas específicos, que se encontram na faixa de excitação da luz ultravioleta (UV). A dopagem com níquel oferece uma redução deste espectro de absorção, ocasionando que a ação destas NPs seja reduzida para o espectro de luz visível. Desta maneira a redução do comprimento de onda a ser absorvido permitiria um aumento da sua atividade, sem a necessidade da exposição à luz UV de forma direta, assim evitando problemas de saúde associados a ação dos radiação UV (Freitas, 2018).

2.2 TOXICIDADE DAS NANOPARTÍCULAS DE ÓXIDO DE ZINCO

De maneira contraria a já relatada por diferentes estudos que indicam a biossegurança no uso das nanopartículas de óxido de zinco (Senthamarai e Malaikozhundan, 2022; Basith *et al.*, 2014; Lokapur *et al.*, 2022). Tem-se a descrição dos efeitos tóxicos das nanopartículas de oxido de zinco a diferentes células eucarióticas de forma robusta (El-Kalliny *et al.*, 2023; Gupta *et al.*, 2017; Koner *et al.*,

2021a). Fernández *et al.*, (2013) verificaram citotoxicidade elevada, *in vitro,* nas linhagens TG-2, RTH-149 e RTL-W1. Outros estudos demonstraram a ação tóxica que as NPs-ZnO podem apresentar em diferentes espécies como *Drosophila melanogaster* (Sood et al., 2019); *Lithobates catesbeianus* (Gonçalves et al., 2020); *Eisenia fetida* (Zhang *et al.*, 2022); e *Daphnia magna* (Santos-Rasera *et al.*, 2022).

De maneira contrária Xia e colaboradores (2011) demonstraram que as NPs-ZnO dopadas com ferro (Fe) apresentam toxicidade reduzida em embriões de *Danio rerio* e em pulmões de roedores, concluindo que neste estado, essas partículas podem vir a ser menos nocivas. Os autores sugerem que menor toxicidade se dá pela dopagem com Fe reduzir a concentração de Zn^{2+} em solução. Contudo, outro trabalho realizado por Li *et al.*, (2011), promoveram testes em bactérias com NPs-ZnO não dopadas e dopadas com ferro e concluíram não haver diferenças significativas em quesito de toxicidade.

Embora resultados contrários tenham sido observados por diferentes estudos de toxicidade de nanopartículas, concorda-se que as propriedades intrínsecas de cada material, métodos de fabricação, mecanismos de contaminação e a natureza do sistema teste são fatores que influenciam os resultados (Bondarenko *et al.*, 2013; Kerin *et al.*, 2023). Neste sentido, se faz necessário que as pesquisas sejam intensificadas com o objetivo de se averiguar o verdadeiro impacto de cada variável.

2.3 TOXICIDADE EM PEIXES

A qualidade de vida de um organismo vivo está diretamente ligada ao ambiente em que ele vive. Desta forma, se um ambiente não está propicio a apresentar uma boa qualidade, seus habitantes são afetados. No ambiente aquático, peixes são vistos como bons organismos representantes da qualidade deste ambiente, já que são altamente responsivos às mudanças, sendo considerados organismos bioindicadores (Ji *et al.*, 2010). Eles podem exibir padrões de respostas fisiológicas e comportamentais a diferentes poluentes, flutuações na temperatura da água, níveis de oxigênio, pH, salinidade além de poderem acumular substâncias nocivas em seus diferentes tecidos (Alkaladi *et al.*, 2020; Aziz *et al.*, 2022a; Recabarren-Villalón *et al.*, 2021).

Considerando a ampla distribuição dos peixes em ecossistemas aquaticos, estes organismos são usados como modelos animais em estudos ecotoxicológicos,

expressando respostas em diferentes níveis (Recabarren-Villalón *et al.*, 2021). Neste contexto, alguns estudos já descreveram a toxicidade das NPs-ZnO em diferentes espécies de peixes como *Oreochromis niloticus* e *Carassius auratus* (Abou-Zeid *et al.*, 2023; Abdel-Daim *et al.*, 2019; Amin *et al.*, 2021; Chen, Lin e Meng, 2014; El-Saadony *et al.*, 2021; Taherian *et al.*, 2020; Valdiglesias *et al.*, 2023; Yin *et al.*, 2017). Dessa maneira, é consenso na área que estes organismos fornecem boas respostas e previsões futuras sobre o impacto dessas partículas podem oferecer aos ecossistemas.

Nos estudos envolvendo peixes e a toxicidade das NPs-ZnO, os principais mecanismos de toxicidade se relacionam diretamente ao estresse oxidativo induzido pelas NPs. Outro mecanismo associado a toxicidade é a liberação de Zn^{2+} em consequência da dissolução das NPs-ZnO em água, esses íons são responsáveis pela perturbação das homeostases dos peixes (Falfushynska *et al.*, 2019; Gubala *et al.*, 2018; Serrà *et al.*, 2020). Alguns estudos a longo prazo já relatam a bioacumulação de íons Zn^{2+} provenientes destas nanopartículas nos tecidos das espécies *Carassius auratus* (Yang *et al.*, 2020) e *Oreochromis niloticus* (Yin *et al.*, 2017*)*, além de essas NPs induzirem deformidades (Mawed *et al.*, 2022).

Assim, considerando os estudos envolvendo peixes como organismo modelo, se torna pertinente a investigação de como espécies endêmicas das américas irão responder diante de uma exposição a este composto de maneira controlada. Desta forma, a espécie *Rhamdia quelen* foi tomada como organismo modelo para este trabalho.

2.3.1 Rhamdia quelen

A espécie *Rhamdia quelen,* popularmente conhecido como Jundiá, é um peixe Siluriforme pertencente à família Heptapteridae, e apresenta distribuição neotropical do sul do México ao centro da Argentina. É uma das espécies nativas cultivadas mais promissoras, e desperta interesse em piscicultura por consequência de seu rápido crescimento, natureza onívora, facilidade de adaptação ao manejo intensivo, alta produtividade em açudes e grande potencial de comercialização (Silvergrip, 1996; Angrizani; Malabarda, 2020).

Este peixe apresenta diferentes nomes vulgares sendo chamado de jundiá, jandiá, bagre, bagre sapo e bagre sul-americano. Apresenta coloração de cinza-

ardósia a avermelhado claro, com pigmentação variável na parte inferior da cabeça. Como principais características morfológicas é observável espinho nas nadadeiras peitorais; lóbulos desiguais na nadadeira caudal; poros sensoriais múltiplos na cabeça; véu da narina posterior aberta, barbilhões maxilares alongados que ultrapassam as brânquias; olhos de tamanho médio podendo ou não conter padrões de manchas (Silvergrip, 1996.; Gomes *et al.* 2000).

São indivíduos de hábito noturno, tendo preferência alimentar por insetos, pequenos crustáceos, detritos orgânicos e vegetais, em ambiente natural optam por se esconder em troncos apodrecidos ou entre pedras. Seu desenvolvimento corporal está diretamente ligado a temperatura, pois em águas mais quentes tendem apresentar dimensões mais avantajadas, estes peixes quando em estado de alevinos podem suportar temperaturas na faixa de 15°C à 31°C, (Nugra *et al.*, 2018., Gomes *et al.* 2000). Os machos tendem a apresentar maior desenvolvimento físico durante os primeiros anos de vida em comparação às fêmeas, situação que muda após o quarto de vida do organismo. O tamanho médio da espécie é de aproximadamente 52 cm para os machos e 66,5 cm para as fêmeas (Gomes *et al.,* 2000).

Esta espécie é altamente adaptável em aquários e possui sensibilidade a diversas substância se mostrando um bom bioindicador em testes toxicológicos (Oya-Silva *et al.*, 2021). O emprego destes animais em bioensaios com avaliações multibiomarcadores já é bem difundida pela literatura (Pereira *et al.*, 2016; Guiloski *et al.*, 2017; Mathias *et al.*, 2018; Perussolo *et al.*, 2019).

FIGURA 2 – EXEMPLAR DE *Rhamdia quelen*

FONTE: O autor (2022)

Não há registros de pesquisas relacionando o uso de nanopartículas de oxido de zinco em *R. quelen*. Assim, o presente estudo escolheu esta espécie como organismo modelo para a detecção dos efeitos ecotoxicologicos que estas NPs podem ocasionar. Outro motivo que levou a utilização desta espécie é seu fácil manejo e aclimatação em condições laboratoriais.

2.4 BIOMARCADORES GENÉTICOS

2.4.1 Ensaio Cometa

O ensaio cometa ou eletroforese de célula única *("single cell gel eletroforesis*") é uma técnica para avaliar danos no DNA em células individualizadas. É uma técnica simples de ser realizada, com o foco em avaliar os danos causados por xenobióticos (Singh, 1988; Møller, 2018). Esta técnica é amplamente utilizada por ser aplicável em qualquer célula eucarionte, sendo aplicada inclusive para células vegetais, avaliando os danos em tecidos específicos (Collins et al, 2023).

Esta técnica pressupõe que o DNA deve migrar em conjunto no gel de eletroforese. Desta forma, deve constituir uma massa única conjunta, enquanto as massas de material genético que sofreram danos deixam fragmentos ao longo da corrida eletroforética, indicando danos ao DNA. A analogia à imagem de um cometa é dada pela formação de uma cauda contendo fragmentos de DNA (Singh *et al.*, 1988; Olive; Banáth; Durand, 1990; Collins, 2023).

2.4.2 Teste do Micronúcleo Písceo (MNP)

Micronúcleo pode ser definido como núcleo adicional e separado do núcleo principal da célula. Este núcleo adicional é formado por pedaços de cromossomos inteiros ou fragmentos de cromossomos que acabam por não serem incorporados ao núcleo principal durante a divisão celular. A formação destas estruturas é decorrente de alterações estruturais espontâneas ou decorrentes da ação de fatores exógenos, ou ainda falhas no fuso mitótico (Schimid, 1973).

Embora tenha sido originalmente desenvolvido para estudar células de mamíferos, Hooftman e Raat (1982) aplicaram com sucesso o teste de micronúcleo em eritrócitos de peixes. A versão do protocolo adaptada para peixes passou a ser

conhecida como Teste do Micronúcleo Písceo (Carrasco *et al.,* 1990), que descreveram e fotografaram as alterações morfológicas encontradas em núcleos de eritrócitos de peixes classificando-as em *Blebbed, Lobed, Vacuolated* e *Notched.*

2.5 BIOMARCADORES BIOQUÍMICOS

2.5.1 Acetilcolinesterase

As colinesterases se referem a um grupo de enzimas esterases que já são bem descritas, dois tipos são bem-compreendidos em vertebrados, a Acetilcolinesterase (AChE) e a butirilcolinesterase (BChE) (Durieux *et al.*, 2011; Martínez-Morcillo *et al.*, 2019). Essas colinesterases são bastante similares no que se refere a estruturas quaternária e terciaria, elas podem ser diferenciadas pela especificidade a seu substrato. A acetilcolinesterase apresenta afinidade pela acetilcolina enquanto a BChE tem afinidade pela butirilcolina (Pope; Brimijoin, 2018). A AChE pode ser encontrada em no tecido nervoso de peixes, enquanto a BChE pode ser detectada em maior quantidade nos tecidos hepático e hematopoiético (Olivares-Rubio; Espinosa-Aguirre, 2021).

A AChE tem como função a hidrolise da acetilcolina em colina e ácido acético nos sítios sinápticos colinérgicos do sistema nervoso. Ela se mostra como um dos principais marcadores bioquímicos para se avaliar a neurotoxicidade de diversas substâncias anticolinesterásicas, como pesticidas organofosforados e carbamatos, metais pesados e contaminantes emergentes, dentre elas as NPs (martínez-Morcillo *et al.*, 2019).

2.5.2 Extresse Oxidativo

A interação com diferentes substâncias pode ser responsável por exercer efeitos citotóxicos no organismo, em decorrência desses danos há a produção de espécies reativas de oxigênio (EROS), e como consequência haverá a indução de danos oxidativos (Li *et al.*, 2010). A membrana celular serve como uma primeira barreira seletiva à diferentes substâncias que um organismo é exposto, ela irá selecionar o que será internalizado para o meio intracelular (NELSON; COX, 2017).

Caso existam fatores que venham a interferir na integridade da membrana, irão ocorrer prejuízos as suas funções (Cooper; Hausman, 2007; Nelson; Cox, 2017).

A partir dos danos causados por espécies reativas de oxigênio (EROS), algumas das propriedades da estrutura da membrana podem ser afetadas, tais como fluidez, permeabilidade e integridade, ocasionando alterações nas interações de diversas enzimas associadas a membrana (Dutra *et al.*, 2008;Li *et al.*, 2010). A Lipoperoxidação (LPO) é uma reação em cadeia de ácidos graxos poli-insaturados nas membranas celulares, que resulta na produção das ROS e radicais livres. A partir da quantificação da LPO é possível verificar se os organismos estão sofrendo algum estresse oxidativo durante as exposições a contaminantes (Cantanhêde *et al.*, 2022; França *et al.*, 2013).

Um sistema que se destaca é o sistema de defesa antioxidante, este sistema é responsável pelo combate e redução dos efeitos causado por radicais livre e espécies reativas de oxigênio. Desta maneira quando o sistema antioxidante se encontra sobrecarregado, há danos mais pronunciadas em biomoléculas como proteínas, lipídeos e DNA (Xue *et al.*, 2023b).

O sistema antioxidante é composto por componentes enzimáticos e não enzimáticos (Hoseinifar *et al.*, 2020). A primeira linha de defesa realizada pelo sistema antioxidante enzimático que envolve reações de redução, hidrolise e oxidação de compostos nocivos. As enzimas envolvidas neste componente de defesa dos organismos são as enzimas: superóxido dismutase (SOD), onde está atua na redução do radical superóxido (O_2^-) em peroxido de hidrogênio (H_2O_2) e Catalase (CAT) que atua na quebra do peroxido de hidrogênio em água (H_2O) e oxigênio (O_2) (Atli *et al.*, 2016).

Na segunda linha de defesa antioxidantes é formada por enzimas que depende da glutationa reduzida (GSH), dentre elas está a glutationa peroxidase (GPx) que de modo semelhante a CAT faz a conversão do H_2O_2 em água e oxigênio; e glutationa-S-tranferase (GST) de maneira que a GST tem a função de detoxificação de intermediários reativos e radicais de oxigênio, mediante a conjugação desta enzima com compostos eletrofílicos (Atli *et al.*, 2016).

