UNIVERSIDADE FEDERAL DE GOIÁS
PROGRAMA DE PÓS-GRADUAÇÃO EM BIOLOGIA DA RELAÇÃO PARASITO-HOSPEDEIRO

MAXWELL BATISTA CAIXETA

Toxicidade reprodutiva e resposta de múltiplos biomarcadores no caramujo *Biomphalaria glabrata* (Say 1818) após exposição crônica às nanopartículas de óxido de ferro (γ-Fe_2O_3) funcionalizadas com ácido glucônico

Goiânia
2020

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MAXWELL BATISTA CAIXETA

Toxicidade reprodutiva e resposta de múltiplos biomarcadores no caramujo *Biomphalaria glabrata* (Say 1818) após exposição crônica às nanopartículas de óxido de ferro (γ-Fe_2O_3) funcionalizadas com ácido glucônico

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UNIVERSIDADE FEDERAL DE GOIÁS

INSTITUTO DE PATOLOGIA TROPICAL E SAÚDE PÚBLICA

ATA DE DEFESA DE DISSERTAÇÃO

ATA DA REUNIÃO DA BANCA EXAMINADORA DA DEFESA DE DISSERTAÇÃO DE MAXWELL BATISTA CAIXETA - Aos dezessete dias do mês de março do ano de 2020 (17/03/2020), às 14:00 horas, reuniram-se os componentes da Banca Examinadora: Profs. Drs. THIAGO LOPES ROCHA (IPTSP/UFG), CAIO MARCIO DE OLIVEIRA MONTEIRO (IPTSP/UFG) e ÉVERTON KORT KAMP FERNANDES (IPTSP/UFG) para, sob a presidência do primeiro, e em sessão pública realizada no INSTITUTO DE PATOLOGIA TROPICAL E SAÚDE PÚBLICA, procederem à avaliação da defesa de dissertação intitulada:" **Toxicidade reprodutiva e resposta de múltiplos biomarcadores no caramujo *Biomphalaria glabrata* (Say 1818) após exposição crônica às nanopartículas de óxido de ferro (γ-Fe2O3) funcionalizadas com ácido glucônico**", em nível de **MESTRADO**, de autoria de **MAXWELL BATISTA CAIXETA**, discente do PROGRAMA DE PÓS-GRADUAÇÃO EM BIOLOGIA DA RELAÇÃO PARASITO-HOSPEDEIRO, da Universidade Federal de Goiás. A sessão foi aberta pelo Orientador, Prof. Dr. THIAGO LOPES ROCHA, que fez a apresentação formal dos membros da Banca e orientou o Candidato sobre como utilizar o tempo durante a apresentação de seu trabalho. A palavra a seguir, foi concedida à autora da dissertação que, em 30 minutos procedeu à apresentação de seu trabalho. Terminada a apresentação, cada membro da Banca arguiu o Candidato, tendo-se adotado o sistema de diálogo sequencial. Terminada a fase de arguição, procedeu-se à avaliação da defesa. Tendo-se em vista o que consta na Resolução nº. 1492/2017 do Conselho de Ensino, Pesquisa, Extensão e Cultura (CEPEC), que regulamenta o Programa de Pós-Graduação em Biologia da Relação Parasito-Hospedeiro a Banca, em sessão secreta, expressou seu Julgamento, considerando o candidato **Aprovado**:

Banca Examinadora

Prof. Dr. Thiago Lopes Rocha	Aprovado
Prof. Dr. Caio Marcio de Oliveira Monteiro	Aprovado
Prof. Dr. Éverton Kort Kamp Fernandes	Aprovado

Em face do resultado obtido, a Banca Examinadora considerou o candidato **Habilitado**, cumprindo todos os requisitos para fins de obtenção do título de **MESTRE EM BIOLOGIA DA RELAÇÃO PARASITO-HOSPEDEIRO**, pela Universidade Federal de Goiás. Cumpridas as formalidades de pauta, às 18 h 30 min, a presidência da mesa encerrou esta sessão de defesa de dissertação e para constar eu, HELOÍSA DE SOUSA VIEIRA, secretária do Programa de Pós-Graduação em Biologia da Relação Parasito-Hospedeiro, lavrei a presente Ata que depois de lida e aprovada, será assinada pelos membros da Banca Examinadora.

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Discente: Maxwell Batista Caixeta

Orientador: Prof. Dr. Thiago Lopes Rocha

Co-orientadora: Profa. Dra. Luciana Damacena Silva

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5. Prof. Dra. Dra. Luciana Damacena Silva (Suplente) – UEG

Data: 17/03/2020

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“Não é sobre chegar no topo do mundo e saber que venceu
É sobre escalar e sentir que o caminho te fortaleceu
É sobre ser abrigo e também ter morada em outros corações
E assim ter amigos contigo em todas as situações...”

- Ana Vilela

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CAPÍTULO I: REVISÃO DA LITERATURA

LISTA DE SÍMBOLOS E ABREVIATURAS

AGL	Ácido glucônico
ANOVA	Análise de multivariância
BOF	Bactérias oxidantes de ferro
BRF	Bactérias redutoras de ferro
CI	*Condition index*
Dh	Diâmetro hidrodinâmico/ *Hydrodynamic diameter*
DLS	Espalhamento de luz dinâmico / *Dynamic Light Scattering*
ELS	Espalhamento Eletroforético de Luz
EROs	Espécies reativas de oxigênio
GR	*Growth rate*
GSI	*Gonadosomatic index*
HI	Hospedeiros intermediários
ICC	Índice de condição corporal
IGS	Índice gonadossomático
IONPs	*Iron oxide nanoparticles*
IPTSP	Instituto de Patologia Tropical e Saúde Pública
IQ	Instituto de Química
MET	Microscopia eletrônica de transmissão / *Transmission Electron Microscopy*
MM	Momento magnético
MON	Matéria orgânica natural
NM	Nanomaterial
NMs	Nanomateriais/ *Nanomaterials*
NOFs	Nanopartículas de óxido de ferro
NPs	Nanopartículas / *Nanoparticles*
OD	Oxigênio dissolvido
OECD	Organização para a Cooperação e Desenvolvimento Econômico
OMS/WHO	Organização Mundial da Saúde / *World Health organization*
PdI	Índice de polidispersão/ *Polidispersion index*
PLA	*Polylactic-acid*
R-OH	Radicais hidroxila
SET	*Snail embryotoxicity test*

RESUMO

A nanotecnologia tem sido aplicada no controle de parasitos e hospedeiros intermediários de agentes etiológicos de doenças globais e tropicais negligenciadas, tal como a esquistossomose. O parasito *Schistosoma mansoni* é o agente etiológico desta doença no Brasil, e caramujos *Biomphalaria* spp. atuam como hospedeiro intermediário. O uso de nanomateriais (NMs) como agentes moluscicidas é promissor devido às suas propriedades específicas que permitem a internalização, maior reatividade e especificidade ao caramujo, além da facilidade de produção, e possibilidade de retirada do ambiente. Neste sentido, o objetivo deste trabalho foi avaliar a potencial atividade moluscicida das nanopartículas de óxido de ferro (NOFs) funcionalizadas com ácido glucônico no caramujo *Biomphalaria glabrata*. Inicialmente, uma análise bibliométrica associada à uma revisão sistemática da literatura identificou que diferentes NMs foram capazes de induzir estresse oxidativo, genotoxicidade, mutagenicidade, embriotoxicidade, toxicidade reprodutiva e transgeracional, imunotoxicidade, mortalidade e alterações comportamentais em diferentes espécies de gastrópodes. Em relação aos bioensaios, após exposição crônica (28 dias) de *B. glabrata* a diferentes concentrações de NOFs e $FeCl_3$ (1,0; 2,5; 6,2 e 15,6 mg L^{-1}), foi evidenciado alta bioacumulação de ferro das NOFs na massa visceral do caramujo em comparação com os íons de ferro e o grupo controle. Da mesma forma, observou-se alta frequência de alterações de comportamento dos caramujos expostos as NOFs quando comparados a sua contrapartida iônica e ao grupo controle. Ambas as formas de Fe reduziram a fecundidade dos caramujos, enquanto a mortalidade e a fertilidade reduzida foram observadas somente após a exposição as NOFs a 15,6 mg L^{-1}. Os resultados gerais indicaram alterações comportamentais e toxicidade reprodutiva associados à bioacumulação das NOFs em *B. glabrata*. Este estudo enfatiza que as nanopartículas à base de metal são potenciais agentes moluscicidas.

Palavras-chave: Esquistossomose; hospedeiro intermediário; ecotoxicologia; embriotoxicidade; biomarcadores.

ABSTRACT

Nanotechnology has been applied to control parasites and intermediate hosts of etiologic agents of neglected global and tropical diseases, such as schistosomiasis. The parasite *Schistosoma mansoni* is the etiological agent of this disease in Brazil, and snails *Biomphalaria* spp. act as intermediate host. The use of nanomaterials (NMs) as molluscicidal agents is promising due to their specific properties that allow internalization, greater reactivity and specificity to the snail, in addition to the ease of production, and the possibility of removal from the environment. In this sense, the aim of this work was to evaluate the potential molluscicidal activity of gluconic acid - functionalized iron oxide nanoparticles (IONPs) in the snail *Biomphalaria glabrata*. Initially, a bibliometric analysis associated with a systematic review of the literature identified that different NMs were able to induce oxidative stress, genotoxicity, mutagenicity, embryotoxicity, reproductive and transgenerational toxicity, immunotoxicity, mortality and behavioural changes in different species of gastropods. Regarding bioassays, after chronic exposure (28 days) of *B. glabrata* to different concentrations of IONPs and $FeCl_3$ (1.0; 2.5; 6.2 and 15.6 mg L^{-1}), a high bioaccumulation of iron by IONPs in visceral mass of the snail compared to the iron ions and the control group was observed. Likewise, there was a high frequency of behavioural changes in snails exposed to IONPs when compared to their ionic counterpart and to the control group. Both forms of Fe reduced fertility, while mortality and reduced snail's fertility were observed only after exposure to IONPs at 15.6 mg L^{-1}. The general results indicated behavioural impairments and reproductive toxicity, associated with the bioaccumulation of IONPs in *B. glabrata*. This study emphasizes that metal-based nanoparticles are potential molluscicidal agents.

Keywords: Schistosomiasis; intermediate host; ecotoxicology; embryotoxicity; biomarkers.

1. CAPÍTULO I: REVISÃO DA LITERATURA

1.1. Nanotecnologia

A nanotecnologia é uma ciência interdisciplinar do século XXI, que teve seu início e ascensão por volta do fim da segunda guerra mundial, com estudos para o desenvolvimento e miniaturização de eletrônicos (Choi and Mody, 2009; Hulla *et al.*, 2015). Esta ciência estuda os materiais em nano escala (1 a 100 nm), e suas novas propriedades físicas, químicas, mecânicas, elétricas entre outras (Linsinger *et al.*, 2012). Um nanomaterial (NM) é definido pela Comissão Europeia (European Comission, 2011) como sendo "um material natural ou manufaturado contendo partículas dispersas ou agregadas, que apresentem distribuição de tamanho das partículas >50 % casos entre 1 a 100 nm em pelo menos uma das suas dimensões". Devido ao seu pequeno tamanho, alta área de superfície e propriedades nanoespecíficas, os NMs diferem do seu mesmo material de composição em macro escala (Linsinger *et al.*, 2012; Mody *et al.*, 2010; Nel *et al.*, 2013). Assim, entender essas propriedades é fundamental para sintetizar NMs com propriedades desejadas para aplicações destinadas aos mais diversos fins.

Atualmente a nanotecnologia é amplamente difundida nas áreas da química, física, biologia, engenharia e biotecnologia, através do estudo e aplicações dos NMs e das nanopartículas (NPs) nos mais diversos setores da sociedade (Figura 1). Sua aplicação vai desde a utilização nos setores industriais e de tecnologia, por exemplo na fabricações de eletrônicos, cerâmicas, plásticos, produtos da indústria têxtil, à agricultura e alimentação, por exemplo através da produção de agentes pesticidas, catalisadores de processamento de alimentos, conservantes entre outros (Siddiqi *et al.*, 2018; Tarafdar *et al.*, 2013; Tsuzuki, 2009). Além disso, encontramos a utilização da nanotecnologia nos setores ambientais e da saúde, tendo como exemplo aplicações na remediação de ambientes poluídos (Corsi *et al.*, 2018; Zou *et al.*, 2016), no desenvolvimento de produtos para terapia, diagnósticos e tratamento de doenças, como por exemplo a produção de vacinas, fármacos (Gupta and Gupta, 2005; Mirzaei and Darroudi, 2017; Verma and Kumar, 2019) e a sua utilização emergente no controle e combate de parasitos (Benelli, 2018a; Li *et al.*, 2018; Moustafa *et al.*, 2018; Tomiotto-Pellissier *et al.*, 2017).

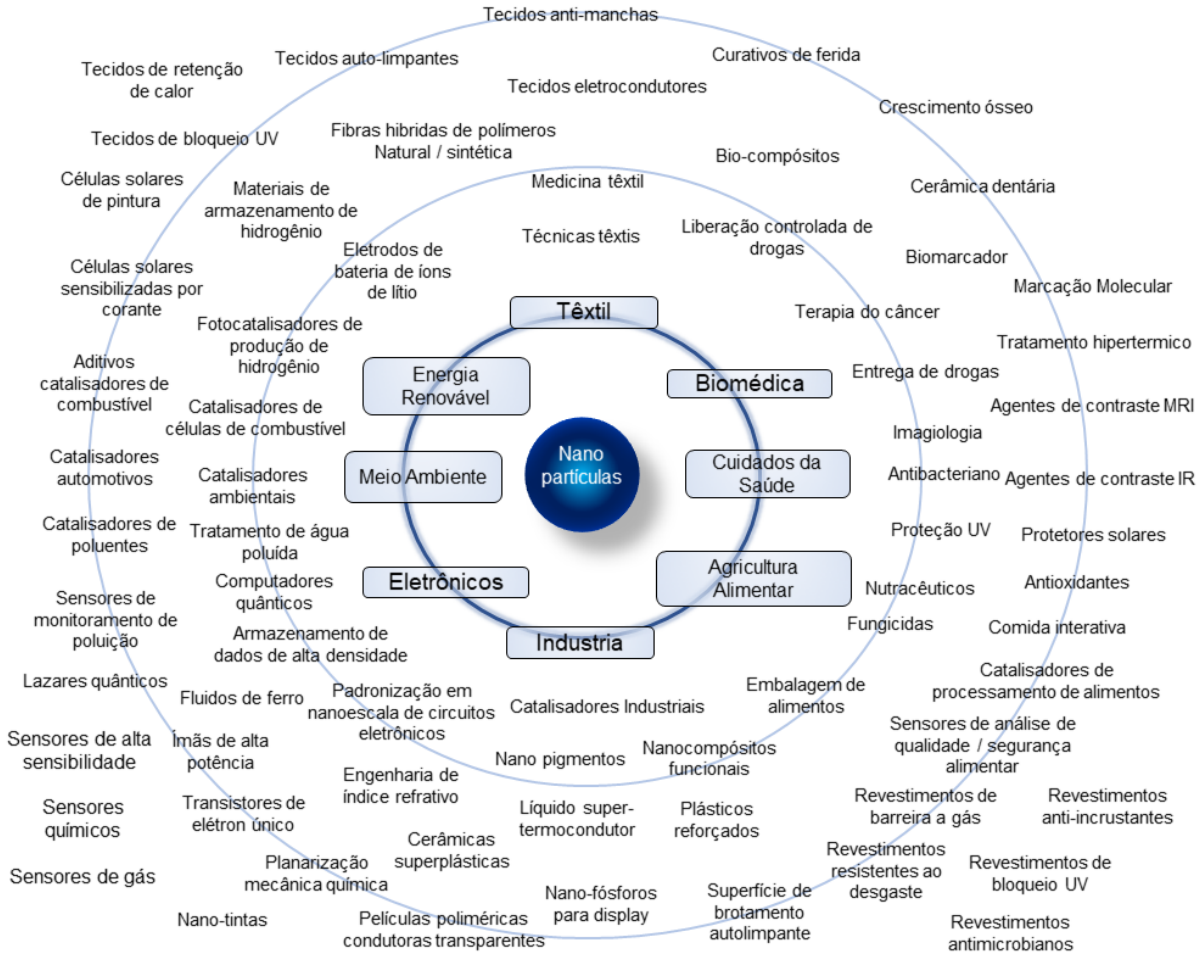


Figura 1. Principais aplicações das nanopartículas inorgânicas. Traduzido e adaptado de Tsuzuki (2009).

Os NMs podem ser divididos quanto às suas dimensões em três categorias: 1) nanoplacas: 1 dimensão; 2) nanotubos: 2 dimensões; ou 3) nanopartículas (NPs): 3 dimensões (Linsinger *et al.*, 2012). Além disso, os NMs podem ser classificados, quanto a natureza química, morfologia, importância comercial, modos de ação fisiológicos, dentre outros. Gaillard *et al.* (2019) categorizaram os NMs para fins de regulamentação em três grupos principais: (*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 (Figura 2).

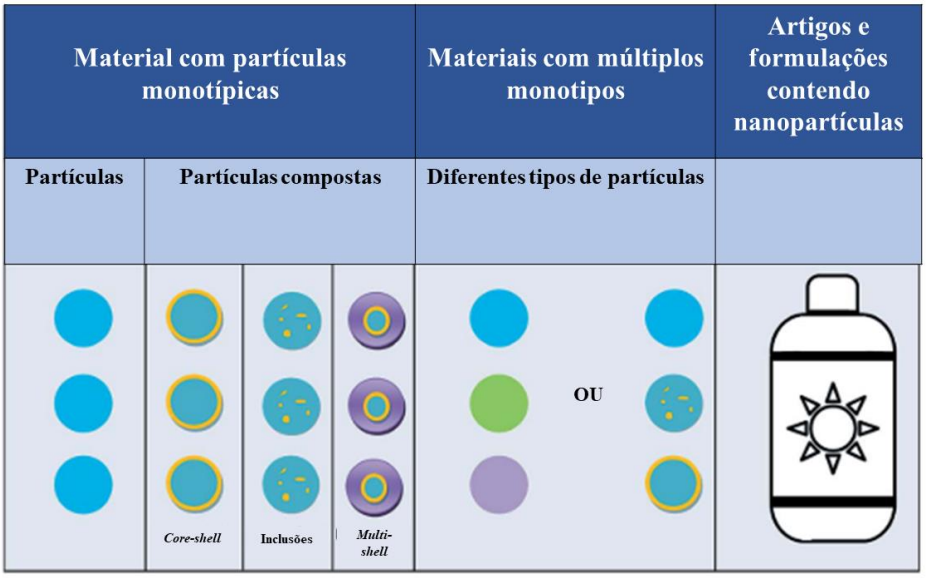


Figura 2. Classificação dos diferentes tipos de partículas e materiais particulados para fins de regulamentação. Fonte: Gaillard *et al.* (2019), com modificações.

Ainda sobre os materiais monotípicos, estes podem ser categorizados de acordo com Gaillard *et al.*, (2019) quanto:

- A sua composição química em: orgânicos, inorgânicos, biológicos, baseados em nanotubos de carbono, compostos por uma ou múltiplas camadas.
- Quanto à dispersibilidade e estabilidade em: monodisperso, polidisperso, disperso em meio aquoso, polar, ou materiais específicos.
- As propriedades específicas: condutividade elétrica, propriedades magnéticas, capacidade de adsorção e tipo de funcionalização.

1.2. Nanopartículas de óxido de ferro (NOFs)

As nanopartículas de óxido de ferro (NOFs) são amplamente conhecidas e muito utilizadas devido às suas propriedades magnéticas, biocompatibilidade, alta capacidade de adsorção e facilidade de síntese, além do baixo custo de produção em larga escala em relação à outros NMs, como por exemplo Ag NPs (Arularasu *et al.*, 2018; Kwon *et al.*, 2018; Zhang *et al.*, 2016). Elas são aplicadas em diversos setores da indústria, como na produção de eletrônicos, mídias de gravação, nas indústrias de pigmentos e tintas, em remediação ambiental para remoção de poluentes da água, e também na área da saúde

como contrastes de ressonância magnética, tratamento de câncer por hipertermia, e como sistemas de entrega de fármacos (Cornell, 2003; Gupta and Gupta, 2005; Zou *et al.*, 2016).

Existem cerca de 16 tipos de óxidos de ferro diferentes. Eles são formados a princípio, naturalmente no ambiente, através da degradação de rochas magmáticas por mecanismos físicos e químicos do intemperismo e oxidação. Por outro lado, elas podem ser também sintetizados em laboratórios para diversos fins (Cornell, 2003). Dentre os quatro principais óxidos magnéticos mais utilizados na produção de NPs para as aplicações biomédicas devido a sua estabilidade, estão a hematita (α-Fe_2O_3), a magnetita (Fe_2O_4), a maghemita (γ-Fe_2O_3) e *wüstite* (FeO), com destaque para magnetita e maghemita devido ao seu pequeno tamanho e sua biocompatibilidade em mamíferos dependendo das propriedades físicas e funcionalização (Oliveira *et al.*, 2013; Teja and Koh, 2009;Ling and Hyeon, 2013; Ma *et al.*, 2012; Rivas *et al.*, 2012; Wang *et al.*, 2018). A Tabela 1 resume as principais características destes 4 tipos de óxidos magnéticos.

Tabela 1. Propriedades das principais nanopartículas de óxido de ferro (NOFs). Adaptado de Cornell (2003).

Nanopartículas (NPs)	**Morfologia cristalina**	**Cor**	**Dimensões celulares (nm)**	**Densidade (g cm^{-3})**	**Tipo de magnetismo**
Hematita (α-Fe_2O_3 NPs)	Hexagonal	Vermelha	0,5 x 1,3	5,26	Antiferromagnético ou ferrimagnético
Magnetita (Fe_3O_4 NPs)	Cúbica	Preta	0,8	5,18	Ferrimagnético
Maghemita (γ-Fe_2O_3 NPs)	Cúbica ou tetragonal	Marrom-avermelhado	0,8	4,87	Ferrimagnético
Wüstite (FeO NPs)	Cúbica	Preta	0,4	5,9	Antiferromagnético

A estrutura cristalina dos óxidos se ferro é formada por ânions de oxigênio e cátions de ferro ligados de forma tetraédrica ou octaédrica (Figura 3). A magnetita possui íons Fe^{3+} distribuídos aleatoriamente entre interstícios dos sítios octaédrico e tetraédricos, e íons Fe^{2+} entre os interstícios dos sítios octaédricos. Tais íons são responsáveis pela sua magnetização. A maghemita é muito similar a magnetita, porém a principal diferença é a presença de Fe^{3+} disposto regularmente, constituindo cerca de 2/3 de toda sua estrutura,

sendo seguidos por um local vago. No caso da maghemita suas propriedades magnéticas dependem do tamanho da partícula (Oliveira *et al.*, 2013; Teja and Koh, 2009).

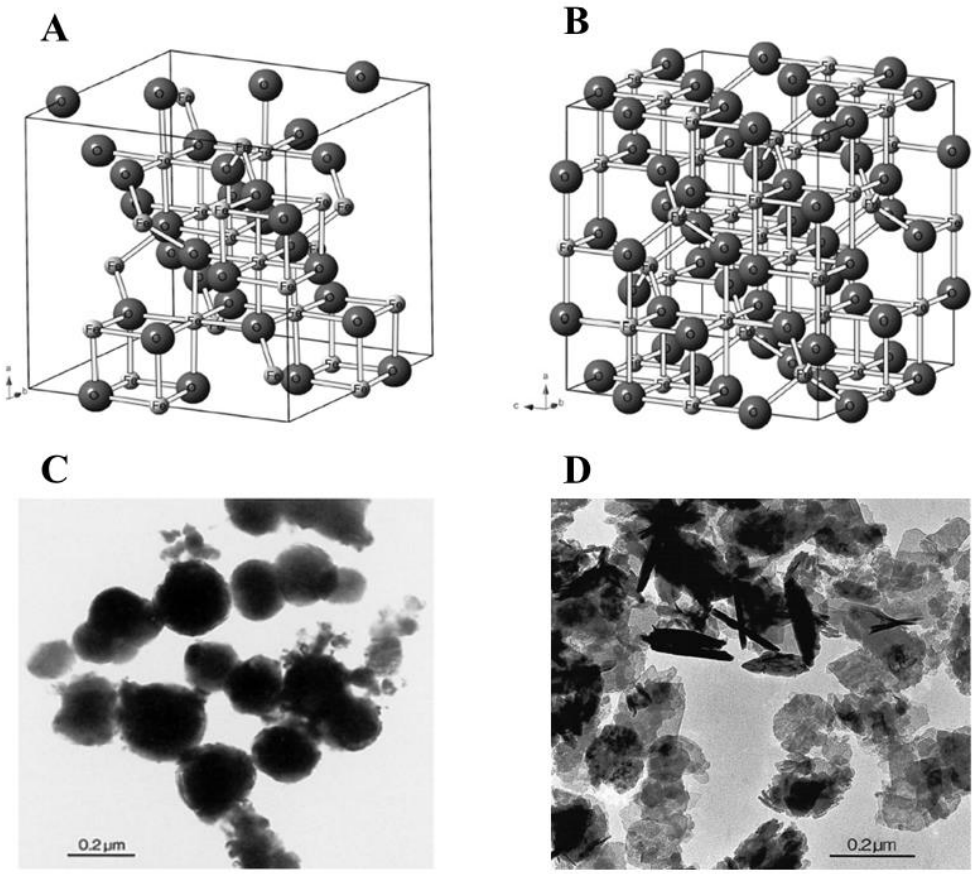


Figura 3. Estrutura cristalina das nanopartículas de óxido de ferro (NOFs). A) Magnetita. B) Maghemita. Adaptado de Oliveira *et al.* (2013). Microscopia eletrônica de transmissão (MET) de partículas de C) Magnetita e D) Maghemita. Fonte: Cornell (2003).

Para fins de aplicações biológicas e ambientais dos NMs, faz-se necessário compreender as propriedades das NPs, tais como comportamento magnético, tamanho, carga de superfície, diâmetro hidrodinâmico (Dh), índice de polidispersão (PDI) e o tipo de funcionalização, pois estas propriedades podem influenciar significativamente na toxicidade e biodisponibilidade do NM dependendo do intuito da aplicação (Lei *et al.*, 2018; Mahmoudi *et al.*, 2009; Teja and Koh, 2009; Zhang *et al.*, 2019).

1.3. Propriedades das NOFs

1.3.1. Comportamento magnético

Quando um campo magnético é aplicado sobre um material, através da mudança nos polos magnéticos, ocorre uma magnetização ou momento magnético (MM). O átomo de ferro tem um forte momento magnético devido a quatro elétrons não pareados em seus orbitais 3d. Quando um macrocristal é formado por átomos de ferro, ele geralmente se divide em múltiplos momentos magnéticos (Figura 4A). Conforme esses cristais vão diminuindo em escala nanométrica (ex. nanopartículas), há a tendência da formação do monodomínios magnéticos, diferindo assim do material em macroescala (Figura 4B) (Laurent *et al.*, 2008; Teja and Koh, 2009).

Conforme Teja e Koh (2009) um metal magnético baseado em ferro pode apresentar monodomíneos (Figura 4B):

- Paramagnético: Os MM atômicos individuais são organizados de forma aleatória, e a magnetização é nula, caso não haja um campo magnético que alinhe esses MM.
- Ferromagnético: Todos os MM são alinhados, mesmo sem um campo magnético externo.
- Ferrimagnético: Os MM são alinhados em dois estados de forma antiparalela, com magnitudes diferentes.
- Antiferromagnético: Os MM são alinhados em dois estados de forma antiparalela de mesma magnitude.

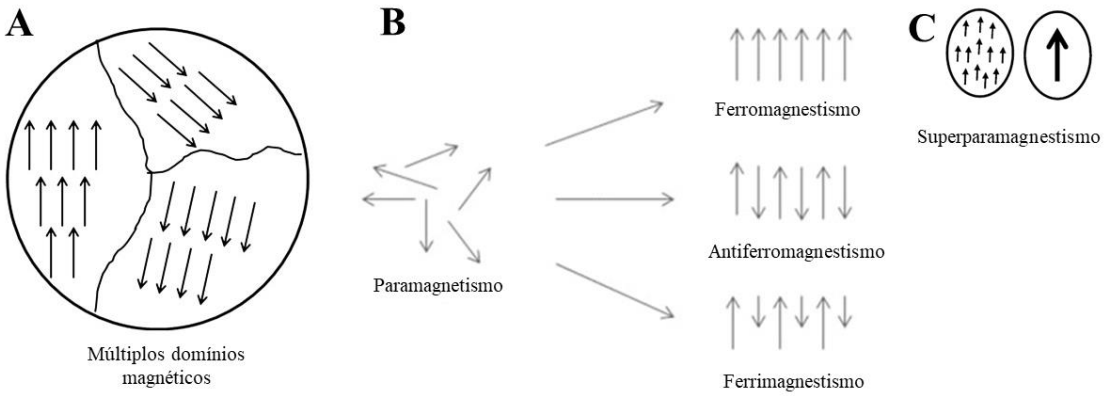


Figura 4. Momentos magnéticos. A) Macrocristal compostos por diferentes momentos magnéticos. B) Monodomíneos magnéticos. C) Monodomídeos ferromagnéticos constituem o superparamagnetismo. Adaptado de Teja e Koh (2009).

Para as NPs de magnetita e maghemita esses monodomíneos são ferromagnéticos, e quando possuem um tamanho < 20 nm apresentam um comportamento especial conhecido como superparamagnetismo (Figura 4C). Quanto mais alinhados estão estes domíneos magnéticos, como é o caso do superparamagnetismo, maior será a magnetização geral. Ao se aplicar um campo magnético em uma partícula que não seja superparamagnética, a tendência é restar um magnetismo residual, que pode ser prejudicial em aplicações biológicas dentro do corpo, pois essas partículas tendem a se aglomerar, o que normalmente não ocorrem com partículas com domíneos alinhados, como é o caso das superparamagnéticas (Laurent *et al.*, 2008; Ling and Hyeon, 2013; Teja and Koh, 2009).

1.3.2. Tamanho

Os NMs possuem tamanhos variáveis e controláveis. Por exemplo, no caso das NOFs, em geral esse tamanho é < 20 nm, o que as tornam interessantes por serem equiparáveis, ou mesmo apresentar dimensões inferiores as células (10 – 100 μm), proteínas (5 – 50 nm) ou genes (2 nm de largura e 10 – 100 nm de comprimento), o que torna sua aplicação interessante pela possibilidade de transpassarem ou se ligarem a estas unidades biológicas, melhorar a biodistribuição através dos tecidos, e poderem ser direcionadas através da magnetização ou funcionalização (Gupta and Gupta, 2005; Kwon *et al.*, 2018).

Estudos de captação celular das NOFs *in vitro* em células do sistema de defesa e células tumorais de mamíferos, indicam que o menor tamanho dessas NPs, implicaram em uma maior captação, distribuição e citotoxicidade, dependendo ainda do tipo de funcionalização que foi utilizado, da carga de superfície, diâmetro hidrodinâmico e o índice de polidispersão (Feng *et al.*, 2018; Schweiger *et al.*, 2012; Xiao *et al.*, 2011). Portanto explorar estas propriedades das NPs é importante para compreender seu o comportamento no meio de dispersão, e crucial para entender a interação das NPs com as células.

1.3.3. Carga de superfície (potencial zeta), diâmetro hidrodinâmico (Dh) e índice de polidispersão (PDI)

O potencial zeta (ζ) refere-se a carga de superfície formada ao redor do NM nos diferentes meios de dispersão. É a medida da magnitude da atração ou repulsão eletrostática, ou cargas das partículas, e pode influenciar na estabilidade dessa suspensão levando ou não a agregação, floculação e sedimentação dessas partículas. Geralmente um potencial zeta > [± 30 mV] é ideal para que a partícula permaneça em suspensão em meio aquoso (Bhattacharjee, 2016). A carga de superfície dependerá dos grupos funcionais dos materiais utilizados para o revestimento (funcionalização) das NPs, o que influenciará os seus subsequentes comportamentos no meio biológico. Geralmente NPs que se apresentam altamente carregadas (positiva ou negativamente), são massivamente incorporadas pelas células (Schweiger *et al.*, 2012; Xiao *et al.*, 2011).

NPs carregadas positivamente, são incorporadas eficientemente através da interação com glicocálix carregado negativamente e internalizadas, principalmente via endocitose adsortiva, sendo relacionadas a um acúmulo de membrana e concentração específica de NPs, levando a um padrão de absorção limitado (saturável), estável, mas não-linear (Martin *et al.*, 2008; Schweiger *et al.*, 2012). Por outro lado, NPs carregadas negativamente tendem a interagir com proteínas séricas, e formar uma camada de proteínas chamada *corona,* que são internalizadas via endocitose comum, ou mesmo por endocitose mediada por receptor proteína específico. Além disso, a captação parece ser constante e ao longo do tempo, independente do acúmulo de membrana (Lundqvist *et al.*, 2008; Schweiger *et al.*, 2012).

