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PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS AMBIENTAIS

DIOGO FERREIRA DO AMARAL

Efeitos genotóxicos, mutagênicos e comportamentais de nanopartículas de dióxido detitânio em girinos de Dendropsophus minutus (Hylidae, Anura)

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DIOGO FERREIRA DO AMARAL

Efeitos genotóxicos, mutagênicos e comportamentais de nanopartículas de dióxido de titânio em girinos de Dendropsophus minutus (Hylidae, Anura)

Tese de doutorado apresentada ao Programa de Pós-Graduação em Ciências Ambientais(CIAMB) na área de concentração em Estrutura e Dinâmica Ambiental, Pró-Reitoria de Pós-Graduação (PRPG), Universidade Federal de Goiás (UFG), como requisito para obtenção do título de Doutor em Ciências Ambientais.

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Ata da sessão de Defesa de Tese de **DIOGO FERREIRA DO AMARAL,** que confere o título de Doutor(a) em **Ciências Ambientais**, na área de concentração em **Estrutura e Dinâmica Ambiental.**

Aos **vinte dias do mês de agosto do ano de 2021**, a partir das **14:00h**, por **videoconferência** no **Google Meet: <https://meet.google.com/rdp-fxst-xma>**, realizou-se a sessão pública de Defesa de Tese intitulada **"Efeitos genotóxicos, mutagênicos e comportamentais de nanopartículas de dióxido de titânio em girinos de *Dendropsophus minutus* (Hylidae, Anura)"**. Os trabalhos foram instalados pela Orientadora, Professora Doutora **Daniela de Melo e Silva (ICB/UFG)** e Coorientador Professor Doutor **Thiago Lopes Rocha (IPTSP/ UFG)** com a participação dos demais membros da Banca Examinadora: Professora Doutora **Raquel Fernanda Salla Jacob (IB/UFSCAR)**, membro titular externo; Professor Doutor **Cláudio Carlos da Silva (PUC-GO e UEG)**, membro titular externo, Professor Doutor **Fausto Nomura (ICB/UFG)**, membro titular externo; Professor Doutor **Klebber Teodomiro Formiga (EECA/UFG)**, membro titular interno. Durante a arguição os membros da banca **não fizeram** sugestão de alteração do título do **trabalho**. A Banca Examinadora reuniu-se em sessão secreta a fim de concluir o julgamento da Tese tendo sido o candidato **aprovado** pelos seus membros. Proclamados os resultados pela Professora Doutora **Daniela de Melo e Silva**, Presidente da Banca Examinadora, foram encerrados os trabalhos e, para constar, lavrou-se a presente ata que é assinada pelos Membros da Banca Examinadora, aos **vinte dias do mês de agosto do ano de 2021**.

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DEDICATÓRIA

"Chega um momento em sua vida, que você sabe:
Quem é imprescindível para você,
Quem nunca foi,
Quem não é mais,
Quem será sempre."
Charles Chaplin

Aos meus pais, Wanderlan Amaral e Cleusa Ferreira do Amaral, que muitas vezes se doaram e renunciaram aos seus sonhos, para que eu pudesse realizar os meus. Quero dizer que essa conquista não é só minha, mas nossa. Tudo que consegui só foi possível graças ao amor, apoio e dedicação que vocês sempre tiveram por mim. Sempre me ensinaram agir com respeito, simplicidade, dignidade, honestidade e amor ao próximo. E graças às suas orações, os obstáculos foram ultrapassados, vitórias foram conquistadas e alegrias divididas. Agradeço a paciência e compreensão com minha ausência durante essa jornada. Muitíssimo obrigado.

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"Cada pessoa que passa em nossa vida, passa sozinha e não nos deixa só,
porque deixa um pouco de si e leva um pouquinho de nós.
Essa é a mais bela responsabilidade da vida
e a prova de que as pessoas não se encontram por acaso."
Charles Chaplin

Antes de tudo, agradeço a **Deus**, por estar comigo em todos os meus dias.

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"Bons alunos aprendem a matemática numérica, alunos fascinantes vão além, aprendem a matemática da emoção, que não tem conta exata e que rompe a regra da lógica. Nessa matemática você só aprende a multiplicar quando aprende a dividir, só consegue ganhar quando aprende a perder, só consegue receber, quando aprende a se doar."
Augusto Cury

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RESUMO

Os nanomateriais (NMs) têm sido usados em um número crescente de produtos comerciais e essa rápida expansão pode levar à sua liberação em ambientes aquáticos. Atualmente a acelerada produção dos diversos tipos de produtos convencionais em nanoescala tem sido fundamental para o progresso econômico de vários países. Muitos tipos de nanopartículas e nanomateriais já estão sendo utilizados e outras variedades estão previstas para aparecer no futuro, no entanto, é escasso o conhecimento sobre o impacto dos NMs na biota, principalmente nos anfíbios. Sendo assim, há a necessidade de classificar e compreender melhor os NMs. A primeira parte de nosso estudo revisou o uso histórico de espécies de anfíbios como sistemas modelo em estudos nanoecotoxicológicos, no qual resumimos os dados disponíveis na literatura científica sobre os efeitos genotóxicos, mutagênicos, histopatológicos, embriotóxicos e reprodutivos de NMs em diferentes grupos de anfíbios. A interação, bioacumulação, modo de ação (MoA) e ecotoxicidade de NMs em anfíbios também foram revisados. Os estudos nanoecotoxicológicos foram realizados com 12 espécies de anfíbios, sendo oito da ordem Anura e três da ordem Caudata. *Xenopus laevis* foi a espécie mais estudada. Os estudos foram conduzidos principalmente com NMs inorgânicos (72%) em comparação com os orgânicos. A nanoecotoxicidade depende do comportamento do NM e da transformação em meio ambiente, bem como dos estágios de desenvolvimento dos anfíbios. O MoA de NMs em anfíbios foi associado principalmente à produção de espécies reativas de oxigênio (ROS), estresse oxidativo, efeitos genotóxicos e mutagênicos. Os resultados enfatizaram a necessidade de mais estudos que testem a ecotoxicidade de diferentes NMs, concentrações e períodos de exposição em abordagens ambientalmente relevantes. Além disso, há a necessidade de protocolos padrão para testes nanoecotoxicológicos com anfíbios. Os dados revisados mostraram que os anfíbios são organismos adequados para avaliar o impacto ambiental dos NMs e indicaram importantes lacunas na pesquisa sobre a ecotoxicidade dos NMs em ecossistemas de água doce e recomendações para pesquisas futuras. Nesse sentido, observamos a partir do primeiro capítulo que entre as nanopartículas mais produzidas e consumidas mundialmente estão as nanopartículas de dióxido de titânio (NPs de TiO_2), cuja liberação no meio ambiente pode induzir efeitos tóxicos já reconhecidos em invertebrados aquáticos e vertebrados. No entanto, o conhecimento de seu impacto sobre os anfíbios neotropicais permanece limitado. Assim, o segundo capítulo teve como objetivo avaliar a toxicidade de NPs de TiO_2 e sua contraparte dissolvida, dióxido de titânio (TiO_2), em uma espécie de anfíbio anuro neotropical (*Dendropsophus minutus*). Danos ao DNA, parâmetros biométricos e mudanças comportamentais foram analisados em girinos após exposição a três concentrações ambientalmente relevantes (0,1, 1,0 e 10 mg L^{-1}) de NPs de TiO_2 e TiO_2 por 7 dias. Como resultado, verificamos danos ao DNA em girinos de *D. minutus* expostos a ambas as formas de Ti em comparação ao grupo controle. Também identificamos uma redução semelhante no

tamanho total, comprimento do corpo, largura e altura da musculatura da cauda em girinos expostos a NPs de TiO_2 e TiO_2 em comparação com girinos não expostos. Em relação ao teste comportamental, os girinos apresentaram menor mobilidade e ficaram mais distantes dos coespecíficos (menos agregados) quando expostos aos NPs. Portanto, o uso simultâneo de múltiplos biomarcadores foi essencial para avaliar os efeitos adversos do nanomaterial e estabelecer uma abordagem confiável para o biomonitoramento de ecossistemas aquáticos. Nosso estudo aumenta o conhecimento sobre os efeitos genotóxicos, morfológicos e comportamentais dos NPs de TiO_2 e TiO_2 em anfíbios anuros, contribuindo para estudos futuros na avaliação de risco ambiental de nanomateriais.

Palavras-chave: anuros; nanopartículas; titânio, ensaio cometa; biomarcadores; comportamento; bibliometria.

ABSTRACT

Nanomaterials (NMs) have been used in an increasing number of commercial products, and its rapid expansion could lead to their release into aquatic environments. The accelerated production of the various types of conventional products at nanoscale has been fundamental for the economic progress of several countries. Many types of nanoparticles and nanomaterials are already being used, and other varieties are expected to appear in the future. However, there is little knowledge about the impact of NMs on biota, especially on amphibians. Therefore, there is a need to better classify and understand NMs. The first part of our study has reviewed the historical use of amphibian species as model systems in nanoecotoxicological studies, which summarized data available in the scientific literature on the genotoxic, mutagenic, histopathological, embryotoxic, and reproductive effects of NMs in different groups of amphibians. The interaction, bioaccumulation, mode of action (MoA), and ecotoxicity of NMs in amphibians were also reviewed. The nanoecotoxicological studies were carried out with 12 species of amphibians, eight from the Anura order and three from the Caudata order. *Xenopus laevis* was the most studied species. Studies were conducted primarily with inorganic NMs (72%) compared to organic ones. Nanoecotoxicity depends on the behavior of the NM, the transformation in the environment, and the developmental stages of the amphibians. The MoA of NMs in amphibians was mainly associated with the production of reactive oxygen species (ROS), oxidative stress, genotoxic and mutagenic effects. The results emphasized the need for more studies that test the ecotoxicity of different NMs, concentrations, and exposure periods in environmentally relevant approaches. In addition, there is a need for standard protocols for nanoecotoxicological testing with amphibians. The reviewed data showed that amphibians are suitable organisms to assess the environmental impact of NMs and indicated essential gaps in research on the ecotoxicity of NMs in freshwater ecosystems, as well as recommendations for future research. In this sense, we observed from the first chapter that the most produced and consumed nanoparticles worldwide are titanium dioxide nanoparticles (TiO_2 NPs); whose release into the environment are known to induce toxic effects in aquatic invertebrates and vertebrates. However, knowledge of its impact on neotropical amphibians remains limited. Thus, the second chapter of this thesis aimed to evaluate the toxicity of TiO_2 NPs and its dissolved counterpart, titanium dioxide (TiO_2), in a species of anuran neotropical amphibian (*Dendropsophus minutus*). DNA damage, biometric parameters, and behavioral changes were analyzed in tadpoles after exposure to three environmentally relevant concentrations (0.1, 1.0, and 10 mg L^{-1}) of TiO_2 and TiO_2 NPs for 7 days. As a result, we verified DNA damage in *D. minutus* tadpoles exposed to both forms of Ti compared to the control group. We also identified a similar reduction in overall size, body length, width, and height of the tail musculature in tadpoles exposed to TiO_2 and TiO_2 NPs compared to unexposed tadpoles.

Regarding the behavioral test, the tadpoles showed less mobility and were more distant from the conspecifics (less aggregated) when exposed to NPs. Therefore, the simultaneous use of multiple biomarkers was essential to assess the adverse effects of the nanomaterial and establish a reliable approach for biomonitoring of aquatic ecosystems. Our study increases the knowledge about the genotoxic, morphological, and behavioral effects of TiO_2 and TiO_2 NPs in anuran amphibians, contributing to future studies in the environmental risk assessment of nanomaterials.

Keywords: anurans; nanoparticles; titanium, comet assay; biomarkers; behavior; bibliometrics.

1. INTRODUÇÃO GERAL

1.1 Nanotecnologia

A nanotecnologia foi descrita como uma das mais importantes fronteiras científicas e como a ciência definidora do século XXI (Anderson, 2007). Atualmente, definida como o estudo ou manipulação da matéria na faixa de tamanho de 1–100 nanômetros (nm), a nanotecnologia abrange várias técnicas para alterar as propriedades das partículas em nível molecular, a fim de obter novas qualidades com uma ampla gama de aplicações (Larsson et al., 2019). Os nanomateriais (NMs) possuem qualidades como alta área superficial, alta reatividade, força, durabilidade, resistência à água, capacidade de absorver luz ultravioleta e maior condutividade (Loeb et al., 2018; Murugadoss et al., 2021). Eles são usados em áreas tão diversas como eletrônicos, revestimentos, cosméticos, têxteis e medicina (Adesina, 2019; Papadopoulos et al., 2019), bem como aplicações ambientais, como por exemplo, a agricultura (Chhipa, 2019).

A nanotecnologia tem se expandido de forma acelerada com o passar dos anos (Makam & Gazit, 2018), sendo associada a promessas de produtos de consumo radicalmente aprimorados, inovação revolucionária nas ciências da vida e nova tecnologia ambiental e prevê-se que contribua para uma produção mais eficiente em termos de recursos, melhor saúde, novos empregos e crescimento econômico (Larsson etal., 2019). Em 2005, houve uma incorporação da nanotecnologia superior a US $ 30 bilhões em produtos manufaturados (Jovanović & Palić, 2012). Após uma década, já em2015, o mercado mundial de produtos com nanotecnologia atingiu seu primeiro trilhão dedólares (Koshovets & Ganichev, 2017). Atualmente, devido ao crescimento da área da nanotecnologia, o uso contínuo de produtos comerciais contento NMs cresceu exponencialmente (Rodriguez et al., 2019).

A acelerada produção dos diversos tipos de produtos convencionais em nanoescala atualmente tem sido fundamental para a economia de vários países (Livingston et al., 2020). Muitos tipos de nanopartículas (NPs) e NMs já estão sendo utilizadas e muitas outras variedades estão previstas para aparecer no futuro (İpek etal., 2020). Sendo assim, houve a necessidade de classificar e compreender os NMs (Jeevanandam et al., 2018).

Entender a nanotecnologia é fundamental para compreender os devidos fins dos NMs. Os NMs podem ser divididos quanto às suas dimensões e serem classificados quanto à natureza química, morfologia, importância comercial, modos de ação fisiológicos, dentre outros. Além disso, os NMs podem ser categorizados para fins de regulamentação em três grupos: (*i*) monotipos, quando apresentam uma mesma composição química ou estrutural; (*ii*) materiais com vários tipos de partículas ou múltiplos monotipos; (*iii*) artigos e formulações, que compreendem materiais monotípicos ou de múltiplas composições com formas, superfície, ou composição estrutural que determina sua função em maior grau que sua composição química (Gaillard et al., 2019). A primeira proposta para a classificação de NM foi dada por Gleiter et al. (2000) (Figura 1).

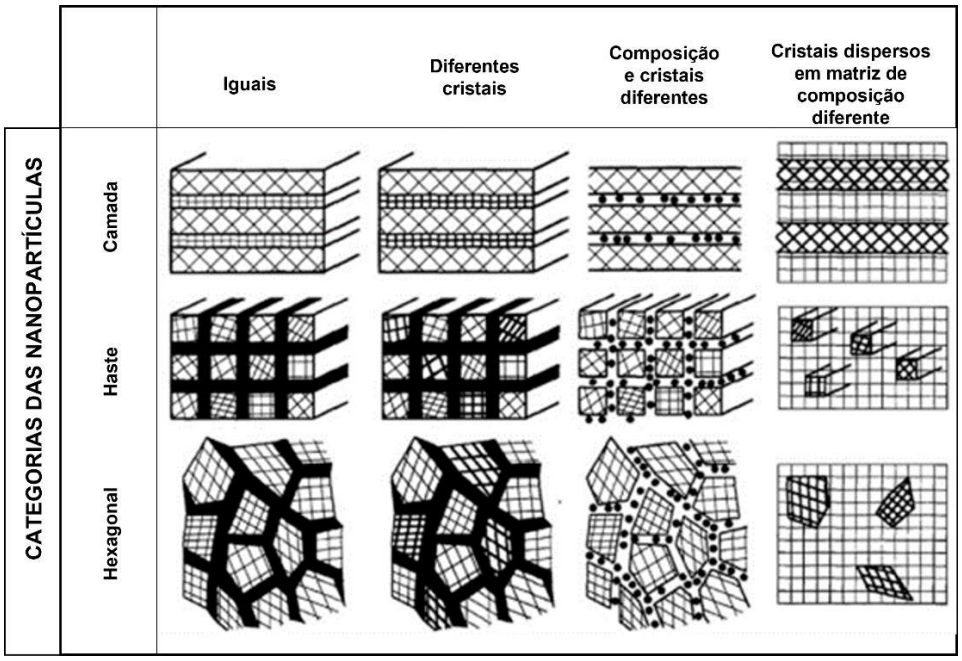

Figura 1. Esquema de classificação dos nanomateriais (NMs) proposto por Gleiter de acordo com sua composição química e a dimensionalidade (forma) dos cristalitos (elementos estruturais) que formam os NMs. Fonte: Pokropivny & Skorokhod, (2007), com modificações.

De acordo com os autores, os NMs foram classificados segundo suas formas cristalinas e composição química. No entanto, o esquema de Gleiter et al. (2000) não estava totalmente completo porque a dimensionalidade dos NPs e NMs não foram consideradas (Tiwari et al., 2012). Em 2007, Pokropivny & Skorokhod (2007) fizeram um novo esquema de classificação para NMs que incluiu as composições recentemente

desenvolvidas como 0D, 1D, 2D e 3D NMs (Figura 2). Esta classificação é altamente dependente do movimento do elétron ao longo das dimensões nos NMs (Livingston et al., 2020). Por exemplo, os elétrons em 0D NMs são aprisionados em um espaço adimensional, enquanto os 1D NMs têm elétrons que podem se mover ao longo do eixo x, que é inferior a 100 nm. Da mesma forma, NMs 2D e 3D têm movimento de elétrons ao longo do eixo x – y e eixo x, y, z respectivamente.

Segundo Livingston et al. (2020), as propriedades dos NMs dependem fortemente dos limites do seu tamanho, conforme mencionado no conceito de "engenharia de limites dos grãos" na classificação de Gleiter (2000). A classificação de Pokropivny e Skorokhod propôs que as características dos NMs sejam atribuídas à forma e dimensionalidade das partículas, conforme o conceito de "engenharia de superfície" e, portanto, classe dos NMs. Assim, essas razões se concentram na engenharia da forma e dimensionalidade das partículas, juntamente com a engenharia de contorno de grão para estender a aplicação de NMs.

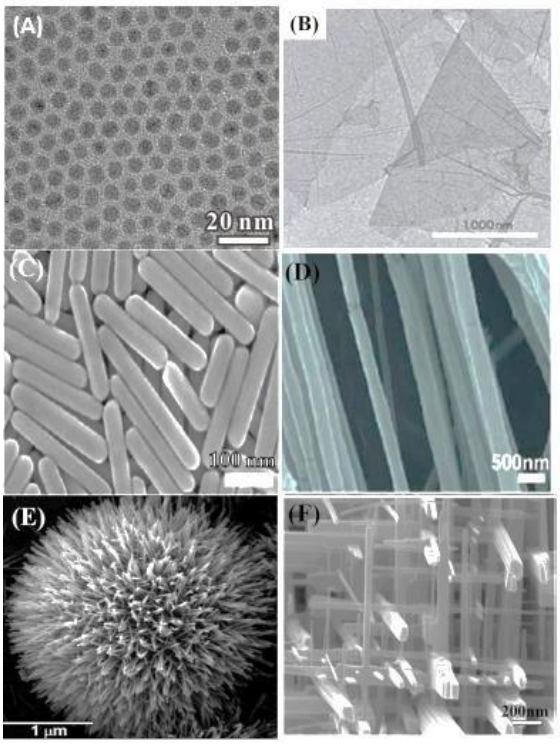

Figura 2: Nanomateriais com morfologias diferentes: (A) NPs de Paládio não porosos (0D) (Zhang et al., 2012); (B) Nanofolhas de grafeno (2D) (Li et al., 2012); (C) NPs de Prata (1D) (Zhang et al., 2011); (D) nanofibras de óxido de polietileno (1D) (Badrossamay et al., 2010); (E) urchin-like ZnO nanowires (3D) (Gokarna et al., 2014); (F) WO3 nanowire network (3D) (Zhou et al., 2005). Fonte: Livingston et al., (2020), com modificações.

1.1.2 Fontes de nanomateriais

As fontes de NMs podem ser classificadas em três categorias que variam de acordo com sua origem: (*i*) NMs produzidos naturalmente, que podem ser encontrados em corpos de organismos, insetos, plantas, animais e corpos humanos, (*ii*) NMs incidentais, que são produzidos incidentalmente como um subproduto de processos industriais, como NPs produzidas a partir de escapamento de motor de veículo, fumos de soldagem, processos de combustão e até mesmo alguns processos naturais como incêndios florestais e (*iii*) NMs manufaturados, que foram fabricados por humanos para ter certas propriedades necessárias para as aplicações desejadas (Jeevanandam et al., 2018).

Uma das principais diferenças entre NMs incidentais e NMs projetados é que a morfologia dos NMs projetados geralmente pode ser melhor controlada em comparação com NMs incidentais. Além disso, NMs projetados podem ser propositadamente delineados para explorar recursos novos que se originam de seu pequeno tamanho (Hochella et al., 2019). Sabe-se que NPs metálicas podem ser geradas espontaneamente a partir de objetos sintéticos, o que implica que os humanos há muito estão em contato direto com NMs sintéticos e que os objetos em macroescala também são uma fonte potencial de NPs incidentais no ambiente (Livingston et al., 2020).

1.2 Nanopartículas de dióxido de titânio (TiO_2 NPs)

As TiO_2 NPs possuem características que permitem transferir energia solar e fotocatálise de compostos tóxicos no meio ambiente (Hoffmann et al., 1995). Além disso, o forte poder oxidante dos orifícios fotogerados, a inércia química e a toxicidade das TiO_2 NPs também o tornaram um fotocatalisador superior (Fu et al., 2001). O uso das TiO_2 NPs como pigmento em tintas e produtos estéticos inspiraram diversos cientistas a renovarem o interesse por esta NP (Reddy et al., 2003). Esse NM pode ser encontrado em várias formas, entre elas as mais abundantes são brookita, rutilo e anatase (Murrray et al., 1993). A fase de brookita é estável apenas em temperaturas muito baixas e, portanto, não é tão útil na prática. Rutilo é aquele que é obtido após calcinação em alta temperatura, e suas propriedades fundamentais como as propriedades elétricas, ópticas e térmicas são bem estudadas. Em contraste, as propriedades da forma anatase não são tão bem

compreendidas. A razão pode ser que o anatase, que é uma fase estável em temperatura comparativamente baixa e ganhou importância somente depois que os materiais nanoestruturados e sua síntese começaram a desempenhar um papel importante na ciência dos materiais (Murrray et al., 1993).

Atualmente, as TiO_2 NPs estão entre as mais utilizadas globalmente (Bind e Kumar, 2019). Essas NPs são testadas pela Organização para Cooperação e Desenvolvimento Econômico (OCDE) em uma ampla variedade de produtos comerciais (Biola-Clier et al., 2020). Os chamados produtos finais estão disponíveis em grandes quantidades e, de acordo com o banco de dados de uso da TiO_2 NP nos EUA, são registradas entre 3800 e 7800 toneladas / ano (Biola-Clier et al., 2020). As TiO_2 NPs são usadas principalmente como componentes em tintas autolimpantes, superfícies autoesterilizantes, cimento e asfalto para construção de estradas, pavimentos e estruturas de túneis (Clemente et al., 2012). Também estão presentes em cosméticos, como protetores solares e sabonetes, pois melhoram a eficácia dos produtos ao aumentar a penetração de substâncias na pele (Jovanović, 2015). Devido ao seu alto uso na indústria moderna, as TiO_2 NPs estão em aproximadamente 9% dos produtos que contêm NPs (Boland et al., 2014).