3 OBJETIVOS

3.1 OBJETIVO GERAL

Avaliar os efeitos de toxicidade aguda de ZnO-NPs em diferentes tecidos da espécie endêmica *Rhadia quelen* a fim de investigar se tais nanopartículas apresentam risco ecotoxicológico no ambiente aquático.

3.2 OBJETIVOS ESPECÍFICOS

a) Testar NPs-ZnO não dopadas e dopadas com Ni em concentrações bactericidas.
b) Avaliar as respostas genotóxicas dos tecidos sanguíneo, cerebral, hepático e branquial utilizando o biomarcador ensaio cometa alcalino.
c) Avaliar a mutagenicidade em amostras de sangue, utilizando o teste do Micronúcleo Písceo (MNP).
d) Avaliar a neurotoxicidade por meio da atividade da enzima acetilcolinesterase (AChE) em tecido cerebral.
e) Avaliar estresse oxidativo em tecidos hepático e branquial utilizando a atividade das enzimas LPO, SOD, CAT, GST e AChE.

CAPÍTULO 1 - ARTIGO DE REVISÃO BIBLIOGRAFICA

Toxicological aspects of ZnO-NPs and their Impact on fishes: a review

ABSTRACT

Nowadays, it is recognized as the nanotech era, mainly due to the growth in nanomaterials development in the last few decades; the impulse in this technological field entails excessive production and consumption of nanomaterials. One of the most utilized is zinc oxide nanoparticles (ZnO-NPs), which are prominent in producing several devices and products. This material attracts attention due to its specific properties and potential applications. The applicability of this material occurs in several fields, such as biomedical, medicine, agriculture, healthcare inputs, antimicrobial, and sensor and photocatalysis development. One crucial factor to be considered regarding the high usage is the toxicological implications of these nanoparticles and how they impact natural environments. Therefore, some research has been performed on the toxicity of those NPs to aquatic organisms, generally using fish species as models. In this literature review, the main characteristics of ZnO-NPs are described, as well as the primary mechanism of toxicity in different species of fish. Various characteristics of NPs directly influenced the toxicity of the compound such as synthesis, size, shape, concentration, dissolution, chemical surface, and sedimentations, thus these features were compared in the studies. The main effects of these nanoparticles caused in fish were a generation of reactive oxygen species (ROS) and the release of Zn2+ ions by dissolution of the material. Therefore, the toxicological effects were an increase in the antioxidant system, the activity of superoxide dismutase (SOD) and catalase (CAT) was increased, as well as the differential expression of genes involved with the oxidative stress system. The alternative synthesis mode was explored in a few studies, besides supplementation with different substances to mitigate the toxic effects of ZnO-NPs.

Keywords: Nanoparticles; Nanotoxicity; Oxidative stress; aquatic organism; Toxic effect

CONTENTS

1 INTRODUCTION

Recently, nanotechnology has become a growing trend in sciences (Smaoui *et al.*, 2023, European Commission, 2011). This field explores the physic-chemical properties of materials that have at least one dimension ranging from 1-100 nanometers(El-Kady *et al.*, 2023). One of the most common nanomaterials emerging as a notable space is zinc oxide nanoparticles (ZnO-NPs), this nanoparticle is the main product used as a potential substitution for titanium dioxide – TiO_2 (Vittal; Ho, 2017).

Given its malleability, which allows modifications to the crystalline structure, shape, size, and surface chemistry, it had a high application (El-Kady *et al.*, 2023). These modifications immediately affect the reactivity of the particle, opening a variety of applications, including agriculture for the manufacture of dairy products, fertilizers, biomedical, health care, and applications in medicine (Mandal *et al.*, 2024; Smaoui *et al.*, 2023; Vittal e Ho, 2017).

It is projected that 30,000 tons of ZnO-NPs are discharged into the environment each year (Hazeem, 2022; Xiao *et al.*, 2024). The increasing usage of ZnO-NPs raises questions concerning their limitations, tolerance, and risk management in aquatic environments (Brown *et al.*, 2018). A significant number of studies have already described the toxicity of ZnO-NPs to different organisms, such as microorganisms (Hou *et al.*, 2018; Nguyen, Moon e Lee, 2020; Xiao *et al.*, 2024) Invertebrates (Dai *et al.*, 2020; Senthamarai e Malaikozhundan, 2022), and vertebrates (Aziz *et al.*, 2020; Gonçalves *et al.*, 2020; Mawed *et al.*, 2022a; Mona *et al.*, 2023; Motta *et al.*, 2020a).

Fish perform an essential part in ecological interactions and are consistently found at the top of aquatic biomes due to their ecological role, they are also crucial for

preserving the equilibrium and making manutention of the food web (Li *et al.*, 2024). Their commercial importance gives an even more significant reason to include them in the toxicological surveys; human aquatic food consumption has already reached 20.2 kg/year, whereas fish consumption is responsible for about 75% of the total, creating a demand for fishing farms to be willing to provide for human consumption worldwide (Khalil *et al.*, 2023).

Considering the toxicity of the ZnO-NPs, it is wise to evaluate how they interact with molecules, their impact on the organism's life, and how they affect the environment. More significant debates about the sustainability of nanotechnology are pertinent, and alignment with green development is worthy of discussion (Jimoh *et al.*, 2022; Shahzad *et al.*, 2013)

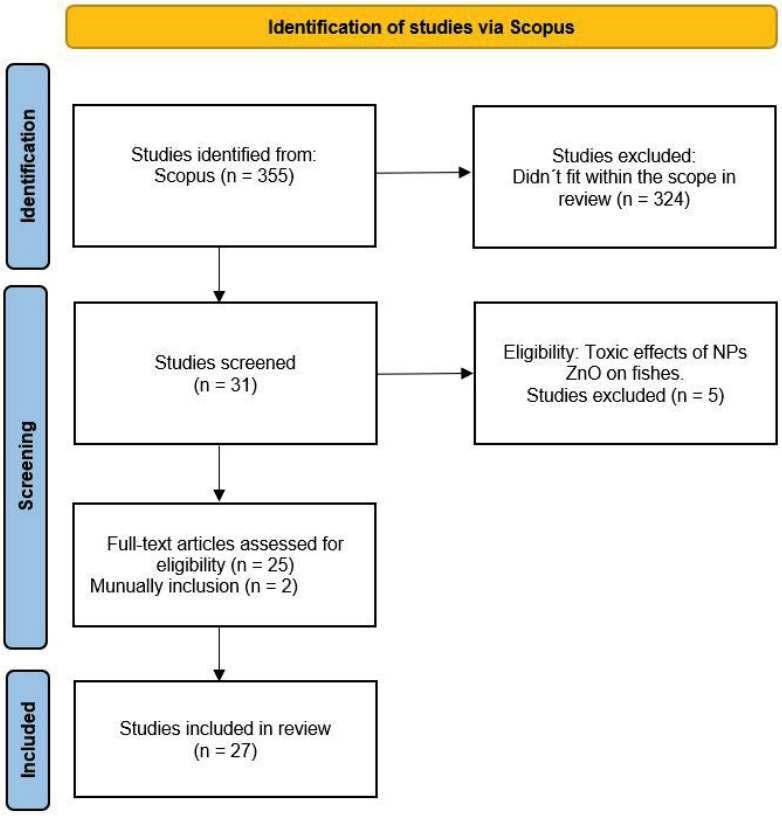
This review aims to summarize the current knowledge about ZnO-NPs' proprieties and main effects on fish and to start discussions about a safer perspective on their production.

2 METHODOLOGY

This review summarizes the main points of investigation regarding the characteristics of ZnO-NPs, exposure, and responses of fish, the data set was carried out using the Scopus database. The publication period of the articles evaluated was fixed from 2017 to 2023. As Keywords were used the following terms: "Zinc Oxide Nanoparticles" OR "ZnO Nanoparticles" AND "Fish" AND "Ecotoxicity" OR "nanotoxicity". The results were filtered for only the original and English language. After the screening, 324 papers were not within the scope of the survey, some are treating different nanoparticles than ZnO-NPs and/or different organisms than fish. After screening 25 articles were selected following this methodology (Figure 1). In addition, two other works (n=2) were included manually.

The next sections will discuss information about zinc oxide nanoparticles' physical and chemical characteristics to better understand the aspects surrounding their toxicity. To find out the impacts that ZnO-NPs can cause on different species of fish, there is a description and summary of the information collected from the 27 studies concerning fish as bioindicators and several biomarkers utilized. Furthermore, it brings information about green synthesis and the lower toxicity obtained from this means of production.

Figure 1. PRISMA flowchart of screening of the papers included in this review.



3 THE ZNO-NPS: AN OVERVIEW

The high production and consumption of nanomaterials (NMs) worldwide are becoming greater as time goes by, and the prediction is that by 2026, the global market will exceed U$12.1 billion (El-Kady *et al.*, 2023). The ZnO-NPs are one of the most used in the nanotechnology market, with a worldwide annual production ranging from about 550 to 33.400 tons, being the second most produced NP (Bordin *et al.*, 2024; Rahman *et al.*, 2022). The application of these nanoparticles is given in different sectors of industry, such as optoelectronics, cosmetics, biomedical equipment, medicaments, fertilizers, food packages, solar cells, and sensors (Islam *et al.*, 2022; Mandal *et al.*, 2024; Mirzaei e Darroudi, 2017; Saratale *et al.*, 2018; Vittal e Ho, 2017).

The widespread use of nanoparticles (NPs) raises concerns about their impact on the aquatic environment (Özgür, 2019; Solano *et al.*, 2021) Manufacturing waste is one of the contributors to the release of this material (Noor *et al.*, 2021). Another direct source is agricultural use, in which ZnO-NPs are employed as a nutrient to increase food production (Yusefi-Tanha *et al.*, 2020). Despite their indirect impact on the aquatic

environment by rainfall and/or runoff, these nanoparticles can be found in paints, tires, and personal care products (Amin *et al.*, 2021; Mandal *et al.*, 2024; Solano *et al.*, 2021; Wong *et al.*, 2020).

Some models already project an average level (1.5ng.L-1) of ZnO-NPs in half of European rivers and over ten times higher concentrations in 10% of the rivers (Arienzo; Ferrara, 2022). The biography already shows the high persistence and bioconcentration of ZnO-NPs in aquatic environments (Khalil *et al.*, 2023), and numerous studies show the impact of these ZnO-NPs on aquatic organisms such as microcrustaceans (Martinez *et al.*, 2022), amphibians (Motta *et al.*, 2020b), and fishes (Koner *et al.*, 2021a; Mahjoubian *et al.*, 2023).

The variation in synthesis parameters interferes directly with the characteristics of the nanomaterial; controlling the parameters in the synthesis of NPs makes it possible to change some variables (e.g., shape, size, crystallinity, and surface chemistry) (Ponnamma *et al.*, 2019; Wu *et al.*, 2019). Different morphologies are capable of being created, regarding the ZnO-NPs, the most common morphology is crystalline wurtzite (Al-Otaibi; Howsawi; Ghrib, 2020; Ponnamma *et al.*, 2019), but different shapes can be generated as a consequence of different synthesis parameters, spherical, conical, and rod shapes were examples of the variety of shapes (Kaya *et al.*, 2017; Rashidian *et al.*, 2021; Saleem *et al.*, 2022).

The interaction of nanoparticles with themselves influences variables such as aggregation, agglomeration, and sedimentation rate, small particles may dissolute more easily than bigger ones (Cardoso *et al.*, 2021). Several variables impact agglomeration: ionic strength, dispersion, pH, shape, size, and surface chemistry all affect the agglomeration rate of ZnO-NPs (Amin *et al.*, 2021).

Environmental conditions can also influence aggregation and agglomeration characteristics, and distinct clusters of ZnO-NPs may emerge according to different patterns, triggering sedimentation. This behavior can be determined by the size of the cluster formed and is influenced by gravity(Lee *et al.*, 2020; Rex M *et al.*, 2023).

When in contact with the aquatic environment, the dissolution of the ZnO-NPs releases Zn2+, an important ion in the living organism on which many enzymes, transcription factors, and proteins are directly dependent (Sloup *et al.*, 2017). However, an excess of these ions could lead to problems linked to cytotoxicity, metabolic alterations, and problems with the enzymatic system (Bordin *et al.*, 2024; El-Kalliny *et al.*, 2023; Sánchez-Argüello, Franco e Fernández, 2023a)

3.1 TOXICOLOGICAL EFFECTS OF ZnO-NPs

Few studies present data on the biosafety of ZnO-NPs and contend that this substance can be employed in medical and pharmaceutical products (Mirzaei; Darroudi, 2017; Senthamarai; Malaikozhundan, 2022). Nonetheless, numerous studies describe the harmful consequences brought on by zinc oxide nanoparticles (Mawed *et al.*, 2022b; Oliviero *et al.*, 2019; Santos-Rasera, Monteiro e Carvalho, de, 2022; Yung *et al.*, 2017). With consideration of the toxicological investigations involving distinct organisms, some regulation organs already consider the nanoparticles as emerging contaminants (El-Kalliny *et al.*, 2023)

According to a study by Bordin *et al.* (2024), organisms' responses to ZnO-NPs vary, and different species may exhibit various effects depending on the environmental circumstances. Invertebrates, bacteria, algae, and vertebrates are the order in which organisms' environmental sensitivity is listed. The mechanisms of action in aquatic organisms are still unclear, because of the interactions of these particles with the environment and their intrinsic proprieties (Kerin; Nagaraj; Kamalesu, 2023).

The mechanism of toxicity of ZnO-NPs could be divided into three main ways, the first one being the physical damage as a result of the size; second the release of ions (Zn^{2+}); and finally, the generation of reactive oxygen species (ROS) (Bordin *et al.*, 2024; Solano *et al.*, 2021). Evaluating the size of the NPs presents greater significance in ecotoxicological surveys, notably because the aggregation pattern can interfere with the internalization of various organisms such as algae, vertebrates, invertebrates, and procaryotes. (Hou *et al.*, 2019; Saxena *et al.*, 2021).

The bioaccumulation and deposition of agglomerates ZnO-NPs on the microcrustaceans' guts of *Ceriodaphnia dubia* was documented in the study by (Bhuvaneshwari *et al.*, 2016) Additional research using fish (Asghar *et al.*, 2018; Goda; Shaheen; Hamed, 2023a), and amphibians (Gonçalves *et al.*, 2020) identifies other effects of ZnO-NPs, like mutagenicity, genotoxicity, and histology.