O diâmetro hidrodinâmico (Dh) de uma NP é a medida do tamanho da partícula em suspensão, levando em conta também a carga superficial conferida pelo meio de dispersão em que a NP se encontra, chamada de camada de solvatação (Bhattacharjee, 2016). Já o índice de polidispersão (PDI) refere-se a variedade da distribuição do tamanho das partículas no meio de dispersão, podendo ser classificadas como monodispersas, moderadamente polidispersas, e polidispersas. Quanto mais homogênea a dispersão, mais monodispersa. Este índice varia de 0 a 1, e geralmente uma dispersão é considerada altamente monodispersa quando PDI $\leq$ 0,3 (Bhattacharjee, 2016; Mahbubul, 2018).

O diâmetro hidrodinâmico é um dos fatores mais importantes na determinação da distribuição e liberação das NPs, uma vez que elas tendem a sofrer *opsonização* (adsorção de proteínas plasmáticas em sua superfície), e aumentar seu tamanho original (Kumar *et*

al., 2011; Torrisi *et al.*, 2014). NOFs com diâmetro > 100 nm são rapidamente fagocitadas pelas células do sistema de defesa e conduzidas ao sistema de depuração do organismo, enquanto NOFs com diâmetro < 50 nm se beneficiam da opsonização e são liberadas mais lentamente (Kumar *et al.*, 2011; Singh *et al.*, 2006). Além disso, a uniformidade no tamanho das NOFs poderá afetar os resultados farmacocinéticos e de biodistribuição, sendo assim um PDI $\leq$ 0,3 (monodisperso) é mais desejável para uma distribuição uniforme *in vivo* (Schweiger *et al.*, 2012).

1.3.4. Funcionalização

A funcionalização das NPs conferem diversas vantagens para a sua conservação e aplicação, como previr sua oxidação pelo ar e aglomeração em meios de dispersão (Bagheri and Julkapli, 2016; Wu *et al.*, 2008), fornece biocompatibilidade por meio da ligação de substâncias inertes que visam minimizar sua reatividade (Torrisi *et al.*, 2014), além de permitir o direcionamento altamente específico a uma célula ou tecido, através da conjugação com enzimas, proteínas e anticorpos (Gupta and Gupta, 2005; Ling and Hyeon, 2013). Além disso, a funcionalização permite a modificação da carga iônica e confere características hidrofílicas ou hidrofóbicas para partículas que apresentariam propriedades iniciais diferentes antes da funcionalização (Wei *et al.*, 2011). Um exemplo de substância que pode ser utilizada como agente de funcionalização das NOFs é o ácido glucônico (Sui *et al.*, 2012).

1.3.4.1 Ácido glucônico

O ácido glucônico (AGL) $C_6H_{12}O_7$ (Figura 5), é um ácido carboxílico derivado da oxidação da glucose, que ocorre naturalmente em diversas espécies de plantas e é transformado através enzimas de fungos e bactérias (Cañete-Rodríguez *et al.*, 2016). É amplamente empregado como conservante em comidas, cosméticos e outros produtos farmacêuticos (Cañete-Rodríguez *et al.*, 2016; Ramachandran *et al.*, 2006). É um ácido orgânico não tóxico, não volátil, não corrosivo, facilmente biodegradável, hidrossolúvel, resistente a oxidação em meio aquoso, e apresenta propriedade quelante a diversos metais, dentre eles o ferro, formando complexos bastante estáveis (PubChem, 2019;

Ramachandran *et al.*, 2006). Devido a todas essas propriedades, em especial pela sua característica hidrofílica e a capacidade de se ligar ao ferro através dos átomos de oxigênio dos radicais hidroxila (R-OH) presente em sua cadeia, sua utilização na funcionalização das NOFs e para aplicações em ambientes aquáticos é desejada (Sui *et al.*, 2012; Wei *et al.*, 2011).

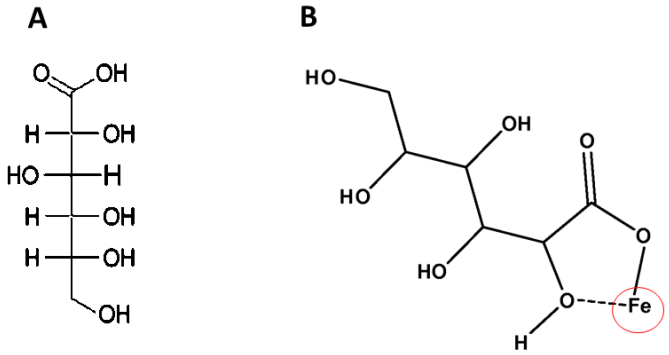


Figura 5. Fórmula estrutural do ácido glugônico isolado (A) e do ácido glucônico (AGL) ligado a um átomo de ferro através do oxigênio dos grupamentos hidroxila. Adaptado de Ramachandran *et al.* (2006) e Wei *et al.* (2011).

1.4. Mecanismos de ação e toxicidade das NOFs

Apesar de amplamente utilizadas em aplicações biomédicas e ambientais, a toxicidade das NOFs foi relatada para diversos organismos, como bactérias, fungos, moluscos, peixes e linhagens de células humanas (Auffan *et al.*, 2008; Diao and Yao, 2009; Qualhato *et al.*, 2018, 2017; Sidiropoulou *et al.*, 2018; Villacis *et al.*, 2017; Wu *et al.*, 2010). A toxicidade provocada pelas NOFs vai desde o nível molecular ao celular e tecidual, sendo que os principais danos destacados na literatura são perda da viabilidade celular, disrrupção da membrana plasmática, alterações do citoesqueleto, danos oxidativos, comprometimento da função mitocondrial, danos ao DNA e apoptose (Laffon *et al.*, 2018; Patil *et al.*, 2018). O principal mecanismo de toxicidade das NOFs é o estresse oxidativo por meio da produção de espécies reativas de oxigênio (EROs).

As NOFs podem ser internalizadas pelas células de diversas maneiras, por meio de difusão passiva, endocitose mediada pelo receptor clatrina, internalização por claveoliva, ou outros tipos de endocitose independentes. Em seguida, elas podem ser degradas pelas enzimas lisossômicas ou sofrem dissolução devido ao pH ácido do interior

dos lisossomos e liberar os íons ferrosos Fe^{2+} que reagem com H_2O_2 presente nas mitocôndrias *(Singh et al.*, 2010) (Figura 6).

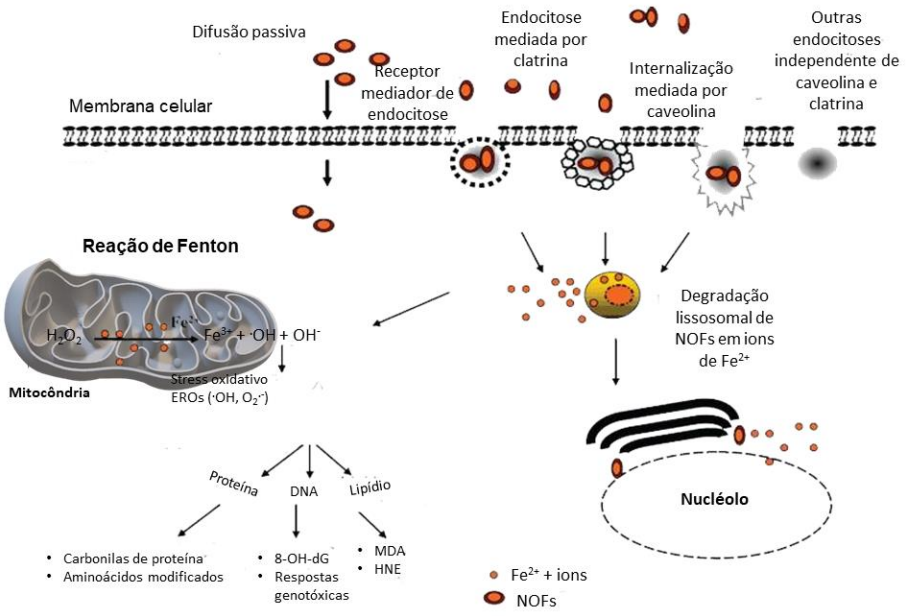


Figura 6. Mecanismos de internalização das nanopartículas de óxido de ferro (NOFs) e geração de espécies reativas de oxigênio. Adaptado de Singh *et al.* (2010).

As reações de Fenton (Equação 1 e 2) e Reação de Haber-Weiss (Equação 3) podem gerar espécies reativas de oxigênio, que afetarão diversas estruturas e organelas celulares (Wang *et al.*, 2013; Wu *et al.*, 2014). Além disso, as NOFs podem se ligar diretamente ao DNA ou a membrana plasmática causando a ruptura dessas estruturas, e se acumular em tecidos, prejudicando sua função ou levando a morte celular (Laffon *et al.*, 2018; Lei *et al.*, 2018; Patil *et al.*, 2018). Sendo assim, diferentes estudos atribuem a toxicidade das NOFs tanto à capacidade de liberação de íons metálicos e produção de EROs, quanto as propriedades nanoespecíficas como tamanho reduzido e afinidade por tecidos, que podem levar a internalização, bioacumulação e a toxicidade.

Equação 1. *Reação de Fenton*

$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + \cdot OH$ ou Fe (IV) + H_2O

Equação 2. *Reação semelhante à de Fenton*

$Fe^{3+} + H_2O_2 \rightarrow FeOOH^{2+} + H^+$

$FeOOH^{2+} \rightarrow Fe^{2+} + HO_2\cdot$

$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + \cdot OH$

Equação 3. *Reação de Haber-Weiss*

$Fe^{3+} + O_2^{\cdot -} \rightarrow Fe^{2+} + O_2$

$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + \cdot OH$

1.5. Transformação das NOFs no ambiente aquático

A toxicidade das NOFs depende do seu comportamento e destino no ambiente aquático (Lei *et al.*, 2018). Diversos fatores podem contribuir para a toxicidade das NOFs no ambiente aquático. Estes fatores estão relacionados à fatores físicos, transformações químicas, e interações biológicas das NOFs no ambiente, sua capacidade dissolução, interação com macromoléculas, deposição por formação de agregados e degradação através de reações de oxirredução (Lei *et al.*, 2018; Lowry *et al.*, 2012) (Figura 7).

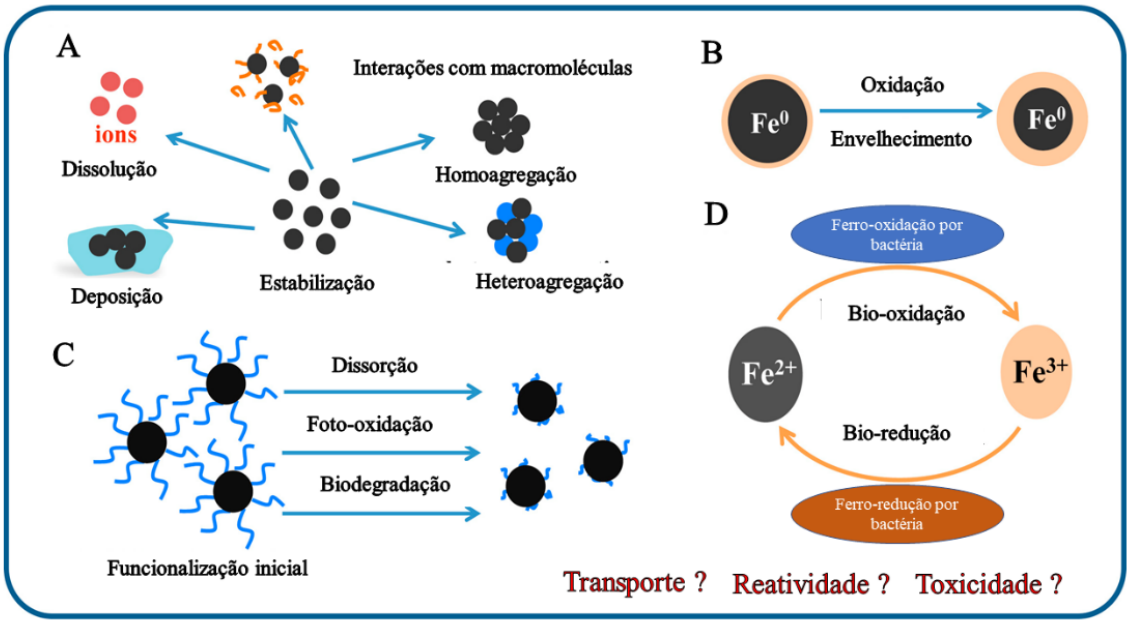


Figura 7. Potenciais transformações das nanopartículas de óxido de ferro (NOFs) no ambiente aquático. Adaptado de Lei *et al.* (2018).

1.5.1. Transformações físicas

Ao serem liberadas no ambiente, as NOFs podem sofrer agregação a partículas similares formando homoagregados, ou com outras substâncias orgânicas e inorgânicas constituindo heteroagregados (Figura 7A). Esta formação de agregados interfere na

diretamente na área de superfície das NOFs, na sua mobilidade e capacidade de permanecer em suspensão, e consequentemente na sua reatividade e no potencial de toxicidade (Batley *et al*., 2013; Dwivedi *et al*., 2015).

O tamanho da partícula, a concentração da dispersão, o pH, a força iônica meio, presença de matéria orgânica natural (MON) e a funcionalização são fatores decisivos para a formação de agregados e estabilização das partículas (Lei *et al.*, 2018). Em geral, quanto menor a partícula, maior a tendência da formação de homoagregados devido alta energia superficial. Por outro lado, partículas muito grandes tendem também a apresentarem maior força magnética e se agregarem (He *et al.*, 2008; Kharisov *et al.*, 2012). Concentrações altas da dispersão resultam em um maior número de colisões das partículas em movimento *browniano* e contribuem para uma maior agregação quando comparados a dispersões menos concentradas (Dwivedi *et al.*, 2015).

O pH e a força iônica estão relacionados com a regulação da carga superficial das NOFs, sendo que quanto mais distantes estiverem da carga (potencial zeta) de ponto zero do NM, melhor será para a dispersão da NP a levando a repulsão eletrostática (Garner and Keller, 2014).

A funcionalização vem como uma estratégia para minimizar a agregação das partículas, e normalmente é feita com partículas poliméricas aniônicas, que interagem com os cátions de ferro e formam uma camada superficial eletricamente negativa que permite uma maior repulsão eletrostática e contribui para melhorar a dispersão (Sui *et al.*, 2012; Wei *et al*., 2011). A escolha do agente para funcionalização depende do interesse da aplicação desejada. É importante considerar alguns fatores como o tipo de ligação entre eles, fotosensibilidade e durabilidade, uma vez que após liberados no ambiente estão sujeitos a dessorção caso encontrem outro ligante de maior afinidade, à foto-oxidação devido aos raios UV e a biodegradação (Figura 7C) (Lei *et al.*, 2018).

A presença de MON e outros materiais em suspensão no meio aquático, tais como algas, bactérias, ácidos orgânicos, açúcares, proteínas, lipídios e argilas, leva a heteroagregação das NOFs e normalmente conferem uma camada de recobrimento chamada Corona que pode contribuir tanto para estabilização das NOFs ao dificultarem a agregação delas com outras partículas, quanto podem levar a deposição dessas partículas se este acúmulo de MON for alto (Bhattacharjee, 2016; Lei *et al.*, 2018; Lowry *et al.*, 2012).

1.5.2. Transformações químicas

As transformações químicas das NOFs ocorrem através de reações de oxidação, redução, dissolução e adsorção de macromoléculas. Lei *et al.*, (2018) relata como processo mais significativo de transformação química, das NOFs zero valentes (ZV) o processo de envelhecimento, que danificam sua estrutura e promovem a perda da reatividade, assim como ilustrado na Figura 7B. Outras NOFs baseados em ferro, como FeO, γ-Fe_2O_3 e Fe_3O_4 são comparativamente mais resistentes a reações redox no meio ambiente (Kim *et al.*, 2012; Yan *et al.*, 2013).

As NOFs ZV, são muito utilizadas na remediação ambiental, para a adsorção de outros metais pesados e posterior remoção por magnetização no ambiente aquático (Diao and Yao, 2009; Zou *et al.*, 2016). As NOFs ZV são constituídas por um núcleo contendo Fe^0 e uma camada superficial de óxidos que elas adiquirem naturalmente após serem sintetizadas devido ao processo de oxidação, essa composição núcleo / camada de superfície é normalmente chamada de *Core-Shell* (Zou *et al.*, 2016). À medida que a camada de óxido se torna mais espessa devido ao envelhecimento e as transformações químicas, o núcleo esférico de ferro desaparece, e essas partículas perdem sua função inicial (Lei *et al.*, 2018; Wang *et al.*, 2010) .

Em geral a corrosão das NOFs ZV ocorrem através de um processo eletroquímico após a dissolulção de Fe^0 e a redução da camada superficial (ou *shell*). Esse processo depende principalmente da presença de oxigênio dissolvido (OD) na água, que forma Fe^{2+} que se difundem do núcleo para a superfície que pode ser descrito pela equação 4. Além disso na ausência de oxigênio, também pode ocorrer a formação de Fe^{2+}, hidroxila e gás hidrogênio em reação com a água (equação 5) (Lei *et al.*, 2018). Dependendo das condições ambientais Fe^{2+} pode ser transformado em Fe_3O_4, γ-Fe_2O_3 e FeOOH (Lefevre *et al.*, 2016).

Equação 4. *Formação de Fe^{2+} a partir de Fe^0 em meio contendo OD*

$$2Fe^0_{(s)} + 4H^+_{(aq)} + O_{2(aq)} \rightarrow 2Fe^{2+} + 2H_2O_{(l)}$$

Equação 5. *Formação de Fe^{2+} a partir de Fe^0 em reação com a água*

$$2Fe^0_{(s)} + 2H_2O_{(l)} \rightarrow 2\,Fe^{2+} + H_{2\,(g)} + 2OH^-_{(aq)}$$

Outros processos de transformação química incluem a presença de ânions e cátions no ambiente aquático. Em geral a presença de ânions como Cl^-, SO_4^{2-}, e Br^- ajuda a previnir o evelhecimento das NOFs ZV pelo fato de contribuirem para a quebra do filme óxido da superfíce, enquanto que os cátions, Mg^{2+}, Mn^{2+}, Co^{2+}, Ni^{2+}, Zn^{2+}, Cu^{2+} contribuem para a degradação e envelhecimento do Fe^0 (Reinsch *et al.*, 2010; Sun *et al.*, 2016).

A formação de íons Fe^{2+} leva a discussão sobre o aumento da toxicidade desses NMs, contudo esta questão é ainda incerta, com alguns estudos demonstrando que a prensença do íon leva o aumento da toxicidade e outros que a toxicidade é proveniente das próprias NPs ou que não existem diferenças significativas da toxicidade entre o íon solúvel e as NPs (Chen *et al.*, 2013; Keenan *et al.*, 2009; D. Wang *et al.*, 2016)

1.5.3. Transformações biológicas

Enquanto na água os principais mecanismos de oxido-redução química dependem da presença de oxigênio, as transformações biológicas reações de oxido-redução podem ocorrer tanto em condições óxicas, quanto anóxicas por bactérias aeróbicas e anaeróbicas (Lei *et al.*, 2018; Roden, 2003).

Nas reações de bioxidação (Figura 7D), as bactérias oxidantes de ferro (BOF) oxidam Fe^{2+} em Fe^{3+} através da doação de elétrons do ferro, o que acelera a corrosão e pode prejudicar a vida útil das NOFs (Emerson *et al.*, 2010). Se isto ocorre por meio bactérias aeróbicas o oxigênio sempre funciona como aceitador de eletróns do ferro durante a respiração celular, já se ocorre por meio de bactérias anaeróbicas a perda de elétrons do ferro pode ser acoplada ao oxigênio do CO_2 por bactérias fotossintetizantes, ou através de nitratos (NO_3^-) por bactérias quimiolitotróficas (Lei *et al.*, 2018; Roden, 2003). Exemplos de BOF fotossíntetizantes *Chlorobium ferrooxidans, Rhodovulum robiginosum, Rhodopseudomonas palustris* e BOFs quimiolitotróficas *Thiobacillus denitrificans, Geobacter metallireducens* (Hedrich *et al.*, 2011; Weber *et al.*, 2006).

Nas reações de ferro redução (Figura 7D), o que ocorre é o oposto Fe^{3+} é biotransformado em Fe^{2+} por bactérias redutoras de ferro (BRF) através do ganho de elétrons. A redução do Fe^{3+} à Fe^{2+} pode ser considerada benéfica no sentido de aumentar a longevidade da reatividade de partículas em estado de oxidação, transformando partículas em processo de envelhecimento em partículas sólidas mais estáveis (Lei *et al.*,

2018; Park *et al.*, 2010; Xie *et al.*, 2017). As BRF reduzem o ferro através da doção de elétrons de átomos de carbono e hidrogênio sob condições anóxicas durante a respiração celular, através da transferência externa direta de elétrons, via citocromos da membrana celular ou através da formação de pili condutores (Lei *et al.*, 2018; Richter *et al.*, 2012; Weber *et al.*, 2006).

1.6. Nanotecnologia aplicada ao controle de parasitos e hospedeiros intermediários

Estudos utilizando as NOFs e outros NMs têm sido desenvolvidos como uma estratégia promissora no controle doenças globais e tropicais negligenciadas, causadas por vírus, bactérias, fungos, protozoários e helmintos, seja através do controle de hospedeiros intermediários, dos próprios agentes etiológicos ou a partir do desenvolvimento de vacinas e melhoria no sistemas entrega de fármacos (Adair, 2009; Benelli, 2018a; Tomiotto-Pellissier *et al.*, 2017). As NOFs e outros NMs foram empregadas por exemplo, no controle de *Influenza* spp., *Staphylococcus* spp., *Candida* spp. (Adair, 2009; Siddiqi *et al.*, 2018), contra o protozoário *Plasmodium* sp. e larvas de mosquitos do gênero *Anopheles* (Benelli, 2018a; Pimentel *et al.*, 2007). Como sistema de entrega do fármaco Anfotericina B contra *Leishmania donovani* e contra larvas de flebotomíneos do gênero *Lutzomyia* (Benelli, 2018a; Kumar *et al.*, 2017). No controle de carrapatos associados a transmissão de *Rickettsia* spp. (Benelli *et al.*, 2017), e também contra diversos estágios de desenvolvimento do parasito *S. mansoni* e seus hospedeiros intermediários, caramujos do gênero *Biomphalaria* (Tomiotto-Pellissier *et al.*, 2017).

Aqui nós fizemos uma revisão sistemática da literatura com a utilização dos NMs em caramujos como sistema modelo para avaliação da toxicidade e ecotoxicidade dos nanomateriais (NMs), e que poderia também, ser empregada no controle de vetores e HI de doenças parasitárias, já que dos 60 artigos analisados e 21 espécies de caramujos encontradas nesta revisão, todas elas participam do ciclo de transmissão de algum parasito de interesse médico ou veterinário. Os resultados serão apresentados de forma mais detalhada no capítulo II.

1.7. Esquistossomose

A esquistossomose, uma das principais doenças tropicais negligenciadas do mundo, é um complexo de infecções aguda e crônica causadas por espécies de

Schistosoma, parasitos intravasculares dos seres humanos (Blanchard, 2004). Esses helmintos pertencem à subclasse Digenea, os quais precisam de dois hospedeiros para completar o ciclo de vida, normalmente, humanos e caramujos de água doce (Steinmann *et al.*, 2006; Worku *et al.*, 2014). Nas Américas, apenas uma espécie constitui problema importante de saúde pública, *Schistosoma mansoni*, enquanto na África, além dessa espécie, há também *Schistosoma haematobium* a destacar e, menos frequentemente, *Schistosoma intercalatum* (Colley *et al.*, 2014).

Até o ano de 2012, em todo o mundo cerca de 390 milhões de pessoas estavam infectadas por *S. mansoni*, enquanto 800 milhões permaneciam sob risco de infecção (WHO, 2013). Para o ano de 2020, estima-se ainda que 240 milhões de pessoas em todo o mundo estão infectas, 218 milhões requerem tratamento preventivo imediato e mais de 700 milhões de pessoas ainda estão em áreas de risco (WHO, 2019). Além disso, mais de 2,2 bilhões de pessoas ainda não possuem acesso a saneamento básico, sendo que 627 milhões praticam defecação em local aberto (WHO/Unicef, 2019).

Atualmente, a esquistossomose afeta mais de 30 mil pessoas por ano no Brasil (DATASUS, 2019), a estimativa de pessoas infectadas é de aproximadamente 1,5 milhões entre os anos de 2000 a 2017 com ocorrência em média de 513 óbitos (Brasil, 2019). Devido aos altos índices de morbidade e mortalidade, a esquistossomose tornou-se problema de saúde pública, e sua transmissão é mais intensa numa faixa de terras contínuas ao longo de quase toda a costa litorânea. Abrange desde a região Nordeste, a partir do Rio Grande do Norte em direção ao Sul, incluídas as zonas quentes e úmidas dos estados da Paraíba, Pernambuco, Alagoas, Sergipe e Bahia, onde se interioriza alcançando Minas Gerais, no Sudeste (Figura 8). Ao todo, são 19 Unidades Federadas com ocorrência da doença (Brasil, 2019).

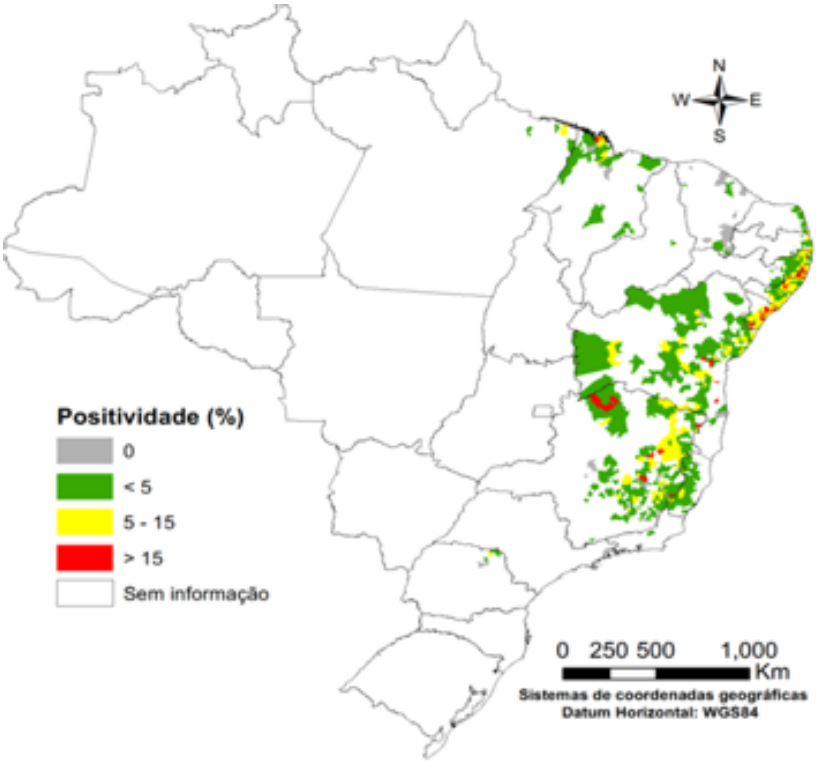


Figura 8. Mapa do Brasil ilustrando a taxa de ocorrência da esquistossomose por município, nos estados de Alagoas, Bahia, Espírito Santo, Maranhão, Minas Gerais, Paraíba, Pernambuco, Rio Grande do Norte, no período compreendido entre 2009 – 2017. (Brasil, 2019).

1.8. Ciclo de vida e transmissão de *Schistosoma mansoni*

No ciclo de *S. mansoni* (Figura 9), os caramujos, hospedeiros intermediários, pertencem ao gênero *Biomphalaria*, os quais são infectados quando os ovos embrionados, liberados juntamente com as fezes humanas, atingem os cursos d'água e o miracídio (larva ciliada), eclode do ovo, e penetra em suas partes moles. O miracídio intramolusco diferencia-se em esporocisto primário e esporocistos secundários, produtores de cercárias, as quais deixam o organismo do caramujo e constituem a forma infectante para o homem e outros vertebrados. Ao entrar em contato com a água contaminada com cercárias, humanos são infectados através da pele ou mucosas. No HD as cercárias se diferenciam em esquistossômulos, ganham a circulação chegando aos pulmões e depois ao fígado onde se diferenciam em vermes adultos machos e fêmeas. O acasalamento resulta na maturação sexual da fêmea; na sequência migram do fígado para as vênulas do intestino, onde a fêmea ovipõe em torno de 300 ovos por dia. Alguns ovos chegam, via circulação, a vários tecidos e ficam retidos nestes (fígado e baço, por exemplo). Uma enzima produzida pelo miracídio em desenvolvimento se difunde através dos poros dos

ovos e, juntamente com a pressão exercida pela postura continuada e pelo espinho dos ovos, facilitam a passagem através das veias mesentéricas. Além disso, ocorre necrose do tecido das vênulas o que facilita a passagem dos ovos para luz intestinal, os quais são eliminados juntamente com as fezes, dando continuidade ao ciclo biológico do *S. mansoni* (Coelho *et al.*, 2008; Gryseels *et al.*, 2006; Pila *et al.*, 2017).

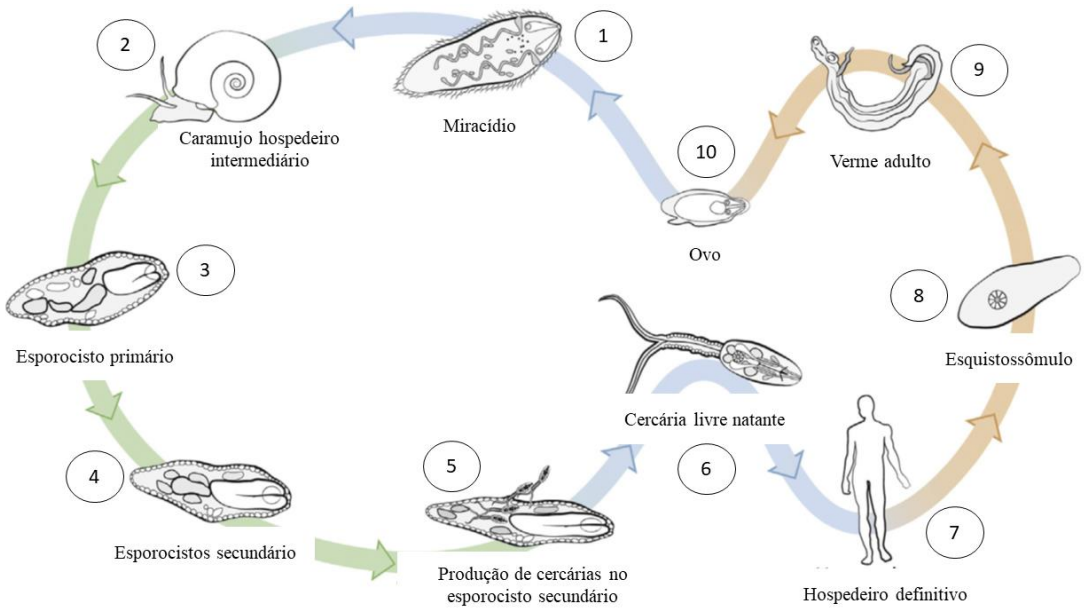


Figura 9. Ciclo de vida de *Schistosoma mansoni*: (1) Os miracídios presentes nos ovos liberados juntamente com as fezes humanas, em contato com a água doce, eclodem e nadam em círculos em busca de caramujos *Biomphalaria* spp. (2). Nos tecidos do caramujo, os miracídios diferenciam-se em esporocisto primário (3) que, por reprodução assexuada produz esporocistos filhos (4) os quais multiplicam-se e resultam em cercárias (5), que saem do caramujo através do tegumento. No ambiente aquático, as cercárias livres natantes (6) podem infectar humanos através da pele ou mucosas (7). Ao penetrar na pele humana as cercárias perdem a cauda bifurcada e diferencia em esquistossômulos (8) que via circulação chegam até o fígado onde se tornam vermes adultos machos e fêmeas (9). Após acasalar-se migram para as vênulas do intestino, onde a fêmea deposita seus ovos (10) os quais podem ganhar a luz intestinal ou migrar para vários tecidos. Os ovos presentes na luz intestinal são eliminados juntamente com as fezes e o ciclo recomeça. As cores azuis, verde, e marrom representam o ciclo de vida do parasito na água, no caramujo e no ser humano respectivamente. Adaptado de Pila *et al.* (2017) .

1.9. Caramujos do gênero *Biomphalaria*

O termo *Biomphalaria* [bis (latim) = duas vezes; omphalos (grego) = umbigo] refere-se aos dois lados da concha serem fundos, como um duplo umbigo, *glabrata* [glabra (latim) = liso] refere-se ao fato de a superfície da concha ser lisa. Estes caramujos

são classificados taxonomicamente no filo Mollusca, classe Gastropoda, superordem Hygrophila e família Planorbidae (MolluscaBase, 2018; Paraense and Deslandes, 1962).