1.3 Destino e transporte dos nanomateriais no meio ambiente

Para avaliar os riscos ecotoxicológicos dos NMs nos diferentes tipos de ambiente, é necessário um melhor conhecimento sobre sua mobilidade, biodisponibilidade e ecotoxicidade (Farré et al., 2009). A emissões antropogênicas de NPs inorgânicos mais do que dobraram o fluxo de NPs na atmosfera (Anastasio & Martin, 2001). A fonte antropogênica dominante é a emissão primária de fontes de combustão e a formação de aerossol secundária a partir da oxidação de espécies como SO_2 gasoso ou compostos orgânicos. Além disso, muitas atividades humanas industriais e domésticas, como cozinhar, manufatura ou transporte rodoviário e aéreo, liberam NPs na atmosfera (Farré et al., 2009). À medida que os NMs fabricados se tornam mais comuns, os cenários em que a exposição é possível aumentarão em relação ao crescimento do financiamento do governo e de empresas privadas em todo o mundo para pesquisa e desenvolvimento em

nanotecnologia (Bermudez et al., 2004). As vias mais importantes envolvendo NPs projetadas no ambiente podem ser observadas na Figura 3:

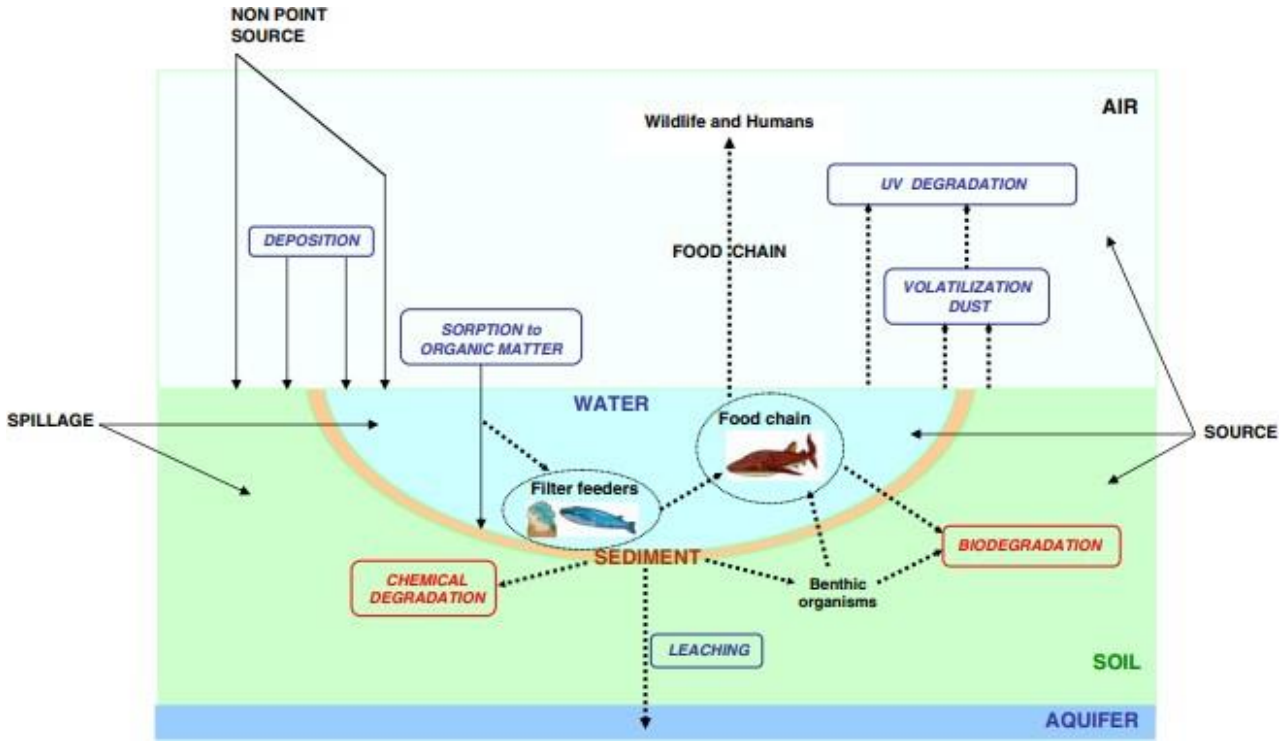

Figura 3: Vias mais importantes envolvendo nanopartículas projetadas no ambiente.
Fonte: Farré et al. (2009), com modificações.

Uma vez lançada no ambiente, as NPs livres tendem a formar agregados, conforme resumido na Figura 4. Os agregados ou NPs adsorvidos são menos móveis, mas podem sofrer absorção por animais filtradores e animais sedimentares (Farré et al., 2009). A agregação é dependente do tamanho, funcionalização e carga superficial da partícula e resulta na remoção eficiente de pequenas partículas em sistemas ambientais, portanto, a estabilidade das NPs é inversamente proporcional à sua tendência de agregação (Mackay et al., 2006). Uma vez que as NPs são liberadas no meio ambiente, elas são afetadas por fatores ambientais, como luz, oxidantes ou microorganismos. No ambiente aquático, os NMs sofrem transformações físicas, químicas e biológicas, ou mesmo, degradação da funcionalização da superfície ou da matriz de incorporação dessas NPs, as quais tambémpodem resultar em NPs livres (Nowack & Bucheli, 2007).

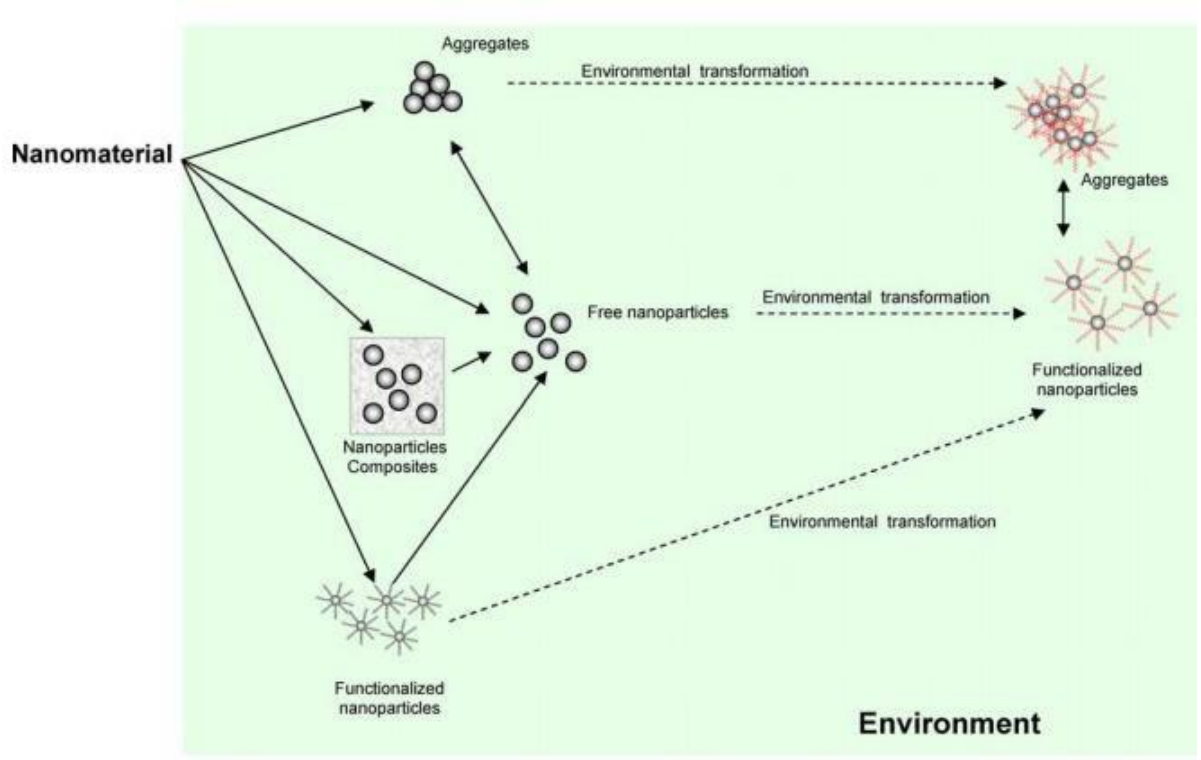

Figura 4: Modificação de nanopartículas no meio ambiente. Fonte: Farré et al. (2009), com modificações.

1.4. Ecotoxicidade das TiO_2 NPs nos organismos aquáticos

Devido à crescente aplicação dos NMs em diversos setores da sociedade, sua rápida expansão inevitavelmente levará ao seu lançamento no ambiente aquático por esgoto, efluentes, afluência de rios ou indiretamente por deposição atmosférica, despejo e escoamento (Rocha et al., 2015). Assim, os NMs podem atingir diferentes tipos de compartimentos aquáticos, como valas de drenagem, rios, lagos, estuários e águas costeiras (Moore, 2006). Nos ecossistemas aquáticos, os NMs podem se acumular nos sedimentos fluviais, ou permanecer suspensos na coluna d'água e transportados para os sistemas marinhos (Navarro et al., 2008). Os organismos aquáticos podem interagir com NMs que sofreram transformações físicas, químicas e biológicas, como agregação, oxidação, dissolução, bio-redução, biodegradação, foto-oxidação, degradação da superfície, interação com macromoléculas e deposição (Rocha et al. 2015).

Sendo assim, há estimativas de que em 2050, ocorrerá um aumento significativo na concentração de NMs em águas doces e marinhas (Giese et al., 2018). Além disso, há uma falta de dados toxicológicos e ecotoxicológicos para produtos comerciais habilitados

para NM, bem como uma crescente preocupação com sua classificação e regulamentação toxicológica (Bundschuh et al., 2018). A aplicação da nanotecnologia em vários campos, como a ciência biomédica e as indústrias eletrônica, cosmética e farmacêutica, tem provocado o aumento da liberação de NPs no meio ambiente (Sun et al., 2017) e os NMs estão se acumulado principalmente no ambiente aquático (Avant et al., 2019). Mesmo sendo escassos os estudos que comprovem seus efeitos tóxicos no ambiente aquático e na saúde humana (Kobayashi et al., 2017), vários artigos de revisão recentes tratam da toxicidade das NPs para organismos aquáticos e enfocam sua absorção, distribuição, metabolismo e excreção, bem como o potencial ecotoxicológico (Jovanović & Palić, 2012; Wang et al., 2019; Ale et al., 2021).

Atualmente as TiO_2 NPs estão entre os NMs mais utilizados (Bind e Kumar, 2019). Esses NMs são fotoativos e produzem espécies reativas de oxigênio (ROS), o que os tornam prejudiciais a muitos organismos, causando alterações fisiológicas, danos oxidativos, alterações celulares e morte (Biola-Clier et al., 2020). A genotoxicidade das TiO_2 NPs está associada à montagem do fuso mitótico, que compromete a progressão do ciclo celular e diminui o reparo do DNA, causando efeitos genotóxicos primários e indiretos (Biola-Clier et al., 2020). Estudos recentes afirmam que TiO_2 NPs quando em contato com organismos aquáticos também são capazes de causar alterações mecânicas, como menor eficiência de filtragem (no caso de organismos de filtro; Bundschuh et al., 2016), menor velocidade de natação e aumento da mortalidade, devido a uma inibição na mudança de mudas, no caso de artrópodes (Hartmann et al., 2019).

Em geral, o potencial ecotoxicológico de TiO_2 NPs foi encontrado em diferentes níveis tróficos, como fitoplâncton (Chiu et al., 2017), zooplâncton (Lu et al., 2018), nematóides (Ratnasekhar et al., 2015), moluscos bivalves (Girardello et al., 2016), crustáceos (Mansfield et al., 2015), mariposas (Zorlu et al., 2018), peixes (Carmo et al., 2018), répteis (Tsukano et al., 2017) e mamíferos (Wang et al., 2019). Embora alguns estudos tenham avaliado a toxicidade de TiO_2 NPs em anfíbios (Hammond et al. 2013), nenhum deles foi realizado com pererecas (Hylidae) ou com espécies de anfíbios Neotropicais.

1.5. Anfíbios como sistema modelo

Os anfíbios podem ser encontrados em praticamente todas as regiões tropicais e temperadas da Terra, com exceção de ilhas oceânicas (Segalla & Langone, 2004). As florestas tropicais possuem a maior biodiversidade de espécies de anfíbios conhecidas (Duellman, 1999). O Brasil é o país de maior biodiversidade desse táxon no mundo, abrigando mais de 1000 espécies, sendo que, aproximadamente 42 espécies estão ameaçadas de extinção, 60% delas endêmicas (Segalla et al., 2016). A grande maioria das espécies são anuros, incluindo 1.093 espécies (2 invasores exóticos) representando 20 famílias e 105 gêneros, seguidos por cecilianos, com 38 espécies em quatro famílias e 12 gêneros, e salamandras, com cinco espécies em uma única família e gênero (SBH, 2010).

Atualmente os anfíbios anuros estão entre os vertebrados que apresentam uma maior diversidade de modos reprodutivos de todo mundo (Haddad & Prado, 2005; Vági et al., 2020). Podem ser encontradas espécies de hábitos reprodutivos generalizados, ou seja, quando os ovos são depositados diretamente na água, até espécies com modos de reprodução mais especializados, com ovos depositados em ambientes terrestres (Pombal Jr. & Haddad, 2005). De acordo com os autores, as estratégias reprodutivas dos anfíbios podem depender de fatores como morfologia, fisiologia e comportamental, associados à certas condições ambientais, o que tornam essas espécies ainda mais importantes de serem estudadas.

Dos trabalhos atuais publicados utilizando os anfíbios como sistema modelo, grande parte estão voltados para os estudos toxicológicos (Slaby et al., 2019). O aumento do interesse científico nesses animais pode estar associado às ações publicitarias levantadas por herpetólogos do mundo todo, que tem sensibilizado a sociedade sobre seu aparente declínio global e sua importância para manutenção da cadeia alimentar (Slaby et al., 2019). São vários os fatores que incentivaram a descoberta e caracterização da diversidade biológica dos anfíbios, entre elas, podemos citar também, a aplicação de métodos moleculares, pois a taxa de descrição de espécies aumentou consideravelmente nas últimas décadas (Gehara et al., 2014).

A maioria das espécies de anfíbios possui ciclo de vida dividido em duas fases (fase larval aquática e fase adulta terrestre). Esses organismos são sensíveis à contaminação da água e do solo e são considerados biomonitores valiosos em estudos

ecotoxicológicos e avaliação de risco ambiental (Do Amaral et al., 2019). De acordo com a Organização para a Cooperação e Desenvolvimento Econômico (OCDE, 2009), os anfíbios são sistemas modelos adequados para analisar substâncias potencialmente tóxicas ao meio ambiente, principalmente compostos que podem interferir no funcionamento normal do eixo tireoidiano do hipotálamo. Sabemos que os anfíbios são modelos adequados para avaliar os modos de ação (anti) estrogênicos e (anti) androgênicos que influenciam a biologia reprodutiva, bem como os modos de ação (anti) tireoidianos que interferem no sistema tireoidiano (Kloas e Lutz, 2006).

Além disso, podemos considerar os anfíbios como: (*i*) bons indicadores de diversidade de habitat, variedade biológica e fatores de estresse no meio ambiente (Burlibaşa e Gavrilacaron, 2011); (*ii*) sistemas modelo para avaliação dos efeitos de diferentes xenobióticos em outros organismos (Denoël et al. 2010); (*iii*) o peptídeo B_2R, que é derivado de secreções de pele dos anuros e tem efeitos citotóxicos nas células cancerosas (Homayouni-Tabrizi et al. 2016); (*iv*) são bons indicadores para avaliar áreas sedimentares (Sansiñena et al. 2018); (*v*) são modelos eficazes para a identificação de algumas doenças que afetam animais e humanos (Whilde et al. 2017) e (*vi*) estão distribuídos ao longo doscontinentes, permitindo a comparação de dados de diferentes áreas (Ficetola et al. 2007).

1.5.1 *Dendropsophus minutus* (Peters, 1872)

Dendropsophus minutus (Peters, 1872) é uma espécie de anfíbio neotropical da família dos hilídeos com cerca de 21 a 28 mm de comprimento (Figura 5), distribuída na América do Sul, incluindo as encostas andinas, a Bacia Amazônica, o Escudo das Guianas, até a Mata Atlântica do sudeste do Brasil, com um recorde de elevação próximo ao nível do mar até 2.000 m (Frost, 2013). Possuem variação na coloração, osteologia, cantos de anúncio e morfologia larval (Donnelly & Myers, 1991), se caracterizando também como anfíbios típico de áreas abertas (Bokermann, 1967). Podendo ser facilmente encontrada inclusive no bioma Cerrado, todas essas características torna a espécie *D. minutus* uma excelente bioindicadora (Gonçalves et al., 2015).

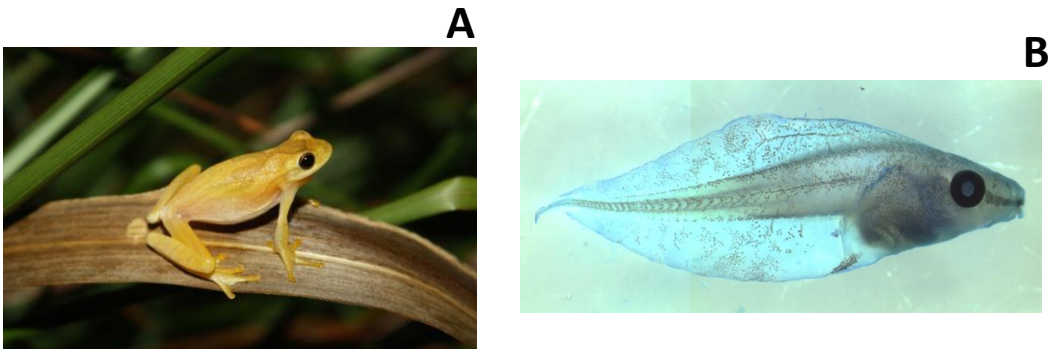

Figura 5: Anfíbio neotropical da família dos hilídeos, espécime adulta (A) e espécime em estado larval (B) de *Dendropsophus minutus* (Peters, 1872).

Vários trabalhos são encontrados na literatura utilizando a *D. minutus* como sistema modelo para análise de estudos ecotoxicológicos de diversos poluentes tradicionais e emergentes, tais como o herbicida atrazina (Gonçalves et al., 2017) e o herbicida a base de glifosato Roundup Original® (Amarante et al., 2002). Gonçalves et al. (2017) analisaram potenciais efeitos mutagênicos e genotóxicos do herbicida atrazina em diferentes estágios de desenvolvimento de girinos de *D. minutus*. Os autorescomprovaram que os estágios pré-metamórficos foram mais sensíveis do que o estágio pro-metamórfico após exposição a quatro concentrações nominais de atrazina (2,25, 4,5,9 e 18 mg/L) por um período de 96 h. Por meio do ensaio cometa e o teste de micronúcleo foram comprovados danos ao DNA em uma curva dependente da concentração. Em conclusão, a atrazina foi genotóxica e mutagênica para *D. minutus* de maneira concentração-sensível, dependente dos estágios de desenvolvimento larval.

Outro herbicida que apresentou efeitos genotóxicos e mutagênicos para girinos de *D. minutus* foi o herbicida a base de glifosato Roundup Original®, o qual é amplamente utilizado nas lavouras brasileiras e comumente encontrado em corpos d'água (Amarante et al., 2002). No ensaio cometa houve um aumento significativo no dano ao DNA em resposta à concentração de 0,28 mg/L para o comprimento da cauda, % de DNA na cauda e momento da cauda de Olive. Para o teste de micronúcleo, não foram encontrados aumentos estatisticamente significativos de micronúcleos em eritrócitos de *D. minutus* após 96 h de exposição ao glifosato (Carvalho et al., 2018).

Portanto, no presente estudo verificamos que não havia na literatura trabalhos com a espécie *D. minutus*, em testes com NPs, mesmo sabendo que esses organismos são sensíveis a alterações ambientais. Por esse motivo destacamos a importância de pesquisas

com diferentes NMs e a utilização dos anfíbios anuros como sistema modelo em estudos toxicológicos.

2. OBJETIVOS

2.1 Objetivo Geral

O principal objetivo do presente estudo foi avaliar os possíveis efeitos negativos da exposição aguda de girinos da espécie *D. minutus* a concentrações ambientalmente relevantes de TiO_2 NPs e TiO_2, com base em testes genotóxicos, parâmetros biométricos e testes comportamentais. Nossa hipótese é que baixas concentrações de TiO_2 NPs e TiO_2 na água podem causar efeitos genotóxicos, alterações morfológicas e distúrbios comportamentais em girinos.

2.2 Objetivos Específicos

- ✓ Revisar o uso histórico de espécies de anfíbios como sistema modelo para estudos nanoecotoxicológicos;
- ✓ Sumarizar e analisar de modo crítico os dados disponíveis na literatura científica sobre os efeitos genotóxicos, mutagênicos, histopatológicos, embriotóxicos e reprodutivos de nanomateriais em diferentes grupos de anfíbios;
- ✓ Avaliar o potencial efeito genotóxico (dano no DNA) das TiO_2 NPs e TiO_2 em girinos de *D. minutus* após exposição aguda (7 dias).
- ✓ Analisar os parâmetros biométricos em girinos após exposição a três concentrações ambientalmente relevantes (0,1, 1,0 e 10 mg L^{-1}) de NPs de TiO_2 e TiO_2 por um período de 7 dias.
- ✓ Observar se há alterações comportamentais após o período de exposição.

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CAPÍTULO II

Ecotoxicity of nanomaterials on amphibians: a critical review

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CAPÍTULO II: Ecotoxicity of nanomaterials on amphibians: a critical review

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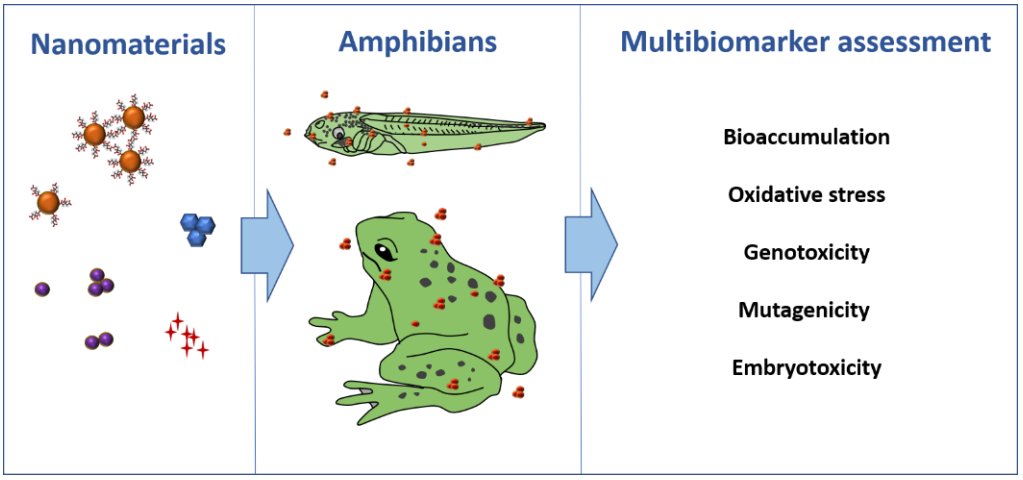
HIGHLIGHTS

- Current advances regarding the amphibians on nanotoxicological studies.
- Mode of action and ecotoxicity of NMs on amphibians are revised.
- Ecotoxicity of NMs on amphibians is mainly associated to oxidative stress.
- Standard protocols for nanoecotoxicological tests using amphibians are required.
- Amphibians are key model systems for nanoecotoxicity assessment.

GRAPHICAL ABSTRACT



ABSTRACT

Nanomaterials (NMs) have been used in a growing number of commercial products and their rapid expansion could lead to their release into the aquatic environments. However, there is a scarce knowledge about the impact of NMs on biota, especially the amphibians. The present study revised the historical use of amphibian species as model system on nanoecotoxicological studies and summarizes the data available in the scientific literature on genotoxic, mutagenic, histopathological, embryotoxic and reproductive effects of NMs in different groups of amphibians. The interaction, bioaccumulation, mode of action (MoA) and ecotoxicity of NMs on amphibians also were revised. The nanoecotoxicological studies were conducted with 12 amphibian species, being eight species of the order Anura and three species of the order Caudata. The *Xenopus laevis* was the most studied species. The studies were conducted mainly with inorganic NMs (72 %) compared to organic ones. The nanoecotoxicity depends on NM behaviour and transformation into environment, as well as the developmental stages of amphibians. The MoA of NMs on amphibians was mainly associated to reactive oxygen species (ROS) production, oxidative stress, genotoxic and mutagenic effects. Results emphasize the need for further studies testing the ecotoxicity of different NMs, concentrations and exposure periods at environmentally relevant approaches. Furthermore, standard protocols for nanoecotoxicological tests using amphibians are required. Revised data showed that amphibians are suitable organisms to assess the environmental impact of NMs and indicated important research gaps concerning the ecotoxicity of NMs on freshwater ecosystems and recommendations for future researches.