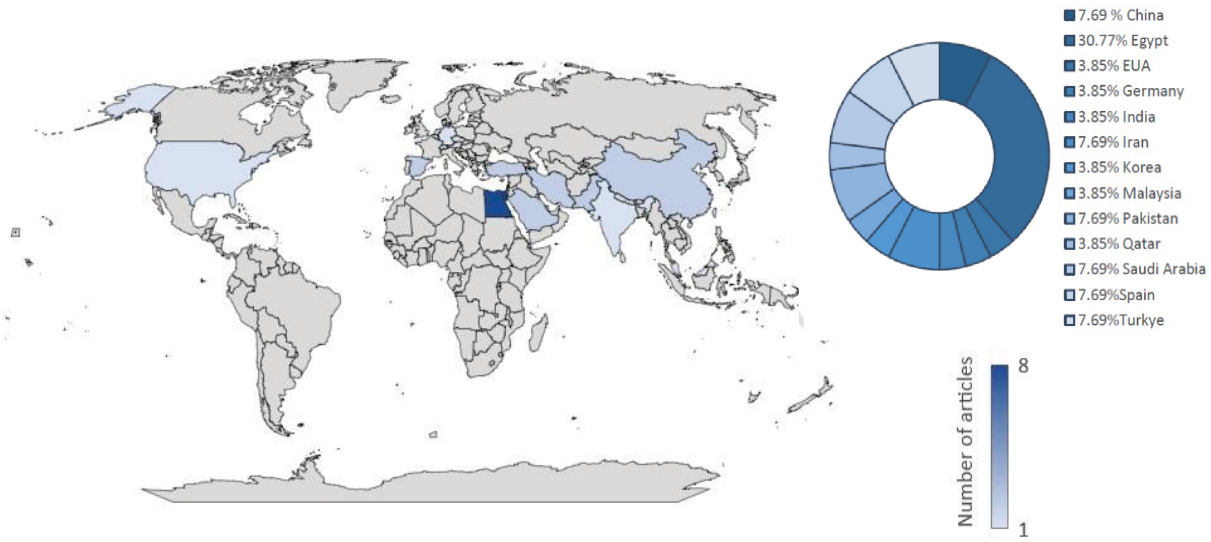
4. RESULTS AND DISCUSSION

4.1 Fishes and ZnO-NPs

As fish are being consumed and employed by humans in several activities, their importance increases the preoccupation with environmental quality (FAO, 2022). Given the purpose of this review, it is possible to identify where a major development of the studies involving ZnO-NPs and fishes occurred. As demonstrated in Figure 2, the studies focused on the European-African-Asian axis, with most of the studies developed in Egypt.

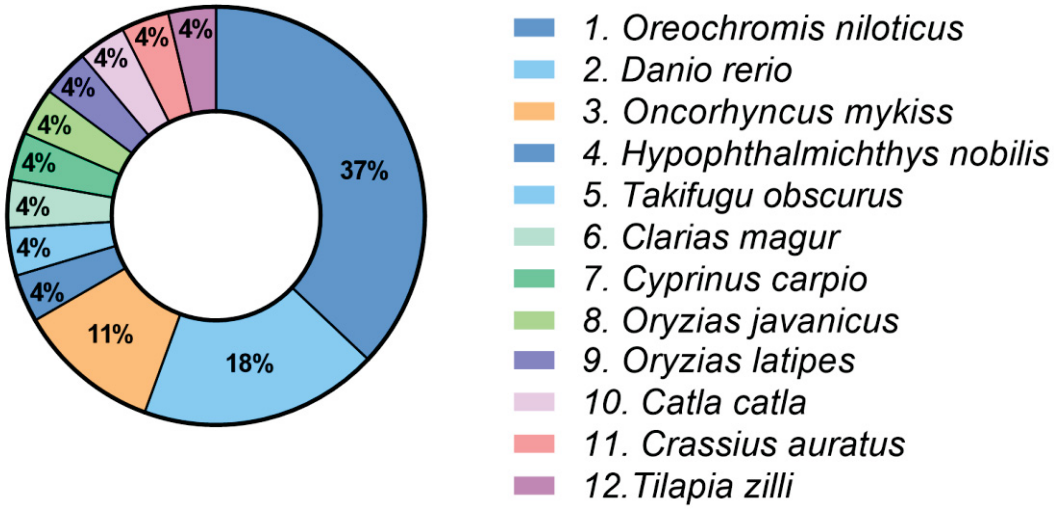
Fish can respond greatly to harmful substances and are known as model organisms (González-fernández *et al.*, 2021). Some already have protocols established, such as *Danio rerio* and *Onchorrynchus mykiss,* which are described in the OTG 236 and OTG 319A, respectively from the Organization for Economic Co-operation and Development (OECD) (OECD, 2013), besides these two species, other fishes, such as *Oreochromis niloticus* and *Carassius auratus* were used in several ecotoxicological studies and monitoring programs as the primary source to provide information on the effects of nanomaterials (Table1) (Massoud *et al.*, 2021; Pereira *et al.*, 2019; Shabrangharehdasht, Mirvaghefi e Farahmand, 2020; Silva Carneiro; Franchi; Rocha, 2023; Yang *et al.*, 2020)

Figure 2. Geographical distribution of the studies involving Zinc Oxide nanoparticles in fish in 2017-2023.



Looking at the richness of species used in the survey, 11 freshwater species were utilized, and only one was marine/estuarine (T*akifugu obscurus*) (Figure 3). In this bibliographic survey, the organism with a high prevalence was *Oreochromis niloticus*, being used in 37% of the studies, followed by *Danio rerio* (18%) and *Oncorhyncos mykiss* (11%) (Figure 3). In agreement with the founds, *O. niloticus* was the species that showed a more expressive appearance in the studies. One reason that could explain the high prevalence of this species in the studies is the commercial bias, where they occupy a large portion of farming fish (Wachira *et al.*, 2021). This species provided excellent know-how about the effects of ZnO-NPs in different biomarkers such as genetics, metabolic ways, histological alterations, and biochemical activity, previewing the possible impact on the environment if ZnO-NPs were discharged into the water bodies (Abou-Zeid *et al.*, 2023; Alkaladi *et al.*, 2020; El-Saadony *et al.*, 2021; Goda; Shaheen; Hamed, 2023; Kaya *et al.*, 2017; Mohamed, Soliman e Ghannam, 2021).

Figure 3. The richness of species in toxicological studies involving zinc oxide nanoparticles and fishes between the years 2017-2023.



Analyzing the data summarized in Table 1, it is possible to observe that a great range of characteristics is responsible for influencing the toxicity of ZnO-NPs. Some characteristics directly affect the toxicity potential in fish species, and the range concentrations of the ZnO-NPs were one factor that deserves attention. The values varied from 0.08 mg. $L^{-1}$to 140 mg. $L^{-1,}$, and some values of LC_{50} (median lethal concentration) were determined for different species such as *Catla catla* (5.6 mg. L^{-1}), *Cyprinus carpio (59.95* mg. L^{-1}*)*, *Oncorhyncus mikiss* (25.50 mg. L^{-1}), *Oreochromis niloticus* (4.1 µg. L^{-1})*,* Oryzias *javanicus* (0.64 mg. L^{-1}), and *Oryzias latipes* (47.31 mg. L^{-1}). In the studies that did not show the IC_{50} value, other effects, such as oxidative stress, morphological alterations, and histological damage, were the most common effects evaluated. Another effect observed in long-term exposures was a high prevalence of bioaccumulation in muscle, gills, liver, and brain (Mohamed *et al.*, 2021; Yin *et al.*, 2017).

The interaction between NPs and biological molecules is already described in the literature, and several harmful effects can be listed on different biological molecules (Table 1). Size is the property that allows the NPs to achieve tissues and cells (Gubala *et al.*, 2018), the effects on the biological systems caused by ZnO-NPs include oxidative stress, bioaccumulation, hatch delay, and histological alterations, (Goda; Shaheen; Hamed, 2023; Koner *et al.*, 2021; Mohamed, Soliman e Ghannam, 2021;

Sánchez-Argüello, Franco e Fernández, 2023; Valdiglesias *et al.*, 2023; Yin *et al.*, 2017).

Table 1. Studies published between 2017 to December 2023, involving toxicity of zinc oxide nanoparticles (NPs-ZnO) and fishes.

	Fish				
Organism	**Size (nm)**	**Concentration (mg. L^{-1})**	**Exposition Time**	**Main effect**	**Reference**
Catla catla	36 - 77	20 - 140	96h	$LC_{50\text{-}96h}$ =5.6mg. L^{-1}	(Asghar *et al.*, 2018)
Clarias magur		10	14 days	Oxidative stress	(Koner *et al.*, 2021b)
Carassius auratus	40	80	30 days	Bioaccumulation	(Yin *et al.*, 2017)
Cyprinus carpio	35	10 - 120	96h	$LC_{50\text{-}96h}$ = 59.95 mg. L^{-1}	(Rashidian *et al.*, 2021)
Danio rerio	>100	0,1 - 100	96h	Hatch delay	(Valdiglesias *et al.*, 2023b)
	89	89	14-30 days	Cytotoxicity	(Mawed *et al.*, 2022b)
	50	2 – 62.5	120h	Hatch delay	(Hansjosten *et al.*, 2022)
	< 100	0.5 - 6	96h	$LC_{50\text{-}96h}$ =3.0mg. L^{-1}	(Giordo *et al.*, 2020)
	50	0.08 - 80	120h	Hatch delay	(Wu; Harper; Harper, 2019)
Hypophthalmichthys nobilis	53	2 - 50	90 days	Oxidative stress	(Aziz *et al.*, 2022b)
Oncorhyncus mikiss	#	1	2h	Motility delay	(Özgür, 2019)
	35	1 - 100	96H	$LC_{50\text{-}96h}$ = 25.50 mg. L^{-1}	(Taherian *et al.*, 2020)
	25	10 - 100	24h	Oxidative stress	(Sánchez-Argüello, Franco e Fernández, 2023)
Oreochromis niloticus	89	1.14	28 days	Oxidative stress	(Abou-Zeid, S.M. *et al.*, 2023)
	25	8	42 days	Histological changes	(Goda, Shaheen e Hamed, 2023)
	31	10.5 - 42	28 days	Bioaccumulation muscle and gills	(Mohamed *et al.*, 2022a)
	89	11.42	28 days	Oxidative stress	(Abou-Zeid, S.M. *et al.*, 2023)
	45	0.5 - 1	70 days	Hormonal imbalance	(El-Saadony *et al.*, 2021)
	<100	1.23 – 2.05 ug/L	96h	LC_{50} = 4.1mg. L^{-1}	(Alkaladi *et al.*, 2020)

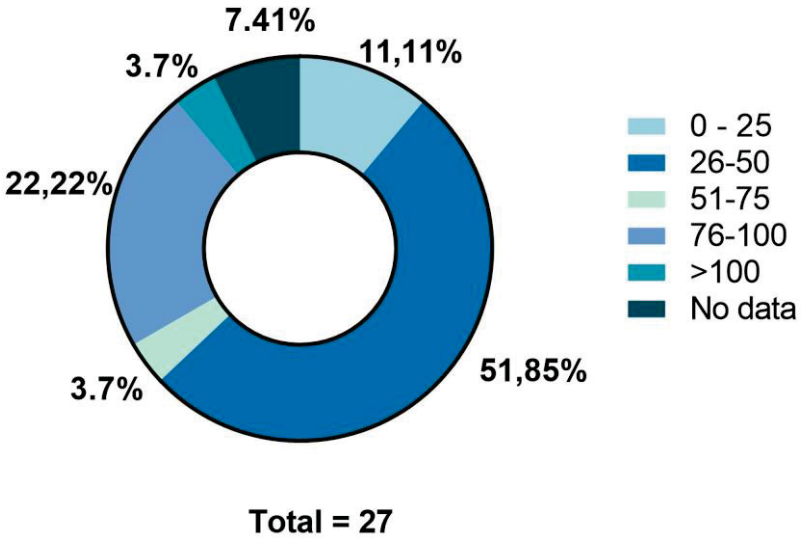
				Hormonal and molecular alteration	
	30	1 -2	15 days	Oxidative stress	(Abdelazim *et al.*, 2018)
	40	0.7 – 2.8	28 days	Oxidative stress	(Mohamed; Soliman; Ghannam, 2021)
	31	0.5 - 2	15 days	Oxidative stress	(Saddick; Afifi; Abu Zinada, 2017)
Oryzias javanicus	26	0.1 - 10	96h	$LC_{50\text{-}96h}$ = 0.64 mg. L^{-1}	(Amin *et al.*, 2021)
Oryzias latipes	36.48	26.9 - 100	96h	$LC_{50\text{-}96h}$ = 47.31 mg. L^{-1}	(Lee *et al.*, 2020b)
Takifugu obscurus	25	0 - 100	96h	Decrease hatch rate and oxidative stress	(Lin *et al.*, 2023)
Tilapia zilli	31	0.5 - 2	15 days	Oxidative stress	(Saddick; Afifi; Abu Zinada, 2017)

LC50: Lethal concentration; #: Data not presented

The interval of the ZnO-NPs size found in the studies obeys the range of 1-100nm (European Commission, 2011). In only one study, the related size was above 100 nm, reaching 238,15 nm (Valdiglesias *et al.*, 2023a). The interval of size with the most representative appearance was 26-50nm (n=14, 51.85), appearing in half of the studies brought in this survey (Figure 4), in 22% of the studies, the size ranged from 76-100nm (n=6), in the others sizes the percentage did not pass 15% in the intervals of 0-25 nm and 51-75 nm (n=3, 11.11%; n=1, 3.7%). Size accomplished with morphology is the most representative parameters that guide the toxicity of ZnO-NPs (Murthy *et al.*, 2022).