A análise filogenética de 23 espécies de *Biomphalaria* (16 neotropicais e sete africanas) tem indicado a origem americana, a partir de um ancestral 'glabrata-like' que produziu doze espécies africanas há cerca de cinco milhões de anos (DeJong *et al.*, 2001). Nas Américas e África são referidas 37 espécies de *Biomphalaria*, das quais nove espécies são consideradas susceptíveis, oito infectaram-se experimentalmente e as demais não foram estudadas quanto à susceptibilidade a infecção por *S. mansoni* (Paraense, 2004). No Brasil, as espécies *Biomphalaria straminea* (Dunker 1848), *Biomphalaria tenagophila* (Orbigny 1835) e *Biomphalaria glabrata* (Say, 1818), são naturalmente infectadas (Goveia *et al.*, 2018). Das três espécies hospedeiras naturais, *B. glabrata* é a mais importante em relação à epidemiologia da doença, por apresentar maior susceptibilidade ao *S. mansoni*, maior produção de cercárias, maior distribuição geográfica além de maior fertilidade e fecundidade (Knight *et al.*, 2000; Scholte *et al.*, 2012).

B. glabrata, inicialmente foi descrito como *Planorbis glabratus* por Thomas Say em 1818, a partir de estudos de sua concha. Em 1819, pesquisadores alemães em uma expedição científica no Brasil, estado da Bahia, também com base em estudos conquiológicos, classificaram algumas espécies de caramujos como *Planorbis olivaceus* (concha olivácea), *P. ferrugineus* (concha de cor ferruginosa), *P. nigricans* (concha de coloração escura), *P. albescens* (concha esbranquiçada) e *P. viridis* (concha esverdeada pela ação de algas). Estudos posteriores revelam semelhanças nas descrições das conchas destas espécies à descrita por Say (1818) (Paraense, 2008). Variações de conchas em diferentes populações e anomalias podem levar a identificações duvidosas (Pereira de Souza and Lima, 1997). Portanto, a morfologia interna dos moluscos é de importância fundamental para a sistemática dos mesmos (Paraense, 2008).

1.9.1. Anatomia externa e interna do caramujo

Anatomicamente, o corpo do caramujo *B. glabrata* é formado pela concha e por partes moles como massa cefalopediosa e a massa visceral (Figura 10) (Paraense and Deslandes, 1962).

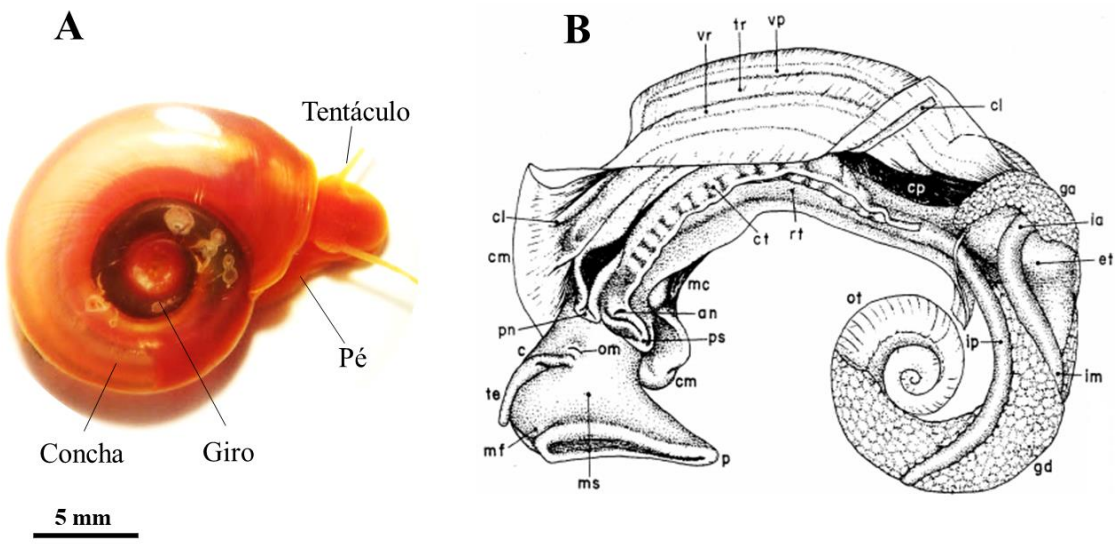


Figura 10. Anatomia externa (A) e interna (B) do caramujo *Biomphalaria glabrata*. an: ânus; c: cabeça; cl: crista dorsolateral; cm: colar do manto; cp: cavidade pulmonar; ct: crista retal; et: estômago; ga: glândula de albúmen; gd: glândula digestiva; ia: intestino anterior; im: intestino médio; ip: intestino posterior; mc: músculo columelar; mf: mufla; ms: massa cefalopodal; om: orifício genital masculino; ot: ovoteste; p: pé, pn: pneumóstoma; ps: pseudobrânquia; rt: reto; te: tentáculo; tr: tubo renal; vp: veia pulmonar; vr: veia renal A) Fonte: o autor. Escala 5 mm. B) Fonte: Paraense (2008).

Na parte superior da massa cefalopediosa estão os olhos situados na base do par de tentáculos que são filiformes e possuem função tátil. A massa visceral é envolta pelo manto que é responsável pela produção da concha que inicia sua formação nos primeiros estágios de desenvolvimento do molusco e seu crescimento é mais ou menos continuo durante toda a vida (Ebanks *et al.*, 2010). O deslocamento dos caramujos é feito através de contrações musculares do pé oblongo. Apresentam glândulas podais que secretam muco sobre o qual o animal move-se e prende-se ao substrato (Paraense and Deslandes, 1955).

A glândula digestiva contém vários túbulos terminados em fundo cego, revestidos por epitélio colunar, cujas células são altas e apresentam microvilosidades na região apical. Este órgão apresenta várias funções, incluindo a digestão intra e extracelular, além de atuar como reservatório de lipídios, glicogênio e minerais. Atua como principal fonte na produção de enzimas digestivas, absorção e armazenamento de nutrientes e excreção. Na glândula digestiva ocorre armazenamento e excreção de reservas inorgânicas, como por exemplo, do íon cálcio (Caceci *et al.*, 1988; Faro *et al.*, 2013; Zarai *et al.*, 2011). A respiração é feita através do saco pulmonar, pseudobrânquias e tegumento. O coração constituído por um átrio e um ventrículo, está contido no pericárdio, e o principal órgão

de excreção é o rim, sendo que a urina é eliminada pelo meato do ureter. O sistema nervoso central é formado por pares de gânglios bucais, cerebrais, pleurais, pedais, parietais e um gânglio visceral, que formam um anel em torno do esôfago, atrás do saco bucal. O sistema digestório é completo, compreendendo a massa bucal, glândulas salivares, esôfago, estômago, glândula digestiva, intestino e ânus (Paraense, 1975).

1.9.2. Reprodução e desenvolvimento dos caramujos

Os caramujos *B. glabrata* são hermafroditas, no entanto, a reprodução por fecundação cruzada é preferencial. O sistema genital é composto por órgãos genitais masculinos e femininos, tendo como órgãos hermafroditas, o ovoteste, órgão produtor de óvulos e espermatozoides, e o ovispermiduto (canal para passagem dos gametas). Os ovos de cor amarela são envolvidos por uma substância gelatinóide muito transparente, o conjunto adquire um aspecto de cápsula amarelada que se denomina massa ovígera (Figura 11). O número de ovos em cada massa ovígera varia de um a mais de 30. A eclosão dos ovos normalmente se inicia sete dias após a postura e com cerca de 30 dias caramujos *Biomphalaria* podem alcançar a maturidade e começam a ovipor, podendo um só indivíduo produzir em poucos meses milhares de descendentes (Pereira de Souza e Lima, 1997).

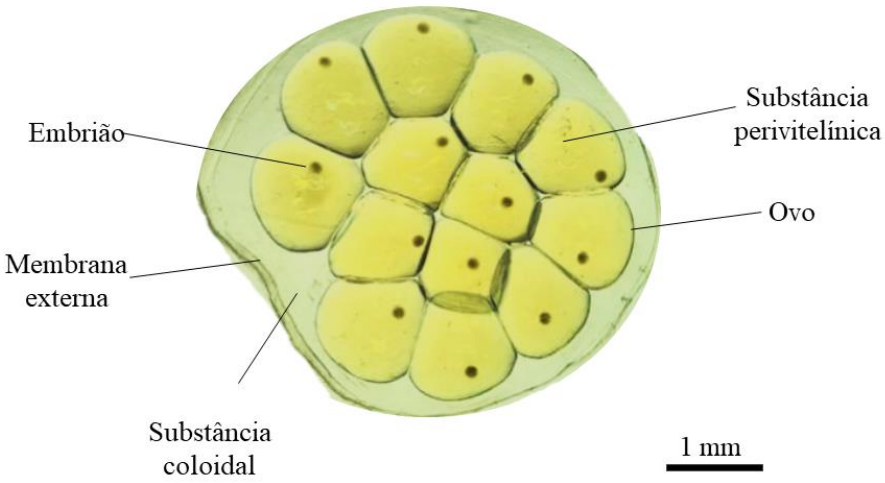


Figura 11. Massa ovígera de *Biomphalaria glabrata.* Fonte: O autor.

Em relação ao desenvolvimento embrionário, ocorre através de sucessivas clivagens espirais, divididas em cinco estádios sequenciais classificados em blástula,

gástrula, trocófora, véliger e hippo estádio (Kawano *et al.*, 2008) (Figura 12). A blástula, ocorre aproximadamente entre a 10ª e 23ª hora após a primeira clivagem, e as divisões mitóticas sem aumento de volume celular (Kawano *et al.*, 2008). A gástrula compreende o período de 24 a 39 horas após a primeira clivagem do ovo, que caracteriza pelo fim da clivagem e início do crescimento, diferenciação e movimentação celular. O estádio trocófora ocorre entre 40ª e 89ª hora após a primeira clivagem, é identificada como a primeira fase larval. Nesta fase inicia-se a movimentação do embrião, a qual inicia lentamente com a progressão de movimentos rápidos (Camey and Verdonk, 1969; Kawano *et al.*, 2008). Os dois últimos estádios são classificados como véliger (120 horas) e hippo estádio (144 horas de idade). Em véliger ocorre a formação e enrolamento da concha, que começa a cobrir uma parte do corpo, desenvolvimento maior do pé, formação dos olhos, aumento do tamanho dos tentáculos. No hippo estádio ocorre a formação completa do embrião e início da eclosão (Kawano *et al*., 2008; Tallarico *et al*., 2014).

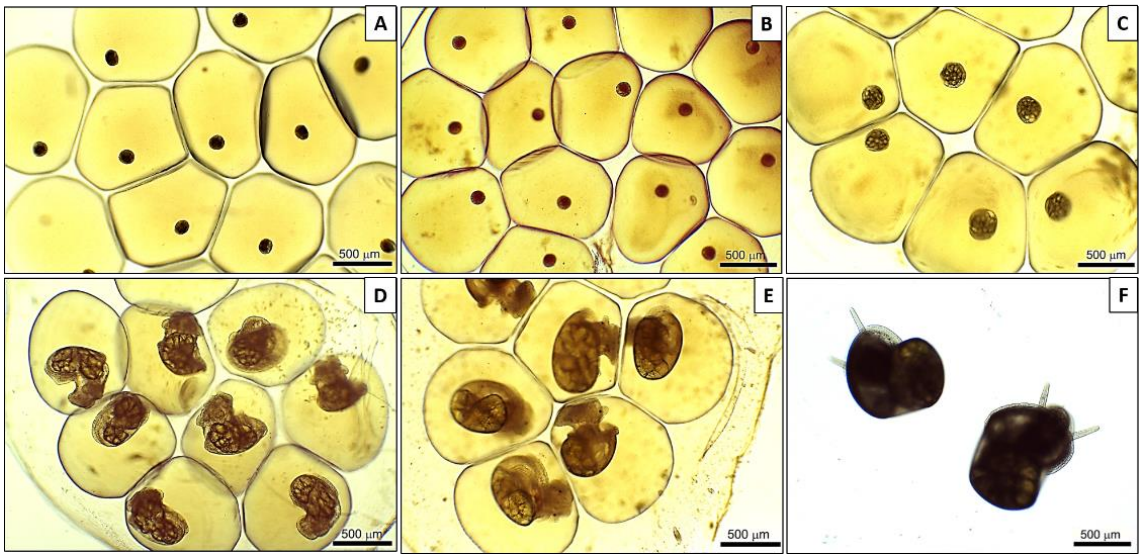


Figura 12. Fases do desenvolvimento de *Biomphalaria glabrata* e os períodos em horas após a primeira clivagem. A) embriões em blástula (10ª e 23ª h); B) embriões em gástrula (24ª a 39ª h); C) embriões em trocófora (40ª e 89ª h); D) embriões em véliger (120 h); E) embriões em hippo estádio (144 h); F) caramujos recém eclodidos. Animais obtidos no Setor de Malacologia do Biotério do Instituto de Patologia Tropical e Saúde Pública (IPTSP) da Universidade Federal de Goiás (UFG). Fonte: o autor. Barra de escala 500 µm.

1.10. Utilização de moluscicidas no controle da esquistossomose

O controle dos caramujos hospedeiros intermediários de *Schistosoma* spp., compreende uma importante medida utilizada nos programas de controle da esquistossomose (King and Bertsch, 2015). Essa prática teve início no Japão em 1918 e desde 1920, aos dias atuais, milhares de substâncias são testadas quanto à atividade moluscicida, como por exemplo, o sulfato de cobre, que teve suas propriedades moluscicidas descobertas sendo utilizado no Egito. Na década de 1940, a cianamida e cálcica pentaclorofenato de sódio também foram utilizadas como moluscicidas, e em 1959, a niclosamida ($C_{13}H_8C_{l2}N_2O_4$) (Baylucide, Bayer, Leverkusen, Alemanha) tornou-se o moluscicida de primeira escolha (Inobaya *et al.*, 2014), sendo nos dias atuais, o único moluscicida comercialmente disponível recomendado pela Organização Mundial da Saúde (OMS) para uso em larga escala em programas de controle da esquistossomose.

No Brasil, a niclosamida é utilizada principalmente no controle do caramujo *B. glabrata*, principal hospedeiro de *S. mansoni*, por apresentar atividade tóxica em todos os estágios de vida do caramujo (Ribeiro *et al.*, 2009). Contudo, há relatos de caramujos que vem sofrendo seleção artificial e propagando linhagens resistentes. Além disso, a niclosamida apresenta um grande impacto ambiental, atingindo outras espécies não alvos, e problema de solubilidade em água (Andrews *et al*., 1982; Buddenborg *et al.*, 2018; Dai *et al.*, 2014; Schall *et al.*, 2001; Zhang *et al.*, 2015). Sendo assim a busca por novas substâncias com potencial moluscicida visando maior especificidade ao caramujo, baixa toxicidade ambiental, além da possibilidade de retirada do ambiente por meio do magnetismo, como é o caso das NOFs, e que sejam economicamente viáveis, poderá contribuir para o controle da esquistossomose em áreas endêmicas, juntamente com outros métodos de controle.

A incorporação de medidas de combate ao caramujo HI, faz parte do plano de diretrizes para o controle da esquistossomose, juntamente com tratamento de doentes, o investimento em infraestruturas de saneamento básico, e a educação em saúde (King and Bertsch, 2015; Savioli and Daumiere, 2012). Esta medida é considerada uma estratégia eficaz, viável, e com efetividade a curto prazo para interromper o ciclo de transmissão da doença em áreas endêmicas com alto risco de infecção, contribuindo assim para redução da morbidade (Li *et al.*, 2019; Secor and Colley, 2018; Sokolow *et al.*, 2016).

Nesse sentido, a nanobiotecnologia e o uso NPs aplicadas ao controle de doenças parasitárias tem sido uma estratégia de grande valia para o tratamento, diagnóstico,

monitoramento e controle dos parasitos e seus hospedeiros intermediários (Zhang *et al.*, 2012). A utilização de nanosistemas se mostrou eficaz na otimização de vacinas e quimioterápicos aplicados ao combate da malária e a toxoplasmose (Assolini *et al.*, 2017; Pimentel *et al.*, 2007), bem como para o reposicionamento de drogas e uso de novos produtos farmacêuticos no combate à esquistossomose (Tomiotto-Pellissier *et al.*, 2017).

Além disso a atividade moluscicida de NMs, tais como Ag NPs, Au NPs, SiO_2 NPs e ZnO NPs foi demonstrada para espécies do gênero *Biomphalaria*, tais como; *B. alexandrina* (Attia *et al.*, 2017; Fahmy *et al.*, 2014; Moustafa *et al.*, 2018), PLA NPs para *B. pfeifferi* (Omobhude *et al.*, 2017), e Ag NPs e CdTe NPs para *B. glabrata* (Oliveira-Filho *et al.*, 2019; Vasconcelos - Lima *et al.*, 2019). Outros efeitos de toxicidade de NMs, tais como; genotoxicidade, mutagenicidade, toxicidade reprodutiva e transgeracional, alterações de comportamento, alterações metabólicas e teciduais, foram verificadas em 21 espécies (aquáticas e terrestres) de gastrópodes HI de importância médica e/ou veterinária, após a exposição a 18 diferentes tipos de NMs, com resultados apresentados em detalhes no capítulo II, , o que suscita a importância da investigação desses NMs como uma nova geração de agentes moluscicidas.

2. JUSTIFICATIVA

A esquistossomose é uma doença tropical negligenciada que afeta mais de 30 mil pessoas no Brasil por ano (DataSUS, 2019) e mais 240 milhões de pessoas no mundo. Além disso, mais de 200 mil mortes foram registradas por ano em todo o mundo (WHO, 2019), e mais de 700 milhões pessoas estão em áreas de risco de infecção. Além disso 2,2 bilhões de pessoas ainda não possuem acesso a saneamento básico e 627 milhões praticam defecação em local aberto (WHO/Unicef, 2019), indicando que o controle desta doença requer um conjunto de ações integradas. O tratamento de doentes, a educação em saúde para a prevenção, e o investimento em infraestruturas de saneamento básico são essenciais para a erradicação da doença, contudo, demandam alto custo financeiro e tempo para implementação, sendo o desenvolvimento de moluscicidas, uma estratégia viável e imediata para interromper o ciclo de transmissão da doença em áreas endêmicas com efetividade a curto prazo (King and Bertsch, 2015).

O desenvolvimento de novos agentes moluscicidas justifica-se pelo fato do único moluscicida presente no mercado atualmente (a niclosamida, recomendada pela OMS desde 1960), apresentar alta toxicidade a biodiversidade aquática, solubilidade limitada em água, e por haver diversos relatos sobre o surgimento de linhagens de caramujos que vem sofrendo seleção artificial e a propagação de linhagens resistentes (Andrews *et al.*, 1982; Buddenborg *et al.*, 2018; Dai *et al.*, 2014; Zhang *et al.*, 2015).

Por apresentar ampla incidência de distribuição, elevada população, e por ser mais suscetível à infecção por *Schistomosoma mansoni* ao apresentar maior taxa de infecção, menor resistência imunológica e maior produção de cercárias, *Biomphalaria glabrata* é considerada a principal espécie hospedeira intermediária do parasito no Brasil (Knight *et al.*, 2000; Scholte *et al.*, 2012). Por este motivo foi escolhida como modelo experimental para avaliação de agentes moluscicidas baseados em NMs.

Os NMs apresentam propriedades altamente interessantes para o desenvolvimento de agentes moluscicidas, como a possibilidade do direcionamento altamente específico a um órgão ou tecido, através da funcionalização ou conjugação com proteínas, anticorpos, lipídeos, enzimas e até mesmo fármacos, além do processo de fabricação relativamente simples e de apresentar um alto rendimento (Kwon *et al.*, 2018; Moustafa *et al.*, 2018; Tomiotto-Pellissier *et al.*, 2017). Apesar disso, estudos básicos ainda são necessários para inferir sua toxicidade e potencial atividade moluscicida.

A utilização de nanopartículas de ferro (NOFs) para o desenvolvimento de compostos moluscicidas justifica-se tanto pelas suas propriedades nanoespecíficas, como pequeno tamanho, alta área de superfície que permitiria o acesso aos tecidos biológicos, além da sua propriedade magnética, que permitiria a retirada do ambiente através da magnetização (Corsi *et al.*, 2018; Lei *et al.*, 2018). Neste sentido, as NOFs apresentam vantagens em relação às AgNPs (que não apresentam propriedades magnéticas), além de apresentar um menor custo para a produção (Kwon *et al.*, 2018; Siddiqi *et al.*, 2018). Além disso, estudos anteriores mostraram toxicidade das NOFs no caramujo terrestre *Cornu aspersum* e aos seus embriões (Besnaci *et al.*, 2019, 2016; Sidiropoulou *et al.*, 2018). Apesar disso, estudos sobre a toxicidade das NOFs em caramujos ainda são bastante limitados.

Sendo assim, estudos básicos para se conhecer a toxicidade das NOFs em caramujos, a fim de possibilitar o desenvolvimento de novos agentes moluscicidas e contribuir para o avanço de estudos posteriores são altamente necessários. Neste sentido, o estudo da toxicidade reprodutiva das NOFs, por meio de uma exposição crônica, poderia contribuir para uma melhor compreensão do efeito da toxicidade dessas NPs a longo prazo no caramujo, bem como, caso venha a apresentar efeito, ser implementada como uma substância para o controle da população dos caramujos *B. glabrata*.

3. OBJETIVO GERAL E OBJETIVOS ESPECÍFICOS

3.1. Objetivo geral

Avaliar a potencial toxicidade reprodutiva e a resposta de múltiplos biomarcadores em *Biomphalaria glabrata* após exposição crônica às nanopartículas de óxido de ferro (NOFs) funcionalizadas com ácido glucônico em comparação com sua contrapartida iônica (cloreto férrico - $FeCl_3$).

3.2. Objetivos específicos

1) Realizar uma análise bibliométrica e uma revisão sistemática da literatura sobre a toxicidade e atividade moluscicida dos nanomateriais (NMs) em diferentes espécies de gastrópodes.

2) Sintetizar e caracterizar as NOFs funcionalizadas com ácido glucônico quanto ao diâmetro individual, potencial zeta e tamanho hidrodinâmico através das técnicas de microscopia eletrônica de transmissão (MET), e espalhamento de luz eletroforético (ELS) e espalhamento de luz dinâmico (DLS).

3) Analisar se a exposição crônica (28 dias) dos caramujos adultos *B. glabrata* às NOFs e ao $FeCl_3$ induz a bioacumulação de ferro na massa visceral e concha dos caramujos por meio da técnica de digestão ácida do tecido e quantificação por espectrometria de absorção atômica.

4) Determinar se a exposição crônica dos caramujos adultos *B. glabrata* às NOFs e ao $FeCl_3$ induz alterações na resposta de múltiplos biomarcadores, tais como na taxa de mortalidade, biometria (taxa de crescimento, índice de condição corporal, índice gonadossomático), alterações no comportamento e fecundidade (número de massas ovígeras/caramujos e ovos/massas).

5) Avaliar se a exposição crônica dos caramujos adultos *B. glabrata* às NOFs e ao $FeCl_3$ induz a toxicidade transgeracional por meio da coleta de massas ovígeras e análise dos

embriões desses caramujos, tendo como biomarcadores: (*i*) taxa de mortalidade (*ii*) taxa de eclosão e (*iii*) frequência de atrasos no desenvolvimento dos embriões.

4. METODOLOGIA DETALHADA

Os resultados do presente trabalho foram divididos em dois artigos. O primeiro artigo foi uma bibliometria associada a uma revisão sistemática da literatura intitulado *"Toxicity of engineered nanomaterials to aquatic and land snails: a scientometric and systematic review"*. O segundo artigo consistiu em um estudo experimental, o qual foi intitulado *"Iron oxide nanoparticles and ferric chloride induce bioaccumulation, behavioral impairments and reproductive toxicity to Biomphalaria glabrata (Say 1818) after chronic exposure"*. A metodologia geral e detalhada destes artigos será apresentada aqui de forma conjunta.

4.1. Metodologia do artigo de revisão

Uma revisão cientométrica e sistemática da literatura foi realizada usando o banco de dados Thomson Reuters ISI Web of Science, ScienceDirect, PubMed, Scopus e Scielo. As palavras-chave "nanoparticles" e "nanomaterials" foram combinadas com "snail" e "gastroprods, de forma singular e plural, para recuperar as informações nas bases de dados. 1873 artigos foram encontrados com os termos de busca, após alguns critérios de inserção e eliminação foram empregados ; teses acadêmicas, relatórios técnicos, artigos de revisão e resumos de eventos científicos foram excluídos desta revisão. Foram selecionados assim, 750 artigos para leitura dos títulos e resumos e 60 artigos publicados de 2010 (primeiro registro) a dezembro de 2019 atenderam o critério de inclusão. Estes artigos foram lidos na integra, e em seguida analisados sistematicamente e resumidos de acordo com os seguintes parâmetros: (*i*) ano de publicação; (*ii*) coordenadas geográficas onde o estudo foi realizado (a origem de cada estudo foi identificada no endereço para correspondência do primeiro autor); (*iii*) espécies e habitat dos caramujos; (*iv*); tipo de NMs (*v*) condições de exposição, como tempo e concentração; (*vi*) órgão ou tecido analisado; (*vii*) bioacumulação e (*viii*) múltiplas respostas de biomarcadores moleculares, bioquímicos, celulares, teciduais, sistêmicos e a nível de organismo, conforme Rocha *et al.* (2017, 2015) (Figura 13). Os NMs foram classificados em três categorias (inorgânica, orgânica e polimérica), de acordo com Gaillard *et al.* (2019).

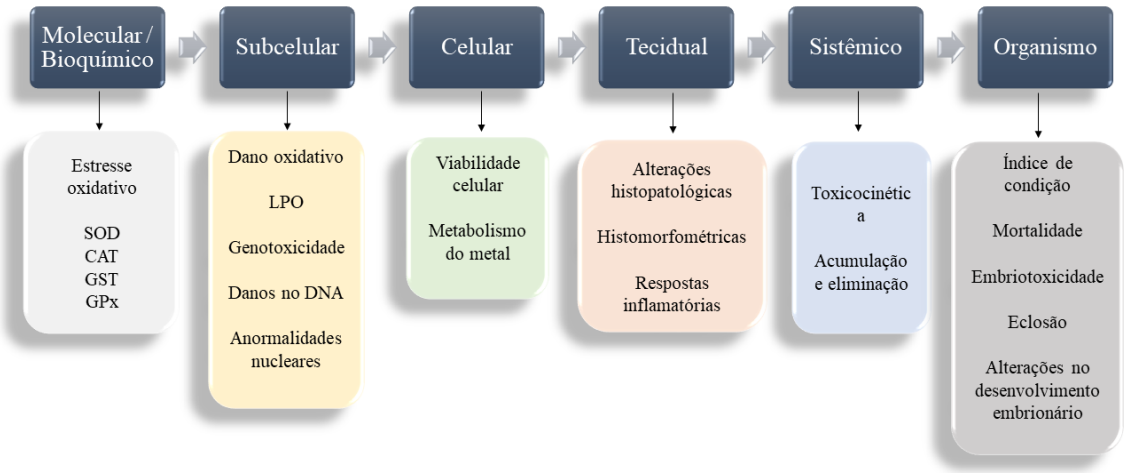


Figura 13. Níveis de organização biológica e os biomarcadores de toxicidade analisados em caramujos expostos a diferentes nanomateriais através da revisão sistemática da literatura. SOD (superóxido dismutase), CAT (Catalase), GST (Glutationa S-transferase), GPx (Glutationa peroxidase), LPO (Peroxidação lipídica).

4.2. Metodologia do artigo experimental

4.2.1. Síntese das NOFs

As NOFs funcionalizados com ácido glucônico (AGL) foram preparados por co-precipitação alcalina no Laboratório de Química de Materiais e Modelagem Molecular da Universidade Estadual de Goiás (UEG), com base nos métodos descritos por Wei *et al.* (2011) e Sui *et al.* (2012) com modificações. Resumidamente, uma solução aquosa de 0,1296 mol de cloreto ferroso ($FeCl_2.4H_2O$) e 0,2591 mol de cloreto férrico ($FeCl_3.6H_2O$), com uma razão molar de Fe^{3+} / Fe^{2+} = 2, foi adicionada à 400 mL de água purificada eletrodeionizada ASTM tipo II. Em seguida, foram adicionados 600 mL de NH_4OH a 2 mol L^{-1} sem agitação e um precipitado preto foi formado instantaneamente. O precipitado foi então decantado magneticamente com um ímã de neodímio e lavado várias vezes com água até o sobrenadante atingir pH 7,0. Uma solução aquosa de 2,3 mmol de sal de ácido glucônico de sódio ($C_6H_{11}NaO_7$) e 12,5 mmol de NaOH em água purificada (100 mL) foi adicionada ao precipitado de NOFs e mantida sob agitação magnética a 80 ° C por 4 horas. O precipitado resultante foi purificado por centrifugação usando etanol a 70% como solvente de lavagem até o sobrenadante atingir pH 7,0. Posteriormente, o material foi seco em um forno a vácuo a 60 ° C por 4 horas, até a

formação de um pó seco (Figura 14). A equação 6 descreve a reação de formação das NOFs (Wei *et al.*, 2011).

Equação 6. *Reação de formação das NOF:*

$$FeCl_2 + 2\ FeCl_3 + 8\ NH_4OH \rightarrow Fe_3O_4 + 8\ NH_4Cl + 4\ H_2O$$

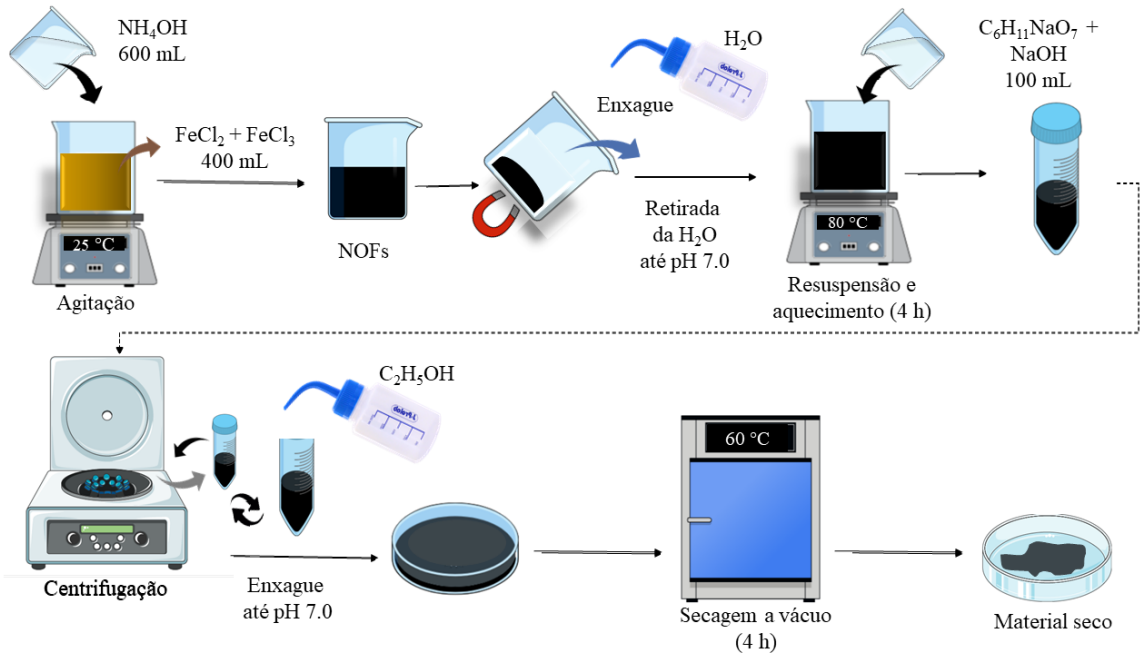


Figura 14. Esquema ilustrativo representando a síntese das nanopartículas de óxido de ferro e a funcionalização com ácido glucônico (AGL). As dimensões são meramente ilustrativas. Fonte: o autor.

Uma dispersão estoque das NOFs a 10000 mg L^{-1} foi preparada em água ultrapura (Milli-Q). Posteriormente, quatro concentrações (1,0; 2,5; 6,2; e 15,6 mg L^{-1}) foram preparadas em água desclorada para utilização no bioensaio de toxicidade, acrescendo ao procedimento a sonicação da dispersão estoque por 10 min em banho ultrassom, antes da "diluição" das dispersões de menor concentração.