Keywords: anura, caudata, nanoparticle, nanoecotoxicity, omarker.

1. Introduction

Nanomaterials (NMs) are structures with at least one dimension in the range of 1 to 100 nanometers (nm) and can occur naturally in the environment or intentionally manufactured (engineered nanomaterials – ENMs) (European Commission, 2011). NMs have been used in an increasing number of commercial products (Jain et al. 2018), particularly in personal care and health products, which is the area of fastest-growing market segments for NM-enabled products (Reed et al. 2014; Rogers et al. 2018). Another segment, which also appears in the market, is an application of NMs in medicine, mainly in the diagnosis, treatment and prevention of diseases (Rizzo et al. 2013), as well as applications in the water treatment, optics, electronics engineering, photovoltaic devices, automotive industry and sports equipment (Freixa et al. 2018; Jackson et al. 2013). These NMs, embedded in different products, can be released throughout the product's life cycle, particularly during its use and disposal (Bressot et al., 2017).

Due to the increasing application of NMs in several sectors of society, its rapid expansion will inevitably lead to its release directly into the aquatic environment by sewage, effluents, river inflow, or indirectly by atmospheric deposition, dump and runoff (Baker et al. 2014; Nowack and Mueller, 2008; Rocha et al. 2017, 2015), reaching different types of aquatic compartments, such as drainage ditches, rivers, lakes, estuaries and coastal waters (Moore, 2006). In the aquatic ecosystems, NMs can be accumulated in the fluvial sediments, or remain suspended in the water column and transported to marine systems (Navarro et al. 2008). Aquatic organisms interact with NMs undergo physical, chemical and biological transformations, such as aggregation, oxidation, dissolution, bio-reduction, biodegradation, photo-oxidation, surface degradation, interaction with macromolecules and deposition (Lei et al. 2018; Minetto et al. 2014; Rocha et al. 2015).

Nowack et al. (2012) examined the fate of several NMs released from different products and concluded that it is not possible to assess environmental risks only by studying the original material and that the environmental transformation processes need to be considered in the specific destination of NMs. Understanding these transformations is essential to correctly extrapolate the fate and effect of NMs during the use and disposal phases, better understanding the likely end points of each material after aging. Thus, considering that the global NM production is expected to increase further in the coming

decades (Landvik et al., 2018), there is little knowledge in the literature about the impact of these NMs on biota, since their effects depend on their nano-specific properties (Colvin, 2003; Rocha et al., 2017).

Ecotoxicity of NMs on aquatic organisms have been reviewed for different trophic levels, such as phytoplankton (Miller et al., 2017; Moreno-Garrido et al., 2015), zooplankton (Lu et al., 2018), bivalves (Rocha et al., 2017, 2015) and fishes (Hund-Rinke et al., 2018; Shaw and Handy, 2011). However, the knowledge about the ecotoxicological effects of NMs on amphibians are scarce. Amphibians are considered key species in the ecosystem, being considered good indicators of habitat diversity, biological variety, stressors in the environment, and efficient models to evaluate the effect of different xenobiotics on other organisms, and also for the identification of some diseases that affect animals (Burlibaşa and Gavrilacaron, 2011; Denoël et al., 2010; Wells, 2007; Whilde et al., 2017). In this context, the present study summarizes the data available in the scientific literature on interaction, mode of action (MoA) and toxicity of NMs on amphibians. Furthermore, the genotoxicity, mutagenicity, histopathology, embryotoxicity and reproductive toxicity are discussed, showing that several future research needs are put forward.

2. Methodology

The literature review on the ecotoxic effects of NMs on amphibians was carried in the database published on the ISI Web of Science website using the following keywords: “amphibians and nanoparticles”, “amphibians and nanomaterials”, “tadpoles and nanoparticles” and “tadpoles and nanomaterials”. The ISI Web of Science was used due to its comprehensiveness regarding the number of publications and the quality of indexed scientific journals. These journals publish articles of broad interest and are focused on the general purpose of toxicology and ecotoxicology. We used only the compound form of the word set, since the terms when separated could indicate an enormous variety of works unrelated to the theme of this study. Scientific papers were retrieved and added to the study database. No other filters were used during the search. A total of 37 studies on the ecotoxic effects of NMs on amphibians were identified for the period 2007-2018 and each publication was compiled according to: (*i*) year of publication; (*ii*) period in which the

study was published; (*iii*) where the study was performed; (*iv*) name of the species (amphibian) used as a model system; (*v*) type of NPs (size and toxicity); (*vi*) exposure conditions of model organisms (exposure time and concentrations); (vii) geographical coordinates where the study was performed; (*viii*) as well as the multi-biomarker response.

3. Amphibians as model system on ecotoxicological studies

As most amphibian species have a two-phase life cycle (aquatic larval phase and adult terrestrial phase), these organisms are sensitive to water and soil contamination and are considered valuable biomonitors in ecotoxicological studies and environmental risk assessment (He et al., 2014). According to the Organization for Economic Co-operation and Development (OECD, 2009), amphibians are organisms designed to analyze substances potentially toxic to the environment, especially compounds that may interfere with the normal functioning of the thyroid axis of the hypothalamus.

Several characteristics make the amphibians especially important as a model system and extensively used as sentinel organisms (Table 1 - TABLE CAPTIONS), mainly because they are: (*i*) sensitive to water and soil contaminants, being adequate models to evaluate the anti-estrogenic and antiandrogenic MoA that influence reproductive biology as well as MoA (anti) thyroid that interfere with the thyroid system (Kloas and Lutz, 2006); (*ii*) widely distributed, thus, good indicators of habitat diversity, biological variety and stressors in the environment (Burlibaşa and Gavrilacaron, 2011); (*iii*) adequate model systems to evaluate the effect of different xenobiotics on other organisms (Denoël et al., 2010); (*iv*) good indicators to evaluate sedimentary areas (Sansiñena et al., 2018); (*v*) effective models for the identification of some diseases that affect other animals (Whilde et al., 2017); and (*vi*) distributed on almost all continents, allowing the comparison of data from different areas (Ficetola et al., 2007), as presented in Table 1.

However, the biomonitoring using amphibians as model organisms may be underused due to the lack of molecular biology data and analytical tools (Ceschin, 2017). To date, only six species of amphibians have been sequenced: *X. tropicalis* (Hellsten et al., 2010), *Nanorana parkeri* (Sun et al., 2015), *X. laevis* (Session et al., 2016), *L.*

catesbeiana (Hammond et al., 2017), *Rhinella marina* (Edwards et al., 2018) and *Ambystoma mexicanum* (Nowoshilow et al., 2018). Even so, amphibians are useful to characterize the environmental impact of new and emerging contaminants in the aquatic environment, as is the case of NMs (Table 2 - TABLE CAPTIONS).

4. Ecotoxicological impact of NMs on amphibians

4.1. Historical perspective

Overall, 24.32 % of studies were conducted by French research centers, 16.22 % were carried out in centers in the USA, followed by Argentina, Canada and Italy, with 8.11 % of studies each. No study with NMs was performed on the African continent. The first two studies addressing the application of NMs in amphibians were performed at the'Laboratoire d'Ecologie Fonctionnelle – EcoLab, Paul Sabatier University, Auzeville-Tolosane, France (Mouchet et al., 2007; Mouchet et al., 2008). The first study conducted by researchers of the United States was published in 2011 (Nations et al., 2011). The last study published according to our search was published by the Department of Biology of the University of Qaboos in Sultan (Al Mahrouqi et al., 2018). The distribution of the studies that tested the effects of MNs on amphibians can be observed in Figure 6.

The ecotoxicological effects of NMs on amphibians are described in Table 2. The first study was conducted by Mouchet et al. (2007), which demonstrated the genotoxic effects of the carbon-based nanotube (CNT) in *Xenopus laevis*. In this study, the frogs were exposed for 12 days at different concentrations of CNT (10, 100 and 500 mg L^{-1}). In 2010, Becker et al. (2010) indicated that amphibians are organisms vulnerable to different types of pollution due to their life cycle. After that, the biological effects of NMs on different species of amphibians were more explored, with 30 % of publications being conducted between 2010 and 2012.

FIGURE CAPTIONS

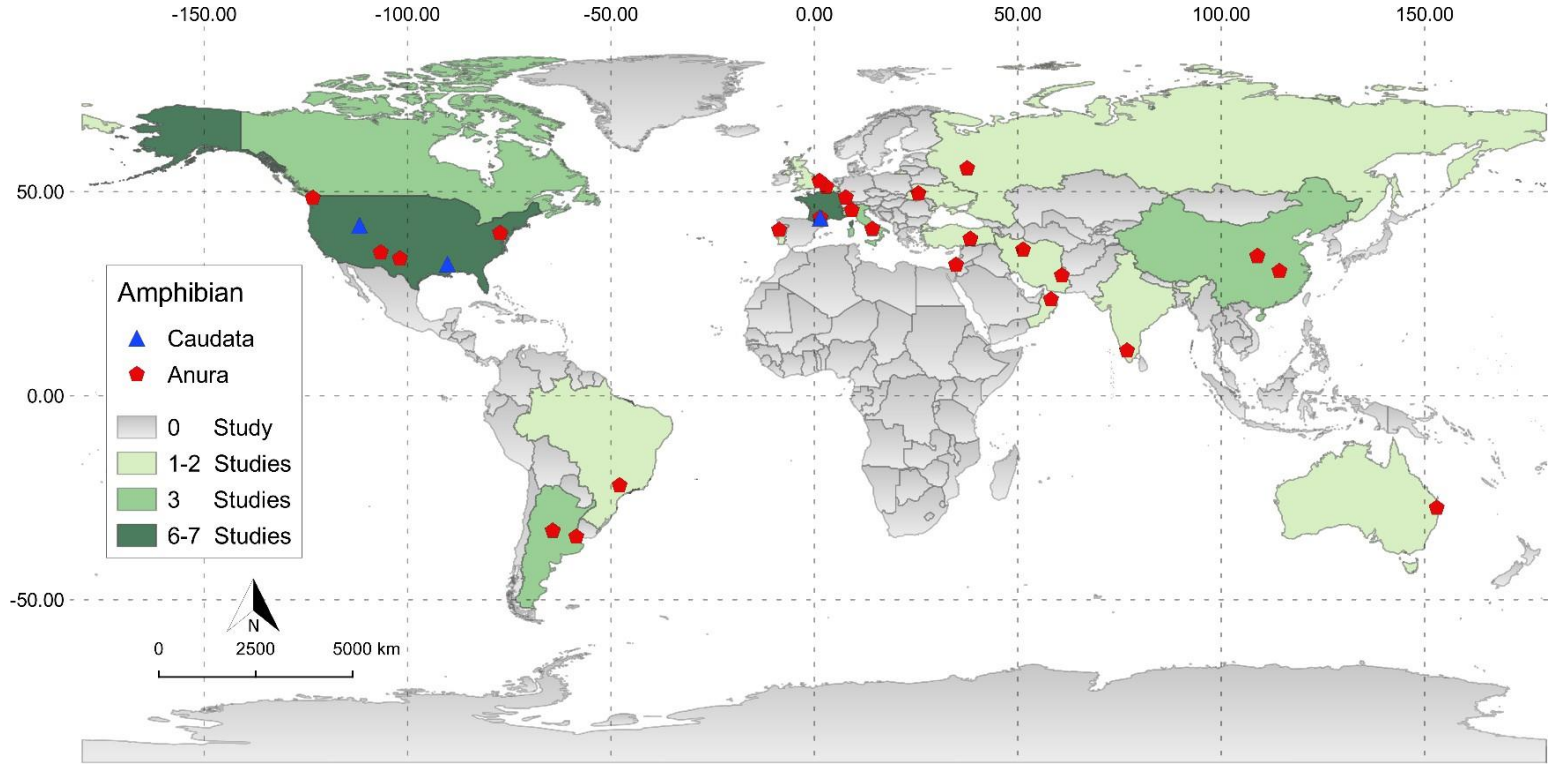


Figure 6: Worldwide distribution of ecotoxicological studies involving nanomaterials on amphibians.

At the same time as the number of NM-induced toxicity studies in amphibians under laboratory conditions progresses (Fig. 7A), several other nanotoxicological issues are raised. The focus of the research has shifted to an environmentally more realistic point of view, including research focused on the interaction of different NPs and the interaction between NPs and other contaminants. Although the revised data showed a growing increase in the number of studies with NMs, which can be explained by the need to understand their ecotoxicological effects on fauna (Rocha et al., 2017), it is noteworthy that no previous studies were performed using amphibians as model organisms. In order to evaluate the effects of the NMs on the aquatic and terrestrial environments, it is important to note that the amphibians may be able to react in contact with the different NMs in natural conditions.

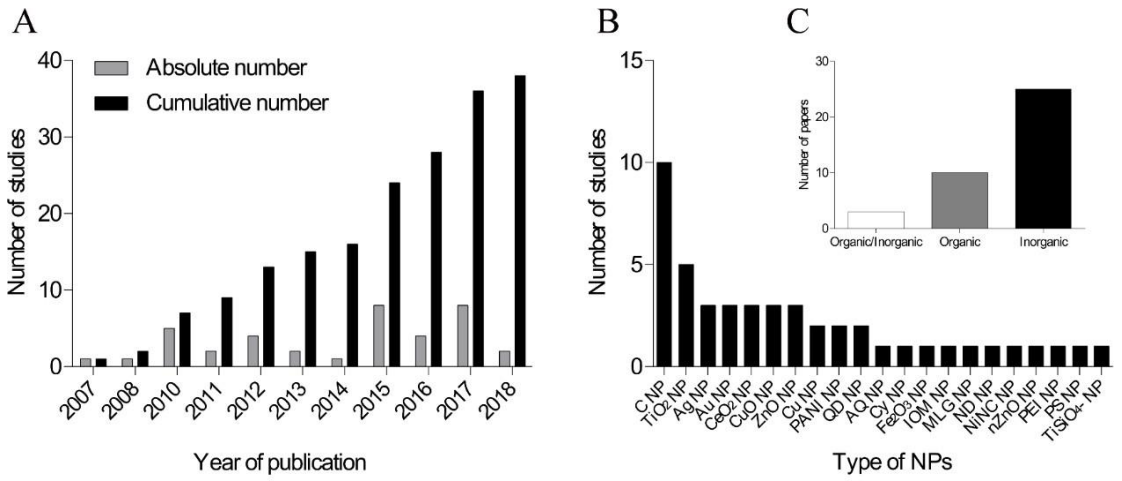


Figure 7: (A) Cumulative and absolute number of NM-induced toxicity studies on amphibians throughout the years; (B) Total number of studies performed on amphibians according to the types of nanomaterials; (C) Total number of studies performed with organic and/or inorganic nanomaterials on amphibians.

4.2. Types of nanomaterials

The reviewed data showed that 72 % of studies were conducted with inorganic NMs, while 28 % were performed with organic NMs (Fig. 7B-C). ZnO NPs (24 %) and Ag NPs (16 %) represent the most studied inorganic NMs, followed by Au NPs (12 %). ZnO NPs, even at low concentrations, such as 0.06 μg L^{-1} (Hinther et al., 2010), may be lethal to the anuran larvae and cause dysfunction of the gills and thyroid hormones,

decreasing the likelihood of larvae reaching adulthood (Falfushynska et al., 2017; He et al., 2014; Perelshtein et al., 2015; Spence et al., 2016). Ag NPs can induce changes in thyroid hormones in amphibians, leading to metamorphosis dysfunction (He et al., 2014; Hinther et al., 2010; Murugan et al., 2015).

The organic NM more investigated was the carbon NPs (CNPs) (40 %), followed by CeO_2 NPs (12 %). Recent studies have showed the toxic effects of CNPs on amphibians (Homayouni-Tabrizi et al., 2016; Lagier et al., 2017; Svart[a] et al., 2017a). According to Homayouni-Tabbrizi (2016), CNPs at small concentrations (0.1 and 0.6 mg L^{-1}) can cause severe cellular alterations in adults of *Rhinella ridibunda* (Anura), producing cellular dysfunction. Svartz et al. (2017a) showed that the exposition of *Rhinella arenarum* larvaes to two alumina-based nanoceramics (γ-Al_2O_3 and Ni/γ-Al_2O_3) cause rupture and alteration in the tadpole cavities, with edema, hyperkinesia anddecrease in movements. Lagier et al. (2017) confirmed that *X. laevis* (Anura) tadpoles exposed to carbon NMs at 10 mg L^{-1} for 12 days reduced their growth, making them morevulnerable to predators.

In relation to CeO_2 NPs, these NMs may present different effects depending on the species studied (Bour et al., 2015). The exposure of *X. laevis* larvae to CeO_2 NPs (0.1, 1, and 10 mg L^{-1}; 4 and 12 days) did not induced genotoxic effects, while similar exposure produced DNA damage in *Pleurodeles walt* larvae; although both species reduced growth rates (Bour et al., 2015). Similarly, *P. waltl* larvae (Caudata) exposed for 12 days at two types of CeO_2 NPs (uncoated and citrate-coated NPs) at 0.1, 1. and 10 mg L^{-1} exhibited genotoxic effects for both NMs (Bour et al., 2017). However, greater genotoxicity was observed in the uncoated CeO_2 NPs, proving that the NP functionalization reduced its genotoxic effects in amphibians (Bour et al., 2017).

Inorganic NMs, such as CuO NPs, ZnO NPs, TiO_2 NPs among others have recently been included in the OECD priority list for toxicological testing (OECD, 2001). According to previous studies CuO NMs may cause deleterious effects on fish, including reduction in survival and retardation in growth (Clearwater et al., 2002). In amphibians, chronic exposure to CuO NPs can cause mortality, embryonic deformity, delay in the metamorphic process and even reduction in body size (Chai et al., 2014). The ecotoxicological effects of ZnO and TiO_2 NPs on amphibians remain unknown. Few studies have used NMs that are included in this priority list (see Bacchetta et al., 2012;

Nations et al., 2011), indicating that further studies are needed for a better clarification about their effects.

4.3. Species

The nanoecotoxicological studies were conducted with 12 amphibian species, being 81.82 % of them performed using eight species of the order Anura, while 27.27 % were performed using three species of the order Caudata (Fig. 8). The order Anura has represented by four species of the family Ranidae (*Lithobates sylvaticus* – 4.17 %, *Lithobates catesbeianus* – 18.75 %, *Pelophylax perezi* – 2.08 % and *Pelophylax ridibundus* – 2.08 %), tree species representing the family Bufonidae (*Bufo gargarizans* – 2.08 %, *Rhinella arenarum* – 8.33 % and *Sclerophrys arabica* – 2.08 %), one species of the family Dicroglossidae (*Hoplobatrachus tigerinus* – 2.08 %), and one species of the family Pipidae (*Xenopus laevis* – 47.92 %). The order Caudata has represented by two species of the family Salamandridae (*Pleurodeles waltl* – 4.17 % and *Taricha granulosa* – 2.08 %) and one species of the family Ambystomatidae (*Ambystoma mexicanum* – 2.08 %). No study was performed using species of the order Gymnophiona. Gymnophionas are amphibians that have a fossorial habit and live in underground tunnels or in freshwater and estuarine environments (Lambertini et al., 2017). Thus, studies on the ecotoxicological effects of emerging contaminants, such as NMs, on these organisms are urgent and necessary.

In relation to Anurans, the *X. laevis* and *L. catesbeianus* represented 47.92 % and 18.75 % of studied species, respectively, followed by *R. arenarum* (8.33 %), *L. sylvaticus* (4.17 %) and *P. ridibundus* (4.17 %) (Fig. 8). This result is associated with the fact that both species are domesticated and have been breed in the laboratory throughout the world, being also commercial species and that can reproduce throughout the year by induced way (i.e. using injections of human chorionic gonadotrophin) (Amaral et al., 2018; OECD, 2001). Besides, both species have aquatic larvae, facilitating the execution of experimental studies under laboratory conditions (Du Pasquier and Weiss, 1973). The wide use of *X. laevis* in experimental studies may be related to FETAX (Frog Embryo Teratogenesis Assay-*Xenopus*), standardized by the American Society for Testing and Materials in 1998 (Ok Song et al., 2003), which have been recently applied in studies on some NMs (Nations et al., 2011). The species *L catesbeianus* is an anuran native to North

America and has a large body size, besides being a species easily breed in captivity (Pasmans et al., 2017), which makes it a good model organism in experimental studies.

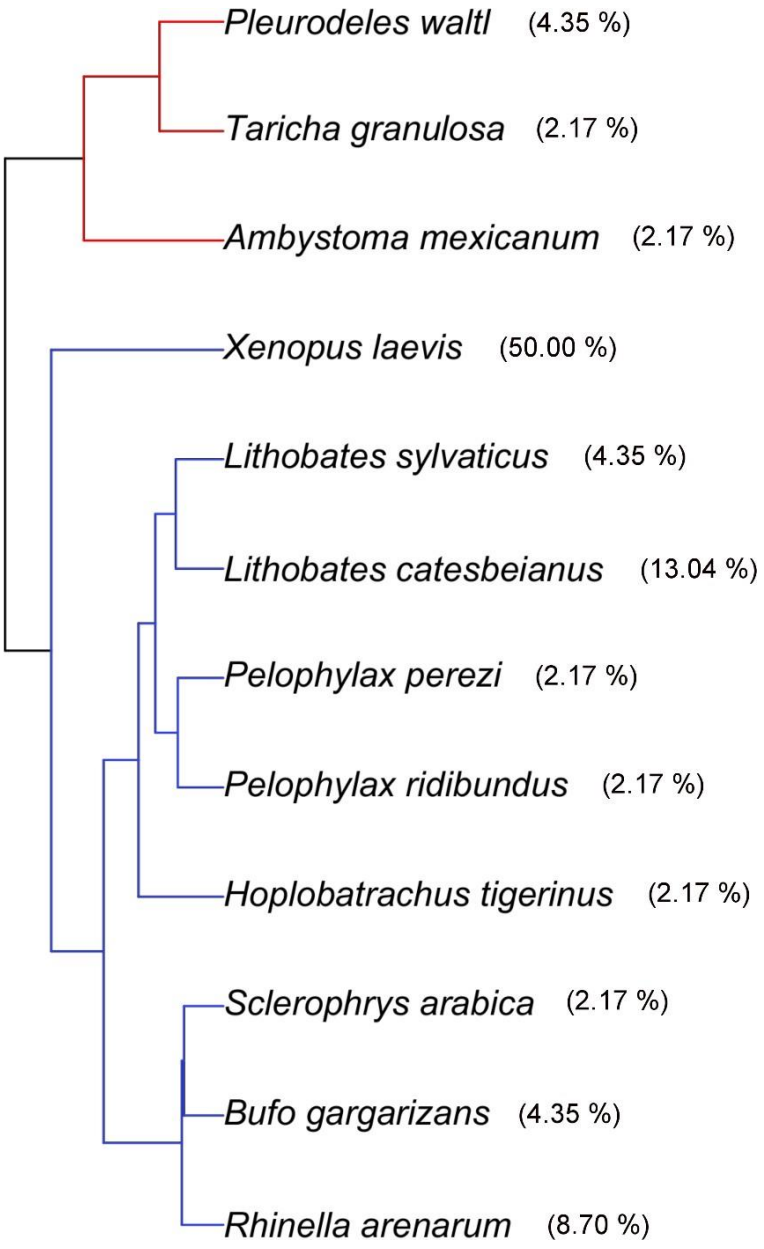


Figure 8: Relative frequency of amphibian species used as model organisms in studies with nanomaterials. Phylogenetic tree adapted from data obtained in Pyron and Wiens (2011).

The first study associating NPs with amphibians was carried out using the species *Xenopus tropicalis* (Mouchet et al., 2007). This species originates in Africa and presents an exclusively aquatic life cycle (larval and adult of aquatic phase), demonstrating that it is a great model organism for studies with NPs and other chemical substances diluted in water (Mouchet et al., 2007; 2008). In addition, it was the first amphibian species with the genome sequenced (Hellsten et al., 2010). The decoding of the genome of *X. tropicalis* may have opened up the possibility of further molecular and genomic studies in amphibians, as well as studying the mutagenic effects of chemicals that may cause environmental contamination (Hellsten et al., 2010). This publication may also have influenced the increase in the number of studies published since 2010 (97.5 %).