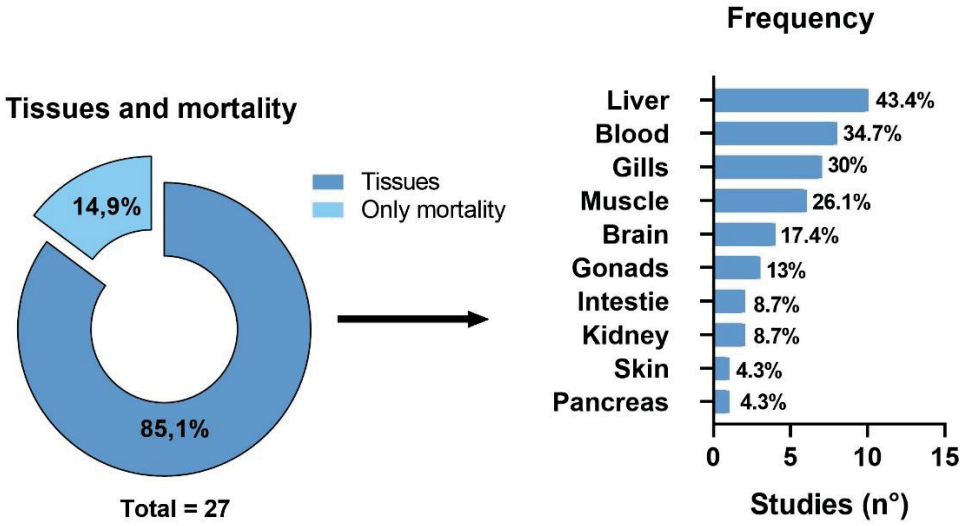
Regarding fishes' response to ZnO-NPs, the nanoparticles induce several effects on different fish organs. However, some reports did not use organs as biomarkers but just analyzed the mortality caused by ZnO-NPs in fish. Mortality was evaluated in 9 of 27 studies. Nonetheless, some biomarkers were evaluated in 5 of these 9 studies that evaluated mortality. Figure 5 exhibits the percentual occurrence of different tissues in 23 studies.

Figure 4. Size (nm) of ZnO-NPs used in studies involving fishes between 2017-2023.



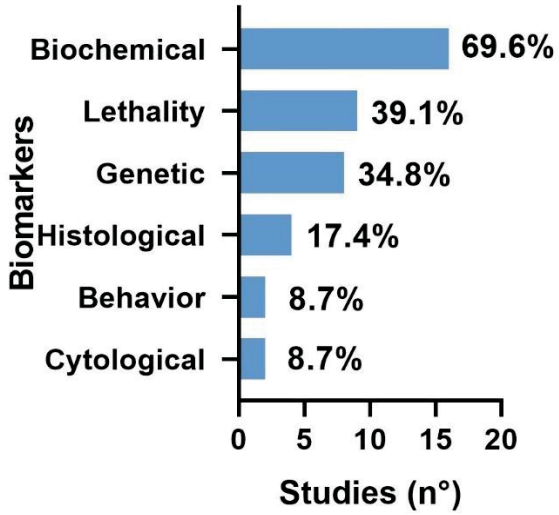
Biochemical biomarkers were the most common in studies involving zinc oxide nanoparticles and fish; about 70% of the investigations included a certain type of biochemical biomarker (Figure 6). The sample of studies evaluated in this review observed a histological analysis as the second most common, followed by genetic biomarker evaluation.

Figure 5. Frequency of studies that evaluated mortality and the percentage of tissues exposed to ZnO-NPs.



In fish, the presence of ZnO-NPs in the environment activates different biochemical responses. When in contact with the cell environment, the NPs could follow two different pathways: first, in direct contact with cells, tissues, and membranes, and second, indirectly, leading to the release of Zn^{2+} ions due to the dissolution and photocorrosion of particles (Gonçalves *et al.*; 2020).

Figure 6. Distribution of the biomarkers in the studies involving ZnO-NPs and fish.



Oxidative damage occurs by the imbalance caused by a high production of reactive oxygen species (ROS) and other molecules such as superoxide radicals (0_2^-), hydroxyl

(OH-), and hydrogen peroxide (H_2O_2) (Bordin *et al.*, 2024; El-Kady *et al.*, 2023). In response, the organisms elevate the production of enzymes that regulate homeostasis, protecting the organism against oxidative damage (Mohamed *et al.*, 2022a). An elevated number of molecules represents the antioxidant system. However, the main response occurs by enzymatic action of superoxide dismutase (SOD) and catalase (CAT) and nonenzymatic of reduced-glutathione (GSH) and glutathione S-transferase (GST) (Abdel-Daim *et al.*, 2019; Murthy *et al.*, 2022).

The summarized results of biochemical biomarkers tend to show an increase in activity, mainly in the SOD and CAT activities (Abdelazim *et al.*, 2018; Abou-Zeid, S.M. *et al.*, 2023; Asghar *et al.*, 2018; Aziz *et al.*, 2020; Koner *et al.*, 2021a; Lin *et al.*, 2023; Mohamed, Soliman e Ghannam, 2021; Saddick, Afifi e Abu Zinada, 2017). The pattern of activity increment was maintained even in different species and tissues utilized in the studies, presenting that it was not an exclusive response to only one species.

The effects evaluated for genetics biomarkers regard the mutagenicity, genotoxicity, and gene expression. The study of Abdelazim *et al.*, 2018 and Mawed *et al.*, 2022 evaluates the genes (SOD, CAT, GPx, and GST) related to the oxidative stress system using Real-Time PCR. The results of both studies demonstrate a decrease in the expression of these genes in a concentration-dependent manner in muscle and ovary cells. Nevertheless, the results obtained by Konner *et al.* (2021), present an elevation of the expression in the muscles, liver, kidney, brain, and gills using the same biomarkers. Some parameters can influence the response of the gene expression in the studies, the first studies used a concentration of the ZnO-NPs 1 and 2 mg. L^{-1} for 15 days and 0.69 mg. L^{-1} for 15 and 30 days, while (Koner *et al.*, 2021) utilized 10 mg. L^{-1} for 30 days. The response will vary not only in reason of the size and concentration, but the species also must be considered, like other characteristics of the nanoparticles. This comparison elucidated the difficulty to compare ZnO-NPs exposure responses.

Other studies involving DNA damage provide information on genotoxicological parameters such as comet assay and micronucleus (40 mg. L^{-1}) (Asghar *et al.*, 2018) and supply information on the downregulation of genes linked to the production of insulin and growth hormone (Alkaladi *et al.*, 2020). Taking an evolutive approach to the data, the paper of Koner *et al.* (2021) proposes fitting in the phylogenetic trees for *Clarias Magur.*

Histopathological assessments of the *O. Niloticus* liver revealed effects from the ZnO-NPs (8 mg. L^{-1}) exposure over six weeks, primarily affecting the organism's liver and causing severe congestion, necrosis, and apoptosis in microscopy analyses in live animals, hemorrhages, thromboses, and perivascular hepatic necrose were evident (Goda, Shaheen e Hamed, 2023). The data describing the main effects on *Danio rerio*, demonstrate the effects on embryos and adult organisms, in the embryos were related to cell death (Hansjosten *et al.*, 2022; Mawed *et al.*, 2022a). In adult organisms, the deformation of the oocysts to a long-term exposition, where the oocytes are maintained in a state of dormancy, and the oocytes in a growing state die before reaching maturity. The effects on the cell structures include deformation of the nucleus, rupture in the mitochondrial membrane, and presenting apoptotic features (Mawed *et al.*, 2022a).

4.2 GREEN SYNTHESIS AND SUPPLEMENTATION

The toxicological features provided by NPs are mainly attributed to their synthesis production; chemical and physical synthesis are the most common ways to produce this material, and toxicity seems to improve by using several substances to stabilize and modify intrinsic characteristics (Sharma; Tripathi, 2021).

Bringing an alternative solution to substitute the chemical NPs, the green synthesized materials offer an alternative to produce eco-friendly materials (Rashidian *et al.*, 2021). The NPs produced by green synthesis present several benefits (low cost, biodegradation accelerates, easy-to-insert modifications, etc). In our research, the studies of (Taherian *et al.*, 2020) and (Rashidian *et al.*, 2021), and (El-Saadony *et al.*, 2021) produced the ZnO-NPs using plants *Thymus pubescent* and *Satureja hortensis* and fungal *Aspergilus niger* in the production of NPs. The results demonstrate better tolerability by green NPs, where the LC_{50}-96h to *O. niloticus* was determined in 25.50 mg. L^{-1} to green nanoparticles produced by *Aspergilus niger* and 3.1 mg. $L^{-1}$for chemical nanoparticles by sol-gel (El-Saadony *et al.*, 2021). The same value of LC_{50}-96h was found for *O. mykiss* (25.50 mg. L^{-1}) for green nanoparticles produced by *Satureja hortensis* (Taherian *et al.*, 2020). On *Cyprinus carpio*, the values encountered for green by *Thymus pubscent* and chemical synthesis were 59.95 mg. L^{-1} and 78.9 mg. L^{-1}, respectively (Rashidian *et al.*, 2021).

Since ZnO-NPs is a substance used in some commercial food in aquaculture farms, supplementing food with different substances shows a decrease in the toxic

effect of ZnO-NPs. (Sharma; Tripathi, 2021). Supplementation of food with selenium (Asghar *et al.*, 2018) and vitamins C and E (Mohamed; Soliman; Ghannam, 2021) was responsible for providing a protective effect against the toxicity of ZnO-NPs. It boosts the immune system and has a protective effect on the liver and gills by supplementing thymol in *O. niloticus* (Abou-Zeid *et al.*, 2023; Khalil *et al.*, 2023). Resveratrol is a natural antioxidant substance. The employment of resveratrol in *Danio rerio* embryos reduced lethality and avoided severe damage during development (Giordo *et al.*, 2020).

5 CONCLUSIONS

In modern society, nanomaterials are becoming part of the daily human routine and contribute to many sectors of industries. The production of nanomaterials occurs by different methodologies, which give them different inputs and exclusive proprieties because of the variation in the physical-chemical parameters. The incorrect discharge of NPs leads to a concern on aquatic environments, and these spaces end up being the destination of several substances of anthropic activity. However, discussion about the destination of these materials must be established, and alternative solutions must be developed to cause a minor effect of this particle in the aquatic environment.

It is evident from the studies that the ZnO-NPs can be considered a harmful compound to be released into the environment, and the variation in parameters can result in different responses from fish. The results summarized in this research provide information for starting discussions about NP's discard regularization and legislation in aquatic systems, aiming to protect and conserve this habitat to guarantee water quality and sustainability. Besides, green synthesis aims to bring a more eco-friendly and sustainable industry development. The remediation of some substances is a short-term solution to improve the quality of environments that already suffer of ZnO-NPs contamination.

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CAPÍTULO 2 - ARTIGO EXPERIMENTAL

Ecogenotoxicological evaluation of undoped and nickel-doped Zinc Oxide Nanoparticles in the native fish *Rhamdia quelen*

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ABSTRACT

Nanomaterials have exponentially increased their production and application within various technological, biomedical, agricultural, and food industries. With the increase in the use of these materials, there is a growing concern about their discharge into aquatic ecosystems and future damage to the inhabitants of these locations. A nanomaterial that stands out today is zinc oxide nanoparticles, which cover the production of various dairy products and show antimicrobial activity. However, studies involving neotropic organisms and zinc oxide nanoparticles (ZnO-NPs) are still scarce. In this sense, this study pioneered analyzing the ecogenotoxicological effects caused by ZnO-NPs in two different states (not doped and nickel doped) in native Latin American fish, Rhamdia quelen. The organisms were exposed to different concentrations of nanoparticles at antimicrobial concentrations (0.4, 4, and 40 mg.L-1) in 96h water bioassay. Genotoxicity was analyzed by comet assay of different tissues, while mutagenicity was assessed via fish micronuclei and erythrocyte nuclear abnormalities (ENA). Biochemical markers were evaluated, including the activity of acetylcholinesterase (AChE), superoxide dismutase (SOD), catalase (CAT), and

glutathione S-transferase (GST). Also, the quantification of hydroperoxides was carried out to assess lipoperoxidation (LPO). The mean size obtained for ZnO-NPs and ZnO-NPs (Ni) was 25.9 nm for ZnO-NPs, with the crystalline wurtzite form. The results obtained by the genetic biomarkers indicate that mutagenic effects were not observed for both nanoparticles. However, genotoxic effects on *R. quelen* blood, brain, liver, and gill caused by ZnO-NPs (Ni) were observed. Also, there was an alteration in the activity of the enzymes SOD and GST on the liver and gill for the concentrations of ZnO-NPs I and II. No changes were observed in the activity for CAT, AChE, and LPO.

Keywords: ZnO-NPs, Genotoxicity, metal nanoparticle, Nanotoxicology, Aquatic toxicology

1. INTRODUCTION

One of the nanoparticles with wide use in industries are those of Zinc Oxide (ZnO-Ps). These NPs are widely used in electronic materials, mainly in the production of semiconductorsin photocatalysts (Feng *et al.*, 2020; Zhang *et al.*, 2019). One of the uses that brings visibility to nanoparticles is antibiotic action, acting directly in the fight against both gram-negative and gram-positive bacteria (Barani *et al.*, 2021; Li, Zhu e Lin, 2011; Senthamarai e Malaikozhundan, 2022). This property stands out, as such nanomaterials can be used in the fight against different microorganisms (Freitas et al, 2018; Ozdal; Gurkok, 2022).

As a result of the extensive production and use of ZnO-NPs, there is growing concern about their disposal in aquatic environments, as these NPs may pose risks to these ecosystems (Aziz *et al.*, 2022; Ma, Williams e Diamond, 2013). ZnO-NPs, when released, directly affect the water quality and life of aquatic organisms, as well as cluster and confinement in sediments (Caldelas *et al.*, 2021; Sibiya *et al.*, 2022; Wu, Harper e Harper, 2019). Different studies describe the toxicity of ZnO-NPs in invertebrate organisms, such as *Drosophila melanogaster* (Anand *et al.*, 2017), *Eisenia fetida* (Hu *et al.*, 2010), and vertebrates such as *Lithobates catesbeianus* (Motta *et al.*, 2020a), and *Danio rerio* (Chen; Lin; Meng, 2014; Mahjoubian *et al.*, 2023)

Studies involving ZnO-NPs and fish are still poorly explored since this compound is an emerging contaminant (Pastorino e Ginebreda, 2021; Zhao *et al.*, 2019). In the species *Cyprinus carpio*, changes in enzymes of the antioxidant system,

histopathological changes in the gills and liver, as well as bioaccumulation in the muscles were observed (Rajkumar *et al.* 2022). For fish of the species *Oreochromis mossambicus*, damage to the gills (breaking and shortening of the lamella) and liver (dissolution of the nuclear membrane and infiltration of lymphocytes) has been observed (Abid *et al.*, 2022). However, studies with fish native to the Neotropical region are little carried out, demanding the best research on the toxicity of these substances in native species.