4.2.2. Caracterização das NOFs

A morfologia e o diâmetro individual das NOFs foram caracterizados por Microscopia Eletrônica de Transmissão (MET) no Laboratório Multiusuário de Microscopia de Alta Resolução (LabMIC) do Instituto de Física da Universidade Federal de Goiás (UFG). Uma gota da solução estoque de NOFs (0,3 mg L^{-1}) foi depositada em

uma grade de malha de cobre revestida com uma camada de carbono e seca à temperatura ambiente (25 ° C). As micrografias eletrônicas foram obtidas em um microscópio JEOL (JEM-2100), utilizando o software Scandium da Olympus Soft Imaging Solutions GmbH. O diâmetro individual das NOFs (n = 250 partículas) foi determinado usando o software ImageJ (National Institute of Health, EUA). A carga superficial (potencial zeta) e o diâmetro hidrodinâmico (Dh) das NOFs (0,3 mg L^{-1}) em água ultrapura (água Milli-Q) e água desclorada (meio de exposição) foram analisados por espalhamento dinâmico de luz (DLS) e dispersão luz eletroforética (ELS), respectivamente, usando um Malvern ZetaSizer (Nano-ZS90) no Laboratório Central Analítica do Instituto de Química da UFG. Para essas análises, a solução de NOFs foi previamente sonicada por 10 minutos, como relatado por Qualhato *et al.* (2017).

A funcionalização das NOFs pelo AGL foi confirmada por espectroscopia no infravermelho em pastilha de KBr (IR-KBR) no Laboratório de Análise Instrumental da UEG. As pastilhas de NOFs foram preparadas com amostras de (p / p) em brometo de potássio (KBr). Os espectros foram registrados na faixa correspondente à região do infravermelho médio (400 - 4000 cm^{-1}) com uma resolução de 4 cm^{-1} usando o espectrômetro FT-IR (Perkin-Elmer). Além disso, as NOFs em pó foram caracterizados por difração de raios X (DRX) no Instituto de Geociências da Universidade de Brasília (UnB), usando um difratômetro de raios X modelo Rigaku D / Max-2ª / C com radiação CuKα (λ = 1,54184 Å), operando a 15 mA e 35 Kv, varredura velocidade de 2,0 ° min^{-1}, com dados medidos a cada 0,01 ° na faixa de 10 ° ± 2 ° a 80 ° As NOFs também foram caracterizadas por espectroscopia Mössbauer no Laboratório de Ciências de Materiais do Instituto de Física da UnB, usando um espectrômetro Wissel. As medições de transmissão de Mössbauer foram realizadas usando uma fonte de radiação ^{57}Co na matriz de ródio (Rh). As medidas foram realizadas em temperatura ambiente (25 °C), conforme recomendado por Dutra *et al.* (2017).

4.2.3. Cloreto férrico ($FeCl_3$)

O cloreto férrico ($FeCl_3 . 6H_2O$) (CAS n° 10025-77-1) foi obtido comercialmente da empresa Labsynth Produtos para Laboratórios Ltda (São Paulo, Brasil). Uma solução estoque de 10000 mg L^{-1} foi preparada com água ultrapura (Milli-Q). A seguir, quatro

concentrações (1,0; 2,5; 6,2; e 15,6 mg L^{-1}) foram diluídas a partir da solução estoque para utilização no bioensaio de toxicidade.

4.2.4. Obtenção dos animais

Caramujos adultos *Biomphalaria glabrata* com 4 meses de vida, (peso total 0.28 ± 0.04 g e diâmetro da concha de 10 ± 2 mm) utilizados neste estudo foram obtidos no Setor de malacologia do Biotério do Instituto de Patologia Tropical e Saúde Pública (IPTSP) da UFG. Os caramujos eram mantidos sobre condições laboratoriais controladas conforme Jannotti-Passos *et al.*, (2008), e recomendações da Organização para a Cooperação e Desenvolvimento Econômico (OECD) *guideline* nº 243 (OECD, 2016). Os animais eram mantidos em tanques com capacidade de 40 L preenchidos com 30 L de água desclorada (3 caramujos L^{-1}) sob fotoperíodo controlado (12 h claro / 12 h escuro), temperatura (25 ± 1 ºC) e pH (7,0 ± 1). Os caramujos eram alimentados *ad libitum* com folhas de alface orgânica (*Lactuca sativa*).

4.2.5. *Desing* Experimental

Os caramujos foram expostos as NOFs funcionalizados com AGL e sua contrapartida dissolvida ($FeCl_3$) em diferentes concentrações de ferro (1,0, 2,5, 6,2 e 15,6 mg L^{-1}) em aquários de vidro de 3 L (19,5 x 9,5 x 14,5 cm) contendo 2 L de dispersão / solução final (10 animais por aquário ; 5 caramujos L^{-1}), em conjunto com um grupo controle mantido em água desclorada, durante 28 dias. A exposição foi realizada em triplicata (10 caramujos por réplica, 30 caramujos por grupo experimental), mantidos sob condições ambientais controladas (temperatura: 25 ± 1 ºC e 12/12 h ciclo claro / escuro).

O meio de exposição foi trocado a cada três dias com reposição das duas concentrações de ferro (OECD, 2016; Oliveira-filho *et al.*, 2016). Os caramujos foram alimentados com folhas de alface (*L. sativa*) (100 mg por caramujo) a cada três dias. Os resíduos alimentares e fecais foram removidos em cada troca de meio.

As concentrações de NOFs utilizadas estão de acordo com a concentração de ferro detectada no ambiente aquático (até 5 mg L^{-1}), de acordo com o Conselho Nacional do Meio Ambiente (CONAMA), Resolução nº 357 (Brasil, 2005) e as quantidades de ferro permitidas para descarte de efluentes no Brasil (até 15 mg L^{-1}), Resolução CONAMA nº

430 (Brasil, 2011). Nos baseamos ainda em estudos prévios que analisaram concentrações semelhantes durante a exposição aguda e a longo prazo de organismos aquáticos expostos as NOFs (Kaloyianni *et al.*, 2020; Oliveira-filho *et al.*, 2016; Villacis *et al.*, 2017).

Para análise da influência das NOFs na reprodução dos caramujos, um pedaço de isopor (5 x 10 cm) foi colocado na superfície dos aquáriose massas ovígeras foram coletas durante o período de exposição , como relatado por Duarte *et al.* (2015). Análsies da mortalidade, comportamento, biomarcadores somáticos (taxa de crescimento, índice de condição, índice gonadossomático), e reprodução (fencundidade) de caramujos adultos e o desenvolvimento de seus embriões (fertilidade) foram realizados diariamente durante 28 dias. Ao final do período de exposição (28 dias) foi analisada a bioacumulação de metal nos caramujos. A Figura 15 descreve de modo resumido o delineamento experimental e as análises realizadas no presente estudo.

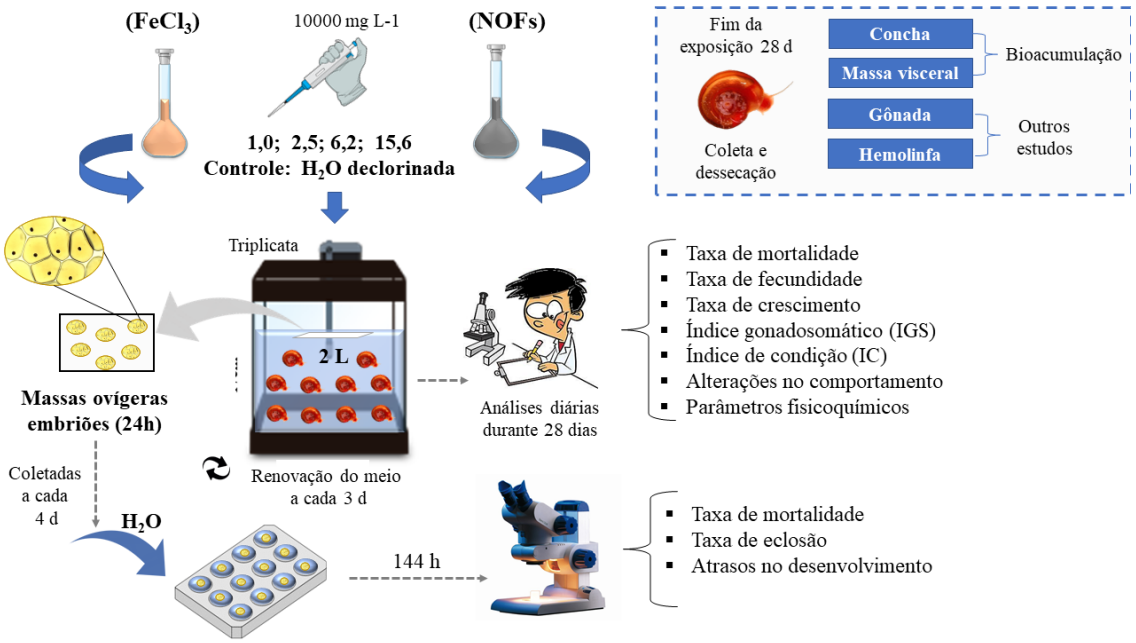


Figura 15. Esquema ilustrativo representando o delineamento experimental para análise da toxicidade reprodutiva e respostas de múltiplos biomarcadores no caramujo *Biomphalaria glabrata* após exposição crônica (28 dias) às nanopartículas de óxido de ferro (NOFs) funcionalizadas com ácido glucônico e sua contrapartida dissolvida ($FeCl_3$). As cores e dimensões são meramente ilustrativas. Fonte: o autor.

4.2.6. Bioacumulação

Tecidos moles e conchas de caramujos adultos foram coletados ao final do período de exposição (28 dias), lavados em água ultrapura e divididos em 3 *pools* (cada *pools* contendo 3 caramujos; 9 caramujos por condição experimental). As amostras foram desidratadas a 70 ºC por 48 h , pulverizados com almofariz e pistilo e tamizado com uma peneira de malha de 80 *mesh* (0,18 mm) conforme Silva *et al.* (2010). De cada amostra utilizou-se 0,03 g do pulverizado, os quais foram submetidas a digestão ácida em 1 mL de ácido nítrico (HNO_3) e 100 µL de H_2O_2 em tubo de ensaio do tipo DQO à 150 °C em um bloco digestor por 30 min com a tampa do tubo fechada. Após a completa digestão, os tubos foram abertos até a evaporação do ácido nítrico próximo à secura. Os tubos foram então lavados várias vezes com água ultrapura e seu conteúdo transferido para um balão de 25 mL para aferição do volume. A concentração de ferro foi determinada por espectrometria de absorção atômica e uma curva padrão analisada com diferentes concentrações de ferro (0,125, 0,25, 0,5, 1, 2, 4, 8 ppm) de metal de referência certificado (PA Fe 1000 ppm, Qhemis High Purity NIST Test: # 822 / 275197-07). As concentrações de ferro foram expressas em µg mg^{-1} de peso seco (média mais desvio padrão de cada conjunto de triplicatas). As vidrarias utilizadas neste experimento foram previamente preparadas da seguinte forma: Submersas em solução HNO_3 10 % por 24 h, posteriormente lavadas em água deionizada e colocadas para secar em temperatura ambiente.

4.2.7. Mortalidade

A taxa de mortalidade acumulativa (TM) dos caramujos adultos foi determinada diariamente durante os 28 dias de exposição de acordo com a equação (7) (OECD, 2016):

Equação 7. *Fórmula para o cálculo da taxa de mortalidade:*

$$TM = \frac{CVI}{MC} \text{ x } 100$$

onde TM é a taxa de mortalidade acumulativa, CVI o número de caramujos vivos iniciais, e MC é a mortalidade acumulativa. Como critérios de mortalidade dos caramujos foram

utilizados os seguintes parâmetros: evidente estado de decomposição, liberação de hemolinfa, e quando recluso ausência de batimentos cardíacos, assim como realizado por Oliveira-filho *et al.* (2010, 2016), Rapado *et al.* (2011) e Melo *et al.* (2019).

4.2.8. Biomarcadores somáticos

No início do experimento (T= 0) e após 15 e 28 dias de exposição, a taxa de crescimento (TC) foi calculada pela diferença entre o diâmetro inicial da concha e o diâmetro final. Além disso, o índice de condição corporal (ICC) foi calculado de acordo com equação 8, sendo PC = peso corporal total (g) e DC = diâmetro da concha (mm) aos 28 dias da exposição (Avila-Poveda, 2013). O índice gonadossomático (IGS) dos caramujos foi calculado usando equação 9, sendo PG= Peso da gônada (g) e PC = peso corporal total (g) (Devlaming *et al.*, 1982).

Equação 8. *Fórmula para o cálculo do índice de condição corporal*

$$ICC = \frac{PC}{DC}\ X\ 100$$

Equação 9. *Fórmula para o cálculo do índice gonadossomático*

$$IGS = \frac{PG}{PC}\ X\ 100$$

4.2.9. Alterações no comportamento

As alterações comportamentais dos caramujos adultos foram analisadas diariamente durante o período de exposição (28 dias), no período da manhã, antes dos procedimentos de rotina, como a remoção de massas ovígeras, troca de água ou alimentação. A observação comportamental foi feita em cada aquário, aproximadamente 1 minuto por indivíduo, totalizando 10 min por réplica e 30 min por condição experimental. A frequência (%) das seguintes alterações comportamentais foi determinada: nadar na superfície da água, reclusão na concha, evitar a água, letargia e desprender por completo da concha seguido de morte (Fig .16) (Jurberg *et al*., 1988; Pieri

and Jurberg, 1981). Os resultados foram apresentados como a média semanal dessas alterações comportamentais.

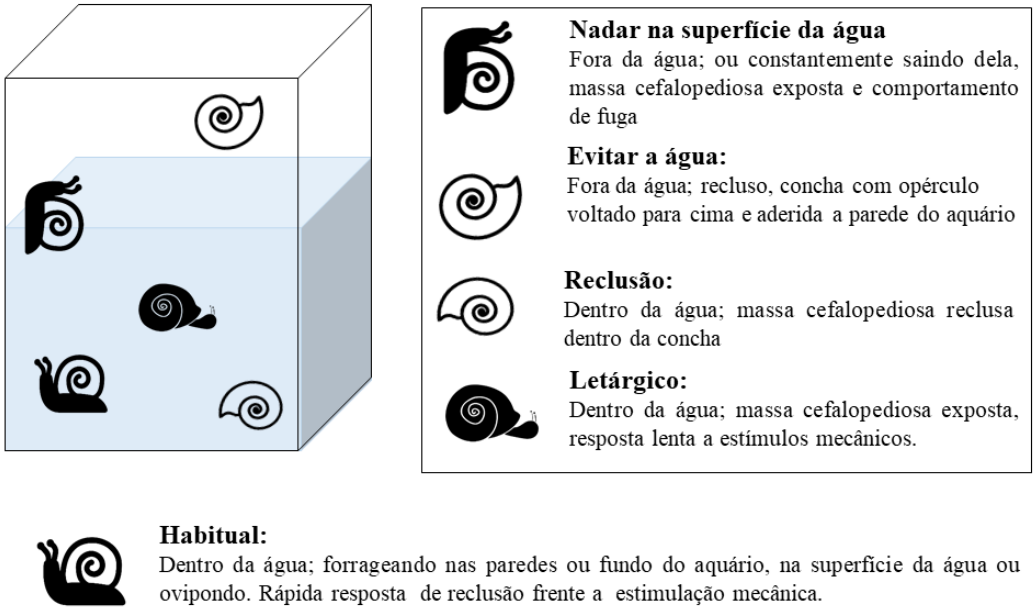


Figura 16. Alterações no comportamento dos caramujos adultos *Biomphalaria glabrata*, avaliados durante 28 dias de exposição às NOFs e ao $FeCl_3$.

4.2.10. Fecundidade

A fecundidade é definida pelo teste de reprodução nº 243 da OCDE, como "taxa reprodutiva real de organismos, medida pelo número de massas ovígeras ou ovos", enquanto a fertilidade ou reprodução a partir da "produção de filhotes por animais parentais durante o período de teste" (OECD, 2016; Schmitt *et al.*, 2010). Nesse sentido, as massas ovígeras dos caramujos dos parentais (geração F_0) expostas às duas formas ferro, e aos grupos controle durante 28 dias, foram coletadas diariamente e analisadas em estereomicroscópio (Zeiss, Stemi DV4) para os seguintes parâmetros: número de massas ovígeras por caramujo vivo (F1), número de ovos por massa ovígeras (F2). A fecundidade foi determinada usando as equações 10 e 11 (OECD, 2016), os dados foram apresentados como valor acumulativo.

Equação 10. *Fórmula para o cálculo do número de ovos por caramujo vivo:*

$$F1 = \frac{CV\ (tx)}{MO\ (tx)}$$

Equação 11. *Fórmula para o cálculo do número de ovos por massa ovígeras:*

$$F2 = \frac{NTO\ (tx)}{EM\ (tx)}$$

sendo CV = número de caramujos vivos, MO = massas ovígeras produzidas, NTO = número total de ovos (viáveis e inviáveis), TOI = total de ovos inviáveis e tx = valores no dia de exposição "X".

4.2.11. Toxicidade no desenvolvimento da primeira geração (fertilidade)

No início do experimento e a cada quatro dias (4, 8, 12, 16, 20 e 24 dias), massas ovígeras (n = 3 por réplica; n = 9 por condição experimental) de caramujos parentais (expostos a ferro e grupo controle) foram coletadas (Figura 15), transferidas para microplacas de 12 poços (1 massa ovígeras por poço) contendo 5 mL de água desclorada e mantidas sob condições controladas de temperatura (27 ± 0,5 °C), umidade (75 ± 5 %) e fotoperíodo (12h / claro: 12h / escuro) utilizando uma incubadora BOD (SL-224). As massas ovígeras foram analisadas diariamente usando um microscópio (Leica DM750) associado à câmera Leica modelo ICC50 HD e ao software LAS EZ. Os biomarcadores para toxicidade do desenvolvimento foram: taxa de mortalidade embrionária, taxa de eclosão e inibição nos estágios de desenvolvimento.

A taxa de mortalidade embrionária (%) foi determinada de acordo com a equação 12 (OECD, 2016; de Oliveira Melo *et al.*, 2019). Sendo TME a taxa de mortalidade dos embriões, CM os caramujos mortos ao fim de 144h, e EVI o total de embriões vivos iniciais, na massa ovígera. Embriões mortos foram identificados de acordo com Oliveira-filho *et al.* (2010): formas embrionárias em desintegração, embrião sem movimentos rotacionais, sem movimentos dos pés ou com ausência de batimentos cardíacos.

Equação 12. *Fórmula do cálculo da taxa de mortalidade dos embriões*

$$TME = \frac{CM}{EVI} \text{ x } 100$$

A taxa de eclosão (TE) (%) foi determinada de acordo com a equação 13, sendo CE= caramujos eclodidos após 144 h e EVI = total de embriões vivos iniciais (OECD, 2016; de Oliveira Melo *et al.*, 2019). Os estágios iniciais de desenvolvimento foram classificados conforme descrito por Rapado *et al.* (2013) e Melo *et al.* (2019): blástula, gástrula, trocófora, véliger, hipoestádio e animais eclodidos (Figura 12), e a frequência relativa de cada estágio de desenvolvimento por massa ovígera foi determinada.

Equação 13. *Fórmula para o cálculo da taxa de eclosão:*

$$TE = \frac{CE}{EVI} \text{ x } 100$$

4.2.12. Análises estatísticas

As análises estatíticas foram conduzidas no software RStudio (RStudio team, 2015). Testes de normalidade e homocedasticidade foram realizados utilizando o teste de Shapiro-Wilk e Levene, respectivamente. Para os parâmetros IC, IGS e fecundidade (F1 e F2), os dados foram paramétricos portanto, foi executado anova two-way com teste *a posteriori* de Tukey. Para a análise de TC, foi realizado o teste não paramétrico de Kruskall Wallis. Para os parâmetros mortalidade, bioacumulação, toxicidade no desenvolvimento da primeira geração e biomarcadores comportamentais, os dados foram não paramétricos, sendo assim foi realizado o teste ANOVA Robusta de dois fatores (Kloke & McKean, 2015) com o teste a *posteriori* de Dunn (Fig.17). Os resultados são apresentados como média e desvio padrão das réplicas de cada experimento e o nível de significância de $p < 0,05$ foi adotado para todas as análises.

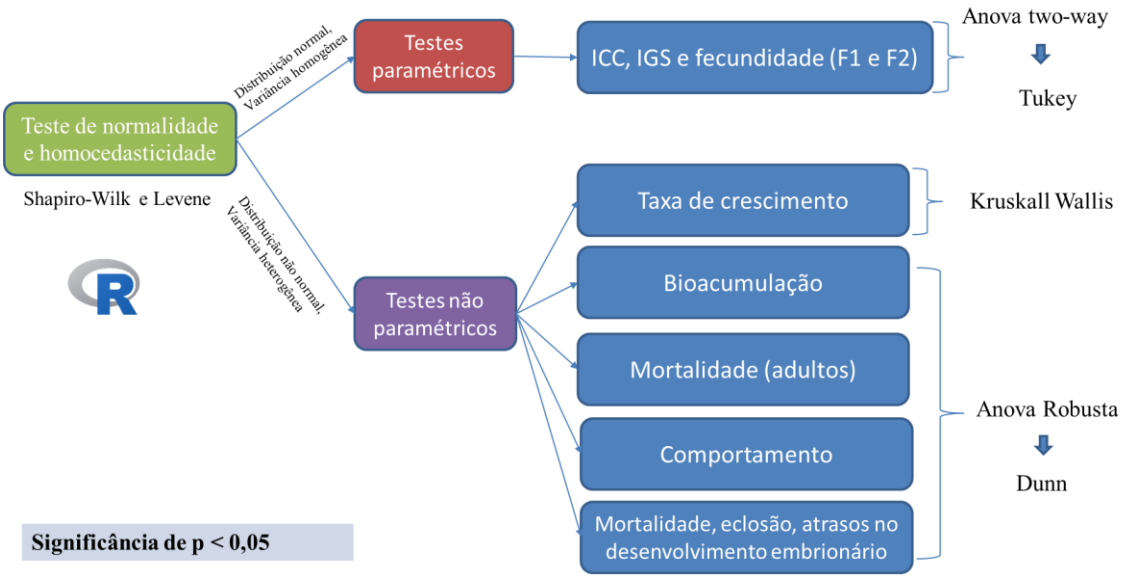
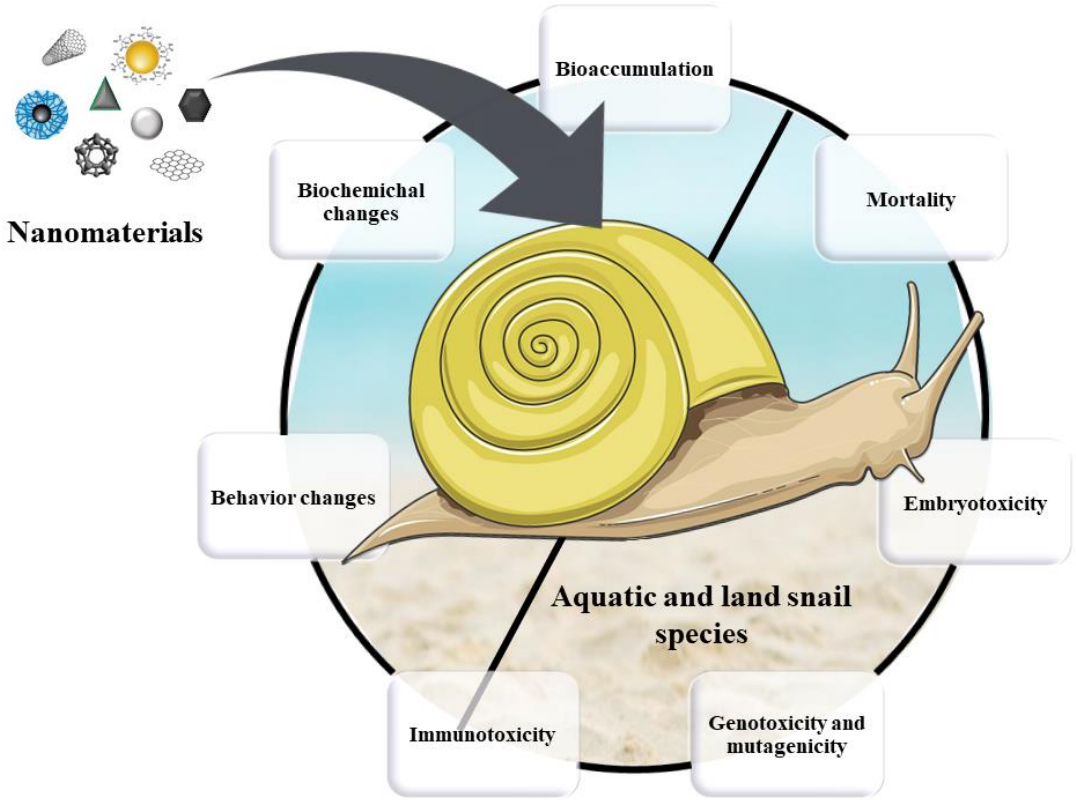


Figura 17. Esquema ilustrativo representando as análises estatísticas realizadas para cada biomarcador do caramujo *Biomphalaria glabrata*. ICC (índice de condição corporal), IGS (índice gonadossomático).

5. CAPÍTULO II: ARTIGO DE REVISÃO

CAPÍTULO II

Toxicity of engineered nanomaterials to aquatic and land snails: a scientometric and systematic review



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Toxicity of engineered nanomaterials to aquatic and land snails: a scientometric and systematic review

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Highligths

- State-of-the-art review of the nanomaterial (NM) toxicity in snails.
- Mechanism of action and toxicity of NMs in snails are revised.
- NMs as potential molluscicide.
- Snail as suitable model system to assess the NM toxicity.

ABSTRACT

The emerging growth of nanotechnology has attracted great attention due to its application in the parasite and intermediate host control. However, the interest use of nanomaterials (NMs) in the snail control is just starting, while the knowledge concerning their mechanism of action (MoA) and toxicity to snails remain unclear. In this context, the present study revised the historical use of snails as experimental models in nanotoxicological studies and summarized the MoA and toxicity of NMs in aquatic and land snails. The data concerning the bioaccumulation, reproductive and transgenerational toxicity, embryotoxicity, genotoxicity and potential molluscicidal activity of NMs were revised. Furthermore, the data about the experimental conditions, such as exposure time, concentrations, cell and tissue-specific responses, snail species and nanoparticle types are discussed. Revised data showed that the toxic effects of NMs were reported for 21 snail species with medical, veterinary and ecological importance. The toxicity of NMs to snail is dependent on the physical and chemical properties of NMs, as well as their environmental transformation and experimental design. The bioaccumulation of NM on snails was related to several toxic effects, such as reactive oxygen species (ROS) production, oxidative stress, following by oxidative damage to DNA, lipids and protein, while the NM metabolism in snails remain unknown. Futhermore, significant research gaps and recommendations for future researches are indicated. The present study is the first scientometric and systematic review concerning the toxic effects NMs to snail species and confirms that snails are suitable model system to assess the nanotoxicity.

Key-words: nanotechnology; nanotoxicology; nanoparticle; molluscicide; ecotoxicity; biomarker.

5.1. Introduction

A nanomaterial (NM) is defined as "a natural or manufactured material containing disseminated or aggregated particles with a particle size distribution according with > 50 % cases between 1 to 100 nm in one dimension, at least" (European Commission, 2011). Currently, NMs are used in various industries and society sectors, such as the textile, agricultural, construction and electronic businesses, notably in healthcare companies with extensive applications, such as the development of several therapy, disease diagnosis, prevention, and treatment technologies, vaccines and drugs progress and cosmetics with antimicrobial properties (Corsi *et al.*, 2018; Gupta and Gupta, 2005; Tarafdar *et al.*, 2013).

Due to the increasing application of NMs in human health and in various segments of society, numerous studies on toxicity and ecotoxicity of these NMs have gained scientific distinction, mainly related to human and environmental health (Chaturvedi and Dave, 2018; Fadeel *et al.*, 2018; Nel *et al.*, 2013). The toxicity of NMs to vertebrate and invertebrate models have been reviewed (Amaral *et al.*, 2019; Bondarenko *et al.*, 2013; Pereira *et al.*, 2019; Rocha *et al.*, 2015; Wu and Tang, 2018), while the knowledge concerning the mechanism of action (MoA) and toxicity of NMs on snails is scarce. Snails (Gastropoda) are invertebrates from terrestrial and aquatic habitats with an ecological and economic importance, such as a food source (Resh and Rosenberg, 2015; Strong *et al.*, 2007). However, some snail species have been considered agricultural pests and intermediate hosts (IH) of parasites of medical and veterinary importance (Barker, 2002; Dunn, 2018; Feshchenko *et al.*, 2019; Lu *et al.*, 2018).

Parasites belonging to the Digenea subclass such as *Schistosoma* spp. (Colley *et al.*, 2014), *Fasciola* spp. (Saba *et al.*, 2004), *Ascocotyle* (*Phagicola*) *longa* (Alda *et al.*, 2015), *Clonorchis sinensis* (Dietrich *et al.*, 2018) and *Clinostomum complanatum* (Simsek *et al.*, 2018), havesnails as the first host in their biological cycle (Cribb *et al.*, 2003), For the metastrongiloids and rabditids from the phylum Nematode, snails are intermediate hosts from at least 61 nematode species which develop an infective larval stage transmissible to vertebrates, with 49 species are Metastrongyloidea (Order: Strongylida), and of the 47 species that use snails as definitive hosts, 33 belong to the Order: Rhabditida Chitwood, 1933 (Grewal *et al.*, 2003). Accordingly, nematodes from the Metastrongyloidea superfamily, before reaching the vertebrate host, develop their initial larval stages in terrestrial and/or freshwater gastropod snails (Malek, 2018).

Several NMs have been indicated to control parasites and IH of global infections and neglected tropical diseases caused by helminths, protozoa, fungi, viruses and bacteria (Adair, 2009; Benelli, 2018a, 2018b; Benelli *et al.*, 2017; Kumar *et al.*, 2017; Siddiqi *et al.*, 2018; Tomiotto-Pellissier *et al.*, 2017). In this context, the aim of this study was to evaluate the use of gastropod snails as experimental models in the nanotoxicity and to summarize the MoA and toxicity of NMs in aquatic and land snails. This review summarizes the data available in the scientific literature concerning the bioaccumulation, reproductive and transgenerational toxicity, embryotoxicity, genotoxicity and potential molluscicidal activity of NMs. Furthermore, the data about the experimental conditions, such as exposure time, concentrations, cell and tissue-specific responses, snail species and nanoparticle types are discussed, as well as significant research gaps and recommendations for future researches are indicated.

5.2. Methodological approach

A scientometric and systematic review was carried using the Thomson Reuters ISI Web of Science database, ScienceDirect, PubMed, Scopus and Scielo. The keywords "nanoparticles" and "nanomaterials" were combined with "snails" and "gastroprods", singular and plural form, to save information in the databases. Some insertion and elimination criteria was employed, and academic theses, technical reports, review articles and scientific events summaries were excluded from this review. A total of 60 papers published from 2010 (first record) to December 2019 were selected, systematically analyzed and summarized according to the following parameters: (*i*) year of publication; (*ii*) geographical coordinates where the study was performed (the origin of each study was identified from the mailing address of the first author); (*iii*) snail species and habitat; (*iv*); type of NMs (*v*) exposure conditions, such as time and concentration; (*vi*) organ or tissue analyzed; (*vii*) bioaccumulation; (*viii*) multiple biomarker responses. NMs were classified into three categories (inorganic, organic and polymeric) according to Gaillard *et al.* (2019).

5.3. Snails as model system

The gastropods have been indicated as suitable invertebrate model-system to assess the toxicity and ecotoxicity of NMs (Amorim *et al.*, 2019; Kaloyianni *et al.*, 2020;

Oliveira-Filho *et al.*, 2017; Salmi *et al.*, 2017; Zhang *et al.*, 2012), specially due to their advantages, such as easy to obtaining and keeping under laboratory conditions, small size, high egg production, short life cycle, adaptation to different environments, some species have transparent embryos and described genome, sensitive to water and sediment contamination, high susceptibility to anthropogenic factors, high distribution worldwide, and the possibility of analysing multiples biomarkers. Furthermore, snails are an ethically acceptable alternative animal model and regulatory biochemical pathways homologous to vertebrate systems (Duft *et al.*, 2007; OECD, 2016; Oliveira-Filho *et al.*, 2017; Ottaviani, 2015; Ruppert *et al.*, 2017). Snails were also used as a model system in immunology studies (Boisseaux *et al.*, 2018, 2017; Coustau *et al.*, 2015; Matozzo and Gagné, 2016), reproductive and developmental biology (Khangarot and Das, 2010; Pirger *et al.*, 2018), neurobiology, especially in learning and memory formation (e.g. *Lymnaea stagnalis*) (Gainutdinova *et al.*, 2005; Kandel, 2001; Young *et al.*, 2017). In neurophysiology and behavioral ecology, *Helix pomatia, Aplysia californica*, terrestrial and marine species, were used as models, respectively, (Leonard and Lukowiak, 1986; Willows, 1973; Zaitseva, 1994).