Among the two species of Caudata used in NMs studies, *Pleurodeles waltl* was the most studied (4.17 %), probably due to their well-established ecotoxicological relevance in a large variety of biomarker studies (Bour et al., 2015). The species *Ambystoma mexicanum* represented only 2.08 % of studies (Fig. 7). Amphibians have shown different levels of sensitivity to chemical substances (Blaustein et al., 2003), with the larval stage being the most vulnerable to NMs (Carew et al., 2015). Although research on NMs is recent, there is a need for further studies on the effects of these materials on amphibians, mainly considering that many species have suffered large population declines or even experienced local extinctions, the group being considered the vertebrate animals most threatened by human activities around the world (Stuart et al., 2004, 2002).

4.4. Bioaccumulation and effects

NMs in the aquatic environment can affect multiple trophic levels simultaneously (Bour et al., 2015), which may interfere with bioaccumulation, trophic transfer and/or biomagnification. Amphibians are vulnerable to toxic contaminants, being subject to accumulation of adverse substances present in their natural habitats, either in the water in its larval stage or even in the soil in its adult phase (Wilbur, 1980). As they have a biphasic life cycle with abrupt changes in morphology, physiology and behavior, and most often as herbivores when larvae and carnivores as adults, amphibians become even more susceptible to the process of bioaccumulation (Weltje et al., 2018). In this sense, the interaction, bioaccumulation and ecotoxicity of NMs on amphibians depend on their developmental stages.

Bacchetta et al. (2012) performed one of the first studies addressing the bioaccumulation of NMs in amphibians. In this study, *X. laevis* embryos were exposed to CNPs for 96 h (1–500 mg L^{-1}) and evaluated for lethality, malformations and growth inhibition. The results showed that the stomach and intestine were the main organs for CNP bioaccumulation (Bacchetta et al., 2012). Galdiero et al. (2017) demonstrated that the exposure of *X. laevis* tadpoles to different concentrations of quantum dots (QDs) could cause bioaccumulation and dysfunction of the gills, liver, and intestine, compromising the development of organisms. Thompson et al. (2017) exposed tadpoles of two species of anurans of the family Ranidae (*L. catesbeianus* and *L. sylvaticus*) to Au NPs, demonstrating that these NMs can accumulate mainly in the oral cavity and gills.

The NM bioaccumulation in larvae and adults of amphibians is shown in the Fig. 9. The larvae of most amphibians develop in aquatic environments, being able to absorb NMs through feeding via the digestive system or by skin and gills (Fig. 9A). Tadpoles are extremely sensitive to a stressor environment because their skin is responsible for regulating fluid balance and ion transport (McCoy and Peralta, 2018). Once in the organism of the amphibian larvaes, NMs are distributed in the bloodstream and can accumulate in the skin, which has a colonization of specific microbes, which, once altered due to the presence of stressors can influence the suppression of infectious diseases (McCoy and Peralta, 2018). NMs can also accumulate in the intestine, which represents the largest internal structure of tadpoles. Furthermore, NMs can induced changes in the intestinal flora of these organisms (Zhang et al., 2016).

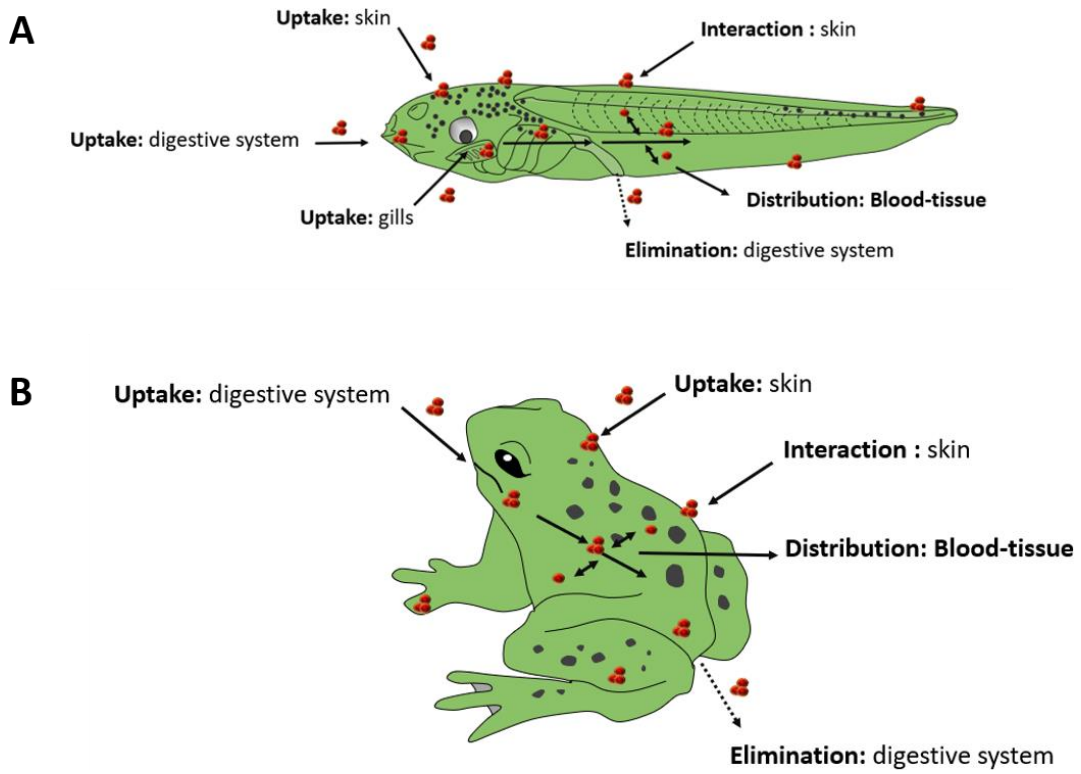


Figure 9: General scheme of toxicokinetics of nanomaterials (NMs) in tadpoles (A) and adults (B) of amphibians. The interaction, uptake, bioaccumulation, tissue distribution and elimination processes of NMs are summarised.

Bioaccumulation of NMs may also occur in the gills. According to Spence et al. (2016), environmentally relevant concentrations (0.1 – 100 mg L^{-1}) of ZnO NPs (> 40 nm) are capable of causing gross degradation in gills of the *Taricha granulosa* larvae – a salamander species of the family Salamandriae (Caudata). In addition, chronic exposure to different NMs may interfere with the metamorphosis process (Chai et al., 2017; Fong et al., 2016), which may cause lung dysfunction (Galdiero et al., 2017), increased mortality (Bacchetta et al., 2012; Bour et al., 2015; Spence et al., 2016) and affect the growth of larvae (Perelshtein et al., 2015). *X. laevis* tadpoles exposed to iron oxide magnetic nanoparticles (IOMNPs) at 0.25, 0.5, 0.75 and 1 mg L^{-1} for 3 days showed malformation in the embryonic column (Marín-Barba et al., 2018), which consequently threatens the growth of the species and interferes ecologically in different trophic niches.

The NM uptake by adult amphibians occurs mainly through the digestive system and skin and is subsequently distributed through the bloodstream (Fig. 9B). For example, adult amphibians exposed to ZnO NPs had elevated zinc levels on the liver in addition to

a considerable increase in DNA fragmentation (Falfushynska et al., 2017). NMs can also accumulate in the skin and alter the intestinal microbiota of adult amphibians (Zhang et al., 2016), making them more susceptible to infectious diseases (McCoy and Peralta, 2018). In addition, ZnO NPs (< 40 nm) induced lethal and sublethal effects at all stages of amphibian life when exposed for 1 to 14 days at 0.1, 1, 10 and 100 mg L^{-1} (Spence et al., 2016). Because they are poikilothermic animals, amphibians tend to accumulate toxic substances, reducing the elimination of bioaccumulative compounds when compared with other organisms (Ortiz-Santaliestra and Marco, 2015). When eliminated, bioaccumulative compounds are released through the skin, excretory system, and through gaseous exchanges in the tadpoles gills, while adults eliminate through the skin and scrotal system (Falfushynska et al., 2017; McCoy and Peralta, 2018).

4.5. Multibiomarker response

Currently, biomarkers are considered useful tools for monitoring the biological effects of pollutants and environmental stress (Huggett et al., 2018), since they are capable of providing important information in *in situ* or *in vitro* tests, and are used to measure wide range of physiological responses to toxic products at different biological levels (Beliaeff and Burgeot, 2002). During the embryonic development of amphibians, physiological and morphological changes are the most frequently related effects of exposure to different NMs (Table 2). However, these biomarkers have low sensitivity compared to biochemical and molecular changes, since the sensitivity of biochemical biomarkers to chronic chemical exposure depends on the MoA of a chemical (Jemec et al., 2008).

The developmental alterations on amphibians exposed to NMs is in Table 2. For example, Mouchet et al. (2008) and Krysanov et al. (2010) described the CNT accumulation in the gills and in the digestive tract of *X. laevis* tadpoles. The exposure to different NMs induced changes in the gill formation, reduced weight and size, altered time to metamorphosis, reduced head formation and induced edema in the anterior ventral region, as well as disturbances in pigmentation (Tussellino et al., 2015).

Ag NPs (10 nm) at low concentrations (0.018, 0.18 and 1.8 µg L^{-1}) increased THE action of mRNA, besides interrupting thyroid hormone-dependent responses (TH), which

may interfere in the endocrine regulation of tadpoles and alter the metamorphosis process (Carew et al., 2015). In addition, Ag NPs and QDs changed the gene expression in the presence or absence of 3,3', 5'-triiodothyronine (T3) (Hinther et al., 2010) and high concentrations of TiO_2 NPs (320 ppm) increased the acetylcholinesterase (AChE) and carboxylesterase (CaE) activities (Birhanli et al., 2014). In conclusion, the use of multiple biomarkers is necessary to link exposure to response and provide better predictive tools for protection of the amphibian species, especially for those endangered species (Barriga-Vallejo et al., 2016).

4.5.1. Genotoxicity

The mechanisms of genotoxicity (direct and indirectly) induced by NPs on amphibians is shown in Fig. 10. The direct mechanism demonstrates that NPs have a considerable capacity for penetration in cells and this is mainly due to their nanometric diameter (1 – 100 nm) and physicochemical properties, which can damage the biomolecules of the organisms, specially DNA, resulting in cellular stress (Homayouni-Tabrizi et al., 2016; Ibarra et al., 2015). Different NMs, such as TiO_2, Fe_2O_3 (Nations et al., 2011), QDs (Galdiero et al., 2017), C NPs (Krysanov et al., 2010) and ZnO NPs (Spence et al., 2016) can penetrate and accumulate in the intracellular environment. In relation to the indirect mechanism, the reactive oxygen species (ROS) generation and oxidative damage is thought to play an important role in many of the biological responses to NPs. Several factors as the size, surface area, and surface chemistry (reactive groups) of NPs are thought to play a role in the ROS production (Rim et al., 2013).

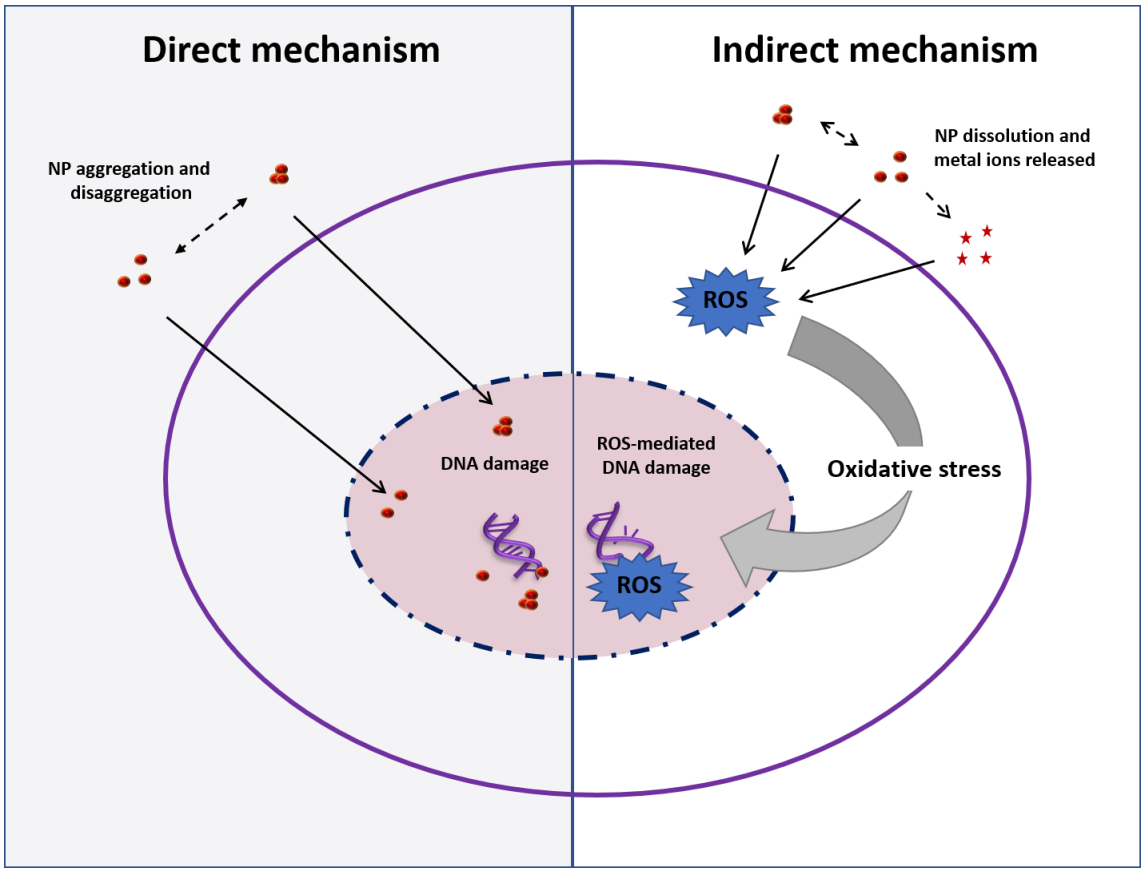


Figure 10: General scheme illustrating the direct and indirect mechanisms of genotoxicity induced by metal-based nanoparticles (NPs) in amphibian cells. ROS = reactive oxygen species.

As a result of alterations in cellular metabolism, NPs are capable of causing genotoxic damage or even apoptosis in cells (Galdiero et al., 2017), due to: (*i*) internalization mechanisms; (*ii*) distribution and accumulation in tissues and organelles; (*iii*) direct interaction with DNA or correlated proteins; (*iv*) the release of free radicals, considered as an indirect mechanism. Thus, all these factors are able to result in the ROS production and consequently cause oxidative damage (Carew et al., 2015; Falfushynska et al., 2017; Fong et al., 2016; Xia et al., 2012) (Fig. 10).

Several studies have shown effects of genotoxicity as a consequence of the contact of the organism with NMs, whether directly or indirectly due to oxidative stress, which also depends on the exposure time, species, developmental stage, NM concentrations, nano-specific properties and environmental factors (Bacchetta et al., 2012; Hammond et al., 2013). For example, one of the mechanisms of ROS formation, generated by metallic NMs, such as ZnO NPs and Ag NPs), occurs by oxidative

dissolution following by ions release and consequent impairment in structure and cellular content in *S. arabica* tadpoles (Al Mahrouqi et al., 2018).

The Ag NPs can induced oxidative stress, influencing transcription responses (Kahru and Dubourguier, 2010). Thus, besides genotoxicity being caused by ROS, is related to the direct interaction of NPs with DNA, with proteins and phospholipids (Carew et al., 2015). Even at low concentrations, Ag NPs (0.018, 0.18, 1.8 μg L^{-1}) can cause oxidative stress and affect tissue formation by disrupting thyroid hormone signalling of *R. catesbeiana* tadpoles exposed for 28 days (Carew et al., 2015).

The effects of NMs on the organism also depend on their interaction with other pollutants, as observed in adult amphibians of *P. ridibundus*, which suffered oxidative damage when exposed to ZnO NPs and Zn^{2+} for 14 days, in the absence of the drug nifedipine (Nfd). During the exposure, the individuals did not show relevant toxicity, due to the suppression of ROS production. However, the same NP combined with Nfd (ZnO NPs-Nfd) was able to interrupt the activation of the defense system by the detection of molecular biomarkers, also causing changes in thyroid hormones (TH), as well as high DNA damage and induction of apoptotic proteases (Falfushynska et al., 2017).

4.5.2. Mutagenicity

Emerging research was conducted to clarify the acute toxicity and mutagenicity of different NMs in amphibians, and the micronucleus (MN) test is an important technique to evaluate adverse genomic mutations in these organisms (He et al., 2014). The MN test is a sensitive tool used to confirm the mutagenicity of NMs on amphibians. In tadpoles, as in most eukaryotes, genomic mutations result in MN, which are disorders of the mitotic apparatus (Mouchet et al., 2008). This method, in amphibians, was first standardized on the species *X. laevis*, in France (AFNOR, 2000), followed by international recommendations (ISO, 2006), whose main function is to provide an early warning signal of significant biological effects, such as changes at the genetic and molecular level. Therefore, the use of the MN test can provide an important tool for predicting the potential long-term effects of amphibians in the environment (Mouchet et al., 2011). Double-walled carbon nanotubes (DWNTs) induced mutagenic effects *X. laevis* tadpoles after 12 days of exposure at 10 and 500 mg L^{-1}. Other species of amphibians have been widely used to perform the MN test, such as *A. mexicanum* and *P. waltl*, demonstrating sensitivity to the method (Mouchet et al., 2011).

The mutagenicity induced by NM exposure depends on amphibian species. Two hypotheses have been presented to explain the variability between species: (*i*) a difference in NP pharmacokinetics in the amphibian organism associated with the physiology of the species studied and (*ii*) differences in hematopoiesis among species, since MN induction depends on the sensitivity of the organs where hematopoiesis occurs (Bour et al., 2017, 2015). In general, toxicity and mutagenicity can occur in different ways, not only related to the species studied, but also to various parameters of NPs, such as size, shape, whether or not they aggregate with other substances and their binding power in the target organism (Bour et al., 2017).

4.5.3. Histopathological biomarkers

In the last decades, researchers from different regions have been concerned to assess the causal relationship between exposure to contaminants and biological effects observable in aquatic organisms, as a wide range of etiologies are increasingly being used as indicators of environmental stress (Stentiford et al., 2003). Histopathological biomarkers provide powerful tools to detect and characterize the biological endpoints of acute and chronic exposure to toxic and carcinogenic agents (Moore and Simpson, 1992). Several laboratory and mesocosm studies have demonstrated causal links between exposure to xenobiotics and the development of histological damages (Vethaak et al., 1996).

Previous studies have demonstrated the importance of histopathological biomarkers in the identification of abnormalities caused by exposure to NMs (e.g. Xia et al., 2012). Tadpoles of *Bufo gargarizans* exposed for 4 days to copper NPs (9 to 10 nm) presented melanocytes on the surface of the olfactory epithelium, eyes alterations (i.e. irregular spherical lenses and partially dissolved retinal cells), DNA damage and apoptosis (Xia et al., 2012). The olfactory systems are sensitive to heavy metals because the olfactory epithelium is exposed directly to the environment, and copper has the power to stimulate lysosomes in the olfactory cells and lead to the accumulation of pigments in olfactory cells (Arraes et al., 2009).

Several NPs, such as CuO NPs, TiO_2 NPs and ZnO NPs induced malformations in *X. laevis* tadpoles, causing histopathological lesions in the kidney and intestine, as well

as intestinal malfunction and abdominal edema (Bacchetta et al., 2012; Mouchet et al., 2008). Bacchetta et al. (2012) demonstrated that CNPs may accumulate in the intestine of *X. laevis* tadpoles, being the digestive system the most affected by these NPs. Histopathological analysis confirms the high distribution of NPs in exposed tadpoles, emphasizing the need for additional research to better clarify the short- and long-term effects of NMs in contact with different species of amphibians, both in the larval and adult stages.

4.5.4. Embryotoxicity

The Frog Embryo Teratogenesis Assay (FETAX) has become one of the most well-known and highly sensitive tools for assessing the effects of embryotoxicity on amphibians (Dumont et al., 1983), since the FETAX assay is designed to observe the organism from the organogenesis (Nations et al., 2011). Thus, FETAX has been applied in studies on NMs, mainly the metal-based NMs (Nations et al., 2015). The first study to evaluate the ecotoxicity of metal-based NMs in amphibians was carried out by Nations et al. (2011). The authors exposed *X. laevis* tadpoles for 4 days to TiO_2 NPs, Fe_2O_3 NPs, ZnO NPs and CuO NPs using the FETAX solution as exposure medium at different concentrations (0.001 to 1000 mg L^{-1}) (Nations et al., 2011). As a result, was observed inhibition of larval growth at all concentrations tested and a high mortality and increasing embryonic malformation frequency at the highest concentrations.

Several studies on NM toxicity have evaluated sublethal and lethal concentrations, gene expression and embryotoxicity. According to Krysanov et al. (2010), C60 and C70 fullerenes at 200 μg L^{-1} increased the malformation and mortality frequency in the embryos. In a cell viability study, Marcon et al. (2010) found that nanocomposite particles (ND) may have a negative impact on the gastrulation and neurulation stages, inducing phenotypic abnormalities and high mortality in *X. laevis* embryos. Recently, Marín-Barba et al. (2018) demonstrated that the exposure to IOMNPs (< 200 nm) for 3 days at different concentrations (0.25, 0.5, 0.75 and 1 mg L^{-1}) changed the development of *X. laevis* tadpoles.

Bacchetta et al. (2012) exposed embryos of *X. laevis* to CNPs at 1, 10, 100 and 500 mg L^{-1} and evaluated for lethality, malformations and growth inhibition. The results

showed that CNPs are capable of influencing the embryonic development of amphibians, which can cause malformation, growth disturbance and in cases of higher concentrations, mortality. Embryo-toxicological assays with CuO and ZnO NPs were also performed in *Xenopus* embryos, using concentrations of 0.1 to 100 mg L^{-1} for both NPs (Perelshtein et al., 2015). Although none of the NPs induced mortality, were observed significant embryotoxic effects (malformations and inhibition of growth) after exposure to ZnO NPs at 10 and 100 mg L^{-1}. Thus, ZnO NPs showed high embryotoxic effects to *Xenopus* tadpoles when compared to CuO NPs (Perelshtein et al., 2015).

Falfushynska et al. (2017) studied the effect of ZnO NPs and a Ca nifedipine channel blocker (Nfd) on adult male amphibians of the species *Pelophylax ridibundus*. Males were exposed for 14 days to 3.1 μm nZnO and 10 μM Nfd. Exposure to nZnO in the absence of the Nfd drug caused minimal physiological and cytological disturbances of the exposed amphibians, indicating that environmentally relevant concentrations of this isolated polymer are not a concern. However, exposures at the same concentrations containing ZnO and Nfd caused an increase in DNA fragmentation reflecting in liver cytotoxicity. Therefore, it is important to quantify the toxicity of NPs both isolated and associated with other substances at the embryonic stage, since in most cases the concentrations that can affect the embryos are very different from those affecting adults (Yslas et al., 2012). The ZnO NPs were the most used for embryotoxic studies, with growth retardation and morphological malformation being the highest damage observed in exposed larvae. Revised studies indicated the need for further studies testing the toxicity of NPs using different concentrations and exposure periods in different species and developmental stages of amphibians.

5. Conclusions and perspectives

Revised data showed that there is a large knowledge gap regarding the ecotoxicological effects of NMs on amphibians. Until 2010 there were only three studies evaluating the negative consequences of NPs on amphibians, showing that most of the research has been carried out in the last eight years. Considering that the increase in the number of publications on a given subject is one of the main measures to quantify the progress and evolution of science (Verbeek et al., 2002), the increase in the number of

studies reflects the greater interest of society and researchers in assessing the ecotoxicological effects of NPs on different organisms. With greater investment in research it is possible to seek alternatives and technologies to try to minimize the disposal of these materials directly in aquatic ecosystems, or even to evaluate those matters that have the least negative effects on fauna and flora.