The *Rhamdia quelen* fish is a species with a wide distribution throughout Latin America, where it presents itself as an organism of importance for the understanding of studies involving the neotropical region (Bordin *et al.*, 2024; Mazzoni; Bombardelli; Quagio-Grassiotto, 2020). Its use as a model organism in ecotoxicological studies is well described in the literature (Delmond *et al.*, 2019; Kitamura *et al.*, 2022; Klingelfus *et al.*, 2019; Mazzoni, Bombardelli; Quagio-Grassiotto, 2020; Oya-Silva *et al.*, 2021; Piancini *et al.*, 2015). This species has also been used in studies that evaluated different nanoparticles, such as silver (Lopez-Barrera *et al.*, 2021), titanium and lead (Oya-Silva *et al.*, 2021). So far, no ecotoxicological evaluation has been carried out with ZnO-NPs.

The main objective was to evaluate the ecotoxicological effects of undoped and nickel-doped ZnO-NP at antimicrobial concentrations, according to studies by Bondarenko *et al.*, (2013) and Dasari; Pathakoti; Hwang, (2013) aiming to evaluate the ecogenotoxic effects on *Rhamdia quelen* as indicator organisms using a multi-biomarker approach during a 96h exposition.

2. MATERIAL AND METHODS

2.1 Synthesis and characterization of ZnO and 1%Ni-ZnO nanoparticles

The nanoparticles ZnO and ZnO dopped with 1% Ni (ZnO-NPs (Ni)) were synthesized by a simple sol-gel methodology in an open system, in which are added distilled water at 80°C, the metal or metals acetates, and mono ethylene glycol. The systems were maintained heated for 30 min, and then allowed to cool until room temperature. The precipitates were centrifuged, washed three times with isopropyl

alcohol, and oven-dried at 60°C. Then they were calcined at 400°C for 1h and free cooled overnight to room temperature. Subsequently were macerated in a pestle until they became a fine powder. The elemental analysis and composition were obtained using Energy-dispersive X-ray spectroscopy (EDS), the size and the shape were measured using techniques of transmission electron microscopy (TEM), and the crystallographic analysis by selected area electron diffraction (SAED). The potential zeta, polydispersity index and hydrodynamic diameter was measured by Zetasizer® Nano Series ZS90 (Malvern Instruments, Worcestershire, UK).

2.2 Fish acclimatation

The Animal Experimentation Ethics Committee of Federal University of Paraná approved the present study under number 1408/2021. The juvenile specimens of *Rhamdia quelen* (length: 14.96 cm ± 2.76 cm; Weight: 27.52 g ± 22.35 g), (Fish: Heptapeteridae) were purchased from a fish farm from psychculture located in Instituto de Pesquisa em Agricultura Ambiental (InPAA), localizado na Universidade estadual do Oeste do Paraná (Unioeste) (Paraná, Brazil). The fish were acclimatized for 90 days at a temperature of 25°C in tanks (500 L), with a photoperiod of 12:12h (clear/dark), in decongested and filtered water, pH 7.0 and with constant aeration. The fish were fed every 48 hours (Acqua line, 42%protein).

After the acclimatization period, the fish were randomly distributed into aquariums (100 L) (n = 15 per treatment), containing different concentrations of Zinc Oxide Nanoparticles (ZnO-NPs) and nickel-doped Nanoparticle (ZnO-NPs (Ni). The exposition was composed of 8 groups (Table 1). For exposure, the concentrations of nanoparticles are defined based on the study of Dasari *et al.* (2013), taking as a reference the lowest concentration (0.04 mg. L^{-1}), testing the multiples of 10, 100, and 1000.

For exposure, ZnO-NPs and ZnO-NPs (Ni) solutions at different concentrations were previously sonicated for 30 minutes in an ultrasonic water bath (Schuster®, f = 42 KHz) to promote particle disaggregation. Soon after, they were added to the aquariums, and after 48 hours of exposure, the ZnO-NPs were repositioned, where two-thirds of the water was removed and filled again until the initial volume with a new amount of the NPs to ensure that the concentration remained the same as the initial.

Table 1. Groups and concentrations of Zinc Oxide Nanoparticles (ZnO-NPs) doped and non-doped with nickel (Ni) exposed in *Rhamdia quelen* fish

Treatment	Concentration (mg. L-1)
Negative Control (CN)	Only water
ZnO-NPs I	**0.4**
ZnO-NPs II	**4**
ZnO-NPs III	**40**
ZnO-NPs (Ni) I	**0.4**
ZnO-NPs (Ni) II	**4**
ZnO-NPs (Ni) III	**40**

Note: (NC) Negative Control; ZnO-NPs I (0,4 mg. L^{-1}); ZnO-NPs II (4 mg.L^{-1}); ZnO-NPs III (40 mg.L^{-1}); ZnO-NPs (Ni) I (0,4 mg.L^{-1}); ZnO-NPs (Ni) II (4 mg.L^{-1}); ZnO-NPs (Ni) III (40 mg.L^{-1}).

At the time 48h, before repositioning the concentrations, and 96h, before the euthanasia, a sample of water at the highest concentrations was collected for Zetasizer analysis. After 96 hours of exposure, the fish were anesthetized with benzocaine (10%) dissolved in ethyl alcohol (ethyl p-aminobenzoate; CAS no. 94-09-7 – 15 mg. L^{-1}) (Gontijo *et al.*, 2003). Blood was collected via caudal vein, with 5 µL intended for the assembly of two micronuclear blades, and about 10 µL added to fetal bovine serum (FBS) for carrying out the comet assay Ramsdorf *et al.* (2009). Shortly after the blood was collected, the euthanasia was carried out via the medullar section. For biochemical analysis, samples were collected from the gills, brain, liver, and muscle, and stored at -80 °C until the analysis. For the genetic biomarkers, samples of gills, brain, liver were used, which were added in Eppendorf-type microtubes containing 500 µL of cold FBS (Ghisi *et al.*, 2011; Benincá *et al.*, 2012).

2.4 Biochemical Biomarkers

For the evaluation of the biochemical markers superoxide dismutase (SOD), catalase (CAT), glutathione S-transferase (GST) and lipoperoxidation (LPO) were used in the tissues of the gills and liver. The tissues were weighed, homogenized in pH 7.0 (0.1 M) potassium phosphate buffer, and centrifuged at 15,000 x g for 30 minutes at 4 °C. The brain and muscle were weighed and homogenized in potassium phosphate buffer pH 7.5 (0.1 M), after homogenization for 30 minutes at 12000 x g at

4 °C and the supernatant was used for the enzymatic analysis of acetylcholinesterase (AChE).

The activity of the enzyme acetylcholinesterase (AChE) was determined according to the protocol established by Ellman *et al.* (1961), with modifications by Silva de Assis *et al.* (1998).

For Superoxide dismutase (SOD) activity, the supernatant was diluted in a 1:10 (v/v) ratio in potassium buffer (pH 7.0; 0.1 M). Subsequently, 40 µL sample and 885 uL buffer (1M Tris / 5mM EDTA, pH 8.0) were added to the microtube and shaken. After stirring, 50 uL of pirogalol (15 mM) was added and the samples were incubated for 30 minutes. The reaction was stopped with the addition of 25 were µL of HCl (1 N). The absorption reading was performed at 400 nm (Gao *et al.*, 1998).

Catalase (CAT) activity was determined by the degradation of hydrogen peroxide (H2O2) using the method described by Aebi (1984). Thus, 5 µL of the supernatant from the samples was added to microplates along with 295 µL from the reaction medium (20 mM H2O2, 50 mM Tris-base, 0.25 mM EDTA, pH 8.0). The absorption was measured at 240 nm for 2 min at 27 °C.

Glutathione S-transferase (GST) activity was determined using glutathione (GSH) and 1-chlorine-2,4-dinitrobenzen (CNDB) reduction as a substrate. Before this, 20 L of the supernatant was added in microplack together with 180 µL reaction solution (GSH 3 mM, CDNB 3mM 0.1 phosphate buffer, pH 6.5). The absorption reading was performed at 320 nm for 3 minutes (Keen et al, 1976).

For the determination of Lipoperoxidation (LPO), 100 µL of the samples were resuspended in methanol (1:1 (v/v) and subsequently centrifugated for 10 minutes at 5,000 x g, at 5 °C. 100 µL of this supernatant were added to the reaction solution (Xylenol orange 0.1 M, H2SO4 25 mM, hydroxytoluene butylated 4 mM) and $FeSO_4NH_4$ (ferrous ammonium sulfate 0.25 mM - added in this order in 90% metanol). The samples were incubated for 30 minutes sheltered from light and at room temperature, and the absorption reading was performed at 570 nm.

2.5 Genetic Biomarkers

The comet assay was carried out with the gills, brain, liver, and blood according to the methodology described by Singh (1988) with adaptations for blood by Cestari *et*

al. (2004) and Ferraro *et al.* (2004) and tissues by Ghisi *et al.*, (2011) Benincá *et al.*, 2012.

The mutagenic effect was measured micronucleus test, were performed using the methodology described by Ueda *et al.* (1992). The frequencies of micronucleus and nuclear erythrocytal abnormalities (NEA) were analyzed according to the methodology proposed by Carrasco *et al.* (1990) and Çavas and Ergene,G ̈ozükara (2005), following the classification of the abnormality in: Micronucleo (Mn); Blebed (Bd); Lobed (Lb); Notched (Nt) and Vacuolated (Vc).

2.6 Statistical analysis

The data were analyzed for normality via the Kolmogorov-Smirnov test with Liliefors correction. Data that presented parametric distribution were analyzed using ANOVA from a pathway with post-Tukey test. Data in which the distribution was non-parametric were submitted to the Kruskal-Wallis test followed by Dunn's post-test. The 95% significance level ($p < 0.05$) was considered for statistical analysis. The data were analyzed using the software GraphPad Prism 8.0.1

3. RESULTS

3.1 Chemical and physical characterization of ZnO-NPs

The samples of ZnO-NPs and ZnO-NPs (Ni) presented a crystalline hexagonal structure, corresponding respectively to the zinc oxide (JCPDS PDF n° 36-1451) and Ni-dopped zinc oxide (JCPDS PDF n° 77-191) standards. The nanoparticle diameter of 350 particles was measured for each sample directly from TEM micrographs using ImageJ software (Rasband, 2016). Through these measures, a histogram gauss fitting displayed was also calculated. The obtained particle size distribution of [10-65] nm and * the full width at half maximum (FWHM) of 18 nm and * and the weighted average size of 25.9 nm (σ = 0.4 nm) and * were obtained for the ZnO-NPs and ZnO-NPs (Ni), respectively. The TEM-EDS investigation exhibited for the ZnO-NPs a zinc content of 41±3 at% and oxygen content of 59±5 at%, indicating that the methodology employed favors the formation of oxygen-rich nanoparticles. As for the ZnO-NPs (Ni), were

obtained a zinc content of 51±3 at%, oxygen content of 46±2 at%, and a nickel content of 1.1±0.3 at%, indicating oxygen vacancies and substitution induced by Ni atom.

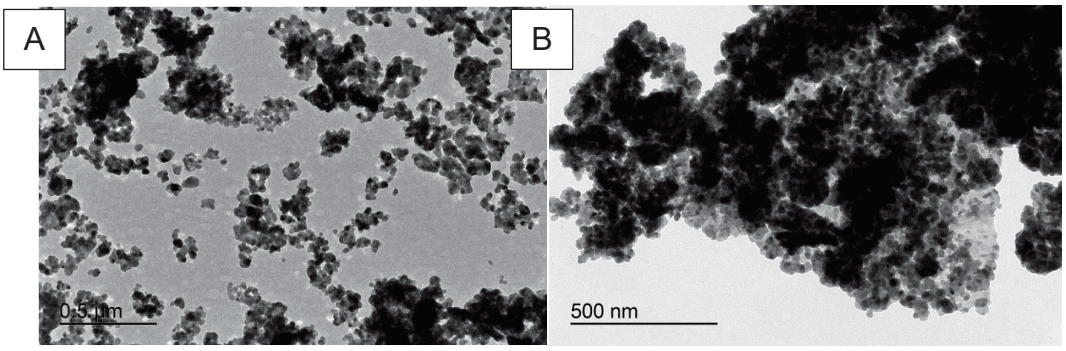

Figure 1. Transmission electron microscopy (TEM) of aggregates of ZnO-NPs (A) and Zno-NPs (Ni) (B).

Table 2. Characterization of ZnO-NPs suspensions, measured by Zetasizer®

Treatments	Time (h)	Hydrodinamic diameter (nm)	Polidispesion Index (%)	Zeta Potential (mV)
ZnO-NPs III	48	661.70	92	-35.5665
ZnO-NPs III	96	692.3	89	-10.7274
ZnO-NPs (Ni) III	48	558.70	98	-28.5723
ZnO-NPs (Ni) III	48	530.00	96	-28.1135

Note: mV = milivolts.

3.2 Biochemical biomarkers

The fish exposed to ZnO -NPS and ZnO-NPs (Ni) TO 96h did not show significant differences between the control group ($p > 0.05$) in the brain for AChE activity. However, in the muscle, the group ZnO-NPs III (40 mg. L-1), showed an increase in the activity of AChE ($p = 0.0013$) (Figure 3B). CAT and LPO did not show statistical differences between treatments and the control group ($p > 0.05$). (Supplementary material 1 and 2).