Terrestrial gastropods are considered agricultural pests, causing economic losses by attacking horticulture, ornamental plants and forestry, while several snail species have medical and veterinary importance because they act as intermediary hosts in the cycle of parasitic helminths that cause various diseases in humans and other animals. Among the parasites transmitted by snails, several helminthiases are caused by representatives of the subclass Digenea, such as schistosomiasis, which has as an intermediate host snails of the genus *Biomphalaria*, *Fasciola* sp. transmitted by *Lymnaea* spp., as well as other parasitosess: Fasciolasis (Alda *et al.*, 2015), Clonorchiasis (Dietrich *et al.*, 2018), Clinostomiasis (Simsek *et al.*, 2018), in addition to the nematode *Angiostrongylus*, transmitted by terrestrial and freshwater snails (Brummaier *et al.*, 2019; Howe *et al.*, 2019; Tunholi-Alves *et al.*, 2019). The Table 1. *Snail species analysed in this review study and their medical or veterinary importance, as well the importance as a pest species in agriculture or as food resource.*, summarizes all species of snails found in this review and their relation with human importance like transmission of parasites of medical or veterinary relevance, and also it's importance as a pest species in agriculture or as feeding utilization.

Table 1. Snail species analysed in this review study and their medical or veterinary importance, as well the importance as a pest species in agriculture or as food resource.

Snail species	Family	Importance[#]	Main parasites species[+]	Reference
Freshwater species				
Ampullaceana balthica (Linneaus, 1758) *	Lymnaeidae	M, V	*Trichobilharzia* spp. (T), *Fasciola* spp. (T), *Echinostoma* spp. (T) *Moliniella anceps* (T), *Diplostomum* spp. (T),	Huňová *et al.* (2012)
Lymnaea stagnalis (Linneaus, 1758)	Lymnaeidae	M, V, A	*Echinostoma* spp (T).	McClelland and Bourns (1969)
Lymnaea sp. Lamark 1799	Lymnaeidae	M, V, A	*Echinostoma* spp. (T)	Aziz and Raut (1996); McClelland and Bourns (1969)
Racesina luteola (Lamarck, 1822) *	Lymnaeidae	M, V, A	*Schistosoma* spp. (T), *Fasciola* spp. (T), *Echinostoma* spp. (T)	Aziz and Raut (1996)
Radix sp. Montfort, 1810	Lymnaeidae	M, V	*Trichobilharzia* spp (T)., *Fasciola* spp (T)., *Echinostoma* spp. (T)	Huňová *et al.* (2012)
Biomphalaria alexandrina (Ehrenberg, 1831)	Planorbidae	M	*Schistosoma mansoni* (N)	DeJong *et al.* (2001)
Biomphalaria glabrata (Say, 1818)	Planorbidae	M	*Schistosoma mansoni* (T)	Morgan *et al.* (2001)

Biomphalaria pfeifferi (Krauss, 1848)	Planorbidae	M	*Schistosoma mansoni* (T)	Morgan *et al.* (2001)
Physella acuta (Draparnaud, 1822) *	Physidae	M, V	*Angiostrongylus cantonensis* (N), *Echinostoma revolutum* (T)	Li *et al.* (2018)
Physella sp. Haldeman, 1842	Physidae	M, V	*Angiostrongylus cantonensis* (N)	Lu *et al.* (2018)
Potamopyrgus antipodarum (Gray, 1843)	Tateidae	V, A	*Microphallus* spp. (T),	Jokela and Lively (1995)
Bellamya aeruginosa (Reeve, 1863)	Viviparidae	M, V, F	*Angiostrongylus cantonensis* (N)	Shan *et al.* (2008)
Bellamya purificata (Heude, 1890)	Viviparidae	M, V, F	*Angiostrongylus cantonensis* (N)	Li *et al.* (2018)
Cipangopaludina cathayensis (Heude, 1890)	Viviparidae	M, V, F	*Aspidogaster conchicola* (T)	Alves *et al.* (2015); Köhler *et al.* (2012)
Cipangopaludina chinensis (Gray, Griffth and Pidgeon, 1833)	Viviparidae	M, V, F	*Echinostoma cinetorchis* (T)	Li *et al.* (2018)
Estuarine species				
Peringia ulvae (Pennant, 1777)	Hydrobiidae	V	*Maritrema* spp. (T)	Rothschild (1941)

Marine species

Littorina littorea (Linneaus, 1758)	Littorinidae	M, V	*Cryptocotyle lingua* (T)	Seaman and Briffa (2015)

Land species

Lissachatina fulica (Bowdich, 1822) *	Achatinidae	M, V, A	*Angiostrongylus* spp. (N)	Graeff-Teixeira (2007)
Cornu aspersum (Muller, 1774) *	Helicidae	M, V, A, F	*Angiostrongylus* spp. (N)	Grewal *et al.* (2003)
Eobania vermiculata (Michelotte, 1840)	Helicidae	V, A, F	*Dollfusinus frontalis* (T)	Mas-Coma and Montoliu (1987)
Theba pisana (Muller, 1774)	Helicidae	V, A	*Miilerius capillaris* (N)	Baker and Vogelzang (1988)

*Scientific names were updated according to MolluscaBase (http://www.molluscabase.org/): *Ampullaceana balthica* (before *Radix balthica*), *Physella acuta* (before *Physa acuta*), *Racesina luteola* (before *Lymnaea luteola*), *Cornu aspersum* (before *Helix aspersa*), *Lissachatina fulica* (before *Achatina fulica*),

M= Medical importance (The snail is the intermediate host of parasites that the man is the definitive or accidental host). # V= Veterinary importance (The snail is the intermediate host of parasites that cause economic losses (diseases or death) in species of veterinary interest, such as fish, birds or mammals). #F= The snail is used for feeding . #A= Agricultutal pest.

+ (N) = Nematoda, (T) = Trematoda.

5.4. Historical perspective

The absolute and cumulative number of studies concerning the toxicity of NMs in snail species are in Fig. 1. The studies using snails as a model system to assess the NMs (eco)toxicity are recent and correspond to (88.3 %) of the studies reviewed in Table 2, while the number of studies aimed at the development of NM-based molluscicides corresponds only to (11.6%). The first study was conducted in 2010, using the freshwater snail *Physella acuta* exposed to different sediment spiked metal-based NP (α-alumina, γ-alumina, and TiO_2 NPs) showed the usability of this gastropod in NMs ecotoxicological assessment, through the sensibility on the reduction of the hatching rate and induction of developmental alterations on embryos of these snails after 96 h of exposure (Musee *et al.*, 2010).

The first study proposing to use NMs for snail control was conducted in 2015, using AgNPs (30 ppm) on the land snail *Eobania vermiculata* (considered a crop pest), and showed a reduction of 20% in snail viability, besides to tissue damage (Ali *et al*., 2015c). In 2016, the Organisation for Economic Co-Operation and Development (OECD) published the guideline nº 243 entitled "*Lymnaea stagnalis* reproduction test" (OECD, 2016), which described the procedures for assess the effects of chemicals on the reproduction and survival of the freshwater snail *L. stagnalis*, confirming their importance in the reproductive toxicology.

Furthermore, the genome of the *Biomphalaria glabrata* was sequenced in 2017 (Adema *et al.*, 2017), indicating the possibility of future studies on molecular and genetic changes induced by NMs on snails. Although the increasing number of articles on snail NM toxicity has grown exponentially, the absolute number of articles has remained similar over the years (Fig. 1), probably due to the new research area and the few researches groups. Recently a protocol for the isotopic marking of NMs for environmental and biological screening in aquatic organisms (snails and mussels) was published by Zhang *et al*. (2019), which supports the need to establish appropriate procedures for studies on NM toxicity, due to its risks to human and environmental health.

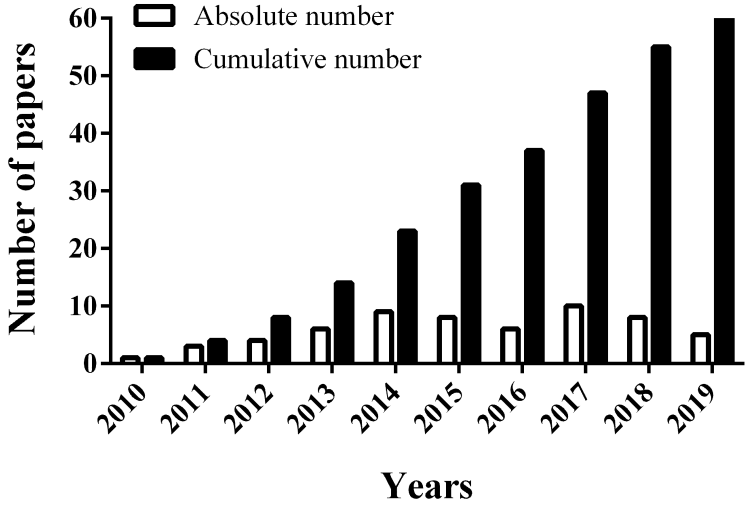

Figure 1: Number of studies (absolute and cumulative) concerning the toxicity of nanomaterials (NMs) to snails until December 2019

Table 2. Toxic effects of nanomaterials (NMs) to snails according to species, type of nanomaterial and experimental design.

Species	Nanomaterials				Exposure conditions		Cell/ tissue [d]	Accumulation [e]	Effects [f]	Reference
	Type[a]	Capping layer[b]	Size (nm)	*Dh* (nm)	Concentration [c]	Time				
Freshwater species										
Ampullaceana balthica *	C_{60} NPs	nd	100 - 200	nd	3 μg L^{-1}	21 d	S	na	No mortality, reproduction, feeding and BAF. The grazing activity was affected on first week.	López-Doval *et al.* (2019)
Bellamya aeruginosa	Ag NPs	nd	20, 40 and 80	nd	1, 10 and 100 μg g^{-1} $_{DS}$	14 d	DG, Go, M, VM	DG > Go = VM > M	↑Oxidative stress, ↑GSH (DG; M), ↑SOD (DG, Go, VM), ↓POD (DG), ↑POD (Go, VM, M). ↑CAT (DG, Go, VM, M), toxicity dependent Ag NP size.	Bao *et al.* (2018)
	CuO NPs	nd	41.6 ± 4.6	nd	180 μg g^{-1} $_{DS}$	28 d	DG Go, M	DG > Go > M; $CuSO_4$ > CuO NPs > CuO MP	↑Oxidative stress, ↑SOD, ↑CAT, ↑GST, ↑MDA. Toxicity was time dependent. NP had specific effect compared to Cu ions.	Ma *et al.* (2016)
	TiO_2 NPs,	nd	11.6 ± 2.4	nd	TiO_2 NPs 1 g kg^{-1} $_{DS}$	21 d	DG	Cd on DG at 5 mg Kg^{-1}	TiO_2 NPs: no effect. Cd NPs: ↑LPO, ↑PC, ↓Na^+/K^+-ATPase. DNA Damage.	Ma *et al.* (2017)
	Cd NPs	nd	nd	nd	Cd 5 and 25 mg Kg^{-1} $_{DS}$					
Bellamya purificata,	CeO_2 NPs	nd	25	103	60 mg L^{-1}	15 d	S	S	Snail showed high bioaccumulation factor due to their feeding mode. No mortality was observed.	Zhang *et al.* (2012)
Biomphalaria alexandrina	Ag NPs,	nd	nd	nd	3 - 100 μg mL^{-1}	24 h	S, Mi, Ce,	na	Molluscicide, cercaricide, and anti-parasitic effect. Modulation and prevention of the infectivity of cercariae and miracidia. LC_{50}: AgNPs (9.68 μg mL^{-1}) < AuNPs (133.7 μg mL^{-1}).	Moustafa *et al.* (2018)
	Au NPs	nd	nd	nd	100 - 200 μg mL^{-1}					

	SiO_2 NPs	nd	80	nd	50, 100, 200, 400, 600, 800, 1000, 1200 ppm	3- 36 h	S, EM	na	Molluscicidal effect to the S and EM. LC_{50}: snails (590 ppm/6 h), non-embryonated egg masses (1400 ppm/24 h), embryonated pre-hatched one (1450 ppm/12 h).	Attia *et al.* (2017)
	ZnO NPs	nd	17.5	71.11	25 - 600 μg mL^{-1} 7 and 35 μg mL^{-1} ($LC_{10 \text{ and } 25}$)	24 h 21 d	S, H, VM	na	Molluscicidal activity (LC_{50} = 145 and LC_{90} = 2,700 μg mL^{-1}). Morphological alterations. ↑MDA, ↑NO, ↓GSH, ↓GST, ↓SOD, ↓tPTN, ↓Alb, ↑tL, ↑Ch, ↑AST, ↑ALT, ↑ALP, ↑oxidative stress. ↑CAT in VM and ↓CAT in H. concentration–response dependent.	Fahmy *et al.* (2014)
Biomphalaria glabrata	Ag NPs	PVP	115.17 ± 55.57		1.0, 2.5, and 5.0 mg L^{-1}	30 d + 35 (depuration)	S, VM	VM even after 35 depuration	$LC_{50,96h}$: 18.57 mg L^{-1}. ↓Reproduction rate (30 d); ↓Egg *per* egg masses, ↓Egg masses production *per* snail.	Oliveira-Filho *et al.* (2019)
	γ-Fe_2O_3	DMSA	5.7	46.4 ± 8.8	1.0, 10, 100 mg L^{-1}	10 d 28 d	S, EM	In VM, eliminated after 30 d depuration	No effect on fecundity, fertility, mortality, of adults neither hatchability of eggs, nor malformation in embryos.	Oliveira-filho *et al.* (2016)
	CdTe NPs	nd	3	nd	1.2, 2.5, 5, 10, and 20 nM (embryos) 50, 100, 200, 400 nM (adults)	24 h	S, EM, H	DG	↑Malformations and mortality of embryos and adult snails depending on the concentration. ↑Cytotoxicity (hemocyte apoptosis).	Vasconcelos - Lima *et al.* (2019)
Biomphalaria Pfeifferi	PLA + Cur Nis	nd	284.0 ± 17.9	0.166 ± 0.03	21.88, 87.5, 350, 175, ppm	96 h (EM), 7 d (S)	S, EM	na	Molluscicidal effect. LC_{50}: EM hippo-stage (1072.7 ppm), juvenile (277.9 ppm), adult (339.1 ppm). ↓Egg hatchability and egg laying.	Omobhude *et al.* (2017)
Cipangopaludina cathayensis	FLG	nd	60 - 590 x 1.05 - 4.05		158 ± 6 μg L^{-1} (With or without 5 mg L^{-1} ALG) Static exposure	72 h	S	FLG > FLG + ALG in Gut	The interaction of graphene with alginate increased NM stabilization and increased the exposure and absorption by snails. Snail acts as a vector by transferring FLG into aquatic environments via food chains.	Su *et al.* (2018)

Cipangopaludina chinensis	Ag NPs	CT; PVP	20-60	nd	20 and 60 μg L^{-1}	14 d	S	TT	↑Bioaccumulation on biofilm. NP impacts on ecological receptors and food chains.	Park *et al.* (2018)
	TiO_2 NPs,	nd	5 - 10,	nd	1818.2 μg L^{-1}	17 d	S	TiO_2 NPs > TiO_2 NTs (TT)	Bioaccumulation through trophic transfer during plant consumption. ↑uptake and bioaccumulation.	Yeo and Nam (2013)
	TiO_2 NTs	nd	7 - 9 x 2 x 6	nd						
	TiO_2	nd	10 - 20	nd	2 and 6 mg L^{-1}	14 d	S	TT	Biomagnified through aquatic food chains. NPs shows greater movement in the sediment than in the water in a simplified food chain. ↑bioaccumulation in the semi-static exposition.	(Kim *et al.*, 2016)
Lymnaea stagnalis	Ag NPs	HA, CT	13 ± 3 17 ± 5	nd	Wb: 0.6 – 87 nM and 1 - 72 nM, Db: 17 a 187 nM g^{-1} and 7 - 250 nM g^{-1}	24 h (Wb) 2 and 4 h (Db)	VM	Efficiently in both Wb and Db exposure	For both exposure routes, uptake rates were faster for Ag^{+} than for Ag NPs.	Croteau *et al.* (2011a)
	Ag NPs	PVP and PEG	10.3 ± 3.4 12.8 ± 4.4	nd	25 nM L^{-1}	24 h	VM	$AgNO_3$>Ag NPs PVP > Ag NPs PEG	The presence HA↑uptake AgNPs PVP in contrast with cysteine but did not eliminate uptake of 25 nM L^{-1} Ag as $AgNO_3$, PVP AgNPs or PEG AgNPs.	Luoma *et al.* (2016)
	Ag NPs	PVP	11.4 ± 2.6	36 ± 1	412 a 586 nM g^{-1} (Food)	2 - 4 h	S, VM, F	↑ Ag uptake in VM with dietary exposure concentrations	Ag was efficiently assimilated from the PVP-Ag NPs mixed with the diatoms. The water hardness and humic acids has no influence on the dietary uptake of PVP-Ag NP by snails.	Oliver *et al.* (2014)
	Ag NPs	PVP and PEG	11.3 ± 2.6 (PVP) and 12.5 ± 3.6 (PEG)	34 ± 2 44	1 to 100 nM	24 h	VM	Dependent of Ag form and transformation	Bioaccumulation is affected by transformation factors that alter bioavailability. Aggregation, dissolution and other uptake factors should be considered in environmental studies.	Stoiber *et al.* (2015)

	Ag NPs	nd	100	nd	5, 10 e 50 μg L^{-1}	72 h	S	na	↑Memory formation (10 μg L^{-1}). Blocks memory formation (50 μg L^{-1}). Memory recall is context specific, thus snails trained in Ag NPs do not exhibit memory when tested in $AgNO_3$ and vice-versa.	Young *et al.* (2017)
	CuO NPs	nd	7	77 ± 5	Db: 4 - 50 μmol g^1 and 50 -175 nmol g^{-1} Wb: 4 -16 nM to 31 μM	3-5 h (Db) 24 (Wb)	VM, F	Db > Wb exposure	Bioaccumulation associated to toxicity. Toxicity: Db exposures > Wb exposure.	Croteau *et al.* (2014)
	ZnO NPs	nd	20 - 70	nd	10 and 1000 mg L^{-1}	3 and 4 (Db) h; 7 d (Wb)	S	TF	Bioaccumulation. Damage digestion: snails ate less, defecated less and inefficiently processed the ingested food.	Croteau *et al.* (2011b)
	ZnO NPs	nd	2 - 125	245	Db: 10 - 5000 mg L^{-1}	3 and 4 h	S	VM	The stable enriched isotopic technique is highly sensitive to determine the rate of Zn uptake in *L. stagnalis*.	Dybowska *et al.* (2011)
Physella acuta *	Ag NPs	nd	24 - 190	nd	0.001, 0.01, 0.1, 1, 10, 100 μg L^{-1}; and 0.001, 0.01, 0.05, 0.1, 0.5, and 1 μg L^{-1}	96 h and 28 d	S, EM	na	↑Mortality; Survival was greater in the presence of sediment (LC_{50} without sediment = 2.18 μg L^{-1}) than in its absence (LC_{50} > 10 μg L^{-1}); ↓egg production; ↓snail size at first reproduction, Behaviour changes.	Bernot and Brandenburg (2013)
	Ag NPs	CB	1.0 – 10.0	nd	0.03 and 30 μg L^{-1}	24 h	S	na	Nonlethal concentrations affect animal behaviour measured even in absence of predator.	Justice and Bernot (2014)
	Ag NPs	nd	100	nd	2000 to 10000 μg L^{-1} 3.7 - 120 μg L^{-1}	96 h 13 d	S EM	na	Exposure media influenced on NPs size and toxicity effect. Molluscicidal effects. LC_{50} in APW: embryos (81.6 μg L^{-1}), juvenile (158.1 - 458 μg L^{-1}), adult (5360 - 9560 μg L^{-1}).	Gonçalves *et al.* (2017)

	α- and γ-alumina ; C-TiO_2, M-TiO_2	nd	20 - 50, 80 - 400, 40 - 60, 7 - 11	nd	0.005, 0.05, or 0.5 g kg^{-1} DS	96 h 28 d	S, EM	na	↓Embryo growth rate and embryo hatchability, ↑deformities for α and γ alumina. Behaviour changes (avoid sediment). TiO_2 and C-TiO_2: no effects.	Musee *et al.* (2010)
	CeO_2 NPs	UC	50	nd	200 mg mL^{-1}	10 m	S	TF and TT	Biomagnification of CeO_2 NPs through the food web. BAF *S. borealis* > *C. japonica* > *P. stratiotes* > *C. fluminea* > *O. latipes* > *D. magna* > *P. acuta*.	Zhao *et al.* (2017)
Physella sp. and *Lymnaea* sp.	CeO_2 NPs	nd	3.8 ± 1.1; 185 ± 60	20 and >100	19.23 mg 1x week during 9 m. 250 L H_2O	9 m	S	plants>snail>insects	Accumulation, transport, transformation, bioavailability and trophic transfer was particle size dependent showing great results to small NPs.	Geitner *et al.* (2018)
Potamopyrgus antipodarum	Ag NPs	PVP	nd	54.5 ± 0.35	0.10 - 1000 µg L^{-1}	28 d	S	na	↓Reproduction was concentration dependent (EC_{10}: 5.57 µg L^{-1}, EC_{50}: 15,0 µg L^{-1}). Ag NPs in low concentrations can modulate 17α-ethynylestradiol activity.	Völker *et al.* (2014)
	CuO NPs	nd	6 ± 1	nd	0, 30, 60, 120 and 240 µg g^{-1} DS	8 wk	S, F	CuO NPs 6 nm > Cu_{aq}> CuO NPs 100 nm > CuO MPs.	↓Growth rate, ↓feeding rate, ↓reproduction, and ↑bioaccumulation higher in NPs than micro CuO or aqueous copper.	Pang *et al.* (2012)
	CuO NPs	nd	6 and 100	19 and 204 ± 1	0, 30, 60, 120 and 240 µg g^{-1} DS	2 - 8 wk	S	S, Sh, VM CuO (100 nm)	↓Juvenile growth. Bioavailability was affected by copper form.	Pang *et al.* (2013)
	CuO NPs	nd	Sph: 7 ± 1. Ro: 8 ± 1 x 40 ± 10. Pl: 1.14 ± 0.24 ×	nd	240 µg g^{-1} DS	9 wk	S	↑ $Cu_{(aq)}$. CuO NPs in VM (Sph and Pl), Sh (Pl)	↓Reproduction and ↓growth influenced by shape. Feeding wasn't affected.	Ramskov *et al.* (2014)

			270 ± 50 × 30 ± 10							
	CuO NPs	nd	Sph: 7 ± 1. Ro: 8 ±1 x 40 ± 10. Pl: 1.14 ± 0.24 × 270 ± 50 × 30 ± 10	nd	207 μg g^{-1} $_{DS}$	14 d	S, Sh, VM	No differences between shapes, or form accumulation in Sh or VM	↓Growth rate for Cu NPs spheres and platelets. The form of Cu did not affect either Cu accumulation into VM or Cu sorption to the Sh. No depuration.	Ramskov *et al.* (2015)
Racesina luteola *	Ag NPs	nd	32.4 ± 2.6	260.5 ± 26	1, 5, 10, 20, 40, 60 and 100 μg L^{-1} 4.01, 12.03 and 24.05 μg L^{-1}	96 h 96 h	S H	na	Molluscicidal effect. LC_{50}= 48.10 μg L^{-1}, ↓CAT (lower concentration), ↑CAT (96 h, higher concentration); ↓GST; ↓GSH at lower concentration, but it slightly increased at higher concentration; ↑LPO; ↑Apoptosis; ↑DNA damage (dose and time dependent).	Ali (2014a)
	Ag NPs	nd	32.4 ± 2.6	260.5 ± 26	4.01, 12.03 24.05 and 36.08 μg L^{-1}	96	DG	na	LC_{50}-96 h: 48.10 μg L^{-1}; ↑Oxidative stress; ↑CAT; ↓GSH; ↓GST; ↓GPx; ↑MDA; ↑DNA damage	Ali *et al.* (2014)
	CuO NPs	nd	43.5 ± 1.5	194.2 ± 14	1, 10, 30, 60, 120, and 240 μg L^{-1} 7 and 21 μg L^{-1}	96 h 5 d	DG	na	Molluscicidal effect (LC_{50} = 83.6 μg L^{-1}). ↑Oxidative stress; ↓GSH, ↓GPx, ↓GST. ↑LPO, ↑SOD (lower concentration, 1 day); ↓SOD (5 days). ↓CAT (2 days); ↑CAT (5 days, lower concentration). DNA damage mediated by oxidative stress.	Ali and Ali (2015)
	MgO NPs	nd	35 ± 4.0	154 ± 6.0	10, 20, 40, 80, 120 and 200 μg mL^{-1} 0, 17, 34, 51 μg mL^{-1}	96 h 4 d	S DG	na	Molluscicidal effect. EC_{50} = 66.8 μg mL^{-1}. ↑MDA, ↑GPx, ↓GSH (1 and 4 days). ↑CAT, ↑GST (51 μg mL^{-1}); ↓CAT, ↓GST (34 μg mL^{-1}). ↑oxidative stress, ↑genotoxicity.	Ali *et al.* (2016)

	TiO_2 NPs	nd	34.1 ± 2.7	190.5 ± 3.4	5, 15, 30, 60, 120, or 200 μg mL^{-1}	96 h	S	na	Molluscicidal effect. LC_{50}:112 μg mL^{-1}. ↓GSH, ↓GST, ↑MDA, ↓SOD, ↓CAT (9 μg mL^{-1}), ↑CAT (28 μg mL^{-1}). ↑Oxidative stress in DG. Toxicity was dose and time dependent.	Ali (2014a)
					9 and 28 μg mL^{-1}	7 d	DG			
	TiO_2 NPs	nd	34.1 ± 2.70	190.5 ± 3.4	5, 15, 30, 60, 120, or 200 μg mL^{-1}	96 h	S	na	Molluscicidal effect. LC_{50}:112 μg mL^{-1}. ↓GSH, ↓GST, ↑Oxidative stress, ↑MDA, ↑ROS, ↑apoptosis, ↑cytotoxicity, ↑DNA damage.	Ali *et al.* (2015b)
					28, 56, 84 μg mL^{-1}	96 h	H			
	SWCNTs	nd	<1.2 - 1.7	nd	0.05, 0.1, 0.3, 0.5, 1.0, 1.2, and 1.5 mg L^{-1}	96 h	S	na	Molluscicidal effect. LC_{50}: 0.61 mg L^{-1}. Behaviour changes. ↓GSH, ↓GST, ↓GPx, ↑LPO, ↑CAT, ↑DNA damage, ↑hemocyte death.	Ali *et al.* (2015a)
					0.05, 0.15, 0.30, 0.46 mg L^{-1}	4 d	DG			
	ZnO NPs	nd	22	264.8	0, 5, 10, 20, 40, 60, 80 and 100 μg mL^{-1}	96 h	S	na	Molluscicidal effect. LC_{50}: 42.67 μg mL^{-1}, ↓GSH, ↓GST, ↓GPx, ↑MDA, ↑CAT. Genotoxicity mediated by oxidative stress.	Ali *et al.* (2012)
					10, 21 and 32 μg mL^{-1}	96 h	DG			
Radix spp.	Ag NPs	PVP	6.0 ± 1.7	26.7 ± 2.5	500 μg L^{-1}	90 d	S	TT	Surface layer of sediment was the main sink of Ag originating from both AgNPs and $AgNO_3$. Ag accumulation. Changes in the nitrogen cycle.	Jiang *et al.* (2017)
Estuarine species										
Peringia ulvae	Ag NPs	CT	16.5 ± 4.5	32 ± 2 -339 ± 6	1.25 - 200 μg L^{-1}	24 h	S, VM	nd	Uptake from both Ag forms demonstrated saturation at the higher exposure concentrations; Biphasic loss dynamics revealed the faster elimination of Ag from Ag NPs at the start of depuration, but similar slow efflux rate constants.	Khan *et al.* (2012)
					20 - 50 μg L^{-1}	21 d				
	Ag NPs	CT	18.9 ± 5.1	79 ± 13	150 μg L^{-1}	6 and 24 h	VM	nd	Uptake is achieved via multiple pathways. NP uptake via different endocytic pathways.	Khan *et al.* (2014)

	CdS CdSe NPs	OA	3.1± 0.4 4.2 ± 0.8	nd	50, 100, 200 and 400 μg g^{-1}	12 h of feedin g	S	CdS NPs in VM	↓Feeding rate. QD uptake and efflux kinetics are distinct from those of Cd^{2+} indicating an NP-specific influence. Availability, persistence and possible accumulation, toxicity and food web transfer.	Khan *et al.* (2013b)
	ZnO NPs	nd	7.8 ± 1.2	38.4 ± 0.7	20 μg L^{-1}	7 d	S (VM, Sh)	ZnO bulk > Zn > ZnO NPs	Influx rate was ↓ in ZnO bulk than Zn and ZnO NPs. NP aggregation in estuarine water doesn't prevent Zn release. Bioaccumulation is dependent of Zn dissolution.	Khan *et al.* (2013a)
Marine species										
Littorina littorea	Ag NPs	nd	59 ± 19	nd	10 and 20 μg L^{-1}	5 d	S, F	Head, Gill	Ag is most available in aqueous form than NPs. Accumulation: Wb > Db exposure. Ag was only detected in the head and gill in one treatment suggests the association may well be a physical (adherent).	Li *et al.* (2013)
Land species										
Cornu aspersum *	CdSe NPs	nd	nd	nd	3.6 and 7.2 μg g^{-1} (injection with micro - syringe)	2 d	DG	NA	↑GST, ↑CAT, ↑MDA, ↓GSH, ↓GPx, ↑ mitochondrial swelling, ↓respiratory level. Oxidative stress.	Salmi *et al.* (2017)
	IONPs, IONPZ	nd	≈ 20	nd	0.05 and 1 mg mL^{-1}	20 d	H	H. IONPs> IONPZ	↑Oxidative stress, ↑ROS, ↑LPO, ↑Ub, ↑Cas, ↓DNA integrity.	Sidiropoulou *et al.* (2018)
	IONPs (Fe_2O_3)	nd	nd	nd	1.25, 1.5, 2 mg mL^{-1}	12 d	EM	At the back of egg	↑Embryotoxicity, ↑Mortality, ↓Hatching. Deformation of egg membrane	Besnaci *et al.* (2016)
	IONPs (Fe_2O_3)	nd	26	nd	1, 2, 3 mg g^{-1} (food)	6 w	S	na	↑Oxidative stress, ↑GSH, ↑GST, ↑GPx, ↑MDA, ↓CAT. Renal tissue damage	Besnaci *et al.* (2019)

	ZnO NPs	nd	59.10	nd	500, 1000, 5000, 10000, 15000 µg g^{-1} (food)	28 d	S	na	↓Growth, ↓Body and shell weight, ↑Mortality (LOEC = 5000 µg g^{-1}, NOEC = 1000µg g^{-1}	Nedjoud *et al.* (2015)
	ZnO NPs	nd	59.10	nd	500, 1000, 5000, 10000, 15000 µg g^{-1} (food)	28 d	S	nd	↓Shell growth, ↓weight of DG and K, ↓Feeding rate, behavioural changes	Nedjoud *et al.* (2017)
	TiO_2 NPs	UC	25.0 ± 5.7		500 mg kg^{-1} (soil) Terrestrial Mc	10 d	F, M, VM	No	No mortality, soil consumption, uptake or accumulation.	Vijayaraj *et al.* (2018)
Eobania vermiculata	Ag NPs	nd	6 - 38	nd	30 ppm	5 d	DG, K	K > DG	↓Viability, ↑histological alterations in the kidney and digestive gland.	Ali *et al.* (2015c)
Lissachatina fulica *	Ag NPs	PVP	14.8 ± 4.0	47.1 ± 4.3	0.80 to 250 mg g^{-1} (Db) 0.5 - 20 mg L^{-1} (Wb)	4 h 8 h	S	Db > Wb for Ag NPs	↓Ku, ↑Ke for Ag NPs assimilation Wb exposure. Db uptake is the dominant route for Ag accumulation in the case of Ag NP exposure compared to $AgNO_3$.	Chen *et al.* (2017)
	CeO_2 NPs	nd	6.9 ± 0.4	40.2 ± 7.2	1.2, 5.5 and 11 mg g^{-1} (plant)	7 d and 30 d	S	GD > F > VM	↑Assimilation efficiency, ↑ingestion rate through the trophic transfer than in direct exposure. Biotransformation of Ce (IV) for Ce (III) occur only in DG.	Ma *et al.* (2018)
Theba pisana	Ag NPs	nd	2.18 - 19.87	nd	1 mM (food)	2 w	H	na	↑Oxidative stress, ↑LPO, ↑CAT, ↑GST, ↓GSH. Immunotoxicity: ↑Cell death and abnormalities, ↑Hemocyanin, ↓Phagocytic activity, ↓Lectins, ↓LMS, ↓O_2^-, ↓POD, ↓PO, ↑DNA damage.	Radwan *et al.* (2019)

* Scientific names updated as MolluscaBase (http://www.molluscabase.org/): *Ampullaceana balthica* (before *Radix balthica*), *Physella acuta* (before *Physa acuta*), *Racesina luteola* (before *Lymnaea luteola*), *Cornu aspersum* (before *Helix aspersa*), *Lissachatina fulica* (before *Achatina fulica*).