Assessing the state of the art of knowledge about the potential ecotoxicological effects of NPs on amphibians it is possible to conclude that NMs can accumulate in different organs and cause various ecotoxic effects (see Table 2). We know that nanotechnology has sought to offer the different industrial and technological sectors the promise of improvements in development and economic growth, health, and environmental remediation (Moore, 2006). Therefore, the scientific community has developed protocols testing and forecasting tools to address environmental impacts risk issues, because from these mitigation measures will make it possible to base decisions that regulate the use and disposal of NMs in the environment. Special attention should be drawn to the potential risks that carbon NMs pose to aquatic organisms, since their origin is diverse and they can be found in different industrialized products (Freixa et al., 2018).

It is widely known that the use of NMs should be controlled due its potential toxicity (Galdiero et al., 2017). Since most amphibians have a two-phase life cycle, they become ideal organisms to evaluate and compare the toxicity of different MNs at different stages of life and also how they may affect the development of organs (e.g. lungs) and limbs during metamorphosis. The present study emphasizes the need for additional research aimed at clarifying the effects of amphibian exposure to NMs in both the short and long term, such as physiological, morphological, embryological and molecular changes, and the histopathological effects that NPs of different source can cause in non-target organisms. This review also highlights the importance of developing new protocols for action against the cytotoxic effects of NMs and that amphibians are suitable model system to evaluate the ecotoxicity of NMs.

Overall, the concepts of dose metrics, evaluation of different types of exposures, identification of the real risks caused by the disposal of NMs in ecosystems and the characterization of risks to exposed amphibians is still unknown. One of the limitations for a comparative assessment of the effects of NMs on species is the lack of studies with more species, since most of the studies were carried out with few species. More studies

could also help guide nanotoxicological researches to develop a complete picture of the risks offered by each type of NM. Furthermore, there is a need for studies evaluating the effect of NMs on native amphibians of different ecosystems, since there may be a great variation in species sensitivity according to the studied environment, as well as other non-studied amphibians, such as gymnophionas, which are fossorial animals – but there are many aquatic species – and mostly with restricted distribution, which also makes them one of the least known vertebrate animal groups (Jared et al., 2018). Aquatic organisms are more vulnerable to exposure to different NMs in ecosystems because they are in direct contact with these materials (Rocha et al., 2015). Considering that most species of amphibians have a larval aquatic phase and that they are the vertebrate organisms most affected by anthropic activities mainly due to their sensitivity to environmental contaminants (Amaral et al., 2018) and the loss and fragmentation of their natural habitats (Almeida-Gomes and Rocha, 2015; Becker et al., 2010; Stuart et al., 2004, 2002), further studies on the effects of NMs on these organisms are urgently needed.

Conflict of interest

No potential conflict of interest was reported by the authors.

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Table 1: Advantages and disadvantages of using amphibians as model organisms in nanoecotoxicological studies.

Advantages	**Reference**	**Disadvantages**	**Reference**
Suitable models for evaluating (anti) estrogenic and (anti) androgenic MOAs influencing reproductive biology, as well as (anti) thyroid MOAs that interfere with the thyroid system.	Kloas and Lutz (2006)	Biomonitoring using amphibians (non-model organisms) is difficult due to the deficiency of molecular biology data and analytical tools.	Ceschin (2017)
Good indicators of habitat diversity, biological variety and stressors in the environment.	Burlibaşa and Gavrilacaron (2011)	To date, genomic sequences of few species have been published.	Richardson et al. (2018)
Model systems for evaluation of effects of different xenobiotics on other organisms.	Denoël et al. (2010)	The lack of monitoring and control of water qualiTY variables (pH, hardness, temperature) can influence the physiology of amphibians, and therefore can mitigate or exacerbate the effects of NMs.	Sparling et al. (2002)
The B2R peptide, which is derived from frog skin secretions, has cytotoxic effects on cancer cells.	Homayouni-Tabrizi et al. (2016)	Exposures to a single NM do not reflect the natural systems, since in natural environments there are synergistic or antagonistic interactions between NMs and other pollutants.	Sparling et al. (2002)
Good indicators to evaluate sedimentary areas.	Sansiñena et al. (2018)	Acute exposures do not reflect the reality of chronic exposure to natural habitats.	Anderson et al. (2015)
Effective models for the identification of some diseases that affect animals and humans.	Whilde et al. (2017)	Abundance of artificial feeding can change variables such as growth or survival, since in the natural environment there may be limited resources.	Sparling et al. (2002)
They are distributed across continents, allowing the comparison of data from different areas	Ficetola et al. (2007)	Generally are used model species with large geographical distribution.	Petranka et al. (2004)

TABELA 2: ECOTOXICOLOGICAL EFFECTS OF NANOMATERIALS ON AMPHIBIANS ACCORDING TO SPECIES, TYPE OF NANOMATERIAL, AND EXPOSURE CONDITIONS.

Species	Nanomaterials				Exposure conditions		Cell/Tissue	Accumulation	Effects	Reference
	Type[a]	Capping layer[b]	Size (nm)	*Dh (nm)*	Concentration (mg L^{-1})	Time [d]				
Ambystoma mexicanum	C NP	10 -20	NI	0.7-2.13	1, 10, 100, 125, 250, 500, 1000	12	B	BC	The CNTs are neither toxic nor genotoxic for the larvae.	Mouchet et al. (2007)
Bufo gargarizans	Cu NP	NI	NI	NI	NI	1, 4, 10, 12	NI	E	↓Weight and length of the embryo, malformation of the fin, curved tail and edema in the yolk sac.	Xia et al. (2012)
Hoplobatrachus tigerinus	Ag NP	Ex-AV	30-70	NI	2, 4, 8, 16, 32 ppm	1-5	La-P	La-P	↑Predatory efficiency of tadpoles against larvae of *Aedes aegypti*.	Murugan et al. (2015)
Lithobates castebeianus	AQ NP	Ca	200-1000	NI	0.5	4	LC	L	↑Hepatic MMs; ↑black pigmentation; ↑lipidose.	Oliveira et al. (2016)

Species	Nanomaterials				Exposure conditions		Cell/Tissue	Accumulation	Effects	Reference
	Type[a]	Capping layer[b]	Size (nm)	*Dh (nm)*	Concentration (mg L^{-1})	Time [d]				
	Au NP	CB	5µM	22±5	2:00 PM	21	C-APR	APR	Concentration of gold nanoparticles in the anterior and posterior region.	Thompson et al. (2017)
	TiO_2 NP	AG-AD	20	NI	0.1, 1.0, 10	2	TF	TH	Affects tissue formation by disrupting thyroid hormone signaling.	Hammond et al. (2013)
		CB	18.1±3.5	60-110	0.05, 0.5, 5 pM	55	O	O	↓Metamorphosis time.	Fong et al. (2016)
Lithobates sylvaticus	Au NP	CB	22±5	NI	NI	21	C-APR	APR	Concentration of gold nanoparticles in the anterior and posterior region.	Thompson et al. (2017)
Pelophylax perezi	$TiSiO_4$ -NP	NI	<50	NI	8.2, 10.2, 12.8, 16, 20	4	CI	I	↑Toxicity due to the additional uptake of ions through the	Salvaterra et al. (2013)

Species	Nanomaterials				Exposure conditions		Cell/Tissue	Accumulation	Effects	Reference
	Type[a]	Capping layer[b]	Size (nm)	*Dh (nm)*	Concentration (mg L^{-1})	Time [d]				
									intestinal epithelium.	
Pelophylax ridibundus	ZnO NP	NI	35	NI	3.1 µM	14	LC	L	Physiological and cytological disorders.	Falfushynska et al. (2017)
		Tri-ammonium citrate	2-5	20, 60	0.1, 1, 10	4, 12	TD	TD	↓Growth; ↑genotoxic effects (DNA damage).	Bour et al. (2015)
Pleurodeles waltl	CeO_2 NP	Citrate	2-5	20, 60	1	12	BC	BC	Genotoxicity was observed with bare and coated NP; ↑MN: naked NP > coated NP.	Bour et al. (2017)
Rana catesbeiana	Ag NP	CF	1.5	2-6 e 10	0.06 µg/L, 5,5	In vitro	NI	E	Ag NP altered the expression of the transcripts bound to the T_3 pathways. ↓altered T_3 signaling.	Hinther et al. (2010)
		CF	1.5	2-10 e 9	0.19, 10	In vitro	NI	E	ZnO NP had no effect.	Hinther et al. (2010)

Species	Nanomaterials				Exposure conditions		Cell/Tissue	Accumulation	Effects	Reference
	Type[a]	Capping layer[b]	Size (nm)	*Dh (nm)*	Concentration (mg L^{-1})	Time[d]				
		CF	20	2-10 e 10-15	0.25 µg/L, 22	In vitro	NI	E	QD altered the expression of the transcripts bound to the T_3 pathways.	Hinther et al. (2010)
Rana ridibunda	C NP	B2R	5.8-1.27	NI	0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6 mg Ml^{-1}	NI	HFLF-pI5*, A549*	CC	CNP-B2R was more potent in the death of the tumor cell line compared to the normal cell line.	Homayouni-Tabrizi et al. (2016)
Rhinella arenarum	CN	NI	200	NI	NC: 21.14 NiNC: 32.12	4	NI	OD	↑Collapsed cavities, edema, axial flexures; ↑hyperkinesia; ↓movements.	Svartz et al. (2017)
	NiNC	NI	200	NI	NC: 21.14 NiNC: 32.12	4	NI	OD	↑Collapsed cavities, edema, axial flexures; ↑hyperkinesia; ↓movements.	Svartz et al. (2017)
	PANI	NI	100	NI	150, 250, 400	4	NI	E	Growth delay at 250 mg/L	Yslas et al. (2012)

Species	Nanomaterials				Exposure conditions		Cell/Tissue	Accumulation	Effects	Reference
	Type[a]	Capping layer[b]	Size (nm)	*Dh (nm)*	Concentration (mg L^{-1})	Time [d]				
	PANI NP	NI	NI	NI	182 a 1240	4	NI	E	exposure and reduction of body growth and tail malformation at 400 mg/L. It did not affect survival, but showed significant teratogenic potential.	Ibarra et al. (2015)
Sclerophrys arabica	Nano-TiO_2	95, 96, 277	NI	NI	1, 10, 100	7, 14	NI	E	↓Growth.	Al Mahrouqi et al. (2018)
	ZnO NP	23, 55, 122	NI	NI	1, 10, 100	7, 14	NI	E	↓Growth.	Al Mahrouqi et al. (2018)
Taricha granulosa	ZnO NP	NI	>40	NI	0.1, 1.0, 10, 100	1, 14	NI	G	↑Mortality; exhibited sublethal effects; ↑gill degradation.	Spence et al. (2016)
Xenopus laevis	Ag NP	NI	10	NI	0.018, 0.18, 1.8 µg/L	28	TH	TH	Alteration TH SIGNaling and mRNA	Carew et al. (2015)

Species	Nanomaterials				Exposure conditions		Cell/Tissue	Accumulation	Effects	Reference
	Type[a]	Capping layer[b]	Size (nm)	*Dh (nm)*	Concentration (mg L^{-1})	Time[d]				
	Cy N	QD	4.1-0.88	NI	NI	NI	E	E	interference during metamorphosis The binding of QD to CyN does not alter the intracellular structures before and during the midblastula transition.	Brandt et al. (2015)
	C NP	NI	50	NI	1, 10, 100, 500	4	S, I	E, I, O	↑Mortality; ↑malformation; ↓growth.	Bacchetta et al. (2012)
		CD	NI	NI	10	12	NI	E	↓Growth.	Lagier et al. (2017)
		NI	NI	<50	1, 10, 100, 500	4	S, I	S, I	↑Concentration was lethal. Concentrations of 100 and 500 caused malformation. Aggregation of C NP in all groups.	Bacchetta et al. (2012)

Species	Nanomaterials				Exposure conditions		Cell/Tissue	Accumulation	Effects	Reference
	Type[a]	Capping layer[b]	Size (nm)	*Dh (nm)*	Concentration (mg L^{-1})	Time [d]				
	C NT	NI	<50	NI	500	NI	CG, CI, Cy, NB	CG, I	Acute toxicity by accumulation of C NP in the gut and gills.	Krysanov et al. (2010)
		NI	0.7-2.2	NI	10, 100, 500	12	CI, CG	I, CG	Physical blockage of the gills and digestive tract.	Mouchet et al., (2008)
		DWNTs	0.7-2.2	NI	0.1, 1.0, 10	12	NI	E	↓Growth; accumulation of NP in the intestinal lumen.	Mouchet et al. (2011)
	CeO_2 NP	Tri-ammonium citrate	2-5	20, 60	0.1, 1.0, 10	4, 12	TD	TD	↑Mortality; ↓growth. No genotoxicity.	Bour et al. (2015)
		NI	23-37	NI	2.1, 10, >1000	4	CI	I	Gastrointestinal, spinal, and other abnormalities, were observed.	Nations et al. (2011)
	CuO NP	NI	57	NI	0.156, 0.313, 0.625, 1.25, 2.5, 0.01875,	14 e 47	NI	E	↑Mortality; ↓affected metamorphosis rate.	Nations et al. (2015)

Species	Nanomaterials				Exposure conditions		Cell/Tissue	Accumulation	Effects	Reference
	Type[a]	Capping layer[b]	Size (nm)	*Dh (nm)*	Concentration (mg L^{-1})	Time [d]				
					0.0375, 0.075, 0.150, 0.3					
		Sono-ZnO, Sono-CuO	<100	NI	0.1, 1, 10, 100	4	AEC	AE	↓Growth; Embryotoxic effects na ↑concentrations (100 mg L^{-1}).	Perelshtein et al. (2015)
	Fe_2O_3	NI	20-40	NI	1000, >1000	4	CI	I	Gastrointestinal, spinal, and other abnormalities, were observed.	Nations et al. (2011)
	IO NP	SC, MC	13, 142	NI	0.25, 0.5, 0.75, 1	3	CI, LC	I, L	Defects in embryo body shape like bent spine or enlarged ventral fin.	Marín-Barba et al. (2018)
	MLG	NI	NI	NI	0.1, 1, 10, 50	12	CI, CG	I, CG	Reduction in the size of tadpoles exposed to 10 and 50 mg/L. No reduced size was	Muzi et al. (2016)

Species	Nanomaterials				Exposure conditions		Cell/Tissue	Accumulation	Effects	Reference
	Type[a]	Capping layer[b]	Size (nm)	*Dh (nm)*	Concentration (mg L^{-1})	Time [d]				
									observed at 0.1 and 1 mg/L.	
	Nano-TiO_2	UV	315-400	5, 10, 32	1, 10, 100, 1000	15	NI	E	Significantly affected the growth of tadpoles, as determined by total body length, muzzle length and development length.	Zhang et al. (2012)
	PS NP	NI	50	NI	4.5, 9, 18	NI	CI	I	Disturbances in the distribution of pigmentation, malformations of the head, intestine and tail, edema in the anterior ventral region; ↓growth.	Tussellino et al. (2015)

Species	Nanomaterials				Exposure conditions		Cell/Tissue	Accumulation	Effects	Reference
	Type[a]	Capping layer[b]	Size (nm)	*Dh (nm)*	Concentration (mg L^{-1})	Time[d]				
	QD	gH625-QDs	120.9±2.37	171.4±1.27	0.1, 0.3, 0.6, 1.2, 2.5, 5, 10	NI	CG, CLg, CI	G, Lg, I	Dysfunction of the gills, lungs and intestines.	Galdiero et al. (2017)
	TiO_2	NI	32	NI	1000, >1000	4	CI	I	Gastrointestinal, spinal, and other abnormalities, were observed.	Nations et al. (2011)
	TiO_2 NP	NI	1-100	NI	0, 5, 10, 20, 40, 80, 160, 320, 640, 1280 ppm	4	NI	E	Alteration of the biomarker enzymes used. Concentrations 160 and 320 ppm; ↑in AChE activity and inhibition of CaE.	Birhanli et al. (2014)
	ND	NI	NI	NI	10, 25, 100, 200	1	AEC	AEC	Alteration in the stages of gastrulation and neurulation, inducing phenotypic abnormalities	Marcon et al. (2010)

Species	Nanomaterials				Exposure conditions		Cell/Tissue	Accumulation	Effects	Reference
	Type[a]	Capping layer[b]	Size (nm)	*Dh (nm)*	Concentration (mg L^{-1})	Time [d]				
									and high mortality.	
	C NP	NI	NI	NI	0.1, 1.0, 10, 50	12	NI	E	↓Growth.	Mouchet et al. (2010)
	PEI NP	DNA	55	NI	0.01, 0.1, 1, 10, 50	1, 2, 3, 4	IN	E	↓Growth, ↓mobility.	Robbens et al. (2010)

[a] C NP (Carbon nanoparticles), CeO_2 NP (Cerium dioxide nanoparticles), Ag NP (Nanosilver particles), Cu NP (Nanoparticle of copper), nZnO (Nano zinc oxide), Zn (Zinc), Au NP (Gold nanoparticles), QD (Quantum dot), CeO NP (Cerium oxide nanoparticle), PANI NP (Polyaniline nanoparticles), CuO NP (Copper oxide nanoparticles),

AQ NPs (Chitosan-alginate nanoparticles), ZnO NPs (Zinc oxide nanoparticles), CuO NPs (Copper oxide nanoparticles), NC (Alumina-based nanoceramics, γ-Al_2O_3), Ni NC (Alumina-based nanoceramics,osi/γ-Al_2O_3), PS NP (Polystyrene nanoparticle), CNT (Carbon nanotubes), ND (Nanodiamond), IO NPs (Iron oxide nanoparticles), PANI (Polyaniline nanofibers), nano-TiO_2 (Titanium dioxide nanoparticle), UV (Ultraviolet), $TiSiO_4$ NP (Titanium silicate nanoparticles), Au NP (Gold nanoparticle), ND (Nanodiamond particles),PEI (Nanoparticle polyethylene imine).

[b] QD (QUAntum points), gH625-QDs (MembranOTropic peptide gH625 derived from the glycoprotein H of herpes simplex vírus), B2R (Brevinin-2R), CD (Chemical dispersant), Ex-AV (Extract of leaves of *Artemisia vulgaris*), CL-AQNP (Clomazone associated with chitosan-alginate nanoparticles), Sono-ZnO (Oxide zinc sonochemically, CB (Cetyltrimethylammonium bromide), Sono-CuO (Copper oxide sonochemically prepared), CF (Carboxyl-functionalized) SC (Single-core nanoparticles), MC (Multicore nanoparticles), MLG (Multi-layer grapheme), Mn-TiO_2 (mn-doped TiO_2, gH625-QD (MembranOTropic peptide gH625 derived from the glycoprotein H of herpes simplex virus), DWNTs (double-walled carbon nanotubes).

[c] E (Stomach), I (Intestine), DT (Digestive tract), B (Blood), L (Liver), G (Gill), Lg (Pulmonary), I (Intestinal), HFLF-pl5 (Normal lineage), A549 (Cancer lineage), AEC (Amphibian embryo), Cy (Cytoplasm), NB (Nuclei of blastomeres), C-AR (Cells of the anterior region).

[d] S (Stomach), I (Intestine), O (Whole organism), DT (Digestive tract), BC (Blood cells), L (Liver), G (Gills), Lg (Lung), CC (Cancer cells), E (embryos), OD (Oral disc), AR (Region anterior).

[e] NP (Nanoparticle), MN (Micronucleus), TH (Thyroid hormone), CE (Chronic exposure), T_3 (3,3′,5′-triiodothyronine).

CAPÍTULO III

Titanium dioxide nanoparticles as a risk factor to the health of neotropical tadpoles: a case study on *Dendropsophus minutus* (Anura: Hylidae)

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CAPÍTULO III: Titanium dioxide nanoparticles as a risk factor to the health of neotropical tadpoles: a case study on *Dendropsophus minutus* (Anura: Hylidae)

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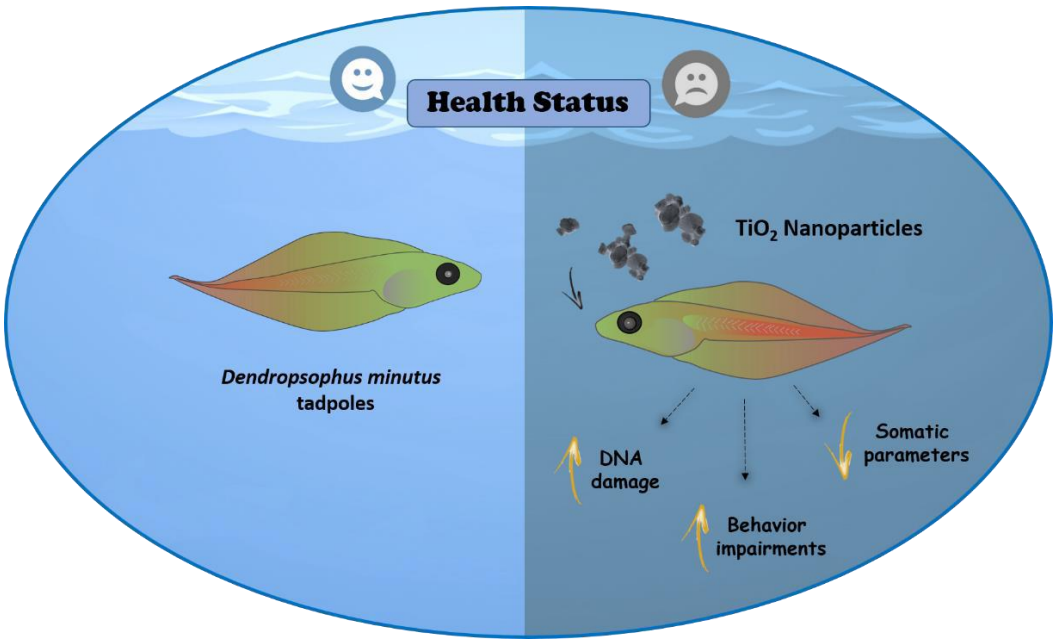
HIGHLIGHTS

- Ecotoxicological impact of TiO_2 NPs on anuran amphibians.
- TiO_2 NPs and TiO_2 induced DNA damage on *D. minutus* tadpoles.
- TiO_2 NPs and TiO_2 altered morphology of the tadpoles.
- *D. minutus* tadpoles exposed to TiO_2 NPs showed behavioral changes.

GRAPHICAL ABSTRACT



ABSTRACT

The production and use of titanium dioxide nanoparticles (TiO_2 NPs) worldwide are rising, while their release into the environment can induce toxic effects in aquatic invertebrates and vertebrates. However, knowledge of its impact on neotropical amphibians remains limited. Thus, the current study aimed to evaluate the toxicity of TiO2 NPs and their dissolved counterpart, titanium dioxide (TiO_2), in a Neotropical anuran species (*Dendropsophus minutus*). DNA damage, biometric parameters, and behavioral changes were analyzed in tadpoles after exposure to three environmentally relevant concentrations (0.1, 1.0, and 10 mg L^{-1}) of TiO_2 NPs and TiO_2 for 7 days. As results, we observed DNA damage in *D. minutus* tadpoles exposed to both Ti forms compared to their control groups. We also identified a similar reduction in total size, body length, width, and height of the tail muscle in tadpoles exposed to TiO_2 NPs and TiO_2 compared to unexposed tadpoles. Regarding the behavioral test, the tadpoles showed less mobility and were more distant from the conspecifics (less aggregated) when exposed to NPs. Therefore, the simultaneous use of multiple biomarkers was essential to assess nanomaterial's adverse effects and establish a reliable approach to biomonitoring aquatic ecosystems. Our study increases knowledge about the genotoxic, morphological, and behavioral effects of TiO_2 NPs and TiO_2 on anuran amphibians, and contribute to future studies on the environmental risk assessment of nanomaterials.

Keywords: amphibians; biomarkers; ecotoxicity; nanomaterial; nanotoxicity.

1. Introduction

Nanomaterials (NMs) can occur naturally or be artificially produced, favoring their use and development in nanotechnology (Wei and Wang, 2013). The disposal of NM residues in the environment can harm biodiversity (Do Amaral et al., 2019a; Bind and Kumar, 2019). Nanoparticles (NPs) and their products can reach the aquatic environment directly (i.e., sewage, effluents, and river inflow) or indirectly (i.e., aerial deposition, dumping, and runoff) (Rocha et al., 2015). Currently, the use of NPs in personal care products, and medical and pharmaceutical industries has increased. The most used NPs are titanium dioxide NPs (TiO_2 NPs), silicon oxide NPs (SiO NPs), aluminum oxide NPs (AlO NPs), zinc oxide NPs (ZnO NPs), carbon nanotube (CNTs), iron oxide NPs (IONPs), and silver NPs (Ag NPs) (Bind and Kumar, 2019).