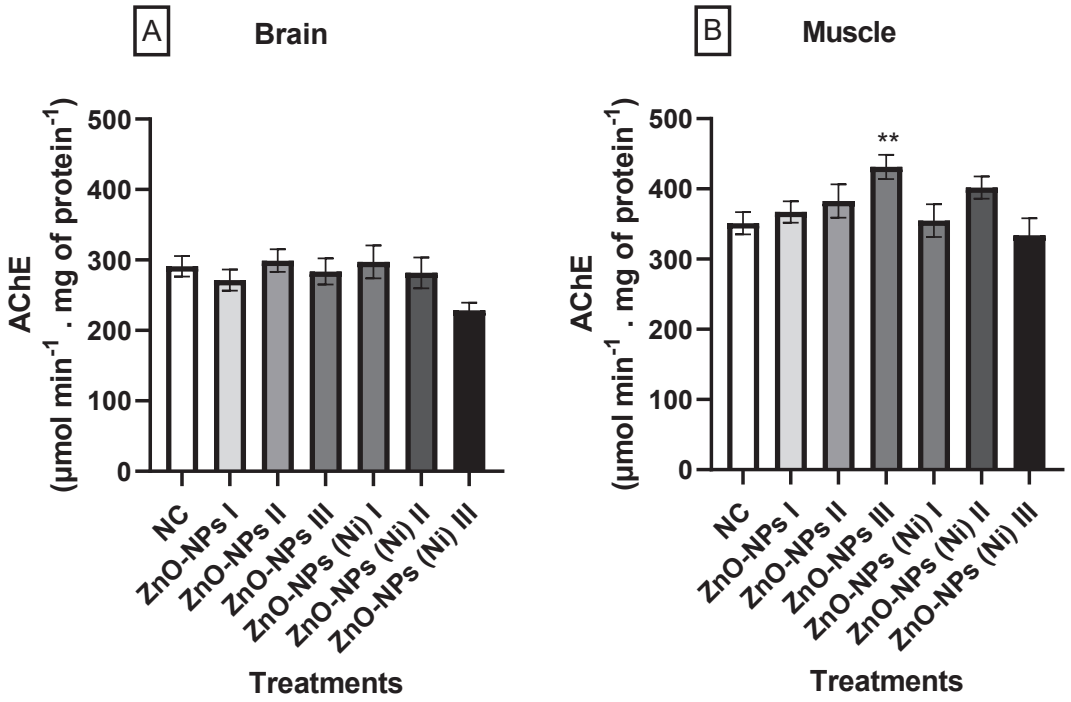

Figure 2. Biochemical biomarkers (mean ± standard error) of *Rhamdia quelen* exposed to 96h to different concentrations of zinc oxide nanoparticles non-doped and doped with nickel. Acetylcholinesterase activity (mean ± standard error) of *Rhamdia quelen* tissues was measured in (A) brain and (B) muscle. Note: Treatments: ZnO-NPs I (0,4 mg. L^{-1}); ZnO-NPs II (4 mg. L^{-1}); ZnO-NPs III (40 mg. L^{-1}); ZnO-NPs (Ni) I (0,4 mg. L^{-1}); ZnO-NPs (Ni) II (4 mg. L^{-1}); ZnO-NPs (Ni) III (40 mg. L^{-1}). Asterisks indicate statistical differences about the negative control by ANOVA test followed by Tukey post-test, where $p<0.05$ = *; $p<0.01$ = **; $p<0.001$ = *** and $p<0.0001$ = ****.

In the groups of ZnO-NPs I and ZnO-NPs there was an increase in the activity of A in the gill ($p = 0.0134$; $p = 0.0246$) and liver ($p<0.0001$; $p = 0.0002$). The GST activity was elevated in the gills at 0.4 mg. ($p = 0.0410$). At the liver, the GST activity increased in the groups ZnO-NPs I ($p = 0.0002$), ZnO-NPs II ($p = 0.0015$), ZnO-NPs (Ni) I ($p<0.0001$), and ZnO-NPs (Ni) II ($p<0.0001$). For the group ZnO-NPs (Ni) III ($p = 0.0002$), there was a significant decrease in the activity of the enzyme in comparison with the control group.

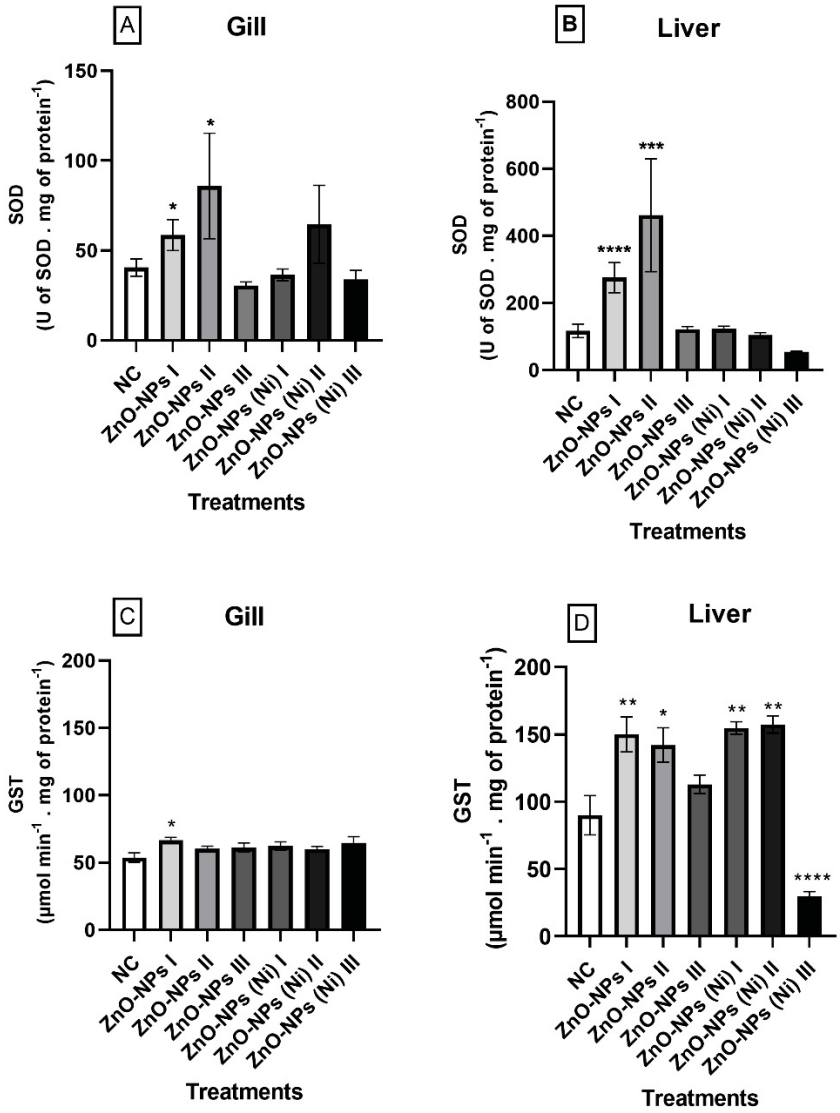

Figure 3. Biochemical biomarkers (mean ± standard error) of *Rhamdia quelen* exposed to 96h to different concentrations of zinc oxide nanoparticles non-doped and doped with nickel. Superoxide dismutase activity in gill (A) and liver(B); Glutathione S-transferase activity gill (C) and liver (D). Note: ZnO-NPs I (0,4 mg. L^{-1}); ZnO-NPs II (4 mg. L^{-1}); ZnO-NPs III (40 mg. L^{-1}); ZnO-NPs (Ni) I (0,4 mg. L^{-1}); ZnO-NPs (Ni) II (4 mg. L^{-1}); ZnO-NPs (Ni) III (40 mg. L^{-1}). Asterisks indicate statistical differences about the negative control by ANOVA-one way test followed by Tukey post-test, where $p<0.05$ = *; $p<0.01$ = **; $p<0.001$ = *** and $p<0.0001$ = ****.

3.3 Genetic biomarkers

Based on the values obtained (Table 3), there was no significant change ($p<0.05$) to MN frequency and ENA in any of the groups exposed to ZnO-NPs or ZnO-NPs. (Ni).

Table 3. Amount of erythrocytic nuclear alterations (ENA) and micronuclei in blood of *Rhamdia quelen* expose to 96h to different concentrations of zinc oxide nanoparticles not dopped (ZnO-NPs) and doped with nickel (Ni). The values indicate the median and first and third quartile (Q1/Q3).

Treatment	Blebbed	Lobed	Vacuolated	Notched	Binúcleus	Micronúcleus	Total ENA
NC	1 (0/1)	1 (0/1)	15 (3/16)	3 (1/4)	0 (0/0)	0 (0/1)	18 (5/23)
ZnO-NPs I	0 (0/0.25)	0 (0/1)	15 (8.75/28)	4.5 (2/6.5)	0 (0/0)	0 (0/0.25)	19 (12/40)
ZnO-NPs II	0 (0/1)	0 (0/1)	18.5 (7.5/41.25)	3 (2/6)	0 (0/0)	0 (0/1)	20 (13/39)
ZnO-NPs III	0(0/1)	0(0/1)	23.5(14.5/30)	1.5(1/5.5)	0(0/1)	1(0.75/2)	25(15/36)
ZnO-NPs (Ni) I	0 (0/0.25)	0 (0/0.25)	7 (5.5/14)	2 (0/7)	0 (0/0)	0(0/1)	11 (6/19)
ZnO-NPs (Ni) II	0 (0/1)	0 (0/1)	10 (5/26)	2 (1/3)	0 (0/0)	0 (0/1)	15(9/33)
ZnO-NPs (Ni) III	1(0/1)	0 (0/1.5)	14(6/26)	5(0.5/7)	0(0/0)	1(0.75/1)	17.5(9/31.5)

Note: (NC) Negative Control; Treatments: ZnO-NPs I (0,4 mg.L^{-1}); ZnO-NPs II (4 mg.L^{-1}); ZnO-NPs III (40 mg.L^{-1}); ZnO-NPs (Ni) I (0,4 mg.L^{-1}); ZnO-NPs (Ni) II (4 mg.L^{-1}); ZnO-NPs (Ni) III (40 mg.L^{-1}).

In the groups exposed to ZnO-NPs and ZnO-NPs (Ni), genotoxic effects were on the DNA of *R. quelen* in all the tissues analyzed. For the blood tissue, there was significant damage in ZnO -NPs (Ni) III group ($p = 0.0001$) (Figure 4A) compared with NC. Already in the brain tissue, there was significant damage in the groups ZnO-NPs III, and ZnO-NPs (Ni) III ($p=0.003$, and $p = 0.0001$, respectively) (Figure 4B) when compared to NC group. For gills, only the higher concentrations of ZnO-NPs III had $p=0.0001$ (Figure 4C). In the liver, there was the presentation of damage in the ZnO-NPs III group ($p=0.0196$) and all ZnO–NPs (Ni) concentrations with greater DNA damage in relation with the negative control (ZnO-NPs (Ni) I, $p=0.0052$; ZnO-NPS(Ni) II, $p= 0.0245$ and ZnO-NPs (Ni) III with $p=0.0001$ (Figure 4D).

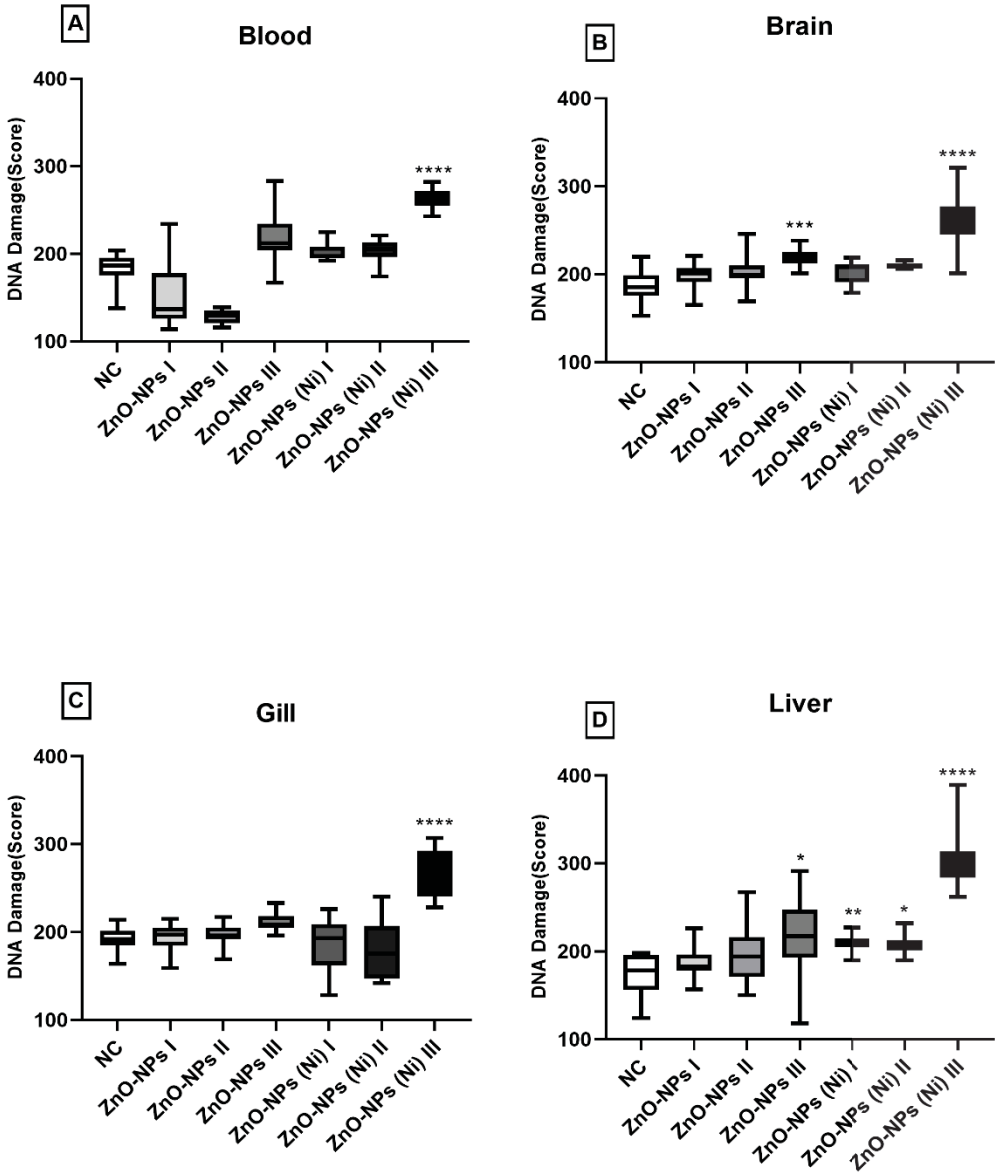

Figure 4. DNA damage score (median ± interquartile range) of *Rhamdia quelen* exposed to 96h to different concentrations of zinc oxide nanoparticles non-doped and doped with nickel: (A) Blood; (B) Brain; (C) Gill; (D) Liver. Note: ZnO-NPs I (0,4 mg. L-1); ZnO-NPs II (4 mg.L-1); ZnO-NPs III (40 mg.L-1); ZnO-NPs (Ni) I (0,4 mg.L-1); ZnO-NPs (Ni) II, and ZnO-NPs (Ni) III. Note: Treatments: ZnO-NPs I (0,4 mg. L^{-1}); ZnO-NPs II (4 mg. L^{-1}); ZnO-NPs III (40 mg. L^{-1}); ZnO-NPs (Ni) I (0,4 mg. L^{-1}); ZnO-NPs (Ni) II (4 mg. L^{-1}); ZnO-NPs (Ni) III (40 mg. L^{-1}). Asterisks indicate statistical differences about the negative control by ANOVA-one way test followed by Tukey post-test, where $p<0.05$ = *; $p<0.01$ = **; $p<0.001$ = *** and $p<0.0001$ = ****.