[a] C_{60} (fullerene), CurNis (curcumin – nisin entrapped), C- TiO_2 (commercial TiO_2), FLG (^{14}C-labeled few-layer graphene), IONPs (oxide iron nanoparticles), IONPZ (oxide iron nanoparticles incorporated into zeolite), NPs (nanoparticles), NT (Nanotubes), SWCNTs (single walled carbon nanotubes), M-TiO_2 (modified TiO_2), PLA (polylactic-acid), TiO_2-NT (TiO_2 nanotubes),

[b] CB (Carboxy-functionalized), CT (citrate), G (gelatine), DMSA (meso-2, 3 dimercaptosuccinic acid), HA (humic acid), nd (not described), OA (oleic acid), PEG (polyethylene-glycol), PVP (polyvinyl-pyrrolidone) UC (Uncoated).

[c] ALG (alginate), DS (dry sediment), Db (dietborne exposure), LC (lethal concentration), Mc (microcosm), Pl (platelets), Ro (rods), Sph (spheres) Wb (waterborne exposure).

[d] Ce (Cercariae), DG (Digestive gland cells, Hepatopancreas), EM (Egg masses), F (Feces), Go (Gonad), H (Hemocyte cells, hemolymph), K (Kidney), M (Muscle, foot muscle), Mi (Miracidia), S (Whole snail), Sh (Shell), VM (Visceral mass, soft mass).

[e] Db (dietborne exposure), DG (digestive gland cells, hepatopancreas), Go (gonad), M (muscle, foot muscle), MPs (microparticles), na (not analysed), nd (not described), S (whole snail), TF (bioaccumulation through feeding), TT (bioaccumulation through trophic transfer), VM (visceral mass, soft mass), Wb (waterborne exposure).

[f] ↑ (Increase, induction), ↓(decrease, Loss), Alb (albumin), ALP (alkaline phosphatase) ALT (alanine aminotransferase), APW (artificial pond water), AST (aspartate aminotransferase), BAF (bioaccumulation factors), Cas (caspase), CAT (catalase), Ch (cholesterol), DG (digestive gland cells, hepatopancreas), DNA (deoxyribonucleic acid), EC (effective concentration), EM (egg masses), Go (gonad), GPx (glutathione peroxidase), GR (glutathione reductase), GSH (glutathione), GST (glutathione-S-transferase), H (hemocyte cells, hemolymph), K (kidney), Ke (elimination coefficient), Ku (uptake coefficient), LC (lethal concentration), LMS (lysosomal membrane stability) LPO (lipidic peroxidation), M (Muscle, foot muscle, MDA (malondialdehyde), MPs (microparticles), NO (nitric oxide), PC (protein carbonylation), PO (phenoloxidase), POD (peroxidase), ROS (reactive oxygen species), SOD (superoxide dismutase), tL(total lipids), tPTN (total proteins), Ub (ubiquitin conjugates), VM (visceral mass, soft mass).

The frequency of studies concerning the NP toxicity to snails worldwide is shown in Fig. 2. The data revealed that the largest number of publications and snail species used is in the European continent (28.3 %), with emphasis on the United Kingdom (UK) (11.6 %), and Denmark (6.6 %), and in the Asian continent (28.3 %) with the largest number of publications in China (15 %) and Saudi Arabia (11.6 %). On the African continent (20 %), most studies were conducted in Egypt and Algeria (8.3 % equally). In American continent (18.3 %), the United States of America (USA) stands out (11.6 %) while in Oceania the only country was Republic Korea (5 %). Studies of the NM toxicity to snails were carried out in 18 countries, namely Denmark (6.6 %) France (1.6 %), Germany (1.6 %), Greece (1.6%), Portugal (1.6 %), Spain (3.3 %) and UK (11.6 %) (in Europe), China (15 %), India (1.6 %), Saudi Arabia (11.6 %) (in Asia), Algeria (8.3 %), Egypt (8.3 %), Nigeria (1.6 %), South Africa (1.6 %) (in Africa), Brazil (5 %), Canada (1.6 %) and USA (11.6 %) (in America) and Republic Korea (5 %) (in Oceania). The revised data showed the need to further nanotoxicological studies in countries with high snail biodiversity and areas with high prevalence of snail-related diseases.

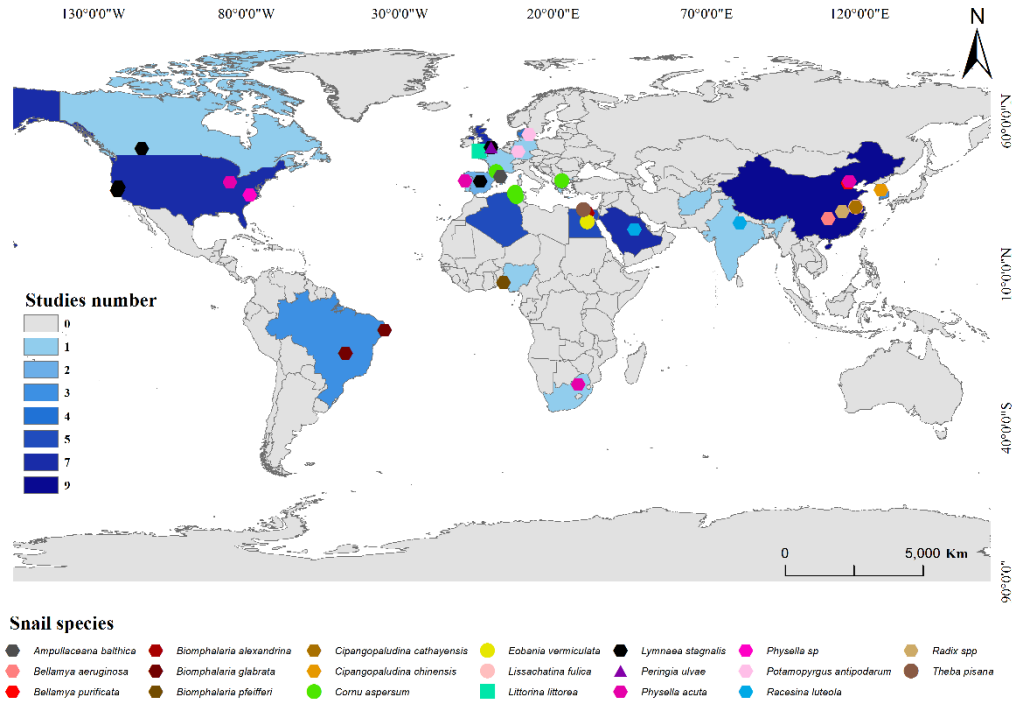

Figure *2.* Worldwide distribution of studies involving nanomaterials and snails, and the distribution of these species according to the first author's affiliation research place. Circles indicate terrestrial species, freshwater hexagons, estuarine environment triangle, and saltwater square

5.5. Snail species

The toxic effects of NMs were investigated in 21 snail species (Tab. 1 and Fig. 3A). *L. stagnalis, R. luteola* and *C. aspersum* are the most studied species (13.1 %, 13.1 and 11.4 % respectively), following by *P. antipodarum* (8.1 %) and *P. acuta* (8.1 %). The family Lymnaeidae, in particular the species *L. stagnalis* and *R. luteola* are composed by freshwater hermaphrodite species, widely distributed over the world, occurring in Asia, Europe, New Zealand, North Africa and North America (Atli and Grosell, 2016). *L. stagnalis* is the key intermediate host of *Fasciola hepatica*, which is considered a snail of medical and veterinary importance (Kendall, 1949), but it is also considered a key-species showing ecological importance for participating in the diet of fish, amphibians, birds and mammals (Amorim *et al.*, 2019).

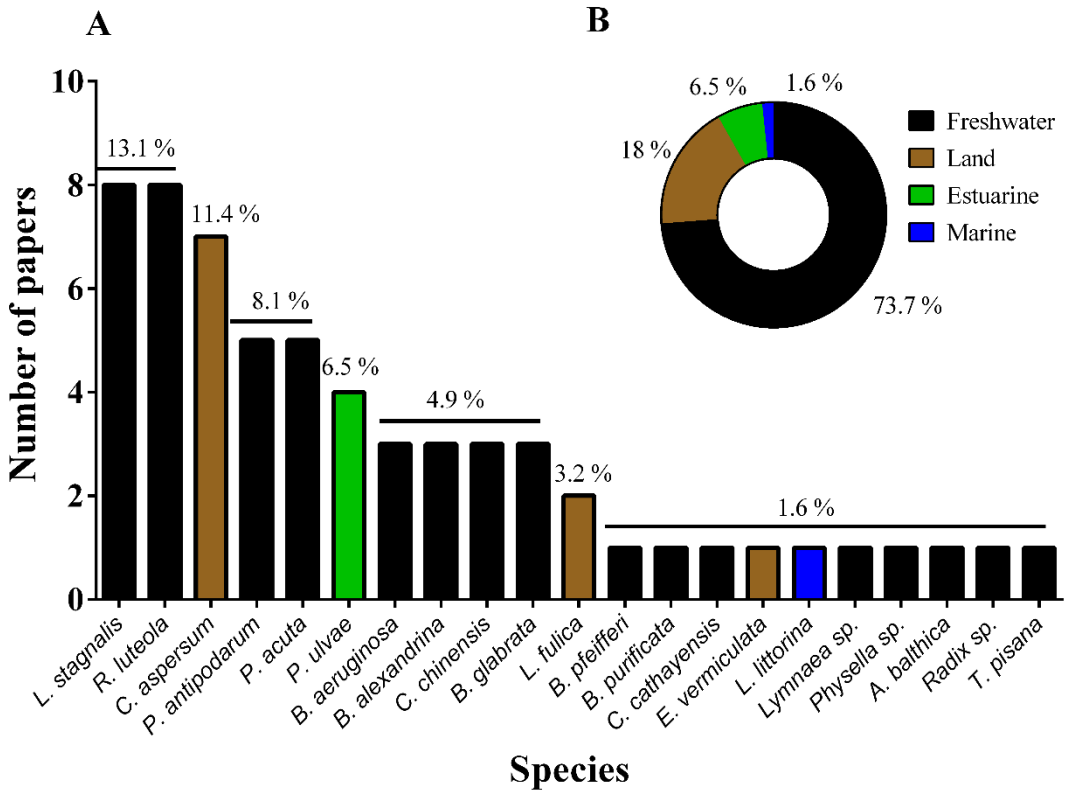

Figure 3. Number of papers about toxicity of nanomaterials (NMs) according to snail species (A) and environment (B) until December 2019.

L. stagnalis has been widely used in the study of toxicity and toxicokinetic of NMs, being a standard model for the study of reproductive toxicity, embryotoxicity and evaluation of endocrine disruptors (Amorim *et al.*, 2019; Mazur *et al.*, 2013; OECD,

2016). *L. stagnalis*, *P. antipodarum* and *P. acuta* have also been widely applied in studies of reproductive toxicity, embryotoxicity and evaluation of endocrine pollutants disruptors (Bernot *et al.*, 2005; Ducrot *et al.*, 2014; Duft *et al.*, 2007). Furthermore, both species are also considered invasive species with global distribution and have medical and/or veterinary importance, acting as IH of parasites (Table 1) (Albrecht *et al.*, 2009; Alonso and Castro-Díez, 2014; Guo *et al.*, 2009; Kerans *et al.*, 2005).

C. aspersum is an herbivorous terrestrial snail, native in the UK and western Europe, considered an invasive species with some medical, veterinary and agricultural importance, also used as a food source as "escargots" (IUCN, 2019). It is the possible vector of the fungus *Phytophthora citrophthora*, which causes plant cancer and acts as an intermediate host of *Angiostrongyllus* spp. (Alvarez *et al.*, 2009; Grewal *et al.*, 2003). In studies using NMs, *C. aspersum* has the third highest number of publications (11.4 %). It is also used as a bioindicator for evaluation of heavy metals in soil and food, using feasibility tests, alteration of energy metabolism, genotoxicity, embryotoxicity and histopathology (Besnaci *et al.*, 2019, 2016; Nedjoud *et al.*, 2017, 2015; Salmi *et al.*, 2017; Sidiropoulou *et al.*, 2018; Vijayaraj *et al.*, 2018).

Studies involving NMs and snails were conducted mainly with freshwater species (73.3 %) when compared to the land species (*C. aspersum*, *L. fulica*, *E. vermiculata* and *T. pisana*) (18 %), estuarine species (*Peringia ulvae*) (6.5 %), and marine species (*Littorina littorea*) (1.6 %) (Fig. 3B), demonstrating that further studies with land, estuarine and marine species are need to evaluated NMs and other pollutants in these environment using gastropod as experimental model.

5.6. NM properties and experimental design

5.6.1. Types of NMs

The potential moluscicidal activity was investigated for 18 types of NMs (Fig. 4A). 93.7 % of studies were performed using inorganic NMs, while 4.6 % used carbon-based NMs and 1.6 % polymeric ones (Fig. 4B). Among the inorganic NMs, Ag NPs were the most studied (34.4 %), following by CuO NPs, TiO_2 NPs and ZnO NPs (each NM with 10.9 %). Due to their antifungal,antibacterial, anthelmintic, inseticide and tickicideproperties, Ag NPs have been applied in personal care and medical or veterinary

products (Abbasi *et al.*, 2014; Ahamed *et al.*, 2010; Benelli, 2018c; Benelli *et al.*, 2017; Chaloupka *et al.*, 2010). The revised data indicated the potential use of Ag NPs in the control of snail intermediate hosts (Fig. 4A, Table 2).

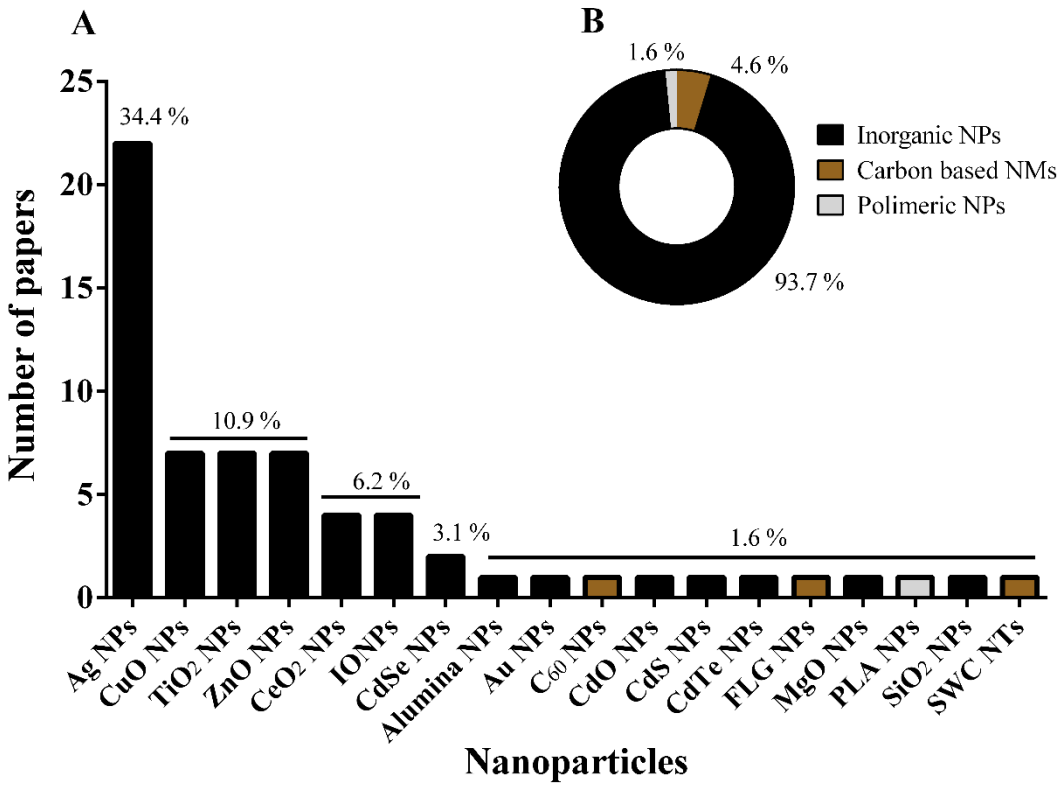

Figure 4. Number of papers about toxicity of nanomaterials (NMs) using snail species according to type (A) and categories of NMs (B) until December 2019. IONP: iron oxide, C_{60} (fullerene), FLG (^{14}C-labeled few-layer graphene), PLA (polylactic-acid), SWCNTs SWCNTs (single walled carbon nanotubes).

CuO NPs has been used in the biomedical application, such as electrochemical sensors for the diagnosis of diabetes and nervous system disorders as Parkinson's and Alzheimer's (Khedekar and Bhanage, 2016; Yazid *et al.*, 2016; Zhang *et al.*, 2011), in cancer therapy (Tisato *et al.*, 2009) and as an antimicrobial agent in ointments and dressings (Ahamed *et al.*, 2014; Ren *et al.*, 2009; Verma and Kumar, 2019). TiO_2 NPs is mainly used in the cosmetics industry, especially in the manufacture of sunscreens, paints and electronic devices (Chen and Mao, 2007; Gupta and Tripathi, 2011), while ZnO NPs have been used in the biomedical area as a bactericidal, fungicidal and inseticide agents, as well as treatment and diagnosis of diseases (Madhumitha *et al.*, 2016; Mirzaei and Darroudi, 2017; Sirelkhatim *et al.*, 2015).

5.6.2. Exposure time

Nanotoxicological studies with snails were conducted mainly under short time exposure compared to long time exposure (Fig. 5). In general, the adults snails were analyzed after short time exposure to NMs (1 - 14 days; 55.84 %), while long time exposure (> 14 – > 180 days; maximum 10 months (Zhao *et al.*, 2017)) represents 46.16 %. On the other hand, the experiments using snail embryos were conducted during 1 - 7 days (Fig. 5, Table 2), indicating that the exposure time to NMs depends on snail developmental stage. Beside, the revised data showed that the toxic effects of NMs to snails depends on the exposure time, as reported for other invertebrate and vertebrate species (Ali, 2014b; 2014a; Ali and Ali, 2015; Amaral *et al.*, 2019; Pereira *et al.*, 2019; Rocha *et al.*, 2017, 2015).

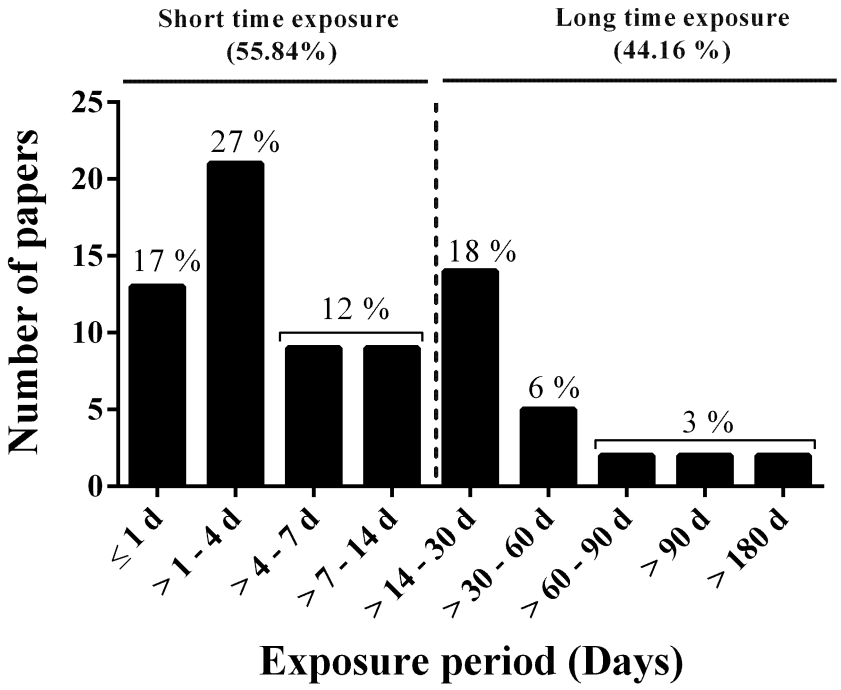

Figure 5. Number of papers about toxicity of nanomaterials (NMs) using snail species according to exposure time**.** The exposure period was classified in short time exposure (≥ 14 days) and long-time exposure (> 14 days).

5.6.3. Experimental design

The nanotoxicological studies using snails as model system have been conducted by differents approaches (Fig. 6): (*i*) Snail embryotoxicity test (SET) (10.4 %); (*ii*). Acute toxicity test with new-hatching snails (3.9 %); (*iii*) multigeneration exposure (3.9 %); (*iv*) direct exposure of adult snails using different environment (freshwater, estuarine and land) (57.1 %); (*v*) dietary exposure of adult snails (15.6 %); (*vi*) micro and mesocosm exposure (9.1 %). Furthermore, different snail developmental stages have been used in nanotoxicological studies, such as embryos (11.4 %) and adults (84.3 %), while the knowledge concerning the toxic effects of NMs to new-hatching snails (4.3 %) remain scarce (Table 2).

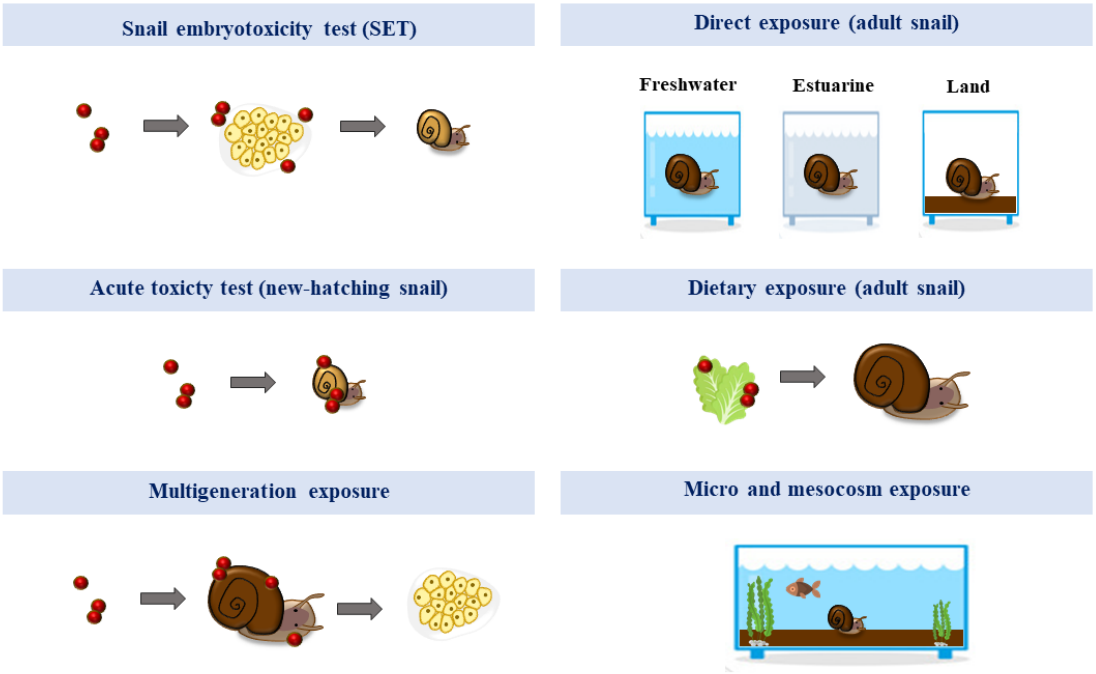

Figure 6. Scheme of different approaches used to study the toxicity of NMs on snail species. This scheme was organized from revised data (Table 2). The color, size and proportions are illustrative and cannot be compared.

The SET consists of the exposure of snail embryos at the early stage of development (i.e. blastula stage) to NMs during 1 to 13 d, while the mortality rate, hatching rate and morphological changes frequency are monitored (Attia *et al.*, 2017; Bernot and Brandenburg, 2013; Besnaci *et al.*, 2016; Gonçalves *et al.*, 2017; Musee *et al.*, 2010; Oliveira-filho *et al.*, 2016; Omobhude *et al.*, 2017; Vasconcelos - Lima *et al.*,

2019). The SET has been used to characterizate the toxicity of water pollutants, pharmaceutical drugs, plants extracts and NPs (Besnaci *et al*., 2016; Mazur *et al*., 2013; Oliveira-Filho *et al*., 2017; Rapado *et al*., 2011; Tallarico, 2016). In similar way, in the acute toxicity test with new-hatching snails, snails after hatching are exposed to NMs during short-time exposure (1 to 4 d) (Gonçalves *et al.*, 2017; Pang *et al.*, 2013).

In the multigeneration exposure, the NM effects over multiple generations were studied by exposing of parental snails and the effects over the next generations were studied. The continuous exposure to NMs over a period of multiple generations induce several generational impairments, such as reproductive toxicity, deformations and death of the embryo, delays in embryonic development, hatching rate reduction and alteration of somatic biomarkers (growth rate, condition index, gonadossomatic index) (Ducrot *et al.*, 2014; OECD, 2016; Ruppert *et al*., 2017; Sarraude, 2019), such as reported for Ag NPs, iron oxides nanoparticles (IONPs) and CuO NPs (Bernot and Brandenburg, 2013; Nedjoud *et al.*, 2017; Oliveira-Filho *et al*., 2019; Oliveira-filho *et al.*, 2016; Pang *et al.*, 2012).

The direct exposure of adults to NMs depends on the exposure medium, specially because different environment factors changed the NM transformation and toxicity, such as salinity, pH, temperature, photoperiod, sediment granulometry, presence of natural organic matter, among others (Musee *et al.*, 2010; Ali *et al.*, 2012; Ali 2014a; Ramskov *et al.*, 2014, 2015; Ali *et al.*, 2015; Ma *et al.*, 2017). In the dietary exposure, the food (e.g. lettuce or diatoms) is previously exposed to NMs and then snails are feeding. . This method allows the analysis of the trophic transfer of NMs to the snails. However, few studies investigated the trophic transfer and biomagnifictaion of NPs using snail species (Geitner *et al.*, 2018; Kim *et al.*, 2016; Park *et al.*, 2018; Yeo and Nam, 2013; Zhao *et al.*, 2017).

Finally, the micro and mesocosm exposure consists in the exposure of multiple species to NMs at environmentally relevant conditions during long-time period (1 to 10 months). In general, the micro and mesocosm containing aquatic and terrestrial compartments and organism from different trophical levels, such as phytoplankton, zooplankton, plants, invertebrates and vertebrates. This aprroaches was used to analyze the bioaccumulation and toxicity of Ag NPs (Jiang *et al.*, 2017), CeO_2 NPs (Geitner *et al.*, 2018; Zhao *et al.*, 2017), CuO NPs (Pang *et al.*, 2013; Ramskov *et al.*, 2014), TiO_2 NPs (Kim *et al.*, 2016; Yeo and Nam, 2013) and ZnO NPs (Dybowska *et al.*, 2011) (Table 2).

5.7. Bioaccumulation and toxicokinetics of NMs in snails

The bioaccumulation and toxicokinetic of NMs in snails were investigated for Ag NPs, CdS NPs, CuO NPs, CeO_2 NPs, IONPs and ZnO NPs (Fig. 7). The uptake and bioaccumulation of NMs on snails depend on the properties and environmental transformations of NMs, snail species and exposure condition (Table 2). The agglomeration and sedimentation of NMs in the aquatic environment decreased their bioavailability, but it does not prevent their uptake and bioaccumulation. After the sedimentation, the NM agglomerates were available for ingestion by snails (Khan *et al.*, 2013b).

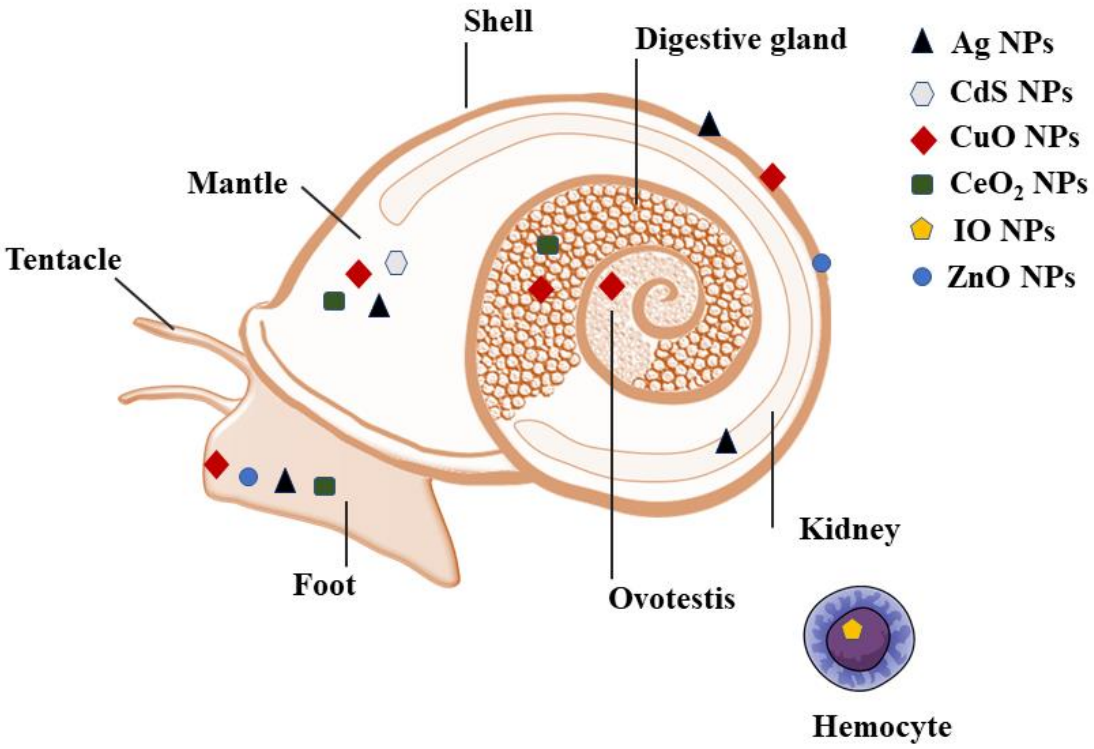

Figure 7. General scheme of the bioaccumulation and tissue distribution of nanoparticles (NPs) in the snail. This scheme was organized from revised data (Table 2). The colour, size and proportions are illustrative and cannot be compared.

In general, the metal-based NMs were less bioavailable to snails when compared to dissolved metal, but both metals forms were bioaccumulated in the snail tissues (Table 2). The *L. stagnalis* exposed to citrate-functionalized Ag NPs (16.5 ± 4.5 nm; 1.25 - 200 µg Ag L^{-1}) and dissolved Ag for 24 h in 17 salinity water showed uptake rate (K_u) of 0.35 ± 0.01 and 1.1 ± 0.1 L^{-1} g^{-1} d^{-1}, respectively (Khan *et al.*, 2012). Similarly, the ZnO NPs

(7.8 ± 1.2 nm; 20 μg Zn L^{-1}) showed low *Ku* (0.042 L^{-1} g^{-1} d^{-1}) compared to dissolved Zn (0.074 L^{-1} g^{-1} d^{-1}) and ZnO bulk (0.085 L^{-1} g^{-1} d^{-1}) in *P. ulvae* after 7 days of exposure (Khan *et al.*, 2013b).

Multiple pathways have been indicated for uptake of NMs by snails. Clathrin- and caveolae-mediated endocytosis was indicated as main uptake pathway of citrate-functionalized Ag NPs (18.9 ± 5.1 nm; 150 μg Ag L^{-1}) by *P. ulvae* after 24 h of exposure (Khan *et al.*, 2014). NMs can interact with the shell and foot of snails, and after the uptake, distributed to different cells like haemocytes and some organs such as the mantle, digestive gland and ovotestis (Fig. 7). The Ag NP bioaccumulation was reported in the shell, foot, mantle and kidney of the *B. aeruginosa, C. chinensis, E. vermiculata* and *L. stagnalis* (Ali *et al.*, 2015; Bao *et al.*, 2018; Croteau *et al.*, 2011; Oliver *et al.*, 2014; Park *et al.*, 2018), while the CuO NPs were observed in the shell, mantle, foot and digestive gland of *B. aeruginosa* , *L. stagnalis* and *P. antipodarum* (Croteau *et al.*, 2014; Ma *et al.*, 2016; Pang *et al.*, 2013, 2012; Ramskov *et al.*, 2015).