One of the NPs tested by the Organization for Economic Cooperation and Development (OECD) is TiO_2 NPs, an additive frequently used in a wide variety of commercial products (Biola-Clier et al., 2020). The so-called final products are available in large quantities, and according to the TiO_2 NP usage database in the USA, between 3800 and 7800 tons/year are registered (Biola-Clier et al., 2020). TiO_2 NPs are mainly used as self-cleaning paints, self-sterilizing surfaces, cement, and asphalt for road construction, pavements, and tunnel structures (Clemente et al., 2012). They are also present in cosmetics, such as sunscreens and soaps, which improve products by increasing the penetration of substances into the skin (Jovanović, 2015). Due to its high use in the modern industry, TiO_2 NPs are in approximately 9% of products containing NPs (Boland et al., 2014).

TiO_2 NPs are photoactive and produce reactive oxygen species (ROS), making them harmful to many organisms, causing cellular and physiological changes, oxidative damage, and death (Fu et al., 2014; Biola-Clier et al., 2020). The genotoxicity of TiO_2

NPs is associated with the mitotic spindle's assembly, which compromises the progression of the cell cycle and decreases DNA repair, causing direct and indirect genotoxic effects (Biola-Clier et al., 2020). Recent studies stated that TiO_2 NPs, when in contact with aquatic organisms, are also capable of causing mechanical changes, such as lower filtering efficiency (in the case of filter organisms; Bundschuh et al., 2016), lower swimming speed, and increased mortality, due to an inhibition in changing seedlings (in the case of arthropods; Hartmann et al., 2019).

In general, the ecotoxicological potential of TiO_2 NPs has been found at different trophic levels, such as phytoplankton (Miller et al., 2012; Chiu et al., 2017), zooplankton (Lu et al., 2018), nematodes (Ratnasekhar et al., 2015), bivalve mollusks (Girardello et al., 2016), crustaceans (Mansfield et al., 2015), moths (Zorlu et al., 2018), fishes (Carmo et al., 2018), reptiles (Tsukano et al., 2017) and mammals (Antsiferova et al., 2018; Wang et al., 2019). Although some studies have evaluated the toxicity of TiO_2 NPs in amphibians (Hammond et al.,2013; Al Mahrouqi et al., 2018; Do Amaral et al., 2019a), none of them have been carried out with tree frogs (Hylidae) or with Neotropical amphibian species.

In amphibians, the bioaccumulation of TiO_2 NPs can cause changes in the formation of the thyroid glands during larval development, interrupting thyroid hormone, which is essential for metamorphosis (Hammond et al., 2013), growth delay (Zhang et al., 2012; Al Mahrouqi et al., 2018), gastrointestinal and vertebral abnormalities in tadpoles, and developmental delay (Nations et al., 2011). Due to their biphasic life cycle and high sensitivity, these organisms are excellent environmental biomonitors (Glinski et al., 2018; Do Amaral et al., 2018a, 2018b). Therefore, they are a suitable model for assessing traditional and emerging pollutants, such as NPs (Do Amaral et al., 2019b).

To improve the knowledge of the effects of NPs on amphibians with a larval stage, it is essential to evaluate the biometric, behavioral and genotoxic effects of species that are exposed to these pollutants in aquatic environments (Do Amaral et al., 2018a; Da Costa Araújo et al., 2020). Thus, the present study aimed to evaluate the impact of TiO_2 NPs and their dissolved counterpart (TiO_2) in tadpoles of the Neotropical amphibian *Dendropsophus minutus* (Peters, 1872). We tested whether, even when exposed to low concentrations for a short period of expusure, TiO_2 NPs could induce genotoxic, morphological, and behavioral changes in *D. minutus* tadpoles.

2. Material and methods

2.1 Preparation and characterization of nanoparticles

TiO_2 NPs (CAS number: 13463-67-7; purity: 99.5%) and TiO_2 (CAS number: 7440-32-6; purity: 99.5%) were obtained from Sigma-Aldrich (St. Loius, MO, USA). For the exposure, TiO_2 NPs were suspended in naturally dechlorinated water and kept stirring for 1 h. Afterward, we sonicated these suspensions for 30 min using a UCD200 Bioruptor (Diagenode Inc., Sparta, NJ, USA).

Transmission Electron Microscopy (TEM) was used to characterize the morphology and individual diameter (DTEM) of TiO_2 NPs. Briefly, powder TiO_2 NPs were deposited onto a mesh copper grid coated with a carbon layer. The electron micrographs were obtained using a JEOL (JEM-2100) microscope associated with the Scandium software (Olympus Soft Imaging Solutions GmbH). The DTEM of 250 particles was measured using the software ImageJ (National Institute of Health, USA).

The hydrodynamic diameter and zeta potential (surface charge) of TiO_2 NPs were analyzed in two aqueous medium (Milli-Q water and naturally dechlorinated water) by

Dynamic Light Scattering (DLS) and Electrophoretic Light Scattering (ELS), respectively, using the ZetaPALS particle sizing software (Malvern ZetaSizer, model Nano-ZS90) and the PALS Zeta Potential analyzer, respectively, as recommended by Qualhato et al. (2017). Both TiO_2 NP suspensions (166 mg L^{-1}) were previously sonicated for 30 min. Disposable polystyrene cuvettes of 12 mm (DTS0012, Malvern, Inc.) were used for DLS measurements, while the zeta potential was determined using a disposable polycarbonate capillary cell (DTS1061, Malvern Inc.). The polydispersity index (PdI) was taken in the same equipment and cuvettes for DLS analysis.

2.2 Sampling of tadpoles

Dendropsophus minutus (Peters, 1872) tadpoles were captured in their natural habitat using a wired dip net with a mesh size of 3 mm and a diameter of 300 mm, in a temporary pond located in the Private Reserve called Fazenda Santa Branca, in the municipality of Terezópolis de Goiás, Goiás State, Brazil (16º25'45.04 "S, 49º06'2.59" W). The fieldwork was carried out in January 2019. A total of 350 tadpoles (Gosner stages 24 to 25; Gosner, 1960) were collected. *D. minutus* is a common anuran species in the Cerrado Biome, and premetamorphic tadpoles (Gosner stage 25 to 33; Gosner, 1960) have been indicated as good bioindicators (Gonçalves et al., 2017; Carvalho et al., 2018). At the same time, collection site and using the same wired dip net, 400 tadpoles of *Scinax fuscovarius* (Lutz, 1925) at Gosner stage 24 to 25 (Gosner, 1960) were collected for the behavioral test. The *S. fuscovarius* tadpoles were selected because they were the most common and abundant interspecific species found on the same site.

After sampling, the *D. minutus* tadpoles were randomly selected and distributed in 5 tanks with a capacity of 10 L, containing 8 L of dechlorinated water, acclimatized

for 20 days with 12/12 h light/dark photoperiod, and continuous oxygenation with submerged pumps, following recommendations by Rodrigues et al. (2018). The tadpoles were fed once a day with commercial food (AlconBASIC®). We sampled all tadpoles under license from the Chico Mendes Institute for Biodiversity Conservation (ICMBio nº 54832) and kept under the approval of the Research Ethics Committee on Animal Use (CEUA-UFG), according to the National Council for the Control of Animal Experimentation (CONCEA) (008/2019).

2.3 Experimental design

After acclimatization, the *D. minutus* tadpoles were distributed in five tanks destined to the negative control group (containing only naturally dechlorinated water), fifteen tanks for the exposure to TiO_2 NPs, and fifteen tanks for TiO_2. Tadpoles were exposed to different concentrations of TiO_2 NPs or TiO_2 (0.1, 1.0, and 10 mg L^{-1}) for seven days. A total of 10 tadpoles were randomly distributed in each tank. The tested concentrations reflect a reasonable level of TiO_2 NPs in aquatic environments (0.01 to 20 mg L^{-1}) (Al Mahrouqi et al., 2018) and allow direct comparison with previously published studies on other types of NPs (e.g., Hinther et al., 2010).

We adopted a semi-static model in which 50% of the total volume of the exposure medium, food scraps, and residues were removed. The same volume of dechlorinated water containing the tested concentrations corresponding to each tank was replaced during each of the seven experimental days. Daily, the water physicochemical parameters, such as temperature, pH, Oxidation Reduction Potential (ORPmV), millisiemens per cm (mS/cm), Nephelometric Turbidity Unit (NTU), and percentage of dissolved oxygen (% OD), were monitored using a HORIBA Water Quality Monitor.

At the end of the exposure (7 days), four tadpoles from each tank (n = 4 per tank, where n = 20 per experimental group) were euthanized by hypothermia, and then we prepared the slides for the Comet Assay. We used some of the remaining tadpoles, n = 5 per experimental tank (n = 5 per tank, where n = 25 per experimental group) for the behavioral tests (aggregation and movement), which provides modifications in tadpoles to recognize potential individuals of the same species and locomotor changes. Then, the animals used in the behavioral test were euthanized using 5% lidocaine for 5 min, fixed by immersion in 4% paraformaldehyde buffered for 24 h at room temperature (Alphatec®, São Paulo, Brazil) and subsequently kept in 70% ethanol, at room temperature for analysis of body structures (measurement of biometric parameters).

2.4 Comet Assay (genotoxicity)

According to Singh et al. (1988), we carried out the genotoxicity test with some modifications proposed by Carvalho et al. (2018). We mixed 15 µl of blood with 0.5% LMP agarose and placed them a standard 1.5% agarose under a slide microscope. The Comet Assay involved at least 3 h of cell lysis using a salt solution (1% Triton X-100, 10% DMSO, lysis stock solution pH = 10; at 4 °C) (Gonçalves et al., 2019). The electrophoretic run was performed for 30 min (dark) at 25 V and 300 mA. The slides were neutralized with 0.4 M Tris buffer solution (pH 7.5) three times (for 5 min and kept in the dark). The slides were washed with distilled water and fixed with absolute ethanol; then, they were dried. Subsequently, the DNA was stained with 0.01 ml of SYBR® Gold (0.02 mg/ml) and TE solution (50 mM Tris-HCl, 500 mM EDTA, pH 7.5). The slides were analyzed with an Epifluorescent Imager D2® microscope (Carl Zeiss, Germany), associated with Comet Imager software version 2.2 (MetaSystems GmbH). We surveyed

100 nucleoids per tadpole (totaling 2,000 cells per experimental group). A single observer conducted the analysis. We selected three parameters to quantify DNA damage: tail length (TL), Olive tail moment (OTM), and percentage of DNA in the tail (% DNA), according to Carvalho et al. (2018).

2.5 Biometric parameters

Body parameters were measured following Costa and Nomura (2016) and Montalvão et al. (2018). All tadpoles separated for biometric measurements were in similar stages of larval development (Gosner stage 26 to 27; Gosner, 1960). For the morphometric analyses, traditional morphometry was used, which studies the variation and covariation of distance measurements between pairs of points and, sometimes, proportions (Klingenberg, 2016). Thus, we obtained seven measurements of each tadpole, five in right side view left and two in dorsal view, following the model of the research project "Anura tadpoles from the Atlantic Forest, Amazon, Pantanal, Cerrado and transition zones: morphological characterization, spatial distribution and patterns of diversity" (SISBIOTA-CNPq /FAPESP).

Through these measurements, five morphometric proportions were calculated for each individual, which also represents ecomorphological attributes related to habitat use: Body Length/Total Size (BL/TS), Body Width/Body Height (BW/BH), Tail Length/Total Size (TL/TS), Tail Musculature Width/Body Width (TMW/BW), Tail Musculature Height/Body Height (TMH/BH) (Fig. 1). It is noteworthy that individuals of a biological species grow isometrically when the proportions between their morphometric measurements remain constant, and these proportions are defined for every two measurements (Santos, 1994). According to that author, the linear relationship between

two measures is indicative of the proportionality between them during the organism's growth. The tadpoles were photographed (n = 4 per tank, where n = 20 per experimental group) in lateral (right side) and dorsal positions in the Leica EZ4 stereomicroscope with an integrated Camera. Morphological measurements were made in the Leica Application Suite software - LAS EZ, version 3.4.0, Copyright© 2016, by a single observer.

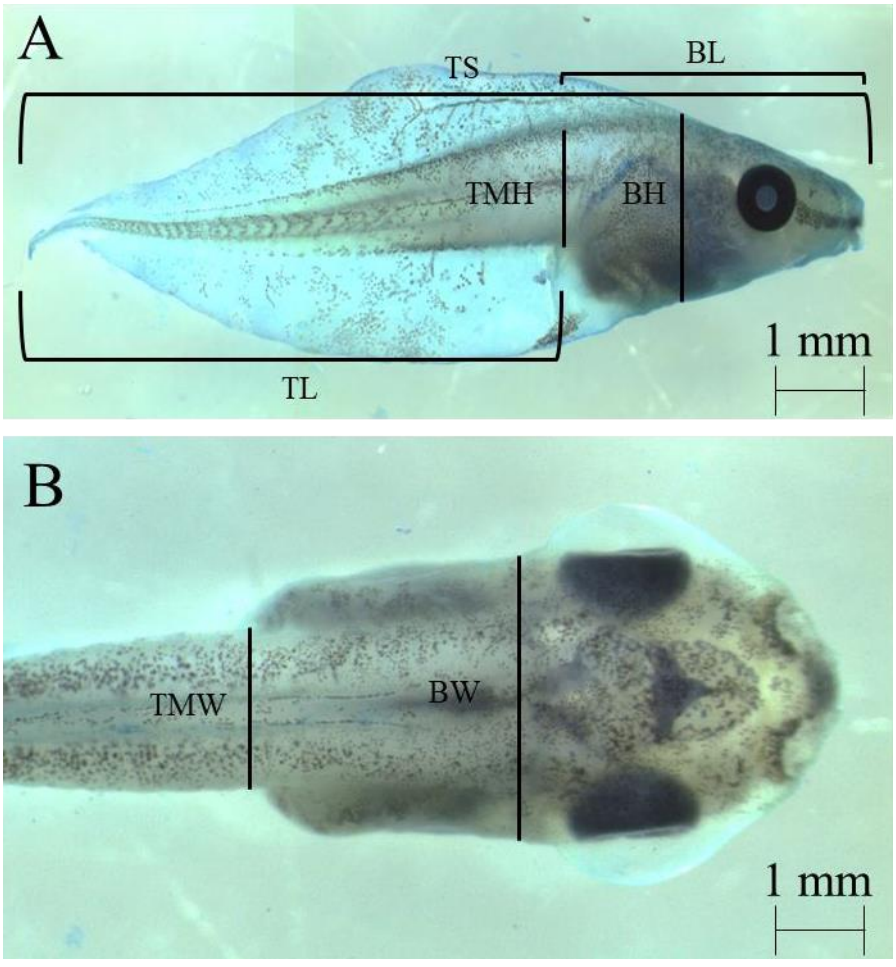


Figure11: Scheme of biometric parameters measured on the body of *D. minutus* tadpoles. A. Lateral visualization: body size (TS), tail musculature width (TMW), body height (BH), and tail length (TL). B. Dorsal visualization: height of the tail musculature (TMH), body width (BW), and body length (BL). Bar scale = 1 mm.

2.6 Behavioral testing

The aggregation test was carried out to verify whether the tadpoles´ exposure to different concentrations of TiO_2 NPs and TiO_2 would affect the aggregation capacity (recognize visual or chemical stimuli emitted by unrelated intraspecific and interspecific tadpoles) and mobility. Thus, we followed the procedures described by Cornell et al. (1989) with some modifications. The experiment was conducted in the same laboratory to avoid factors that could alter the results, such as the stress of transporting the tadpoles between different places. At the end of the experimental period (7 days), the behavioral test was performed using polypropylene trays (28 cm long × 17.5 cm wide × 11.5 cm high) containing 2 L of dechlorinated water (Fig. 2A). The trays had two ends with thin mesh separation walls, which isolated the central region (where we released the tested tadpoles). These walls prevented physical contact between conspecific and/or interspecific tadpoles. However, they allowed visual contact and the passage of chemical stimuli (Fig. 2A). As stated earlier, *S. fuscovarius* tadpoles were used as interspecific species. We have 10 trays in parallel, side by side, to carry out the experiments, which allowed conducting the behavioral test (and the filming) with 10 tadpoles of *D. minutus*, simultaneously. To shoot the test, we used a digital camera Sony Cyber-shot DSC-W530.

Initially (in the session without stimulation), the tadpoles were placed individually in the center of the tray for 5 min. After acclimatization, they were recorded for 10 min, without the conspecific or interspecific tadpoles (Fig. 2B). Then, in the second session (stimulus 1), 10 conspecific or interspecific tadpoles were simultaneously placed at one end of the tray, where they were recorded for another 10 min (Fig. 2C). In the third and last session (stimulus 2), more ten conspecific or interspecific tadpoles were simultaneously placed at the other end of the tray and filmed for 10 min (Fig. 2D). All conspecific and interspecific tadpoles were in similar development stages and were

placed randomly in the different phases of the experiment (stimulus 1 and stimulus 2) to avoid any bias that could interfere with the results.

The entire experimental period lasted 35 min for each tadpole tested (plus acclimatization). After completing the test, we changed the water in the trays and the conspecific and interspecific tadpoles. The videos were analyzed by a single observer using the software LeicaApplication Suite (LES). It was counted the time spent in the test tadpoles (seconds) on each side of the tray (closest to the conspecific or interspecific) and the frequency at which they crossed the centerline of the tray (virtually demarcated) during the entire period, as recommended by Do Amaral et al., 2018a.

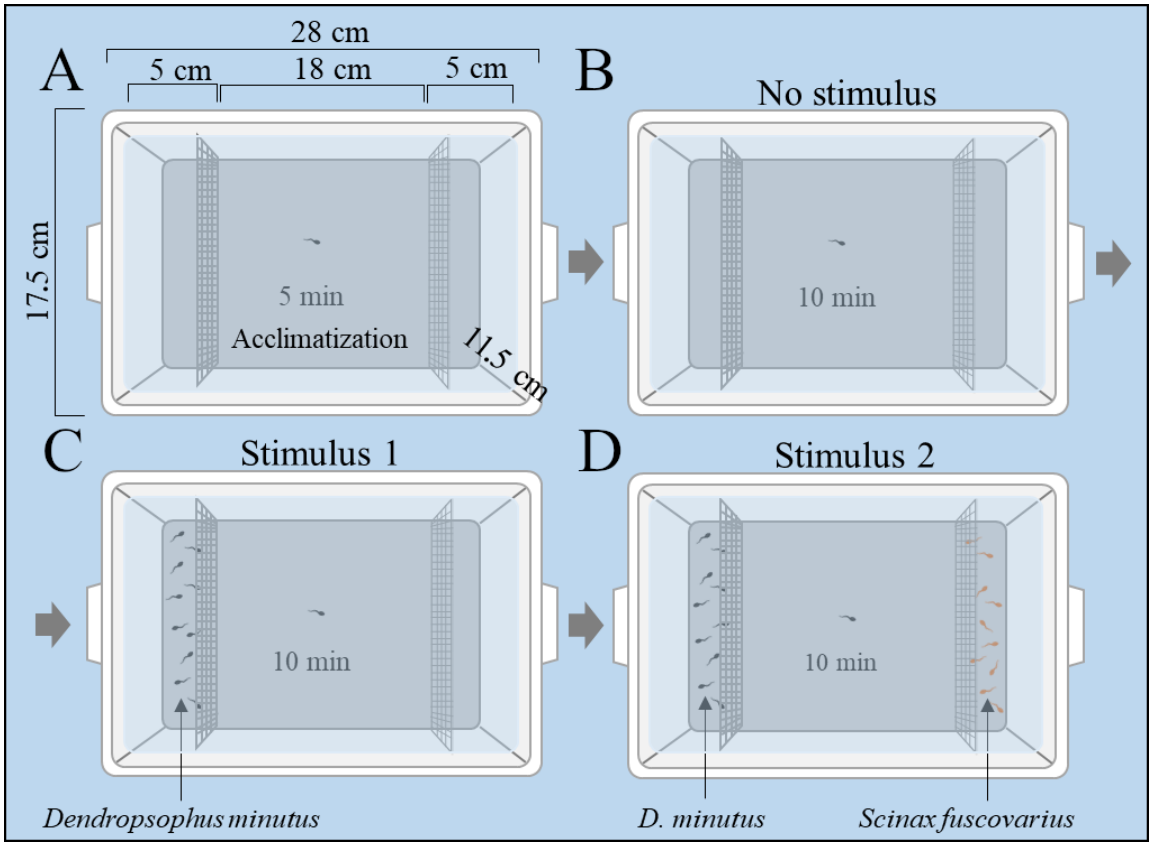


Figure 12: Schematic representation of the apparatus used in the behavioral test. (A) tadpole in acclimatization period; (B) session without stimulation; (C) stimulus session 1, with the presence of 10 conspecific or interspecific tadpoles; (D) stimulus session 2, with the presence of 10 conspecific and 10 interspecific tadpoles.

2.7. Statistical analyses

Physicochemical parameters – To verify if there is a difference between the water parameters in the experimental groups, we performed an analysis of variance (ANOVA), using each parameter as a response variable and the groups as a predictor variable. When the data did not reach the premises of normality and homogeneity, the Kruskal-Wallis non-parametric test was used. Normality was tested using the Shapiro-Wilk normality test, and homogeneity of variances was tested using the Levene statistic. ANOVA and Kruskal-Wallis were performed using the *aov* and *Kruskal.test* functions of the Rsoftware 3.5.3 (R Core Team, 2017).

Larval stages – We performed a generalized linear model (GLM) using the Poisson error distribution to test differences in the developmental stage between the experimental groups. For this model, we randomly selected twelve tadpoles of each experimental group, evaluated the developmental stage, and compared them according to Gosner (1960).

Genotoxicity – We used GLMs with Quasipoisson error distribution to assess whether exposure to TiO_2 NPs and TiO_2 caused DNA damages to *D. minutus* erythrocytes. TL, OTM, and % DNA were the response variables, and the groups were the fixed variables.

Morphology – To assess whether TiO_2 NPs and TiO_2 influenced tadpole morphology, we used GLMs with Gaussian error distribution to test each biometric proportion as a response and the groups as fixed variables. Biometric proportions represents ecomorphological attributes related to habitat use, namely: BL/TS, BW/BH, TL/TS, TMW/BW, and TMH/BH. Since there was no difference in the development stage between groups ($p > 0.05$) and all groups contained the three selected stages, we did not include the development stage in the models.

Aggregation behavior – To evaluate the effect of intraspecific aggregation behavior for groups with TiO_2 NPs and TiO_2, we performed a Generalized Linear Mixed Model (GLMM) with Gaussian error distribution (McCulloch and Neuhaus, 2005). The response variable was the time in seconds that the tadpoles remained on the side where the conspecifics were placed (variable indicating aggregation). We considered two fixed factors: (*i*) groups (considering the concentrations of TiO_2 NPs and TiO_2) and (*ii*) behavioral tests (without stimulation, presence of conspecifics, presence of interspecific, and presence of both); and two random factors (individuals and aquarium). We used the model without interaction between fixed variables, as it presented a better value of AIC (4851.8) than the model with interaction (4859.0) (Zuur et al., 2013). Besides, there was no significant difference in tadpole aggregation time within experimental groups.

Mobility – To assess the effect of TiO_2 NPs and TiO_2 on tadpole mobility, we performed a GLMM with Poisson error distribution (McCulloch and Neuhaus, 2005). The response variable was the number of times (frequency) that the tadpole crossed the centerline of the tray; the fixed and random factors were the same as those used in the aggregation model. As in the previous GLMM, we decided not to use the interaction.

Before all statistical analyses (GLM and GLMM), we conducted graphical data exploration to check for normality and homogeneity by visually inspecting probability plots (Q–Q plots) and the residuals plotted against fitted values (Zuur et al., 2010). We compared the suitability of the GLM models using the ANOVA test. We also carried out the general post hoc linear assumptions (Tukey comparisons of HSD) to determine which group was significantly different. GLMs were performed using the *glm* function in the vegan package (Oksanen et al., 2017), and for post hoc tests, we used the *glht* function of the multcomp package (Hothorn et al., 2008). GLMMs with Gaussian error distribution were performed using the *lmer* function, and the GLMMs with Gaussian error distribution

were performed using the *glmer* function of the lme4 package (Bates et al., 2015). All analyzes were performed using the R software 3.5.3 (R Core Team, 2017). The results were considered statistically significant with $p < 0.05$.