4- Discussion

In the present study, parameters such as size, composition, shape, and surface area were analyzed in the ZnO-NPs non doped and doped with nickel, as well as their properties when in solution, such as Zeta potential, hydrodynamic diameter, and

polyspersion. The pattern of organism responses is directly associated with the characteristics that nanoparticles will present.

The Zeta potential values measured for ZnO-NPs III and ZnO -NPs (Ni) III concentrations were values ranging from -35.56 mV (48h) to -10.72 mV (96h) and -28.57 mV (48h) to -28.11 mV, respectively (96h). Zeta potential values above +30 mV and below -30 mV are indicative of repulsion between the particles when in solution (Lunardi *et al.*, 2021)Thus, it can be inferred that ZnO-NPs (Ni) III had a higher dispersibility before the nanoparticle dosage was replaced, a pattern that was not followed by the same concentration of ZnO-NPs III that kept its potential stable during all exposure.

However, when the polydispersion potential is considered, we have that the indices remained above 89%, so this analysis provides information about the particle's homogeneity in solution. With this, it is possible to see that as much as one of the potential values is above the repulsion range of the molecules, there was a pattern of agglomeration of the particles (Table 2). Another data corroborating this agglomeration is the hydrodynamic diameter, which had mean values of 677 nm and 544.35nm for ZnO-NPs and ZnO-NPs (Ni), respectively.

The induction of micronucleus, as well as the appearance of nuclear erythrocyte abnormalities, are directly related to the life cycle of the cell; the erythrocyte responses to a xenobiotic occur between 1-5 days after exposure (Delmond *et al.*, 2019). The 96h exposure may not have been sufficient to demonstrate the appearance of micronucleus in the erythrocytes of *R. quelen* versus the zinc oxide nanoparticles. One factor that can be considered is the erythrocyte's life cycle since it needs to go through several processes until it is ripe (Witeska, 2013). in the study by Asghar *et al.*, (2018), there was water exposure of ZnO-NPs (40 mg. L^{-1}) in *Catla catla* for 28 days, where after this period, the micronuclear index was significantly increased ($p<0.05$).

Comet assay is a sensitive methodology for finding out the existence of direct damage to DNA in different tissues (Collins *et al.*, 2023), and its ability to detect nanoparticle damage in fish tissues is already described (Klingelfus *et al.*, 2019; Oya-Silva *et al.*, 2021; Vicari *et al.*, 2018)Recalling the results presented in this study, ZnO-NPs caused significant damage to all the tissues evaluated in relation to the control group, mainly in the higher concentrations of both NPs tested.

The comet assay with *R. quelen* erythrocytes demonstrated the existence of significant damage in the ZnO-NPs (Ni) III concentration in relation to the NC. The

erythrocytes are moving cells that carry and distribute various macromolecules and nutrients in organisms, thus carrying them to the vicinity of essential organs such as the brain, where the Blood-brain barrier exists (Persidsky *et al.*, 2006). This physical barrier that exists between the two tissues has the function of protecting the cells against the presence of substances that are harmful to the nerve tissue (Yazdani *et al.*, 2019). Still, there was the exposure of brain tissue, and significant genotoxic damage to this tissue at the higher concentrations of both nanoparticles. In studies by (Saddick; Afifi; Abu Zinada, 2017) there was a reduction in the antioxidant system of *Tilápia zilli* and *O. niloticus* species exposed to 20 mg. L^{-1} of zinc oxide nanoparticles.

The gills show themselves as an essential organ to be evaluated due to being the primary contact interface with the nanoparticles present in water (Kaya *et al.*, 2017). In our study, the direct damage to the DNA of the gills was evaluated using the alkaline comet test, making clear the existence of damage to the gills at the concentrations of 40 mg. L^{-1} of both nanoparticles in a significant way ($p<0.05$). Some studies (Kaya *et al.*, 2017; Koner *et al.*, 2021a; Yang *et al.*, 2020) have already demonstrated that the toxicological effects linked to ZnO-NPs can cause the branchial tissue of *Danio rerio* (5 mg. L^{-1}), *Clarias magur* (10 mg. L^{-1}) and *Oreochromis niloticus* (10 mg. L^{-1}), to alter the functioning of the antioxidant system of these tissues.

The liver is one of the organs responsible for carrying out the detoxification and excretion of harmful compounds to organisms directly or with the help of bile (Wang *et al.*, 2023), It also appears as one of those organs most susceptible to bioaccumulation of metals (Das *et al.*, 2023; Mohamed *et al.*, 2022b). In our study, significant damage (Figure 4D) was observed at all ZnO-NPs (Ni) concentrations and only at the highest concentration of ZnO-NPs when compared to NC.

In the study of (Alkaladi *et al.*, 2020) there was a decrease in the transcription of mRNAs that encode growth hormone by exposure to ZnO-NPs (2.05 µg. L^{-1}) in *O. niloticus*. In another study by Abou-Zeid. *et al.* (2023)Also, in *O. niloticus,* the liver's antioxidant system was less active.

5. CONCLUSIONS

The main objective of this research was to evaluate the ecogenotoxicological effects of the ZnO-NPs not doped and doped with nickel in the native organism *Rhamdia quelen* in bactericidal concentrations during an acute 96h trial, verifying the

incidence of damage to various tissues of this organism. The results obtained through the test biomarkers MN and ENA did not indicate significant differences between the treatments and control group; this may be due to the short exposure period and erythrocyte life cycle. The results obtained using comet testing, show that the concentrations of 40 mg. $L^{-1}$of ZnO-NPs and ZnO-NPs (Ni) were toxic to the genetic material of the brain, blood, gill, and liver significantly when compared to the control group. However, the mechanism of toxicity of these nanoparticles to DNA remains obscure and needs further discussions to be clarified. Other findings relating to this work have been included in the supplementary material and need to be discussed later.

Considering the results obtained in this research, these nanoparticles (doped and non-doped) can negatively impact the genetic material of *R. quelen* in higher doses (40 mg. L-$^{1)}$. Therefore, it becomes essential that future studies explore and pursue to elucidate the toxicity mechanisms of the non-doped and doped nanoparticles.

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APÊNDICE I – METODOLOGIA

No bioensaio hídrico foram utilizados peixes da especíe *Rhamdia quelen* (jundiá). Os peixes foram obtidos do Instituto de Pesquisa em Agricultura Ambiental (InPAA), localizado na Universidade Estadual do Oeste do Paraná (Unioeste). Os animais foram mantidos por 90 dias em tanques de 2000 litros para período de aclimatação, sob condições de pH 7, 25°C, fotoperíodo claro/escuro de 12 horas e aeração constante. Este projeto integra um projeto guarda-chuva já aprovado pela Comissão de Ética para o Uso de Animais (CEUA) da Universidade Federal do Paraná sob o certificado n°1401.

Logo após ao período de aclimatação, os animais foram distribuídos em aquários de 100 litros divididos em grupos de 15 animais. A exposição hídrica foi realizada por meio da dissolução das NpZnO dopadas e não dopadas. Para cada classe de nanopartícula (ZnO não dopada e ZnO dopada com Ni e ZnO, foram testadas 3 concentrações (0.4, 4 e 40 mg. L^{-1}). Os bioensaios tiveram a duração de 96 horas. Os espécimes de *Rhamdia quelen* foram separados em grupos conforme Tabela 1.

Tabela 1 - Grupos experimentais dos testes com nanopartículas de óxido de zinco (NPs-ZnO)

Tratamento	**Concentração (mg. L^{-1})**	**Quantidade**
Controle Negativo (CN)		**16**
ZnO-NPs I	**0.4**	**15**
ZnO-NPs II	**4**	**15**
ZnO-NPs III	**40**	**15**
ZnO-NPs (Ni) I	**0.4**	**15**
ZnO-NPs (Ni) II	**4**	**15**
ZnO-NPs (Ni) III	**40**	**14**
Controle Positivo (CP)	Injeção intraperitoneal de Metilmetanosulfonato 24h antes da eutanásia	15
TOTAL		

FONTE: O autor (2024)

Decorrido o período de exposição de 96 horas, os peixes foram anestesiados em solução contendo benzocaína àcoolica (1%). O sangue foi coletado via artéria caudal, onde aproximadamente 10uL forma destinados para montagem do esfregaço do teste do micronúcleo pisceo, outros 10uL forma adicionados em tubo tipo *eppendrof* contendo soro bovino fetal para realização do ensaio cometa. Amostras de brânquia e fígado foram destinadas para a realização do ensaio cometa e testes bioquímicos (SOD, CAT, LPO e GST). Para a realização dos testes da acetilcolinesterase amostras de músculo axial e cérebro foram coletadas, uma amostra de cérebro também foi destinada para realização do ensaio cometa.

O ensaio cometa de células sanguíneas, cerebrais, hepáticas e branquiais, foi realizado seguindo a metodologia proposta por Speit e Hartmann, (1999), com alterações para eritrócitos proposta por Cestari *et al.*, (2004) e Ferraro *et al.*, (2004), e para tecidos segundo Ramsdorf *et al.*, (2009). Desta forma, os cometas foram analisados e classificados utilizando microscópio de eflorescência Leica seguindo a classificação visual proposta por Collins *et al.* (2004). A classificação visual se baseou na migração dos fragmentos de DNA assim sendo: classe 0 (sem danos aparente), classe 1 (dano pequeno), classe 2 (dano médio), classe 3 (dano extenso) e classe 4 (dano máximo).

Para avaliação dos biomarcadores bioquímicos superóxido dismutase (SOD), catalase (CAT), glutationa S-transferase (GST) e lipoperoxidação (LPO) foram utilizados os tecidos branquiais e hepático. Estes tecidos foram pesados e homogeneizados em tampão fosfato pH 7.0 (0.1 M), centrifugados à 15,000x g por 30 minutos, a 4 °C. Os tecidos cerebrais e musculares foram pesados e homogeneizados em tampão fosfato pH 7.5 (0.1 M), após homogeneização por 30 minutos à 12000xg e 4 °C o sobrenadante foi utilizado para a análise enzimática de acetilcolinesterase (AChE).

A atividade da enzima acetilcolinesterase foi determinada para avaliação do efeito de neurotoxicidade de acordo com o protocolo estabelecido por Ellman *et al.* (1961) com as modificações de Silva de Assis *et al.* (1998).

Para a atividade da SOD, o sobrenadante foi diluído na proporção de 1:10 (v/v) em tampão potássio (pH 7.0; 0.1 M). 40uL da amostra e 885uL de tampão (1M Tris/ 5mM EDTA, pH 8.0) foram adicionados em microtubo e agitado, após agitação 50uL de pirogalol (15 mM), foram adicionados e incubados por 30 minutos. A reação foi

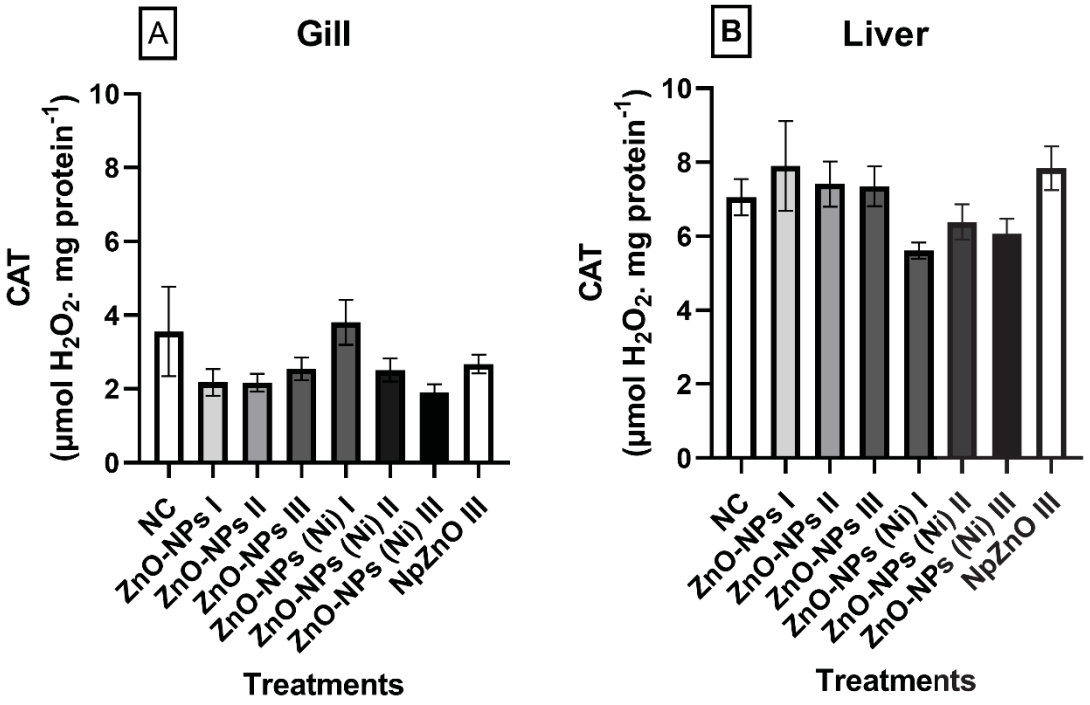
interrompida com a adição de 25 uL de HCl (1 N). A leitura da absorbância foi realizada em 400 nm (Gao *et al.*, 1998).

A atividade da CAT foi determinada através da degradação de peróxido de hidrogênio (H_2O_2), através do método descrito por Aebi (1984). 5 uL do sobrenadante das amostras forma em microplacas juntamente com 295 uL do meio de reação (20 mM H2O2, 50 mM Tris-base, 0.25 mM EDTA, pH 8.0). A absorbância foi medida em 240 nm por 2 min, a 27 °C.