The digestive gland, mantle and foot were considered also the main organs for CeO_2 NP accumulation in *B. purificata, P. acuta* and *L. fulica* (Geitner *et al.*, 2018; Ma *et al.*, 2018; Zhang *et al.*, 2012a; Zhao *et al.*, 2017a). The ZnO NP accumulation was reported in the shell and foot of the *L. stagnalis* and *P. ulvae* (Croteau *et al.*, 2011a; Dybowska *et al.*, 2011; Khan *et al.*, 2013a). Oleic acid-coated CdS NPs were found in gut epithelial cells and mantle of *P. ulvae* after diet borne exposure (Khan *et al.*, 2013b). Only IONPs were observed by transmission electron microscopy in the haemocytes of the *C. aspersum* after 20 days of exposure (Sidiropoulou *et al.*, 2018). In this sense, the revised data showed that the bioaccumulation and tissue distribution of NMs in different snail species deserve further studies, especially from different habitats (freshwater, estuarine and land).

5.8. Mechanism of action and toxicity

The main MoA and toxicity of NMs on snails is in Fig. 8. Revised data indicated that the toxicity of NMs has been associated mainly with ion release from oxidative dissolution of NMs, ROS production, following by oxidative damage (i.e. lipidic peroxidation - LPO, DNA and protein damage) (Table 2).

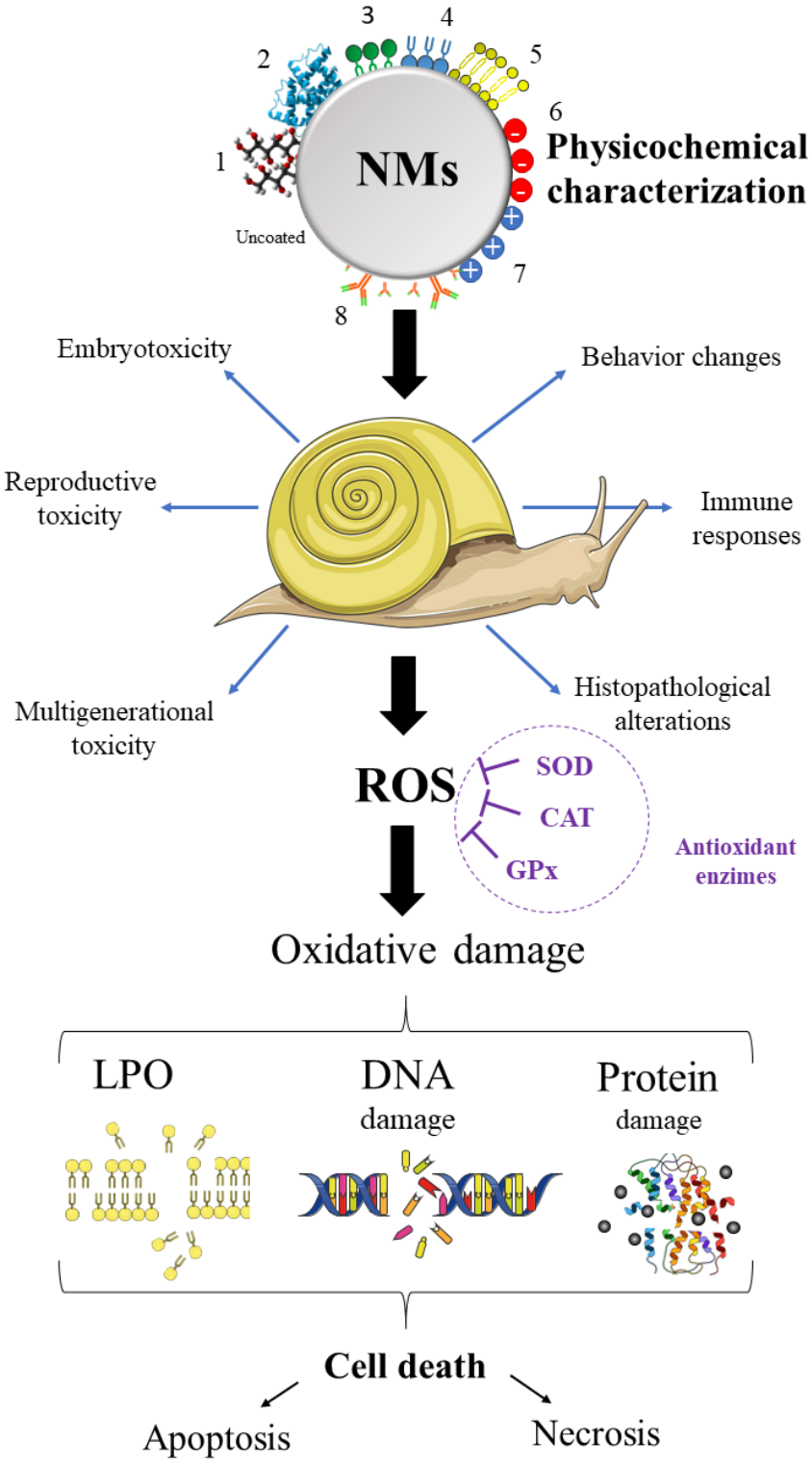

Figure 8. General scheme of the effects of nanomaterials (NMs) on the snail and their main mechanisms of action. ROS (Reactive oxygen species), SOD (Superoxide dismutase), CAT (Catalase), GPx (Glutathione peroxidase), LPO (Lipid peroxidation), DNA (Deoxyribonucleic acid). Organic and inorganic molecules (1), Proteins (2), Lipid monolayers (3 and 4), Lipid bilayers (5), Anions (6), Cations (7), Antibodies (8).

5.8.1. Oxidative stress

The oxidative stress in snails induced by NM exposure was reported for several types of NMs (Ag NPs, Cd NPs, CuO NPs, IONPs, MgO NPs, SWCNTs, TiO_2 NPs and ZnO NPs) and snail species, such as *B. aeruginosa* (Bao *et al.*, 2018; Ma *et al.*, 2017, 2016), *B. alexandrina* (Fahmy *et al.*, 2014), *R. luteola* (Ali *et al.*, 2012; Ali, 2014a, 2014b; Ali *et al.*, 2014; Ali *et al.*, 2015a, 2015b; Ali *et al.*, 2016; Ali and Ali, 2015) *L. stagnalis* (Luoma *et al.*, 2016) and the terrestrial snail *C. aspersum* (Besnaci *et al.*, 2019; Salmi *et al.*, 2017; Sidiropoulou *et al.*, 2018) and *T. phisana* (Radwan *et al.*, 2019) (Table 2).

Oxidative stress was the main toxic effect induced by NMs in gastropods (Fig. 8; Table 2). NMs increased the ROS production, changed the antioxidant defence system, such as superoxide dismutase – SOD, catalase - CAT, glutathione peroxidase - GPx, and glutathione-s-transferase – GST, and increased LPO (i.e. Ali, 2014b; Ali *et al.*, 2012; Ali and Ali, 2015; Bao *et al.*, 2018; Ma *et al.*, 2016); (Table 2). Furthermore, NMs can also induced protein carboxylation (Ma *et al.*, 2017), reduced the total protein content, increased the total lipids and aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) (Fahmy *et al.*, 2014), ubiquitin and caspase (Sidiropoulou *et al.*, 2018), inhibited the Na^+/K^+ ATPase (Ma *et al.*, 2017), induced mitochondrial damage and reduced lysosomal membrane stability (LMS) (Radwan *et al.*, 2019; Salmi *et al.*, 2017), DNA damage (Ali, 2014b, 2014a; Ali *et al.*, 2014; Ali and Ali, 2015; Ma *et al.*, 2017; Radwan *et al.*, 2019; Sidiropoulou *et al.*, 2018) and apoptosis (Ali, 2014b; Radwan *et al.*, 2019).

Toxic effects in snails induced by NMs were dependent on NM size, NM aggregation capacity, and target cell and tissue (Table 2). In general, small NMs have high superficial area and high dissolution potential, as well as the ability to enter the cell membrane and cause oxidative stress (Hou *et al.*, 2017; Rocha *et al.*, 2015). On the other hand, small particles may also have a greater tendency to aggregate (He *et al.*, 2008), or to be more easily adsorbed by organic matter or sediment present in the environment (Hotze *et al.*, 2010; Lei *et al.*, 2018; C. Wang *et al.*, 2016). For example, the toxicity of larger Ag NPs (sizes 40 and 80 nm) to *B. aeruginosa* was higher than small Ag NPs (20 nm) after 14 days (Bao *et al.*, 2018). In opposite, Geitner *et al.* (2018) reported that the small CeO_2 NPs (3.8 vs 185 nm) induced high accumulation rate and trophic transfer potential, since that small NPs were more bioavailable in the water column, whereas the larger NPs remained associated with sediment.

The oxidative damage induced by NM exposure in snails also was dependent on the concentration and exposure time, such as reported for Ag NPs, CuO NPs, TiO_2 NPs, SWCNTs and ZnO NPs (Table 2). In general, longer exposure time and high concentration of NMs induced higher oxidative damage, such as observed in the digestive gland and gonad of the *B. aeruginosa* exposed to 180 μg g^{-1} CuO NPs in dry sediment for 28 days (Ma *et al.*, 2016), and in the visceral mass of *B. alexandrina* exposed to ZnO NPs (7 and 35 μg mL^{-1}) for 21 days (Fahmy *et al.*, 2014). Similarly, the chronic exposure of *C. aspersum* to ZnO NP (28 days) increased the mortality, reduced growth rate, body weight and feed rate (Nedjoud *et al.*, 2017, 2015).

Cell and tissue specific responses to oxidative damage induced by NMs were reported for snails (Table 2). The digestive gland was the main organ analysed in studies concerning oxidative stress induced by NM due to its higher accumulation capacity and role in the metal detoxification. The digestive gland of the *B. aeruginosa* exposed to Ag NPs (40 and 80 nm; 1.10 and 100 μg L^{-1}; 14 days) was more susceptible to oxidative stress than to gonads, visceral mass and foot/muscle (Bao *et al.*, 2018). Similar tissue-specific response also was observed in *B. aeruginosa* exposed to CuO NPs (41.6 nm; 180 μg g^{-1}; 28 days) (Ma *et al.*, 2016). Additionally, the role of the digestive gland in the metal detoxification was indicated in *Lissachatina fulica* after dietary exposure, in a trophic transfer study of CeO_2 NPs (6.9 nm; 1.2, 5.5 and 11 mg g^{-1}) (Ma *et al.*, 2018).

The hemocytes are immune cells of snails and constitute the principal line of defence against external stress factors. The hemocytes represent the second most studied cells to assess the effect of oxidative stress caused by NMs (Table 2). The hemocytes of *B. alexandrina* exposed to ZnO NPs (17.5 nm; 7 and 35 μg mL^{-1}; 21 days) showed oxidative stress after exposure to both concentrations, whereas the visceral mass response was observed only in the highest concentration (Fahmy *et al.*, 2014), indicating that the hemocytes are suitable cells to assess the toxicity of NMs, such as reported for bivalve molluscs (Canesi *et al.*, 2012; Rocha *et al.*, 2015).

The oxidative stress induced by NMs also was associated to DNA damage and apoptosis (Table 2). The snail *R. luteola* exposed to 12 - 24 μg L^{-1} of Ag NPs (32.4 nm) showed LPO, apoptosis and DNA damage associated to ROS production (Ali, 2014a). Different NMs induced oxidative stress in hemocytes by altering detoxification enzymes system, increasing LPO and ROS, such as reported for *R. luteola* exposed to TiO_2 NPs (34 nm, 9 and 28 g mL^{-1}; 96 h) (Ali *et al.*, 2015b), *C. aspersum* exposed to IONPs (20

nm, 0.05 and 1 mg L^{-1}; 20 days) (Sidiropoulou *et al.*, 2018) and *T. pisana* exposed to Ag NPs (2.18 - 19 nm; 1 mM; 2 weeks) (Radwan *et al.*, 2019). On the other hand, the revised data indicated that the oxidative damage to carbohydrates in NM-exposed snails needs to be investigated in future studies.

5.8.2. Genotoxicity and mutagenicity

DNA damage is an important biomarker of NM toxicity in snails (Fig. 8, Table 2). DNA damage is frequently assessed by the alkaline single-cell gel electrophoresis assay, mainly using the hemocytes and digestive gland cells. Given the importance of DNA in the maintenance of cellular homeostasis and the transmission of genetic information between generations, the DNA damage could interfere in the survival and reproduction of snails, especially when this damage is permanent. One way of assessing permanent DNA damage and making nano-genotoxic analysis in a more realistic way is integrating the comet assay (DNA damage) with mutagenicity techniques (i.e. micronucleus tests – MN test, and hemocyte nuclear abnormalities - HNA), as previously reported for other invertebrate and vertebrate species (Amaral *et al.*, 2019; Qualhato *et al.*, 2017; Rocha *et al.*, 2015).

Genotoxic effects induced by NM exposure in snails were reported for Ag NPs (Ali, 2014a; Ali *et al.*, 2014; Luoma *et al.*, 2016; Radwan *et al.*, 2019), CuO NPs (Ali and Ali, 2015) , IONPs (Sidiropoulou *et al.*, 2018), MgO NPs (Ali *et al.*, 2016), TiO_2 NPs (Ali, 2014a; Ali *et al.*, 2015b), ZnO NPs (Ali *et al.*, 2012) and SWCNT (Ali *et al.*, 2015a). However, only two studies analysed the mutagenic effects induced by NMs in snails. *B. glabrata* exposed to CdTe NPs (3 nm; 50 - 200 nM) for 24 h showed increased frequency of micronuclei, binucleated nuclei and apoptotic hemocytes (Vasconcelos - Lima *et al.*, 2019), while *T. pisana* after dietary exposure to Ag NPs (2.1 - 19.8 nm; 1 mM) for 14 days showed high frequency of hemocytes with MN, binucleated nuclei and kidney-shaped nucleus, jointly with reduced DNA content, ROS production and increased LPO (Radwan *et al.*, 2019).

Genotoxicity was dependent on NM type, exposure time, concentration, snail specie and cell/tissue (Table 2). For example *R. luteola* showed higher susceptibility to Ag NPs (32.4 nm; 4.01 - 36.8 µ L^{-1}; 96 h) evidenced by DNA damage in hemocytes (Ali, 2014a) and digestive gland (Ali *et al.*, 2014) at low concentrations than other NMs, such

as CuO NPs (43.5 nm; 7 - 21 µ L^{-1}; 120h) (Ali and Ali, 2015), SWCNTs (< 1.2 - 17 nm; 50 - 460 µg L^{-1}; 96 h) (Ali *et al.*, 2015a), TiO_2 (34.1 nm; 9 - 84 × 10^3 µg L^{-1}; 120 h) (Ali, 2014a; Ali *et al.*, 2015b), MgO NPs (35 nm; 7 - 51 × 10^3 µg L^{-1}; 96 h) (Ali *et al.*, 2016) and ZnO NPs (22 nm; 10 - 32 × 10^3 µg L^{-1}; 96 h) (Ali *et al.*, 2012).

In most cases, the DNA damage induced by NPs increased in a time and concentration-dependent manner, and digestive gland was the most studied tissue. *R. luteola* digestive gland's showed higher sensitivity to TiO_2 NPs (34.1 nm; 9 - 28 × 10^3 µg L^{-1}; 120 h) (Ali, 2014b) than hemocytes exposed to TiO_2 (34.1 nm; 28 - 84) × 10^3 µg L^{-1}, 96h) (Ali *et al.*, 2015b), demonstrating the need for further studies to understand the biodynamics and toxicity of NMs in different tissues.

The nanogenotoxic effects were also confirmed in hemocytes of the terrestrial snail *C. aspersum* exposed to IONPs (20 nm; 0.05 and 1 mg mL^{-1}; 20 d), which showed that the DNA damage induced by NM was time-dependent. These genotoxic effects were associated with NP internalization, ROS production and apoptosis demonstrated by protein carboxylation (Sidiropoulou *et al.*, 2018). Revised data indicated that the genotoxicity induced by NM was related to increased ROS, oxidative stress, or metal ions release and the direct interaction of NPs with nuclear proteins or DNA. On the other hand, the specific mechanism of genotoxicity of NMs (direct or indirect) in snail species remains uncertain.

5.8.3. Developmental toxicity

Several NMs induced toxic effects during the early developmental stages of snails (Table 2), such as Ag NPs, SiO_2 NPs, α alumina and γ-alumina and Fe_2O_3 NPs. The first embryotoxicity study showed that the dimercaptosuccinic acid (DMSA)-functionalized Fe_2O_3 NPs (25 to 250 mg L^{-1}) did not induced embryo mortality, morphological alterations and hatching inhibition due to their physical properties and limited internalization in the egg-clutches (Oliveira-filho *et al.*, 2016). In contrast, *C. aspersum* embryos exposed to Fe_2O_3 NPs (1.25 to 2 mg ml^{-1}) showed morphological alterations (malformation of membrane) reduced egg size and color changes, jointly with NM accumulation in the egg-clutches during the exposure for 12 days (Besnaci *et al.*, 2016).

The comparative embryotoxicity of Ag NPs (3.75 to 120 µg L^{-1}) and its dissolved counterpart $AgNO_3$ (0.94 to 15 µg L^{-1}) was analysed for *P. acuta*, and results showed that

the Ag NPs (81.6 µg L^{-1}) induced low embryotoxic effects than $AgNO_3$ (LC_{50} > 15 µg L^{-1}). This low toxicity of Ag NPs was related to its environmental transformation (aggregation/agglomeration, sedimentation, oxidative dissolution and surface alteration) in aqueous medium (Gonçalves *et al.*, 2017). In contrast, the *B. pfeifferi* embryos exposed to curcumin-nisin polylactic acid NPs with exposure (284.0 ± 17.9 nm; 21.88 at 350 ppm) for 96 h showed hatching inhibition and deformed embryos. In addition, embryos at blastula stage (LC_{50} = 4279.5 ppm) were less susceptible to NM toxicity when compared to embryos at hippo-stage (LC_{50} = 1072.7 ppm) (Omobhude *et al.*, 2017), confirming that the toxicity induced by NM is dependent on snail developmental stages

Hydrophilic nanosilica (80 nm; 50 to 1200 ppm; 48 h) induced embryotoxic effects in *B. alexandrina*, with LC_{50} = 590 ppm at 6 h and 980 ppm at 48 h (Attia *et al.*, 2017), demonstrating time-dependent effects (Attia *et al.*, 2017). Similarly, the exposure of the *B. glabrata* embryos to CdTe NPs (3 nm; 1.2 - 20 nM) for 24 h decreased the hatching rate and induced morphological alterations, specially reduced growth (Lima *et al.*, 2019). These embryotoxic effects was related to the NM interaction with embryos, changes in the animal metabolism and subsequent energy suppression for hatching and development (Vasconcelos - Lima *et al.*, 2019).

The multigenerational exposure of snails to NMs also induced developmental toxicity. *P. acuta* adults exposed to α-alumina and γ-alumina NPs (0.005 - 0.5 g kg^{-1}) for 28 days showed reduced growth, decreased hatching rate and developmental inhibition (Musee *et al.*, 2010). These effects were related to reduced peroxidase activity that can eliminate ROS in adult snails. Similarly, *P. acuta* exposed to Ag NPs (24 - 190 nm; 0.001-1 µg L^{-1}) for 28 days (Bernot and Brandenburg, 2013) and *B. glabrata* exposed to PVP-functionalized Ag NPs (115.17 ± 55.57 nm, 1 - 5 mg L^{-1}) for 30 days showed reduced egg-clutch production and low number of embryos *per* egg-clutches (Oliveira-Filho *et al.*, 2019).

5.8.4. Immunotoxicity

The gastropod immune system is a sensitive target for assessing NM toxicity. Hemocytes are circulating cells in the hemolymph responsible for several defence mechanisms, such as phagocytosis and ROS production, and for transport and metabolism of the xenobiotics (Boisseaux *et al.*, 2017; Loker, 2010; Matozzo and Gagné, 2016). The

immune response of snails to NMs depended on the concentration, exposure time, type of NM, as well the snail species. For example, the exposure of *R. luteola* to Ag NPs (32 nm; 4.01 - 24.05 μg L^{-1}) for 96 h induced a reduction of hemocyte viability and increased apoptosis and necrosis in a time-dependent concentration (Ali, 2014a). Similar results were reported for TiO_2 NPs (34.1 nm, 28 - 84 μg mL^{-1}; 96 h), which decreased circulating hemocytes and increased apoptosis in *R. luteola* in a concentration-time dependent pattern (Ali *et al.*, 2015b)

Chronic exposure of the *Theba pisana* to Ag NPs (2.18 - 19 nm; 1 mM) for 2 weeks decreased the phagocytic activity and LMS, induced the formation of nuclear alterations in hemocytes, such as micronuclei, binucleated and kidney-like nuclei, as well as decreased lectin level, phenoloxidase and peroxidase activities, and increased hemocyanin level (Radwan *et al.*, 2019). *B. alexandrina* exposed to ZnO NPs (17.5 nm; 7 and 35 μg mL^{-1}) for 21 d also showed biochemical changes in the hemolymph, such as reduction of total proteins and albumin, high lipid and total cholesterol levels, as well as increased aminotransferase (alanine aminotransferase - ALT and aspartate aminotransferase - AST) and ALP activities, indicating tissue toxicity changes in the immune system (Fahmy *et al.*, 2014).

IONPs (20 nm; 0.05 and 1 mg L^{-1}) also induced cytotoxicity in *C. aspersum* hemocytes after chronic exposure (20 days), such as increased protein carboxylation, ubiquitin conjugate level and caspase activity (Sidiropoulou *et al.*, 2018). Revised data indicated that the main immune response of snails to NMs was related to increased ROS production and alteration of antioxidant enzymes, leading to the DNA damage, nuclear alterations and cell death. Furthermore, NMs can also alter the phagocytic activity of hemocytes, which may reduce the snail immune response to other external agents and pathogens (Table 2). However, the susceptibility of NM-exposed snails to parasites needs to be further studied, especially due to its importance as an IH (Table 1).

5.8.5. Behavioral impairments

NMs also changed the nervous system of snails and induced several behaviour alterations (Table 2). Revised data demonstrated that behavioural changes in snails induced by NM exposure, such as reduced memory development and ability to detect

predators may affect snail survival strategies, feeding rate, individual energy balance, growth and reproduction, reducing the snail population.

Ag NPs (100 nm; 50 μg L^{-1}; 72 h) reduced memory formation and learning in *L. stagnalis* after 72 h of exposure (Young *et al.*, 2017). Carboxyl-functionalized Ag NPs (1 - 10 nm; 0.03 and 30 μg L^{-1}; 24 h) inhibited the ability to assess prediction risk of *P. acuta* to the natural predator pumpkinseed sunfish (*Lepomis gibbosus*) (Justice and Bernot, 2014), indicating that NMs can alter the chemoreception of snails. ZnO NPs (110 nm; 10 and 1000 mg L^{-1} ; 4 h) induced damage to the digestive system of *L. stagnalis* after dietary exposure to NP-contamined diatoms, resulting in a lower consumption rate, defecation and ability to process food ingested (Croteau *et al.*, 2011a). In addition, the snail *C. aspersum* after dietary exposed to food (wheat flour) contamined by ZnO NPs (59 nm; 500 - 15000 μg g^{-1}) showed a reduction in the feeding rate and behavioural changes, such as inactivity and high refuge behaviour in the first 2 weeks of exposure, with the tendency for behaviour to disappear at the end of exposure time (4 weeks) (Nedjoud *et al.*, 2017).

SWCNTs (<1.2 - 1.7 nm; 0.05 - 1.5 mg L^{-1}) induced escape and swam at the surface of the water behaviour in *R. luteola* after 96 h of exposure (Ali *et al.*, 2014). Similar behaviour alterations were observed in *P. acuta* exposed to Ag NPs (24 - 190 nm; 0.001 - 1 μg L^{-1}) for 28 days (Bernot and Brandenburg, 2013). The behaviour of avoiding the sediment surface was also observed in *P. acuta* exposed to α-alumina and γ-alumina (20 - 50 / 80 - 400 nm; 0.05 g kg^{-1}) for 96 h and 28 days (Musee *et al.*, 2010). Oleic acid-functionalized CdS quantum dots (3 nm; 50 - 400 μg g^{-1}; 12 h) decreased the feeding rate of the *P. ulvae* in a concentration-dependent pattern (Khan *et al.*, 2013b). CuO NPs (6 nm, 30 - 240 μg Cu g^{-1} dry sediment; 8 weeks) also reduced the feeding rate of the *P. antipodarum* (Pang *et al.*, 2012). On the other hand, these behavioural impairments were not observed in the *P. antipodarum* exposed to different forms of CuO NPs at 207 g g^{-1} sediment for 14 days and at 240 μg g^{-1} sediment for 9 week (Ramskov *et al.*, 2015, 2014). C_{60} NPs (fullerene) (100 - 200 nm; 3 μg L^{-1}; 21 days) also reduced the foraging activity of the in *A. balthica* during the first week of exposure, but no significant alteration effect was observed in the feeding rate (López-Doval *et al.*, 2019).

5.9. EMN as new generation of molluscicide

Molluscicidal effects have been reported for Au NPs, CdTe NPs, CuO NPs, MgO NPs, SiO_2 NPs, SWCNTs, TiO_2 NPs, ZnO NPs and Ag NPs, beside to their genotoxic, mutagenic, immunotoxic, embryotoxic, and behavioral effects, that significantly affect the snail survival and reproduction. The molluscicidal activity varied according to NM type, concentration, exposure time, snail species and experimental design (Table 2).

B. alexandrina was more susceptible to Ag NPs, presenting the lowest lethal concentration (LC) ($LC_{50,24h}$ = 9.68 µg mL^{-1}) (Moustafa *et al.*, 2018) when compared to SiO_2 NPs ($LC_{50,36h}$ = 590 µg mL^{-1}) (Attia *et al.*, 2017), ZnO NPs ($LC_{50,24h}$ = 145 µg mL^{-1}) (Fahmy *et al.*, 2014), and Au NPs ($LC_{50,24h}$ = 133.7 µg mL^{-1}) (Moustafa *et al.*, 2018). Similarly, *R. luteola* also was more sensitive to Ag NPs ($LC_{50,96h}$ = 0.0481 µg mL^{-1}) (Ali, 2014a; Ali *et al.*, 2014) than TiO_2 NPs ($LC_{50,96h}$ = 122 µg mL^{-1}) (Ali, 2014a; Ali *et al.*, 2015b), MgO NPs ($EC_{50,96h}$ = 66.8 µg mL^{-1}) (Ali *et al.*, 2016), ZnO NPs ($LC_{5,96h}$ = 42.67 µg mL^{-1}) (Ali *et al.*, 2012), SWCNTs ($LC_{50,96h}$ = 0.61 µg mL^{-1}) (Ali *et al.*, 2015a) and CuO NPs ($LC_{50,96h}$ = 0.0836 µg mL^{-1}) (Ali and Ali, 2015), confirming the potential use of Ag NPs as molluscicide. On the other hand, the role of functionalization on the Ag NP toxicity to snails remains unknown.

Molluscicidal effects were also recorded for CdTe NPs in *B. glabrata*, with 100 % mortality at 400 nM after 24 h (Vasconcelos - Lima *et al.*, 2019). Similar effect was also observed for *C. aspersum* after dietary exposure to ZnO NPs (59.1 nm; 500 - 15000 mg g^{-1}) for 28 days (NOEC = 1000 mg g^{-1} LOEC = 5000 mg g^{-1}) (Nedjoud *et al.*, 2015). However, different concentrations of DMSA-functionalized IONPs (1 to 100 mg L^{-1}) had no effect on the *B. glabrata* snail after chronic exposure (28 days) (Oliveira-filho *et al.*, 2016), confirming that the molluscicidal activity of NMs depend on their composition and functionalization.

Among the species exposed to Ag NPs, *P. acuta* was the most susceptible species, presenting the lowest $LC_{50,\ 96h}$ = 0.0028 µg mL^{-1} via waterborne exposure (Bernot and Brandenburg, 2013). In addition to inorganic NPs, curcumin-nisin polylactic acid NP (284 nm; 21 - 175 µg mL^{-1}) showed a molluscicidal effect for all developmental stages of the snail *B. pfeifferi* (LC_{50} = 1072.7 µg mL^{-1} for hypo-stage embryos, LC_{50} = 277.9 µg mL^{-1} for juvenile snails after 96 h of exposure, and LC_{50} = 339.1 µg mL^{-1} after 7 days of exposure) (Omobhude *et al.*, 2017), showing the potential use of polymeric NPs as molluscicide. However, for the purpose of applying NMs as molluscicidal agents, there

are still a big number of issues that need to be evaluated and answer, such as the chemical, physical and biological transformations of NPs in the environment, as well as the interaction with the exposure medium.

5.10. Conclusions

Nanobiotechnology is a emergent science with wide application for the control of parasites and intermediate hosts, such as snails. In this context, the present study is the first review of a decade of studies concerning the potential use of snails as suitable model system to assess the toxic effects of NMs and the potential use as molluscicide. Accordindly to World Health Organization (WHO), "molluscicides are chemicals, of synthetic or biological origin, used primarily to kill various species of molluscs, including intermediate host snails". However, this concept does not yet contemplate NMs as molluscicide. In this sense, the present study indicates the urgent need to revise and include NMs in the concept of molluscicide.

The molluscicidal activity of NMs to snail is dependent on the physical and chemical properties of NMs, as well as their environmental transformation. Data showed the need to develop standard protocols for assessing the toxic effects of NMs on aquatic and land snail species. Furthermore, revised data indicated some research gaps in order to improve the use of NMs as molluscicide: (*i*) field studies and environmentally relevant approaches are required; (*ii*) analyze the comparative effects of NMs between native snail species and those that act as intermediate hosts; (*iii*) assessment of toxicokinetics, specially the tissue distribution, metabolism and detoxification process of NMs in snails; (*iv*) OMICs technologies, such as transcriptomics, proteomics and metabolomics, may help in understanding the mechanism of action and toxicity of NMs to snails, but studies on the genome description of important snail species are needed; (*v*) analysed the comparative toxicity of NMs during the all developmental stages of snails; (*vi*) investigate the NM toxicity in infected snails; (*vii*) study the toxic effects NMs on parasites inside snails; (viii) studies concerning the toxicity of NMs throughout the life-cycle of parasites that have snails as intermediate hosts.

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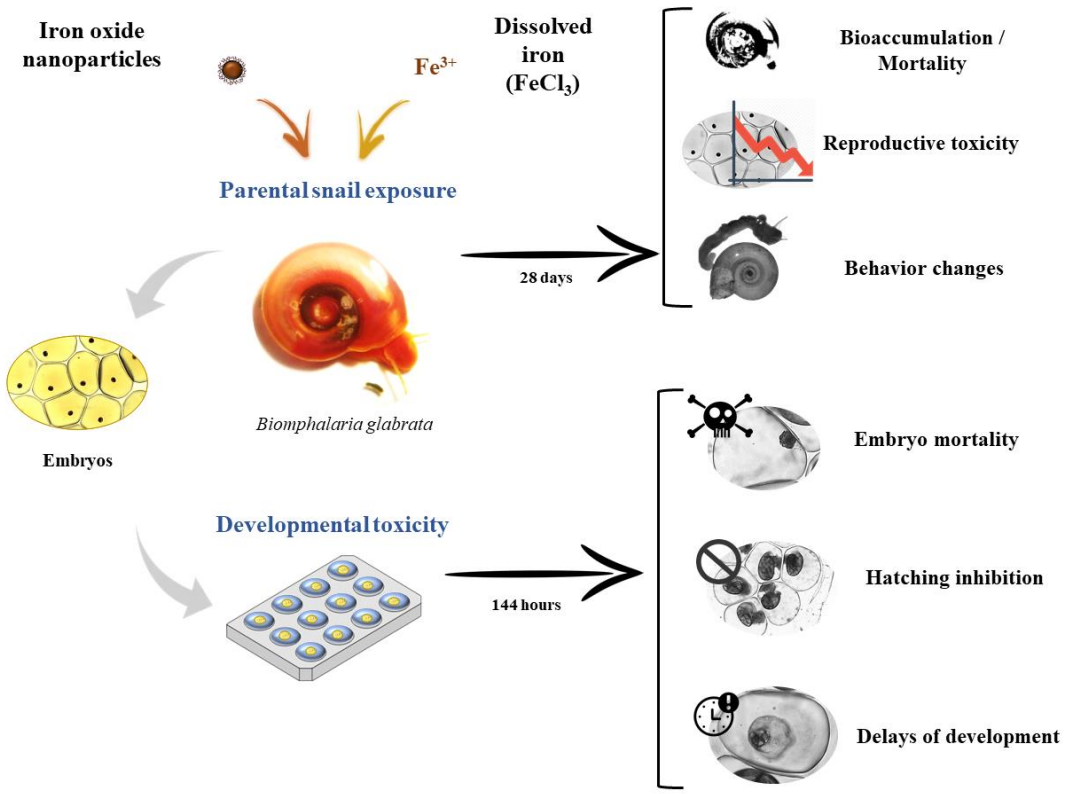
6. CAPÍTULO III: ARTIGO EXPERIMENTAL

CAPÍTULO III

Iron oxide nanoparticles and ferric chloride induce bioaccumulation, behavioural impairments and reproductive toxicity to *Biomphalaria glabrata* (Say, 1818) after chronic exposure



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Iron oxide nanoparticles and ferric chloride induce bioaccumulation, behavioral impairments and reproductive toxicity to *Biomphalaria glabrata* (Say, 1818) after chronic exposure

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Highlights

- Molluscicidal activity of iron oxide nanoparticles (IONPs).
- Differential bioacumulation of IONPs and iron ions in *B. glabrata*.
- Reproductive toxicity induced by chronic exposure to IONPs.
- Behavior impairments in *B. glabrata* induced by IONP chronic exposure.
- Parental exposure to IONPs decreased the fecundity and fertility of snails.