3. Results

3.1. Characterization of nanoparticles

The TEM results showed the spherical TiO_2 NP with an average diameter of 23.61 ± 2.4 nm (Fig. 3A-C). Both in the aqueous medium (Milli-Q and in naturally dechlorinated water), TiO_2 NPs formed aggregates up to 100 nm due to the absence of stabilizing agents (Fig. 3D). The DLS and ELS analyzes showed that TiO_2 NPs had lower Dh and high surface load in Milli-Q water (158.5 nm and 31.2 ± 6.23 mV) compared to dechlorinated water (439.2 nm and 3.57 ± 4.21 mV) (Table 1; Fig. 3). We observed that the Dh was higher in dechlorinated water (439.2 nm) than in Milli-Q water (158.5 nm); the same does not apply for the zeta potential because in the dispersed TiO_2 NPs in the MQ water was higher (31.2 $\pm$ 6.23) compared to the dechlorinated ones (3.57 $\pm$ 4.21). The average diameter was 23.61 $\pm$ 2.4 nm; that is, the lowest zeta potential corresponds with the highest Dh. PdI is characterized as polydispersed since its aggregates have different sizes (PdI was 0.667 and 0.387 in dechlorinated water and Milli-Q water, respectively).

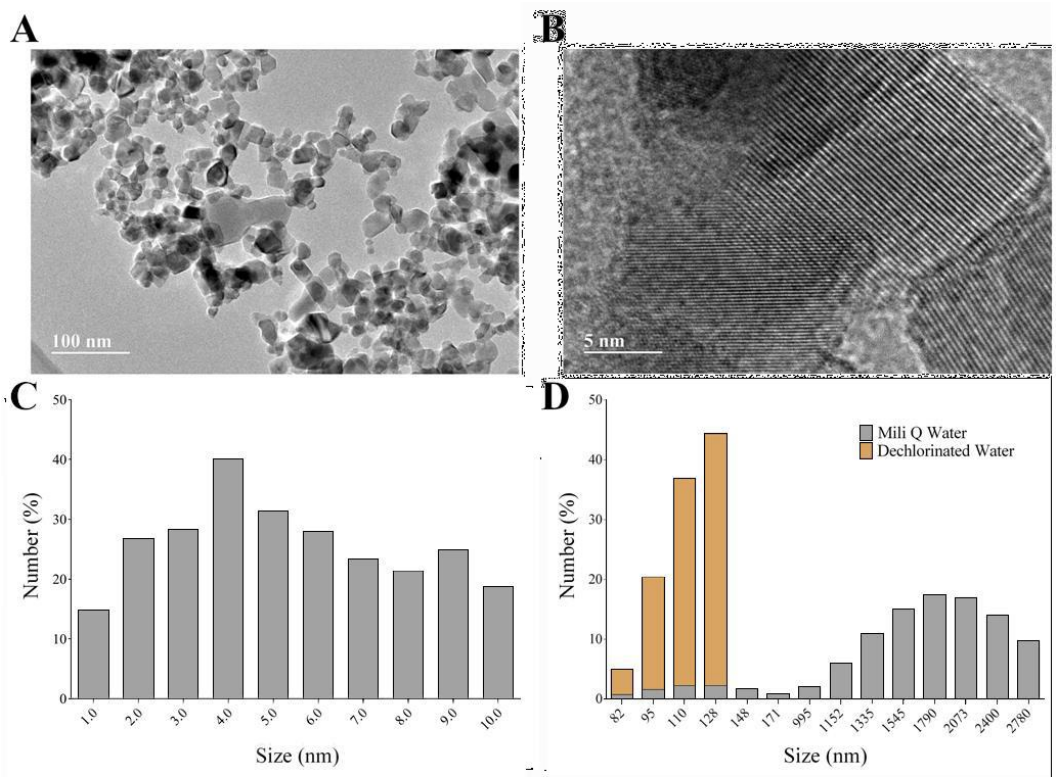


Figure 13: (A and B) Transmission Electron Microscopy (TEM) of titanium dioxide (TiO_2) nanoparticles. (C) individual diameter histogram of TiO_2 NPs isolated from TEM figures and (D) Distribution of the hydrodynamic diameter of the TiO_2 NPs titanium oxide nanoparticles in Milli-Q water (gray) and de-chlorinated water (brown) by dynamic light scattering (DLS).

3.2. Physicochemical parameters

Based on the monitoring of the water's physical-chemical parameters during the experiment, we observed that the oxidation-reduction potential (DF = 37985, Mean Sq = 6331, F = 30.92, $p < 0.001$), electric conductivity (DF = 0.00063, Mean Sq = 1.06e-4, F = 3.705, $p = 0.006$) and turbidity (Kruskal-Wallis chi-squared = 35.63, $p < 0.001$) differed between experimental groups, with higher values in the experimental groups compared to the control group (see Tables S1 and S2 in supplementary material).

3.3. Genotoxicity

Exposures to environmental relevant concentrations of TiO_2 NPs and TiO_2 did not cause mortality of tadpoles in any of the groups. However, genotoxic effects in *D. minutus* tadpoles were evident; both Ti forms induced DNA damage in the three parameters used to quantify damage in the erythrocytes compared with negative controls, namely TL (Estimate=2.73, Sd.Error=0.21, z.value=12.76, p<0.01), OTM (Estimate=0.97, Sd.Error=0.30, z.value=3.18, p=0.02), and %DNA (Estimate=3.00, Sd.Error=0.13, z.value= 2 3.65, $p < 0.01$) (supplementary material Table S3). However, there was no significant difference between TiO_2 NPs or TiO_2 concentrations for any of the three parameters ($p > 0.05$, Fig. 4).

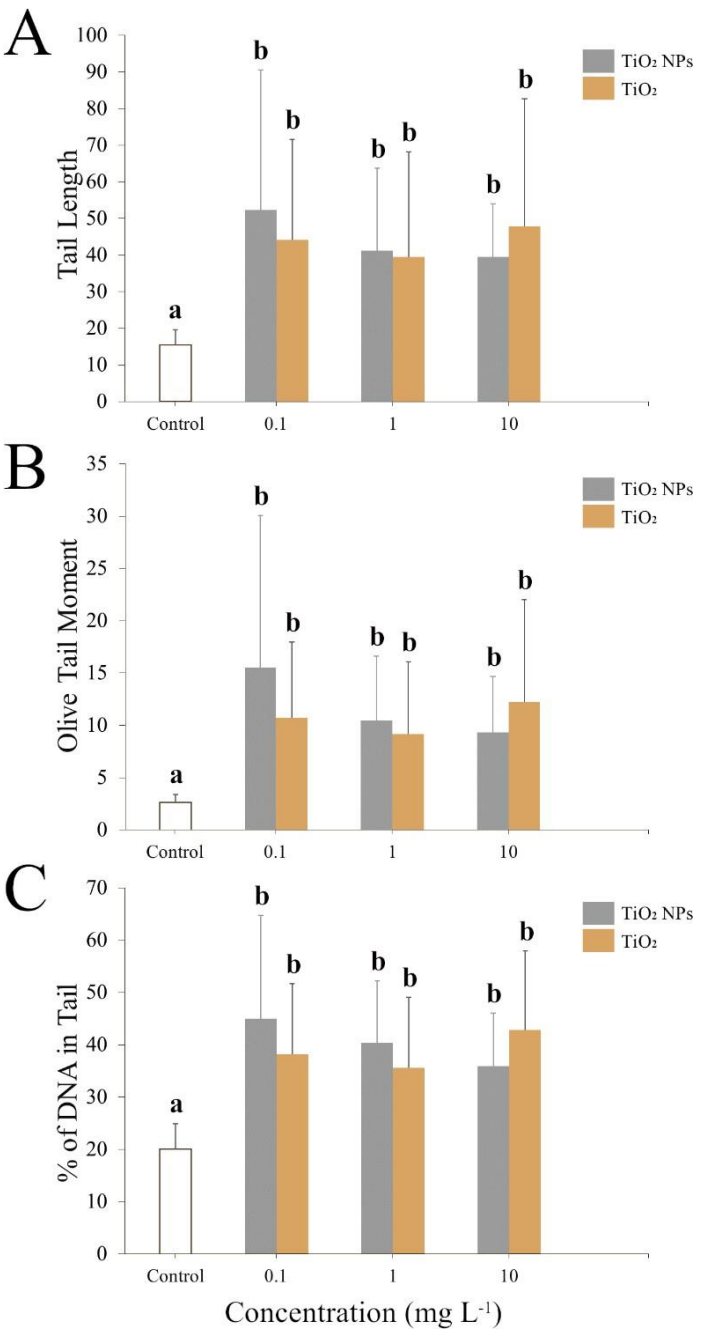


Figure 14: Genotoxic effects expressed as tail length (A), Olive Tail Moment – OTM (B) and % of the DNA in tail (C) of *D. minutus* tadpoles from control group and after exposure to TiO_2 NPs and TiO_2 for seven days. Different letters indicate statistical differences between treatments ($p < 0.05$).

3.4. Biometric parameters

At the end of exposure period, there was no difference in the development stage of tadpoles between treatments ($p > 0.05$). On the other hand, the exposure of *D. minutus*

tadpoles to TiO_2 NPs and TiO_2 for 7 days caused significant morphological changes compared to negative controls (supplementary material Table S4). Only the model for body width (BL/TS) was not significant (ANOVA $p > 0.05$), demonstrating that there was no difference between the control tadpoles and the experimental groups. The tadpoles exposed to TiO_2 NPs and TiO_2 at environmentally relevant concentrations (0.1 to 10 mg L^{-1}) showed significant reductions in total size (TS; Estimate = 21.76, Sd.Error = 0.50, t = 43.33, $p < 0.01$; Fig. 5A), body length (BL/TS; Estimate = 0.358, Sd.Error = 0.005, t = 61.727, $p < 0.01$; Fig. 5B), tail musculature width (TMW/BW; Estimate = 0.540, Sd.Error = 0.014, t = 38.883, $p < 0.01$; Fig. 5E), and tail musculature height (TMH/BH; Estimate = 0.759, Sd.Error = 0.022, t = 35.038, $p < 0.01$; Fig. 5F) when compared to unexposed tadpoles. On the other hand, the tail length of tadpoles exposed to both forms of Ti (TL/TS; Estimate = 0.642, Sd.Error = 0.006, t = 108.548, $p < 0.01$; Fig. 5D) were greater than the control group. We did not find differences in biometric parameters between tadpoles exposed to concentrations of TiO_2 NPs or TiO_2 (Fig. 5).

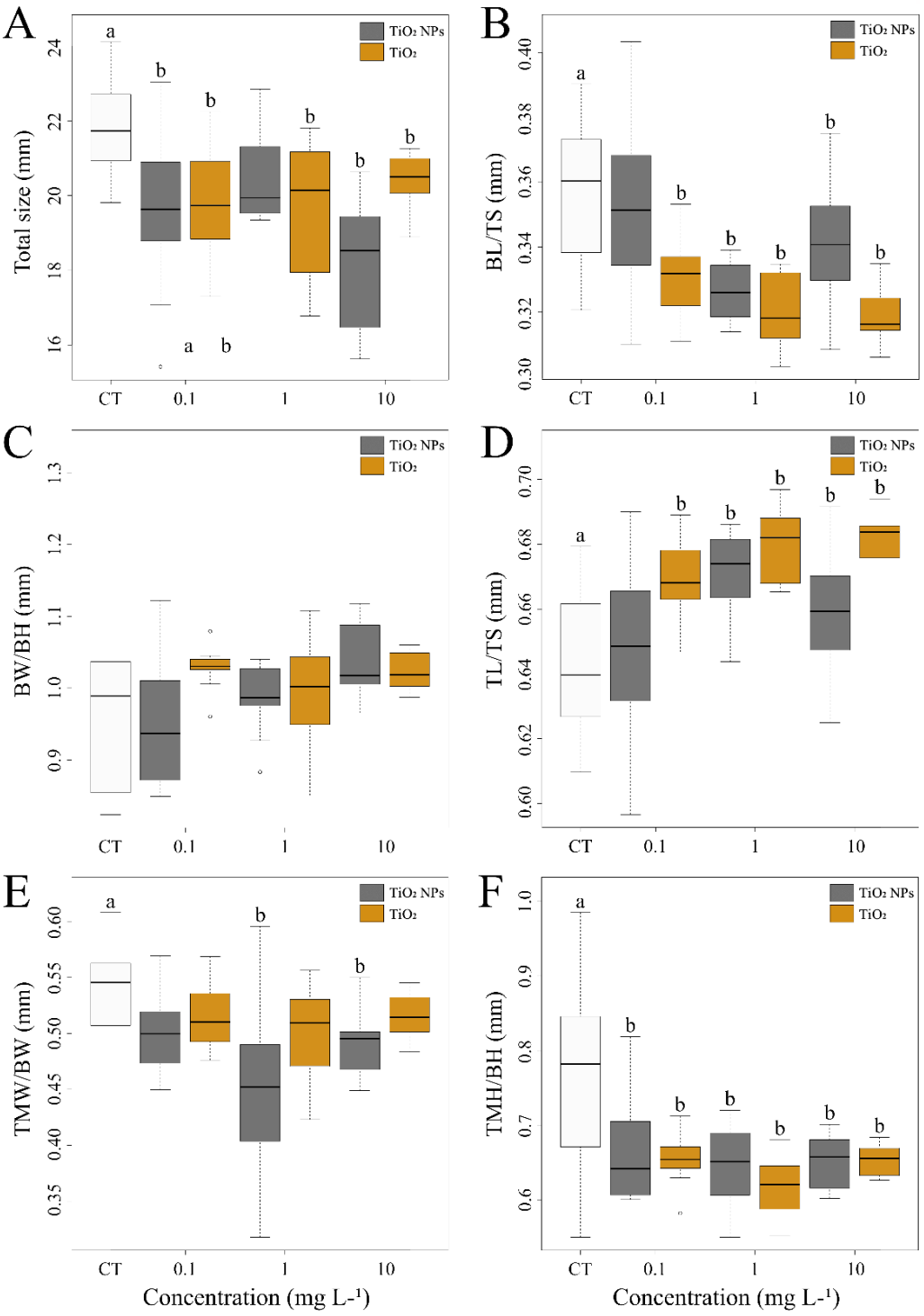


Figure 15: Biometric parameters of *D. minutus* tadpoles from the control group and after exposure to TiO_2 NPs and TiO_2 for seven days. (A) Total size; (B) Body Length/Total Size; (C) Body Width/Body Height; (D) Tail Length/Total Size, (E) Tail Musculature Width/Body Width; and (F) Tail Musculature Height/Body Height. Different letters indicate statistical differences between treatments ($p < 0.05$).

3.5. Behavioral testing

We found a change in aggregation behavior under both TiO_2 NPs and TiO_2 exposure, with tadpoles tending to be more distant from the intraspecific group in all experimental groups (Estimate = 169.53, Sd.Error = 15.09, df = 224.94, t = 11.23, p < 2e-16) compared to the control group (Fig. 6A, supplementary material Table S5). In addition, there was a significant decrease in tadpole mobility in TiO_2 NPs at 0.1 mg L^{-1} (Estimate = -0.83, Sd.Error = 0.31, z.value = -2.62, p < 0.01) and TiO_2 at 0.1 mg L^{-1} (Estimate = -0.75, Sd.Error = 0.31, z.value = -2.38, p = 0.01) groups compared to control (Fig. 6B, supplementary material Table S6). No significant difference was found between the TiO_2 NPs and TiO_2 experimental groups.

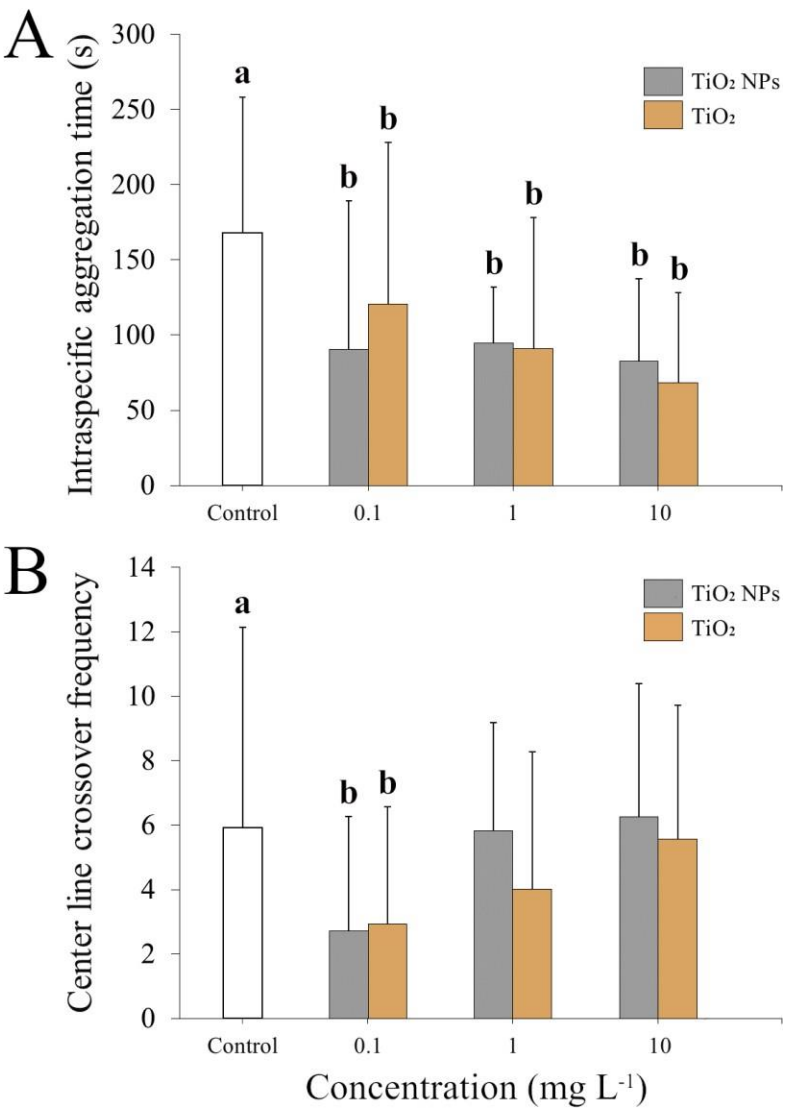


Figure16: Behavioral parameters (aggregation and mobility) of *D. minutus* tadpoles from control group and after exposure to TiO_2 NPs and TiO_2 for seven days. (A) Aggregation test with the tadpole residence time in the area close to the conspecifics. (B) Movement test with the number of times the tadpole crossed the center line. Different letters indicate statistical differences between treatments ($p < 0.05$).

4. Discussion

4.1. Characterization of nanoparticles

DLS and ELS analysis showed that TiO_2 NPs presented lower Dh and high surface charges in Milli-Q water, indicating that tadpoles were exposed to TiO_2 NP aggregates with 439.2 nm and positive surface charge (3.57 ± 4.21 mV). In the exposure medium (dechlorinated water), TiO_2 NPs formed aggregates due to the absence of stabilizing agents. This agglomeration and consequent sedimentation of TiO_2 NPs in the exposure medium was also reported by Larios et al. (2018). Furthermore, TiO_2 NPs can undergo physical and chemical transformations in the aquatic environment, such as oxidative dissolution following ion release and photo-oxidation (Praetorius et al., 2012).

4.2. Physicochemical parameters

There were high ORP, EC, and TU values in the experimental groups compared to the control group. However, the physical-chemical parameters were within the guidelines of water quality established by the American Society for Testing and Materials (Tietge et al., 2000). It is known that TiO_2 NPs can undergo physical-chemical changes in the aquatic environments, such as oxidative dissolution followed by ion release and photo-oxidation (Praetorius et al., 2012), as observed in the ORP parameters of our study. Conductivity is a measure of water's ability to pass an electrical current, which is affected by the presence of inorganic dissolved solids. The increase in electrical conductivity is commonly related to TiO_2 NPs (see review by Minea, 2019). Besides, the high quantities of suspended TiO_2 NPs increased the water turbidity. Previous studies emphasize that NPs are a potential risk to aquatic environments and that we also need to understand better

their effects on the physicochemical parameters of water (Klaine et al., 2008; Minea, 2019).

4.3. Genotoxicity

TiO_2 NPs at environmentally relevant concentrations (0.1 to 10 mg L^{-1}) did not induce mortality in *D. minutus* tadpoles after acute exposure (7 days), such as reported in previous studies with other anuran larvae exposed to TiO_2 NPs (Nations et al., 2011; Zhang et al. 2012). For example, no mortality was observed in *Xenopus laevis* tadpoles exposed to TiO_2 NPs for 4 days (32 nm; 1000 mg L^{-1}; Nations et al., 2011) or 15 days (5, 10 and 32 nm; 1 – 100 mg L^{-1}; Zhang et al., 2012). Similarly, no mortality was reported in *Sclerophrys arabica* tadpoles exposed to TiO_2 NPs for 7 and 14 days (95, 96, and 277 nm; 1, 10, and 100 mg L^{-1}) (Al Mahrouqi et al., 2018). Those results indicated that the toxicity assessment of TiO_2 NPs at environmentally relevant concentrations in Neotropical tadpoles must consider sub-lethal parameters, as observed in the current study.

We observed that TiO_2 NPs and TiO_2 induced DNA damage in *D. minutus* erythrocytes, causing significant changes in the TL, OTM, and % DNA of all exposed groups compared to the negative control. Thus, our study is the first to demonstrate that even the lowest concentrations of TiO_2 NPs and TiO_2 can induce genotoxic effects in amphibians.

Genotoxicity mediated by TiO_2 NPs and TiO_2 is associated with their oxidative potential and their ability to cause genetic alterations (Santonastaso et al., 2020). NPs can internalize in cells via endocytosis or phagocytosis and cause cellular changes (Dombu et al., 2010), including DNA single or double breaks (Rahman et al., 2017; Oliveira et al.,

2019). Genotoxic damage caused in *Lithobates catesbeianus* tadpoles after acute exposure (48 h) to solid lipid NPs loaded with pyride extract (PYR) can potentially lead to necrosis, which induces an inflammatory response potentially mediated by eosinophils recruited by pro-inflammatory cytokines (Breedveld et al., 2017; Oliveira et al., 2019). Therefore, genotoxic changes can affect individuals' fitness and survival, ultimately leading to an ecological imbalance (Wakeel et al., 2020).

We emphasize the need for further studies to clarify the genotoxic effects of amphibian exposure to NMs in the short and long term. One of the limitations for a comparative assessment of the effects of TiO_2 NPs on neotropical amphibians is the lack of studies with more species. Thus, we suggest implementing more experiments evaluating the toxic effects of environmental concentrations of TiO_2 NPs on neotropical amphibian species.

4.4. Biometric parameters

In general, the *D. minutus* tadpoles showed significant reductions in total size, body length, width, and height of the tail musculature. A considerable decrease was also found in the body size of *X. laevis* when exposed to high concentrations of TiO_2 (Nations et al., 2011) and TiO_2 NPs (concentrations between 0.31 and 1.000 mg L^{-1}, NPs with different sizes and presence of white light or UVA; Zhang et al., 2012). Similarly, morphological changes in tadpoles have already been observed for ZnO and CuO NPs. Exposure of *X. laevis* tadpoles to ZnO NPs demonstrated decreased body size (10 and 31.6 mg L^{-1}) compared to the control group, and the exposure to CuO NPs caused morphological changes that reduce growth (Nations et al., 2011).

It was previously demonstrated that *S. arabicas* tadpoles reduced the size due to exposure to ZnO NPs (1, 10, and 100 mg L^{-1}) for 7 and 14 days (Al Mahrouqi et al., 2018), which is similar to the results of our study, since the tadpoles of *D. minutus*, whenexposed to TiO_2 NPs, showed significant reductions in total size. Other studies have alsoshown that NPs can interfere with the biometric parameters of amphibians, such as tadpoles of *X. laevis* when exposed to PEI NP (Nanoparticles of polyethyleneimine) in concentrations between 0.01 – 50 mg L^{-1}, presented a reduction in growth, causing a significant decrease in mobility (Robbens et al., 2010). The same occurred with tadpolesof *P. waltl* exposed to CeO_2 NPs for 4 days (0.1, 1, and 10 mg L^{-1}; Bour et al., 2015). Therefore, like Cd, PEI NP, and CeO_2 NPs, TiO_2 NPs can also cause changes in the biometric parameters of tadpoles, even to environmentally relevant concentrations. Changes in body size can affect the ability to survive since larger organisms are more likely to reach adulthood, while smaller organisms are more likely to be preyed upon (Lefcort et al., 1999).