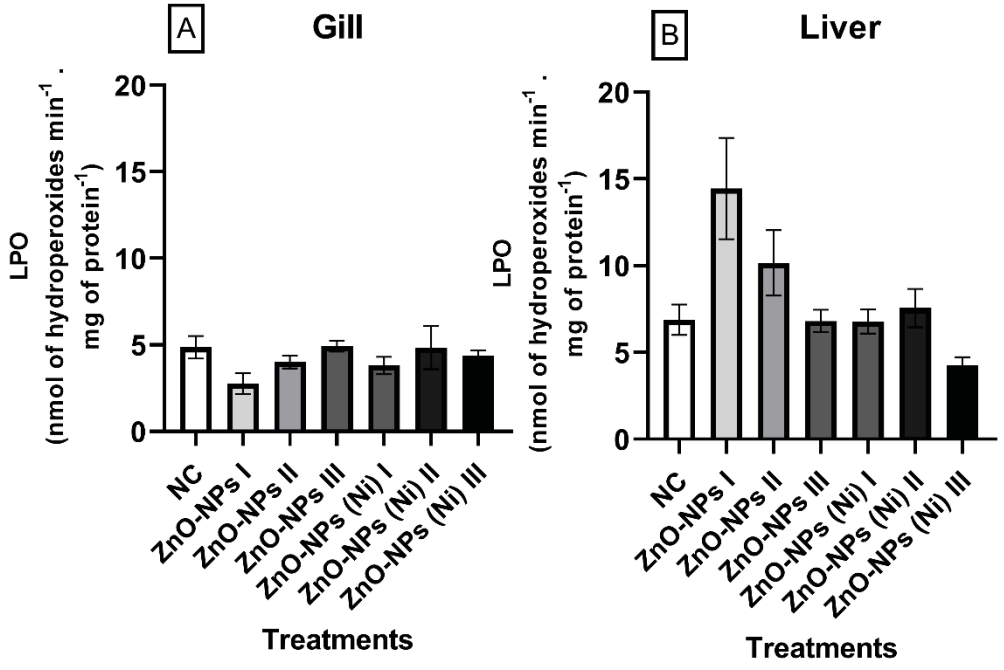
A atividade da GST foi determinada utilizando a redução da glutationa (GSH) e 1-cloro-2,4-dinitrobenzeno (CNDB) como substrato. 20 uL do sobrenadante foi adicionado a microplaca juntamente com 180 uL da solução de reação (GSH 3 mM, CDNB 3 mM 0.1 tampão fosfato, pH 6.5). A leitura da absorbância foi realizada em 320 nm por 3 minutos (Keen et al, 1976).

Para a determinação da LPO, 100 uL das amostras foram ressuspendidas em metanol (1:1 (v/v) e posteriormente centrifugados durante 10 minutos à 5,000x g, à 5 °C. 100 uL deste sobrenadante foram adicionados à solução de reação (laranja de xilenol 0.1 M, $H2_SO_4$ 25 mM, hidroxitolueno butilado) 4 mM e $FeSO_4NH_4$ (sulfato de amônio ferroso 0.25 mM adicionado nesta ordem em metanol 90%). As amostras foram incubadas por 30 minutos abrigados da luz e em temperatura ambiente, a leitura da absorbância foi realizada em 570 nm.

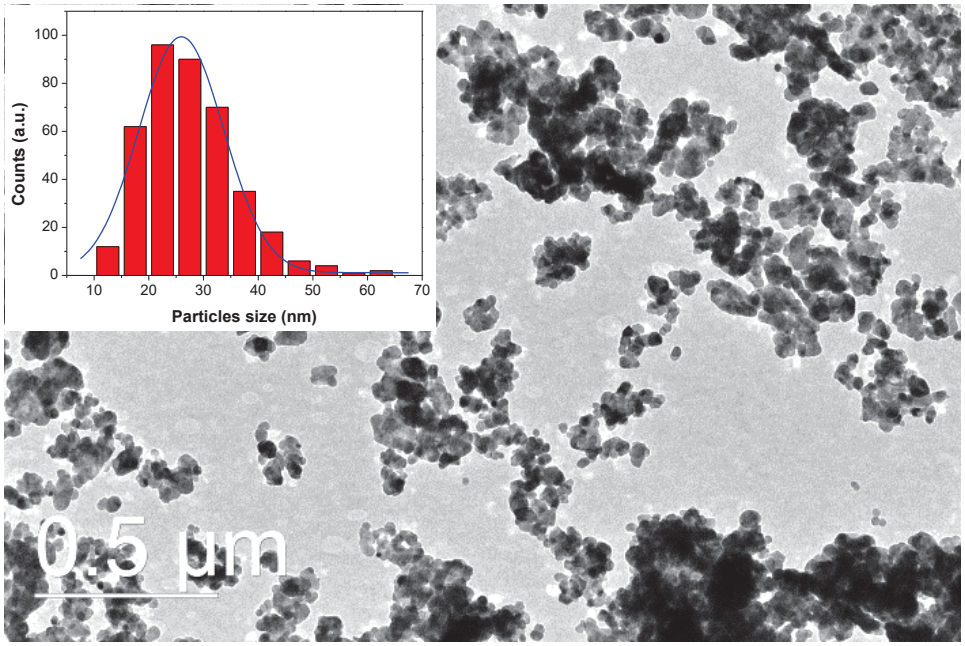
APÊNDICE II - SUPPLEMENTARY MATERIAL



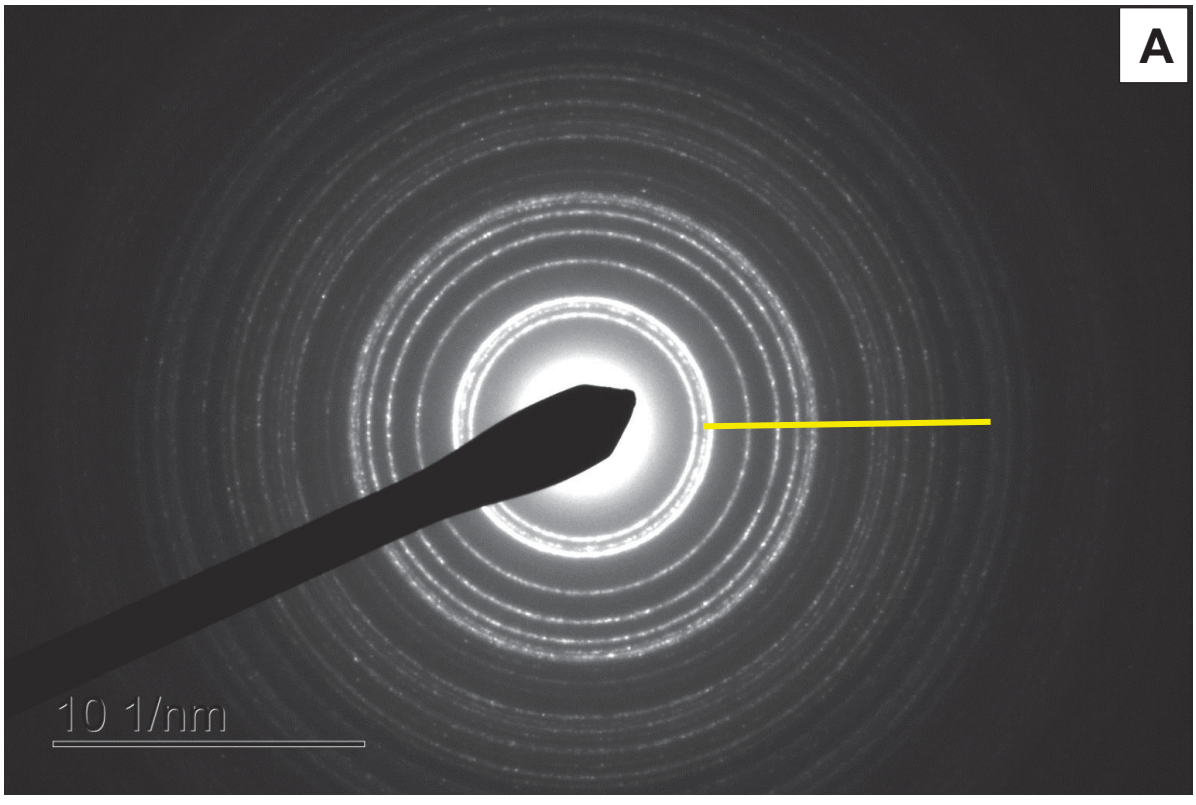
Supplementary material 1. Biochemical biomarkers (mean ± standard error) of *Rhamdia quelen* exposed to 96h to different concentrations of zinc oxide nanoparticles non-doped and doped with nickel. Catalase (CAT) activity in gill (A) and liver(B); Note: ZnO-NPs I (0,4 mg/L); ZnO-NPs II (4 mg/L); ZnO-NPs III (40 mg/L); ZnO-NPs (Ni) I (0,4 mg/L); ZnO-NPs (Ni) II (4 mg/L); ZnO-NPs (Ni) III (40 mg/L).



Supplementary material 2. Biochemical biomarkers (mean ± standard error) of *Rhamdia quelen* exposed to 96h to different concentrations of zinc oxide nanoparticles non-doped and doped with nickel. Liperoxidation (LPO) activity in gill (A) and liver(B); Note: ZnO-NPs I (0,4 mg/L); ZnO-NPs II (4 mg/L); ZnO-NPs III (40 mg/L); ZnO-NPs (Ni) I (0,4 mg/L); ZnO-NPs (Ni) II (4 mg/L); ZnO-NPs (Ni) III (40 mg/L).



Supplementary material 3. ZnO-NPs TEM micrographs at 10 kX with histogram gauss fitting.



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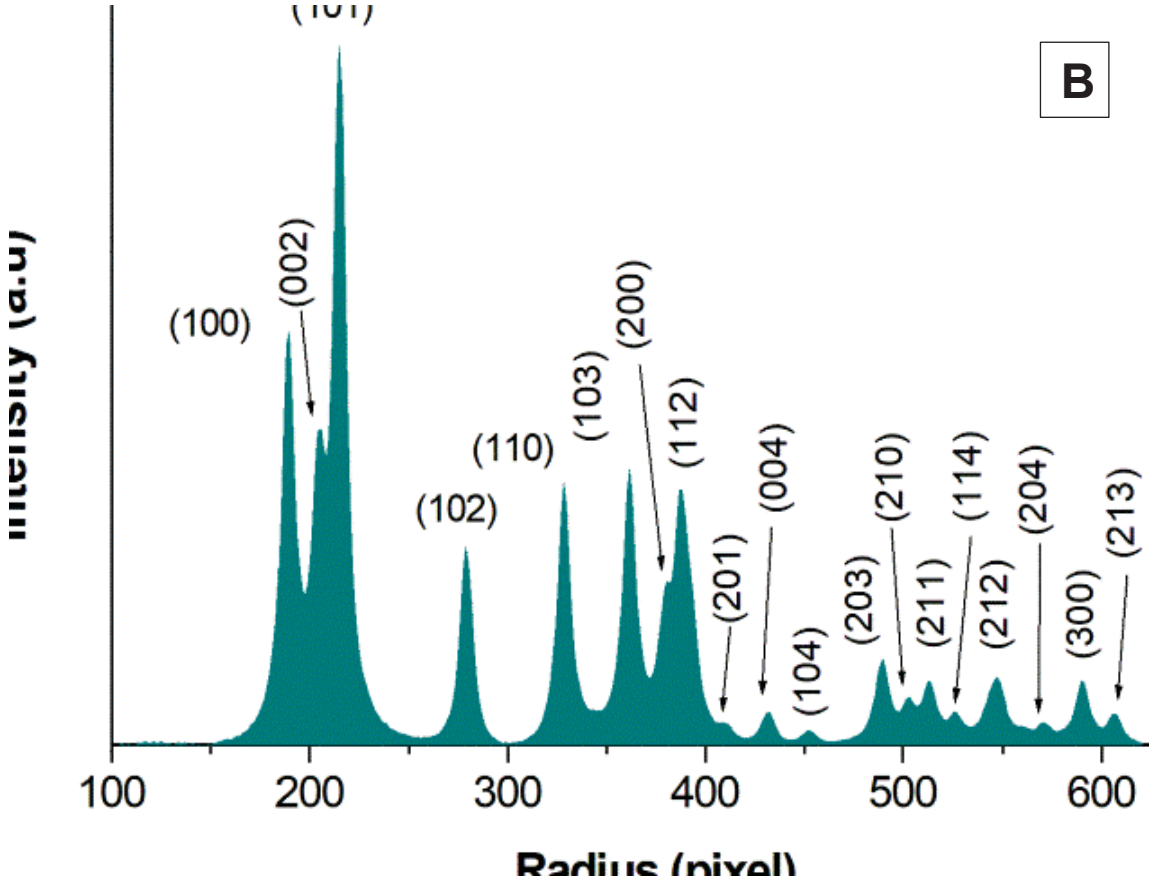

Supplementary material 4. ZnO-NPs TEM micrographs at 10 kX with histogram gauss fitting. TEM-SAED ZnO-NPs analysis (a) SAED ring pattern (b) SAED diffraction pattern with ZnO peak assignment, indicated by the yellow line in A.

ANEXO I

MINISTÉRIO DA EDUCAÇÃO
UNIVERSIDADE FEDERAL DO PARANÁ
SETOR DE CIÊNCIAS BIOLÓGICAS
COMISSÃO DE ÉTICA NO USO DE ANIMAIS

UFPR

Nº 1401

CERTIFICADO

A Comissão de Ética no Uso de Animais do Setor de Ciências Biológicas da Universidade Federal do Paraná (CEUA/BIO – UFPR), instituída pela Resolução Nº 86/11 do Conselho de Ensino Pesquisa e Extensão (CEPE), de 22 de dezembro de 2011, **CERTIFICA** que os procedimentos utilizando animais no projeto de pesquisa abaixo especificado estão de acordo com a Diretriz Brasileira para o Cuidado e a Utilização de Animais para fins Científicos e Didáticos (DBCA) estabelecidas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA) e com as normas internacionais para a experimentação animal.

STATEMENT

The Ethics Committee for Animal Use from the Biological Sciences Section of the Federal University of Paraná (CEUA/BIO – UFPR), established by the Resolution Nº 86/11 of the Teaching Research and Extension Council (CEPE) on December 22nd 2011, **CERTIFIES** that the procedures using animals in the research project specified below are in agreement with the Brazilian Guidelines for Care and Use of Animals for Scientific and Teaching purposes established by the National Council for Control of Animal Experimentation (CONCEA) and with the international guidelines for animal experimentation.

PROCESSO/PROCESS: 23075.008049/2021-75

APROVADO/APPROVAL: 27/04/2021 – R.O. 03/2021

TÍTULO: Avaliação ecotoxicológica de contaminantes emergentes: *in vivo* (peixes nativos) and *in vitro*.

TITLE: An ecotoxicological evaluation of emerging contaminants: *in vivo* (native fish) and *in vitro*.

AUTORES/AUTHORS: Marta Margarete Cestari, William de Almeida, Fellip Rodrigues Marcondes, Luis Phelipe de Souza Miranda.

DEPARTAMENTO/DEPARTAMENT: Genética

Profa. Dra. Katya Naliwaiko
Coordenadora da CEUA

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