Contrary to our expectations, the tail size of tadpoles exposed to both forms of Ti were larger than those of the control group. This could be related to a delay in metamorphosis. Suppose that exposure to TiO_2 NPs inhibits the development, growth and metamorphosis of exposed tadpoles, as shown in our results. In that case, there is likely to be a decrease in the number of organisms that reach sexual maturity to breed and replenish the population within an ecosystem.

4.5. Behavioral testing

There were no differences in the development stages (sensu Gosner) of the *D. minutus* tadpoles between the exposed groups of TiO_2 NPs and TiO_2 and the control group in this study. A similar result was found for *X. laevis* tadpoles exposed to TiO_2 NPs (32

and 45 nm) at 1,000 mg L^{-1} for 4 days (Nations et al., 2011). However, another toxicity experiment with *X. laevis* tadpoles demonstrated that high concentrations of TiO_2 NPs (1,000 mg L^{-1}) delayed the development (Zhang et al., 2012). There is evidence that the exposure to high concentrations of TiO_2 NPs (160 and 320 ppm) increases the activity of the enzyme acetylcholinesterase (AChE) and inhibits the activity of the enzyme carboxylesterase (CaE) in amphibian embryos (Güngördü et al., 2010), mussels (e.g., *Limnoperma fortunei*; Bainy et al., 2006), and fishes (e.g., *Sparus aurata*; Romani et al., 2003). Changes in the enzymatic activity of CaE and AChE can interfere with organisms' development and are considered biomarkers to predict the toxic effects of substances on organisms (Aydin-Sinan et al., 2012).

Our results showed that *D. minutus* tadpoles exposed to TiO_2 NPs and TiO_2 tended to move away from their conspecifics. Specific recognition in anuran amphibian tadpoles is primarily mediated by chemical (released by their conspecific) and visual cues (Blaustein and Waldman, 1992). While visual sense would be used to identify conspecifics, chemical clues are used primarily for kinship recognition (Blaustein and O'Hara, 1987). Besides, the tadpole aggregation behavior can be stimulated by alarm signals (Bateman and Fleming, 2015; Do Amaral et al., 2018b). However, this did not happen in the present study because predatory clues were not used to assess the animal aggregation behavior.

Polettini Neto and Bertoluci (2021) demonstrated that tadpoles could recognize signs of the environment and use this ability for their survival; in addition, recognition mechanisms may be involved in aggregation through the attraction between individuals. According to Blaustein and O'Hara (1987), the aggregation behavior is standard in tadpoles. It may represent an important strategy to survive possible predators in natural environments. As the structure separated the tadpoles allowed visual, acoustic, or

chemical perception, we believe that TiO_2 NPs may have affected the cognitive system preventing the chemical or visual recognition of the tadpoles in their presence intraspecific and interspecific (separately or together).

In general, tadpoles remain aggregated in their natural environments to avoid predation (Blaustein and O'Hara 1987; Do Amaral et al., 2018b). Although *D. minutus* tadpoles do not exhibit the typical aggregation behavior in their natural environments (V.G. pers. obs), control tadpoles tended to remain close to their conspecifics. The experimental situation may have induced stress in the tested individuals, causing aggregation in the unexposed tadpoles. In contrast, the exposed tadpoles moved away from the conspecifics, which leads us to believe that titanium negatively affects the tadpoles. Previous studies have shown that tadpoles' recognition of chemical cues can be strongly influenced by environmental factors, including water quality (Gamboa et al., 1991; Polo-Cavia et al., 2016).

Although most of the water's physical-chemical parameters did not differ between the groups, TiO_2 NPs and TiO_2 may have influenced the chemical constituents of the tracks emitted by the conspecifics and reduced the perception of the tadpoles. Besides, it is tempting to speculate that TiO_2 NPs and TiO_2 may have affected the odorous mechanisms of the tadpole reception or even the neural networks responsible for transmitting the olfactory information received as for processing/displaying the animals' behavior. This hypothesis is corroborated by other studies, which showed that the first action mechanism of NPs necessarily involves the interference of the primary neurotransmitters in the display of behavioral categories by non-target organisms (Durairaj et al., 2018; Chauhan et al., 2020).

According to Hu & Gao (2010), NPs can bypass the blood-brain barrier and accumulate in the brain due to their unique properties. A recent study demonstrated that

TiO_2 NPs are photoactive and induce ROS production (Biola-Clier et al., 2020). Some reports have already observed the neurotoxicity of different NPs after *in vitro* and *in vivo* tests (Pisanic et al., 2007). Also, Veronesi et al. (2005) demonstrated that high levels of ROS were found in the brain of mice exposed to Tuxedo concentrated environmental particles (CAPs), indicating that NPs can reach the central nervous system (CNS). We can assume that possible cerebral neurotoxicity induced by TiO_2 NPs and TiO_2 may partially explain the significant effect of altering the behavior and mobility of *D. minutus* tadpoles. Observed results indicated that further studies concerning the biodistribution and the mechanism of neurotoxicity of TiO_2 NPs on amphibians are needed.

In our study, tadpoles of *D. minutus* decreased mobility when exposed to low concentrations (0.1 mg L^{-1}) of TiO_2 NPs and TiO_2. *Rhinella arenarum* tadpoles also showed decreased movement when exposed to NiNC (Alumina-based nanoceramics, Ni/γ-Al_2O_3) at 32.12 mg L^{-1} for 4 days (Svartz et al., 2017). In contrast, TiO_2 NPs, even in high concentrations (10 and 100 mg L^{-1}), were not able to affect the mobility of *Daphnia magna* (Zhu et al., 2010). When exposed to CeO_2 NPs, the mussel *Mytilus galloprovincialis* showed reduced mobility, but this reduction was not observed when the same species was exposed to ZnO NPs (Montes et al., 2012). Previous studies have also shown that TiO_2 NPs can exert minimal toxicity for *D. magna* over an exposure period of 48 h but can cause high toxicity when the exposure time is extended to 72 h (0.1 to 100mg L^{-1}; Zhu et al., 2010), which proves that the duration of exposure can be a contributing factor in toxicity mediated by NPs, as well as, these results indicate that NP can affect the organisms of different ways or that species have different levels of sensitivity to the constituents of NP.

The amphibians' high susceptibility to different NPs and their semi-aquatic lifestyle makes this taxon particularly vulnerable to environmental stress, partially

explaining the decrease in population sizes worldwide. Future studies exploring the real effects of NPs on amphibian sensory systems will elucidate the relative role of sublethal water pollution in the viability of the aquatic organism population. Based on our results, we believe that the next step is to test the toxicity, especially the bioaccumulation and neurotoxicity of different NPs in tadpoles after long-term exposure periods.

5. Conclusions

Our study demonstrated that *D. minutus* tadpoles are sensitive to the exposure of TiO_2 NPs and their dissolved counterparts. TiO_2 NPs and TiO_2 induce genotoxic effects (DNA damage) in the erythrocytes. Besides, we observed morphological changes, suggesting that TiO_2 NPs and TiO_2 in the environment may reduce some tadpoles' body parameters, such as total size and tail musculature. Also, we observed a change in aggregation behavior, with tadpoles tending to be more distant from the intraspecific group in all experimental groups. The genotoxic, morphological, and behavioral changes induced by TiO_2 NPs and TiO_2 in the *D. minutus* tadpoles suggest its ecotoxicological impact on tadpoles' natural environment since it affects the individual fitness.

Our study is the first one evaluating multiple responses in Neotropical amphibian species after acute exposure to TiO_2 NPs. Neotropical regions are those that contain the highest biodiversity of species on the planet and are those that suffer most due to natural habitats degradation by anthropic actions. In this sense, it is crucial to assess how substances produced by humans are deposited directly in aquatic environments and can affect biodiversity. As NPs are being produced on a full scale in all regions of the world, we emphasize the importance of assessing these nanomaterials' adverse effects on native organisms in unsafe environments, especially those more sensitive such as amphibians.

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7. Conflict of interest

The authors declare that they have no conflict of interest.

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CONCLUSÕES GERAIS

✓ Concluímos por meio dos dados revisados que há uma grande lacuna de conhecimento sobre os efeitos ecotoxicológicos dos NMs em anfíbios. Até o ano de 2010, poucos estudos abordavam as consequências negativas das NPs sobre esse táxon, sendo assim, observamos que que há necessidade de investir e incentivar o interesse da sociedade e de pesquisadores em considerar os efeitos ecotoxicológicos de NPs em diferentes organismos.

✓ Enfatizamos em nosso estudo a necessidade de pesquisas adicionais destinadas a esclarecer os efeitos da exposição de anfíbios a NMs em curto e longo prazo, como alterações fisiológicas, morfológicas, embriológicas e moleculares, e os efeitos histopatológicos que as NPs de diferentes origens podem causar em organismos não-alvo.

✓ Nosso estudo demonstrou que girinos de *D. minutus* são sensíveis à exposição de NPs de TiO_2 e sua contraparte dissolvida. Os NPs de TiO_2 e TiO_2 são capazes de induzir efeitos genotóxicos (danos ao DNA) nos eritrócitos dos organismos após exposição aguda (7 dias).

✓ Além disso, observamos alterações morfológicas, como redução significativa no tamanho total dos girinos expostos, no comprimento do corpo, na largura e altura da musculatura da cauda, sugerindo que NPs de TiO_2 e TiO_2 no ambiente podem interferir em alguns parâmetros corporais de girinos.

✓ Observamos também, uma mudança no comportamento de agregação, e que os girinos expostos tenderam a estar mais distantes do grupo intraespecífico em todos os grupos experimentais.

PERSPECTIVAS

Nosso estudo é pioneiro na análise da resposta de múltiplos biomarcadores em espécies de anfíbios neotropicais após exposição aguda a NPs de TiO_2. Destacamos que as regiões neotropicais são as que apresentam a maior biodiversidade de espécies do planeta e são as que mais sofrem com a degradação dos habitats naturais por ações antrópicas. Nesse sentido, é fundamental avaliar como as substâncias produzidas pelo homem – os NMs por exemplo - são depositadas diretamente nos ambientes aquáticos e podem afetar a biodiversidade. Em virtude da produção em grande escala dos NMs, enfatizamos a importância de avaliar os efeitos adversos dos NMs sobre os organismos nativos em ambientes inseguros, especialmente aqueles mais sensíveis como os anfíbios.

SUPPLEMENTARY MATERIAL

Table 3: Characterization of TiO_2 NPs in different aqueous media (Milli-Q water and dechlorinated water) using multiple techniques. Results are demonstrated as median ± standard deviation. Hydrodynamic Diameter* (Dh), Zeta Potential** (mV) e Polydispersity Index*** (PdI).

Water	Dh* (nm)	Zeta potential** (mV)	PdI***
Milli-Q	158.5	31.2 ± 6.23	0.387
Dechlorinated	439.2	3.57 ± 4.21	0.667

Table 4: Mean and standard deviation of the physicochemical parameters measured in the aquariums during the experiment. WT: Water temperature (°C), ORP: Oxidation-reduction potential, EC: Electric conductivity (μS/cm^2), TU: Turbidity (NTU), DO: Dissolved oxygen (mgL^{-1}), TDS: Total dissolved solids (mg/L).

Parameter	Experimental groups (Mean ± Standard Desviation)						
	Control	TiO_2 0.1	TiO_2 1	TiO_2 10	TiO_2 NP 0.1	TiO_2 NP 1	TiO_2 NP 10
WT (°C)	23.58 ± 0.32	23.32 ± 0.22	23.29 ± 0.16	23.21 ± 0.21	23.15 ± 0.14	23.30 ± 0.34	23.12 ± 0.29
pH	6.86 ± 0.13	6.80 ± 0.17	6.72 ± 0.22	6.72 ± 0.34	6.62 ± 0.39	6.56 ± 0.38	6.56 ± 0.40
ORP	189.17 ± 2.93	217.33 ± 8.04	241.17 ± 18.17	252.33 ± 9.75	265.67 ± 17.85	275.67 ± 16.63	276.83 ± 18.42
EC (μS/cm^2)	0.09 ± 0.00	0.09 ± 0.00	0.09 ± 0.00	0.09 ± 0.00	0.10 ± 0.01	0.09 ± 0.01	0.09 ± 0.01
TU (NTU)	1.25 ± 0.68	2.71 ± 0.72	14.80 ± 1.69	75.90 ± 8.47	2.29 ± 0.80	1.82 ± 0.30	13.45 ± 1.91
DO (mgL^{-1})	11.80 ± 2.90	11.66 ± 3.10	11.70 ± 3.01	12.01 ± 3.13	11.73 ± 3.01	10.99 ± 2.33	11.91 ± 2.84
% DO	139.00 ± 32.16	141.82 ± 38.38	138.38 ± 34.19	139.37 ± 34.40	140.52 ± 36.47	137.12 ± 31.78	138.72 ± 31.27
TDS (mg/L)	0.06 ± 0.00	0.06 ± 0.01	0.06 ± 0.00	0.06 ± 0.00	0.07 ± 0.01	0.06 ± 0.00	0.06 ± 0.00

Table 5: Comparison of physical-chemical parameters of water between experimental groups using parametric (ANOVA) and non-parametric (Kruskal-Wallis test) statistical analyzes. WT: Water temperature (°C), ORP: Oxidation-reduction potential, EC: Electric conductivity (μS/cm^2), TU: Turbidity (NTU), DO: Dissolved oxygen (mgL^{-1}), TDS: Total dissolved solids (mg/L). Significant values (0<0.05) are in bold.

Parameter	Df	Sum Sq	Mean Sq	F value	K chi-squared	Pr(>F)
WT	6	0.8273	0.1379	2.181	–	0.068
pH	6	0.4880	0.0814	0.852	–	0.539
ORP	6	37985	6331	30.920	–	**<0.001**
EC	6	0.0006	1.06e-04	3.705	–	**0.006**
TU	6	–	–	–	35.627	**<0.001**
DO (mgL^{-1})	6	–	–	–	3.147	0.791
% DO	6	–	–	–	1.842	0.934
TDS	6	–	–	–	11.654	0.071

Table 6: Summary of the three generalized linear models fit of comet assay in tadpoles of *Dendropsophus minutus*. The response variables were CC, MCO, %DNA, and the explanatory variables were treatments. Significant values (0<0.05) are in bold.

Variable	Category	Estimate	Standart error	z value	Pr(>|z|)
CC	Intercept	2.73	0.21	12.76	**< 0.001**
	TiO_2 NPs 0.1	1.22	0.29	4.17	**< 0.001**
	TiO_2 NPs 1	0.99	0.29	3.35	**< 0.001**
	TiO_2 NPs 10	0.94	0.29	3.20	**< 0.001**
	TiO_2 0.1	1.05	0.29	3.58	**< 0.001**
	TiO_2 1	0.94	0.29	3.20	**< 0.001**
	TiO_2 10	1.13	0.29	3.86	**< 0.001**
MCO	Intercept	0.97	0.30	3.18	**0.002**
	TiO_2 NPs 0.1	1.78	0.38	4.66	**< 0.001**
	TiO_2 NPs 1	1.38	0.39	3.58	**< 0.001**
	TiO_2 NPs 10	1.26	0.39	3.26	**< 0.001**
	TiO_2 0.1	1.40	0.39	3.64	**< 0.001**
	TiO_2 1	1.25	0.39	3.21	**< 0.001**
	TiO_2 10	1.54	0.38	4.00	**< 0.001**
PDCA	Intercept	3.00	0.13	23.65	**< 0.001**
	TiO_2 NPs 0.1	0.81	0.17	4.76	**< 0.001**
	TiO_2 NPs 1	0.70	0.17	4.10	**< 0.001**
	TiO_2 NPs 10	0.58	0.17	3.40	**< 0.001**
	TiO_2 0.1	0.64	0.17	3.76	**< 0.001**
	TiO_2 1	0.57	0.17	3.34	**< 0.001**
	TiO_2 10	0.76	0.17	4.46	**<0.001**

Table 7: Summary of the generalized linear models fit of morphological data of tadpole of *Dendropsophus minutus*. *Adequacy of the model according to the anova test (0<0.05). Significant values (0<0.05) are in bold.

Variable	Category	Estimate	Standard error	z value	Pr(>|z|)
BS*	Intercept	21.757	0.5022	43.326	**<0.001**
	TiO2 NPs 0.1	-2.082	0.7102	-2.932	**0.005**
	TiO_2 NPs 1	-1.273	0.7296	-1.744	0.086
	TiO_2 NPs 10	-3.599	0.7296	-4.933	**<0.001**
	TiO_2 0.1	-1.909	0.7102	-2.688	**0.009**
	TiO_2 1	-2.121	0.7533	-2.815	**0.007**
	TiO_2 10	-1.428	0.7102	-2.011	**0.049**
TL*	Intercept	7.806	0.2129	36.658	**<0.001**
	TiO_2 NPs 0.1	-0.848	0.3011	-2.816	**0.007**
	TiO_2 NPs 1	-1.005	0.3094	-3.248	**0.002**
	TiO_2 NPs 10	-1.658	0.3094	-5.36	**<0.001**
	TiO_2 0.1	-1.250	0.3011	-4.151	**0.001**
	TiO_2 1	-1.513	0.3194	-4.738	**<0.001**
	TiO_2 10	-1.325	0.3011	-4.4	**<0.001**
BW	Intercept	3.890	0.103	37.772	**<0.001**
	TiO_2 NPs 0.1	-0.309	0.1457	-2.122	**0.038**
	TiO_2 NPs 1	-0.108	0.1496	-0.72	0.474
	TiO_2 NPs 10	-0.372	0.1496	-2.487	**0.016**
	TiO_2 0.1	-0.180	0.1457	-1.236	0.221
	TiO_2 1	-0.257	0.1545	-1.667	0.101

Variable	Category	Estimate	Standard error	z value	Pr(>|z|)
	TiO_2 10	-0.074	0.1457	-0.508	0.613
	Intercept	2.099	0.0817	25.703	**<0.001**
	TiO_2 NPs 0.1	-0.291	0.1155	-2.52	**0.014**
	TiO_2 NPs 1	-0.396	0.1187	-3.335	**0.001**
TMW*	TiO_2 NPs 10	-0.366	0.1187	-3.082	**0.003**
	TiO_2 0.1	-0.188	0.1155	-1.628	0.109
	TiO_2 1	-0.274	0.1225	-2.237	**0.029**
	TiO_2 10	-0.125	0.1155	-1.082	0.283
	Intercept	2.993	0.1151	26.003	**<0.001**
	TiO_2 NPs 0.1	-0.481	0.1628	-2.955	**0.004**
	TiO_2 NPs 1	-0.514	0.1672	-3.074	**0.003**
TMH*	TiO_2 NPs 10	-0.651	0.1672	-3.891	**<0.001**
	TiO_2 0.1	-0.627	0.1628	-3.852	**<0.001**
	TiO_2 1	-0.727	0.1727	-4.209	**<0.001**
	TiO_2 10	-0.556	0.1628	-3.416	**0.001**

Table 8: Effects of TiO_2 NPs and TiO_2 on intraspecific aggregation behavior of *Dendropsophus minutus* tadpoles. Significant values (0<0.05) are in bold.

Fixed effects (Y)	Estimate	Std. Error	df	t value	Pr(>|t|)
(Intercept)	169.53	15.09	224.94	11.23	**<0.001**
TiO_2 NPs 0.1	-77.18	18.38	139.51	-4.20	**<0.001**
TiO_2 NPs 1	-72.87	18.38	139.51	-3.96	**<0.001**
TiO_2 NPs 10	-85.01	18.38	139.51	-4.63	**<0.001**
TiO_2 0.1	-46.53	18.38	139.51	-2.53	**0.012**
TiO_2 1	-76.40	18.38	139.51	-4.16	**<0.001**
TiO_2 10	-98.94	18.38	139.51	-5.38	**<0.001**
Intraspecific	-10.81	12.37	377.72	-0.87	0.383
Intra and interspecific together	-5.27	10.01	318.10	-0.53	0.599
Without tadpoles	5.09	10.01	318.10	0.51	0.611

Table 7: Effects of TiO_2 NPs and TiO_2 on mobility of *Dendropsophus minutus* tadpoles. Significant values (0<0.05) are in bold.

Fixed effects (Y)	Estimate	Std. Error	z value	Pr(>|z|)
(Intercept)	1.52	0.22	6.76	**<0.001**
TiO_2 NPs 0.1	-0.83	0.32	-2.62	**0.008**
TiO_2 NPs 1	0.21	0.31	0.70	0.487
TiO_2 NPs 10	0.22	0.31	0.71	0.479
TiO_2 0.1	-0.75	0.32	-2.38	**0.017**
TiO_2 1	-0.35	0.31	-1.14	0.256
TiO_2 10	0.12	0.31	0.38	0.706
Intraspecific	-0.14	0.09	-1.51	0.130
Intra and interspecific together	-0.02	0.07	-0.23	0.817
Without tadpoles	-0.10	0.07	-1.53	0.127

APROVAÇÃO DO COMITÊ CEUA – UFG, PROJETO 008/19

MINISTÉRIO DA EDUCAÇÃO
UNIVERSIDADE FEDERAL DE GOIÁS
PRÓ-REITORIA DE PESQUISA E INOVAÇÃO
COMISSÃO DE ÉTICA NO USO DE ANIMAIS/CEUA

Goiânia, 18 de março de 2019.

PARECER CONSUBSTANCIADO REFERENTE AO ATENDIMENTO DE PENDÊNCIA DO PROTOCOLO Nº. 008/19

I - Finalidade do projeto de pesquisa:

II - Identificação:

- **Data de apresentação a CEUA:** 06/02/19
- **Data do atendimento da pendência:** 14/02/19
- **Título do projeto:** "Presente fragmentado e futuro incerto: a conservação dos anuros no Cerrado".
- **Pesquisador Responsável/ Unidade:** Rogério Pereira Bastos
- **Pesquisadores Participantes/ Unidade:** Dra. Daniela de Melo e Silva (ICB/UFG); Dr. Paulo De Marco Júnior (ICB/UFG); Dr. Fausto Nomura (ICB/UFG); Dr. Natan Medeiros Maciel (ICB/UFG); Dr. Alessandro Ribeiro de Morais (ICB/UFG); Dra. Karina Simões (ICB/UFG); Dr. Wilian Vaz Silva (Pontifícia Universidade Católica de Goiás); Ms. Flávia Pereira Lima (doutoranda ICB/UFG).
- **Médico Veterinário/CRMV:** Luiz Carlos T. Borges/CRMV-3661
  Unidade onde será realizada a pesquisa: ICB/UFG

III – Respostas as pendências:

Solicitação da CEUA:

1- Inclusão de cronograma mensal do primeiro ano da pesquisa.
2- Descrição da forma da eutanásia no item 17.1 da ficha protocolo.
3- Especificar quantos animais serão necessários para cada procedimento experimental e qual a fonte de obtenção destes anfíbios para cada ensaio, citar a referência ou a estatística utilizada para o cálculo mínimo e máximo da amostragem.
4- Informar qual o tempo de exposição dos girinos aos agrotóxicos, quais agrotóxicos serão utilizados e quais medidas de biossegurança serão tomadas especificamente para este bioensaio;
5- Determinar onde será realizado o descarte dos girinos e da água contaminada com agrotóxicos.

Resposta do Pesquisador Responsável:

1- Todo o cronograma foi refeito. Veja item 3: Adequação do Cronograma, no presente termo de atendimento. Sendo alterado para início do experimento para novembro de 2019.
2- Os anuros adultos serão anestesiados com lidocaína e os girinos em solução de benzocaína 5%. Como estes anestésicos são rapidamente absorvidos, uma vez que a pele dos anuros é permeável, a inconsciência e morte ocorrem em sequência. Posteriormente, os adultos são colocados em solução de formalina 10% e os girinos em solução de formalina 5%;
3- Indivíduos adultos das espécies a serem estudadas serão capturados manualmente e os girinos, com utilização de puçás, em corpos d'água encontrados na natureza. O número de animas necessários