ABSCTRACT

Iron oxide nanoparticles (IONPs) have been applied in several sectors in the biomedical field and its use in the control of parasites and intermediate hosts of neglected tropical diseases is emerging. However, few studies have explored the molluscicidal activities and toxicity of this nanomaterial (NM) after chronic exposure. The present study aimed to analyse the chronic toxicity of gluconic acid-functionalized IONPs and their dissolved counterpart ($FeCl_3$) to freshwater snail *Biomphalaria glabrata*, intermediate host of *Schistosoma mansoni*. IONPs were synthesized and characterized by multiple techniques, and the snails were exposed to both Fe forms (1.0 – 15.6 mg L^{-1}) for 28 days in semi-static systems. During the exposure period, the bioaccumulation, mortality rate, behaviour impairments, morphological alterations, fecundity (production of egg-clutches and embryos) and fertility (mortality rate, hatching and delayed embryo development) of the snails were analysed. Results showed that the IONPs induced high iron bioaccumulation in the whole soft tissue compared to iron ions and control group. Similarly, high frequency of behaviour alterations was observed in the snails exposed to IONPs when compared to its dissolved counterpart and the unexposed ones. Both Fe forms reduced the fecundity, while the mortality and reduced fertility was observed only after the exposure to IONPs at 15.6 mg L^{-1}. Overall results indicated the behavioural impairments and reproductive toxicity associated to bioaccumulation of IONPs in the *B. glabrata*. This study emphasizes that IONPs are potential molluscicidal agents and have an advantage over other NMs due to their magnetic properties, that could allow the withdraw from the environment

Keywords: Schistosomiasis; molluscicide; nanotechnology; biomarkers.

6.1. Introduction

Iron oxide nanoparticles (IONPs) have been applied in several fields of society, ranging from the production of materials in the industry and incorporation into commercial products, such as paints, ceramics, porcelain, and electronics (Cornell, 2003; Teja and Koh, 2009). Furthermore, these engineered nanoparticles have been used as contrast agent for magnetic resonance imaging (Kool *et al.*, 2003; Shen *et al.*, 2016; Yan *et al.*, 2018), in cancer treatment by hyperthermia (Arularasu *et al.*, 2018; Rivas *et al.*, 2012), as systems for drug delivery (Kumar *et al.*, 2017; Ma *et al.*, 2012), in food analysis (Cao *et al.*, 2012; Hernández-Hernández *et al.*, 2017) and in other fields of human and environmental health such in effluent decontamination and withdraw from the environment through the magnetism(Dong *et al.*, 2019; Zhang *et al.*, 2016; Zou *et al.*, 2016). , , , and

Due to its small size, high surface ratio, magnetic properties, and the possibility of highly specific targeting to organs or cells through functionalization or conjugation to drugs, proteins, enzymes, antibodies and nucleotides (Gupta and Gupta, 2005; Ling and Hyeon, 2013), the IONPs have gained special prominence in biomedical applications (Figuerola *et al.*, 2010; Gupta and Gupta, 2005; Kwon *et al.*, 2018). For example, the IONPs have been applied as drugdelivery systems against visceral leishmaniasis and schistosomiasis (Kumar *et al.*, 2017; Tomiotto-Pellissier *et al.*, 2017) besides to show molluscicidal, embryotoxic and genotoxic effects on the land snail *Cornu aspersum* (Müller, 1774) (Besnaci *et al.*, 2016, 2019; Kaloyianni *et al.*, 2020; Sidiropoulou *et al.*, 2018).

The toxicity of metal-based NPs to snail species has been associated to their high uptake and bioacumulation in snail tissues (Croteau *et al.*, 2014, 2011a; Dybowska *et al.*, 2011; Oliver *et al.*, 2014), inducing oxidative stress, lipid peroxidation, protein carbonilation, DNA damage (Ali *et al.*, 2015, 2016, 2012; Ma *et al.*, 2016; Sidiropoulou *et. al*, 2018) and Na^+/K^+ ATPase inhibition (Ma *et al.*, 2017).. Several freshwater snail species have been used in the nanotoxicologial researches, such as *Bellamya aeruginosa* (Reeve, 1863), *Racesina luteola* (Lamarck, 1822), , *Lymnaea stagnalis* (Linneaus, 1758), *Physella acuta* (Draparnaud, 1822), and *Potamopyrgus antipodarum* (Lamarck, 1843). These species are exposed to different nanomaterials(NMs) like; Ag NPs, CeO NPs, CuO NPs, TiO_2 NPs, MgO NPs and ZnO NPs, resulting in genotoxic, immunotoxic, embryotoxic and molluscicidal effects, as well behavioral impairments and reproductive

toxicity (Musee *et al.*, 2010; Ali *et al.*, 2012, 2015, 2016, Croteau *et al.*, 2014, 2011; Ramskov *et al.*, 2015; Ma *et al.*, 2016, 2017) Despite that, the knowledge about the chronic toxicity and the mechanism of action (MoA) of IONPs in snails remain limited (Besnaci *et al.*, 2019, 2016; Kaloyianni *et al.*, 2020; Sidiropoulou *et al.*, 2018), especially in the freshwater snail *Biomphalaria* spp. (Oliveira-filho *et al.*, 2016).

Biomphalaria spp., especially *Biomphalaria glabrata* (Say 1818), is an intermediate host (IH) of *Schistosoma masoni* Sambon 1907, the etiologic agent of schistosomiasis (Scholte *et al.*, 2012). Schistosomiasis affects around 200 million people worldwide and more than 700 million are in an inffection risk area (WHO, 2013). Besides that, 2.2 billion people wordwild still lack access to basic sanitation, and 627 million practice outdoor defecation, which contributes to the parasite and disease spread (WHO/Unicef, 2019). The incorporation of measures to combat the HI snail is a plan part guideline for schistosomiasis control together with patients treatment, funding in basic sanitation structures, and health education (King and Bertsch, 2015; Savioli and Daumiere, 2012). This measure is considered an effective and viable strategy, with short-term effectiveness to interrupt the parasite transmission cycle in endemic areas with high infection rates (Li *et al.*, 2019; Secor and Colley, 2018; Sokolow *et al.*, 2016).

The occurrence of the snail *Biomphalaria* spp. was highly correlated with disease incidence in Brazil appearing in 19 of the 27 states and affecting at least 30 thousand people per year (DataSUS, 2019; Scholte *et al*., 2012).Although it represents a species of interest in public health, *Biomphalaria* spp. has been identified as a potential biomonitor of environmental quality due to its sensitivity to pollutants (Tallarico, 2015; Oliveira-Filho *et al.*, 2017). Multiple reproductive and developmental biomarkers of the *Biomphalaria* spp. has been used in the ecotoxicological assessment of traditional and emerging pollutants (Ducrot *et al.*, 2014; Tallarico, 2015; OECD, 2016), such as nanomaterials (Oliveira-Filho *et al.*, 2017, 2019).

Accordingly, the present study aimed to evaluate the bioaccumulation, mortality, reproductive and developmental toxicity of IONPs using the freshwater snail *B. glabrata* as model system after chronic exposure to different environmentally relevant iron concentration, comparing IONPs to their dissolved counterpart ($FeCl_3$). Given the recent advances in nanotechnology in the control of intermediate hosts and vectors of neotropical diseases (Benelli, 2018b; Benelli *et al*., 2017; P. Li *et al*., 2018; Norouzi, 2017; Tomiotto-Pellissier *et al*., 2017), the hypothesis of this study was that the IONPs could alter the survival, reproduction, development and behaviour of the snail *B. glabrata*

and consequently contribute to reducing the snails population . The proposal of IONPs application in HI control, (if it shows some effect), has the advantage of could be withdrawn from the environment by magnetization, minimizing environmental impacts (Corsi *et al.*, 2018; Zou *et al.*, 2016), lower production cost about other NMs such as Ag NPs (Arularasu *et al.*, 2018; Kwon *et al.*, 2018; Zhang *et al.*, 2016), and the possibility of targeting specific species through functionalization.

To the best of our knowledge, this is the first study concerning the comparative reproductive toxicity of IONPs and $FeCl_3$ in *B. glabrata* snail after chronic exposure. Gluconic acid was chosen as a functionalizing agent due to its hydrophilic property, for ensuring greater stability to IONPs in suspension, and for being a non-toxic acid (PubChem, 2019; Ramachandran *et al*., 2006; Sui *et al*., 2012). Knowing the specific toxicity effects of IONPs, further studies will be able to explore other types of functionalization species-specific.

6.2. Material and methods

6.2.1. IONP synthesis

Gluconic acid (GLA)-functionalized IONPs were prepared by alkaline co-precipitation based on the methods described by Sui *et al.* (2012) and Wei *et al.* (2011) with modifications. Briefly, an aqueous solution of 0.1296 Mmol ferrous chloride ($FeCl_2.4H_2O$) and 0.2591 mol ferric chloride ($FeCl_3.6H_2O$), with a molar ratio of Fe^{3+}/Fe^{2+} = 2, was added to 400 mL of ASTM type II electrodeionized purified water. Then 600 mL of 2 Mmol L^{-1} NH_4OH was added without stirring and a black precipitate was instantaneously formed. The precipitate was then magnetic decanted with a neodymium magnet and rinsed several times with water until the supernatant reached pH 7.0. An aqueous solution of 2.3 Mmol sodium gluconic acid salt ($C_6H_{11}NaO_7$) and 12.5 Mmol NaOH in purified water (100 mL) was added to the IONP precipitate and kept under magnetic stirring at 80 °C for 4 hours. The resulting precipitate was purified by centrifugation using 70 % ethanol as a rinsing solvent until the supernatant reached pH 7.0. Subsequently, the material was dried in a vacuum oven at 60 °C for 4 hours.

6.2.2. IONP characterization

The morphology and individual diameter of IONPs were characterized by Transmission Electron Microscopy (MET). A drop of the IONP stock solution (0.3 mg L^{-1}) was deposited onto a mesh copper grid coated with a carbon layer and dry at room temperature (25 °C). The electron micrographs were obtained in a JEOL (JEM-2100) microscope, using the software Scandium da Olympus Soft Imaging Solutions GmbH. The individual diameter (D_{TEM}) of IONPs (n = 250 particles) was determined using the software ImageJ (National Institute of Health, USA). The surface charge (zeta potential) and hydrodynamic diameter (dh) of IONPs (0.3 mg L^{-1}) in ultrapure water (Milli-Q water) and dechlorinated water (exposure medium) were analysed by Dynamic Light Scattering (DLS) and Electrophoretic Light Scattering (ELS), respectively, using a Malvern ZetaSizer, (Nano-ZS90). For these analyses, the IONP solution was previously sonicated for 10 min, such as reported by Qualhato *et al.* (2017).

The IONP functionalization by the GLA was confirmed using the infrared spectroscopy in KBr pellet (IR-KBR). The IONP pellets were prepared with 1 % (w/w) samples in potassium bromide (KBr). The spectra were recorded in the range corresponding to the mid-infrared region (400 - 4000 cm^{-1}) with a resolution of 4 cm^{-1} using the FT-IR spectrometer (Perkin-Elmer). Furthermore, the powder IONPs were characterized by X-ray diffractions (XRD) using a X-ray diffractometer model Rigaku D/Max-2ª/C with CuKα radiation (λ = 1.54184 Å), operating at 15 mA and 35 Kv, sweep speed of 2.0° min^{-1}, with data measured every 0.01° in the range $10° \leq 2\theta \leq 80°$. The IONPs were also characterized by Mössbauer spectroscopy using a Wissel spectrometer. The Mössbauer transmission measurements were performed using a ^{57}Co radiation source in rhodium (Rh) matrix. Measurements were performed at room temperature (25 °C), as recommended by Dutra *et al.* (2017).

6.2.3. Animals

Four-month-old snails *B. glabrata* (total weight: 0.28 ± 0.04 g; shell diameter: 10 ± 2 mm) were obtained in the breeding stock from Institute of Tropical Pathology and Public Health in the Federal University of Goiás (Goiânia, Goiás, Brazil) and maintained in laboratory conditions according to Organisation for Economic Co-operation and Development (OECD) guideline nº 243 (OECD, 2016). Snails were maintained in 40 L tanks filled with 30 L of dechlorinated water (3 snails L^{-1}) under controled photoperiod

(12h:12h, light/dark cycle), temperature (25 ± 1 ºC) and pH (7.0 ± 1). Snails were fed *ad libitum* with organic lettuce leaves (*Lactuca sativa*).

6.2.4. Experimental design

Snails were exposed to GLA-functionalized IONPs and their dissolved counterpart ($FeCl_3$) at different iron concentrations (1.0, 2.5, 6.2 and 15.6 mg L^{-1}) in 3 L glass tanks (19.5 x 9.5 x 14.5 cm) containing 2 L of final dispersion/solution (10 snails *per* tank; 5 snail L^{-1}), jointly with a control group kept in declorinated water, during 28 days. The exposure was conducted in triplicate (10 snails *per* replica, 30 snails *per* experimental group) under controlled environmental conditions (temperature: 25 ± 1 ºC and 12/12 h light/dark cycle). The exposure medium was changed every three days with redosing of the both iron concentrations (OECD, 2016; Oliveira-Filho *et al.*, 2016). Snails were fed with lettuce leaves (*L. sativa*) (100 mg *per* snail) every three days. The food remains were removed at each medium exchange and the food replacement was done along with the replenishment of the exposure medium.

The IONP concentrations used are in accordance with the concentration of iron detected in the aquatic environment (up to 5 mg L^{-1}), according to Conselho Nacional do Meio Ambiente (CONAMA), Resolution No. 357 (Brasil, 2005) and the quantities of iron authorized to discharge effluents in Brazil (up to 15 mg L^{-1}), CONAMA Resolution No. 430 (Brasil, 2011). Furthermore, previous studies analyzed similar concentrations during the acute and long-term exposure of land and freshwater snails and fish to IONPs (Kaloyianni *et al.*, 2020; Oliveira-filho *et al.*, 2016; Villacis *et al.*, 2017).

To analyze the reproduction and collection of egg-masses during the exposure period, a piece of styrofoam (5 x 10 cm) was placed on the surface of tanks for oviposition, such as reported by Duarte *et al.* (2015). Mortality, somatic biomarkers (growth rate, condition index, gonadossomatic index), behavior and reproduction analyzes (fencundity) of adult snails and the development of their embryos (fertility) were performed daily during 28 days, while the metal bioaccumulation in snails was analyzed at the end of the exposure period (28 days).

6.2.5. Bioaccumulation

Whole soft tissues and shells of snails were collected at the end of exposure period (28 days), washed in ultrapure water, divided into 3 pools (each pool containing 3 snails; 9 snails *per* experimental condition), dried (70 ºC for 48 h), and digested in nitric acid (HNO_3) at 150 ºC for 30 min. The iron concentration was determined by atomic absorption spectrometry and a standard curve analyzed with different iron concentrations (0.125, 0.25, 0.5, 1, 2, 4, 8 ppm) of certified reference metal (PA Fe 1000 ppm, Qhemis High Purity NIST Test: #822/275197-07). The iron concentrations are expressed as μg mg^{-1} of dry weight (mean plus standard deviation of each pool triplicates).

6.2.6. Mortality

Cumulative mortality rate (MR) was determined daily during the exposure period (28 days) using the following equation (OECD, 2016): MR = (LSS/CM), were LSS is the number of live snails at the start of exporuse and CM is the cumulative mortality daily. Dead snails were identified according to the follow parameters: animal in decomposition, release of hemolymph, and when reclused into the shell with absence of heartbeat (Melo *et al.*, 2019; Rapado *et al.*, 2013)

6.2.7. Somatic biomarkers

At the beginning of the experiment (T0) and after 15 and 28 days of exposure, the growth rate (GR) was calculed by diferrence between initial diameter of the shell and final diameter. Furthermore, the condition index (CI) was calculed acoording to following equation: CI = (BW/SD) x 100, were BW is the body weigth (g) and SD is shell diameter (mm) at 28 days of the exposure (Avila-Poveda, 2013). The gonadosomatic index (GSI) of snails were calculed using the equation: GSI = (GW/ BW) x 100, were GW = gonad weith (g) and BW = total body weigth (g) (Devlaming *et al.*, 1982).

6.2.8. Behavioral imparments

Behavioral impairments were analyzed daily during the exposure period (28 days), in the morning, before any interference (removal of egg-masses, water exchange or feeding), during 1 minute for each experimental tank totaling 10 min per replica and 30 min per experimental condition. The frequency (%) of following behavioral changes were determined: swim on the water surface, reclusion into the shell, water avoiding, lethargy and detach from the shell as previosly described (Jurberg *et al.*, 1988; Pieri and Jurberg, 1981). Results were presented as mean of the weekly frequency (%) and standard deviation of the total of altered behaviors, and of each individual behavior.

6.2.9. Fecundity

Fecundity is defined by OECD guideline reproduction test nº 243, as "actual reproductive rate of organisms, measured by the number of egg- clutches or eggs'', while fertitily or reproductive output as "offspring production by parental animals within the test period" (OECD, 2016; Schmitt *et al.*, 2010). In this sense, egg-masses from parental snails (F_0-generation) exposed to both iron forms and control groups during 28 days were collected dialy and analyzed in a stereomicroscope (Zeiss, Stemi DV4) by following parameters: number of egg masses per live snail (F1), number of eggs *per* egg-mass (F2). The fecundity was determined using the Equations 1 and 2 (OECD, 2016), the data were presented as cumulative value:

$$F1 = \frac{LS\,(tx)}{EM\,(tx)} \quad (1)$$

$$F2 = \frac{TNE\,(tx)}{EM\,(tx)} \quad (2)$$

Where LS = number of live snails, EM = egg mass produced, TNE = total number of eggs (viable and unviable), and tx = values on "x" exposure day.

6.2.10. Developmental toxicity in the first generation (fertility)

At the beginning of the experiment (T0) and every four days (4, 8, 12, 16, 20 and 24 days), the egg-clutches (n = 3 *per* replica; n = 9 *per* experimental condition) from parental snails (exposed to both iron forms and control group) were collected, transferred to 12-wells microplates (1 egg-clutche *per* well) containing 5 mL of declorinated water and maintained under controlled conditions of the temperature (27 ± 0.5 °C), humidity (75 ± 5 %) and photoperiod (12h:12h ligth/dark cycle) using the BOD incubator (SL-224). Egg-clutches were analyzed daily using a microscope (Leica DM750) associated with the Leica model ICC50 HD camera and the LAS EZ software.

The embryo mortality rate (EM) (%) was calculated dividing the number of inviable embryos by the total embryos x 100 (OECD, 2016; Melo *et al.*, 2019). Dead embryos were identified according to Oliveira-filho *et al.* (2010): disintegrating embryonic forms, embryo with no rotational movements, no foot movements or abstence of heartbeat. The hatching rate (%) (HR) was determined dividing the number of hatched snails by the total embryos *per* egg-clutches after 144 h (OECD, 2016; Melo *et al.*, 2019), The early developmental stages were classified as described by Rapado *et al.*, (2013) and (Melo *et al.*, 2019): blatulae, gastrulae, trochophore, veliger, hippo stage and hatched and the frequency (%) of each developmental stages was determined.

6.2.11. Statistical analysis

Statistical analyzes were conducted in RStudio software (RStudio team, 2015). Tests of normality and homoscedasticity were performed using the Shapiro-Wilk and Levene test, respectively. For the parameters CI, IGS and fecundity (F1 and F2) the data were parametric, so it was performed Two-way Anova with Tukey's posteriori test. To GR Kruskall Wallis non-parametric test was performed. For parameters MR, bioaccumulation, EM, HR, EDS and behavior the data were non-parametric, and the Robusta ANOVA test was performed. The results are presented as mean and standard deviation of the replicates of each experiment and the significance level of $p < 0.05$ was adopted for all analyzes (Kloke & McKean, 2015) with the Dunn a posteriori test.

6.3. Results and discussion

6.3.1. NP characterization

TEM results showed IONPs with cuboid shape, monocrystalline nature (well defined edges and corners) (Fig. 1 A-B), and individual diameter of 7.5 ± 3.2 nm (Fig. 1 C). Similar morphology and size was reported in previous studies (Kwon *et al.*, 2018; Qualhato *et al.*, 2017; Wei *et al.*, 2011), demonstrating high reproducibility in the synthesis method by alkaline co-precipitation. The DLS and ELS analyses demonstrated that the IONPs presented higher hydrodynamic diameter and lower negative surface charge in dechlorinated water (671.9 ± 12.2 nm; -19.7 ± 3.87 mV) when compared to ultrapure water (156.7 ± 3.1 nm; - 47.8 ± 5.42 mV) (Fig. 1 D), confirming that the GLA functionalization confer a negative surface charge in both aqueous medium. These results corroborates with the chemical structure of gluconic acid, which in aqueous solution tends to deprotonate the hydroxyl groups (Wei *et al.*, 2011). This increase of the surface charge in dechlorinated water can be explained by the presence of cations, such as Ca^{2+} and Mg^{2+}, which partially overcome and neutralize the negative charge provided by the deprotonated hydroxyl group (Bhattacharjee, 2016; Qualhato *et al.*, 2017). The IONPs were moderately polydisperse with a polydispersity index (PdI) of 0.265 and 0.263 in dechlorinated and ultrapure water, respectively. The agglomeration and consequent sedimentation of IONPs in the exposure medium (dechlorinated) was similar to that previously reported for IONPs (Qualhato *et al.*, 2017; Wei *et al.*, 2011).

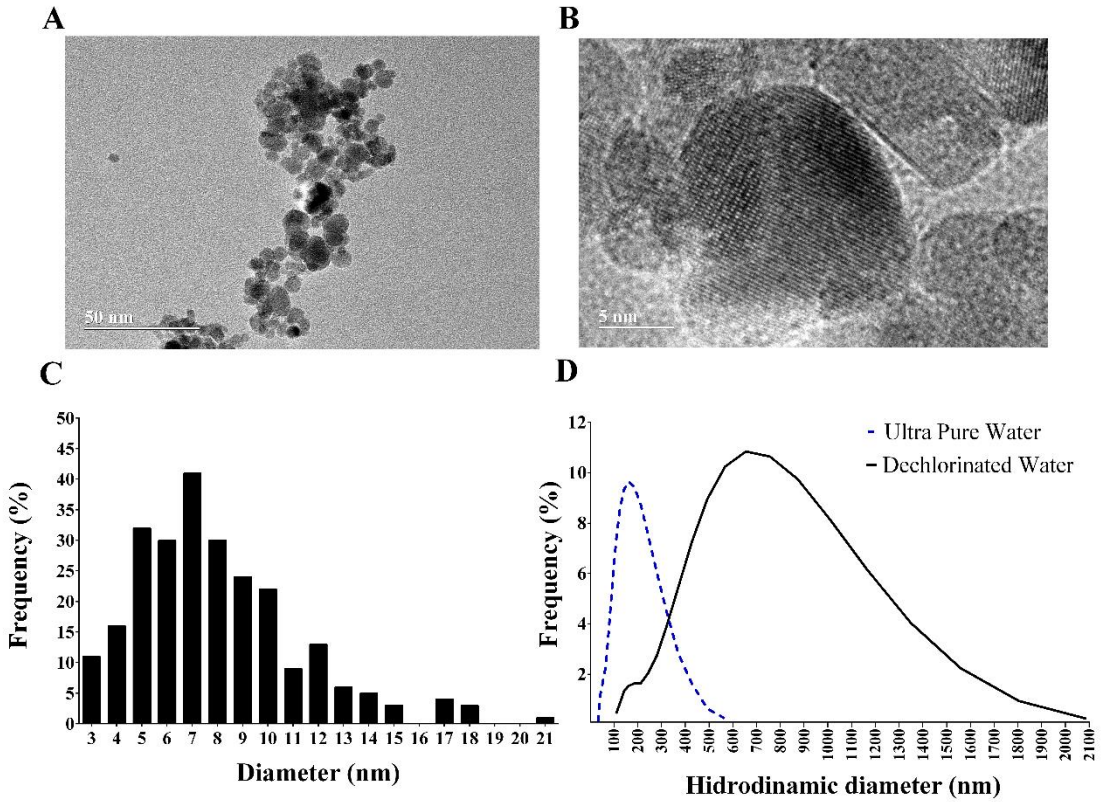

Figure 1. Transmission Electron Microscopic (TEM) images of gluconic acid-functionalized iron oxide (maghemite) nanoparticle (IONPs) (A and B), the individual diameter histogram of IONPs obtained from TEM figures and hydrodynamic diameter in ultrapure and dechlorinated water by Dynamic Light Scattering (DLS) (D). Scale bar = 50 nm (A) and 5 nm (B).

The vibrational absorption spectrum in the IR region of the uncoated IONP and GLA-functionalized IONPs is in Figure 2A. The absorption band at 3419 cm^{-1} (IONP and GLA-IONP) was attributed to symmetrical stretching of O-H bonds from water molecules incorporated into the crystal structure, from hydroxyls on the surface of particles and hydroxyl groups of GLA. The peak at 1626 cm^{-1} (IONP and GLA-IONP) is due to the angular deformation of the O-H bonds of water molecules, and the asymmetric stretching of the GLA carboxylate group bonds (COO-). The low absorption at 1382 cm^{-1} (GLA-IONP) was attributed to the symmetrical stretching of the carboxylate group that overlaps the wagging deformation of C-H in CH_2. At 1082 cm^{-1} the C-O strain of GLA is observed, confirming that the IONPs were functionalized with GLA (Sui *et al.*, 2012; Wei *et al.*, 2011). The absorptions at 630, 580, and 440 cm^{-1} correspond to the vibrational modes of Fe–O bonds in the samples. The peak at 580 cm^{-1} was associated with the mode of stretching the bonds at the tetrahedral and octahedral sites of the inverse spinel structure

and at 440 cm^{-1} related to the mode of stretching the octahedral sites of the magnetite crystal lattice (Fe_3O_4), and also their oxidized form, the maghemite (γ-Fe_2O_3). The peak at 630 cm^{-1} corresponds to the mode of stretching of maghemite bonds (Ercuta and Chirita, 2013; Gotić *et al.*, 2009; Ishii *et al.*, 1972; Stoia *et al.*, 2016; Sui *et al.*, 2012; Wei *et al.*, 2011). These results confirmed that the synthesized IONPs were composed of particles of magnetite partially oxidized to maguemite functionalized with GLA.

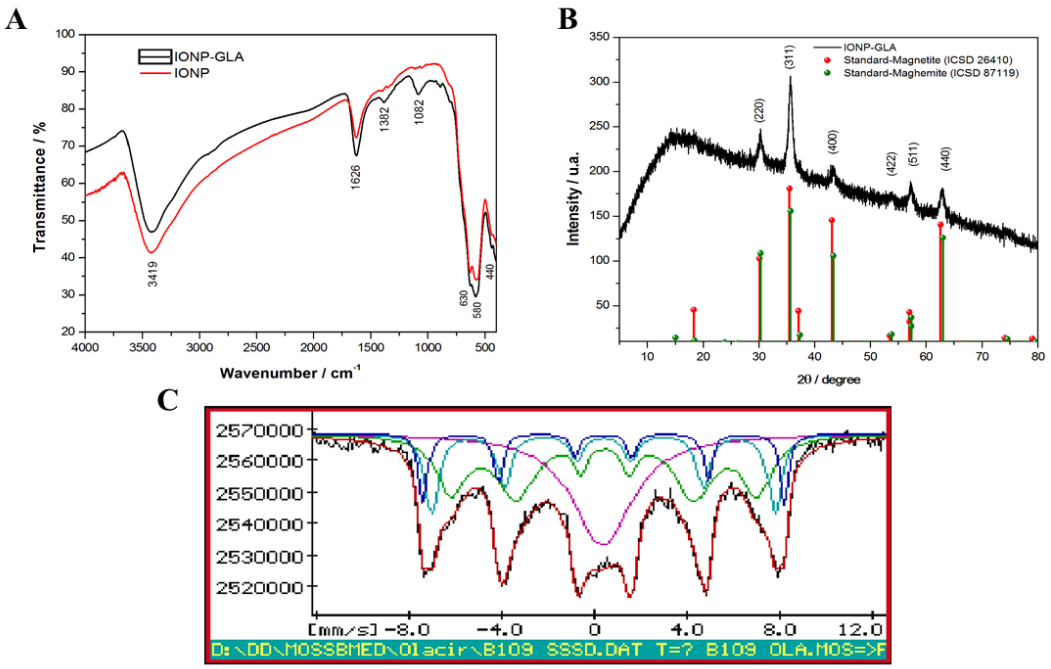

Figure 2. A) Vibrational absorption spectra in the infrared region of the uncoated IONP and gluconic acid (GLA)-functionalized IONPs. B) X-ray diffractogram of the GLA-functionalized IONP compared to magnetite and maghemite standards. C) Mössbauer spectra obtained from gluconic acid (GLA)-functionalized IONP.

XRD results (Fig. 2 B) showed the presence of inverse spinel structure forming a typical centered face cubic network of magnetite (Fe_3O_4) and / or maguemite (γ-Fe_2O_3) compared to Inorganic Crystal Structure Database (ICSD 26410 and 87119) magnetite and maghemite, respectively), with 2θ diffraction peaks = 30.3°, 35.6°, 43.3°, 53.7°, 57.3°, 62.9°, referring to the reflections of the crystalline planes (220), (311), (400), (422), (511) and (440) (Chen *et al.*, 2009; Dutra *et al.*, 2017; Kim *et al.*, 2012). As shown in the reference standards, magnetite and maghemite have nearly identical diffraction patterns, and their phases are very difficult to differentiate by XRD. Thus, the Mössbauer spectroscopy was used as a complementary technique to characterize two phases present in the GLA-IONPs (Fig. 2C and Table 1).

Table 1. Least square fitted at room temperature Mössbauer parameters obtained for gluconic acid (GLA)-functionalized IONP.

Sample	Site	IS / mms^{-1}	QS / mms^{-1}	B_{hf} / T	A / %
IONP-GLA	S_1	0.31738 (0.00759)	0.02123 (0.01525)	48.40105 (0.09525)	11.143
	S_2	0.32299 (0.00917)	-0.01684 (0.01842)	45.98169 (0.16334)	19.060
	S_3	0.36741 (0.01461)	-0.03343 (0.02555)	41.01368 (0.33260)	39.801
	D	0.28521 (0.02070)	0.35687 (2.32295)	-	29.996

S = sextet; D = doublet; B_{hf} = hyperfine magnetic field; IS = isomeric shift/Fe; QS = quadrupolar displacement; A = relative area

Figure 2C shows room temperature Mössbauer spectra obtained from IONP-GLA sample, and the least square fitted room temperature parameters are given in table 1. The spectra were fitted with three magnetic sextets and one doublet. The least square fitted parameters values obtained from the adjusted parameters for the samples differ to the in relation to the bulk of the stoichiometric magnetite parameters. These differences are related to obtaining non-stoichiometric magnetite, due to the partial oxidation of $Fe^{2+} \rightarrow Fe^{3+}$, giving rise to non-stoichiometric maghemite. The sextet 1 and 2 samples were attributed to the presence of the octahedral sites, containing Fe^{3+} and Fe^{2+} ions, and tetrahedral sites containing Fe^{3+} ions, respectively, for the magnetite phase. The sextet 3 corresponds to the maghemite phase. The presence of a doublet is due to the superparamagnetic character with a hyperfine magnetic field equal to 0 and the reduction of the crystallite diameter that was confirmed in the XRD, indicating the presence of some particles below the critical size for superparamagnetic relaxation in the measurement time scale. The Mössbauer spectroscopy results corroborate the FTIR and XRD data, indicating that the OM phase in the hybrids are formed by the magnetite and maghemite phases (Dutra *et al.*, 2017; Neto *et al.*, 2018; Wei *et al.*, 2011).

6.3.2. Bioacumulation

The chronic exposure to both iron forms induced iron accumulation in the snail *B. glabrata*, with high accumulation in the visceral mass (Fig. 3 A) compared to shell (Fig. 3 B). The snail exposed to IONPs for 28 days showed a higher iron concentration starting from 6.2 mg L^{-1} in the visceral mass when compared to control group and those exposed to iron ions ($p < 0.05$; Fig. 3), while similar iron concentration in the shell was observed in the snails exposed to both iron forms compared to control group (Fig. 3 B).

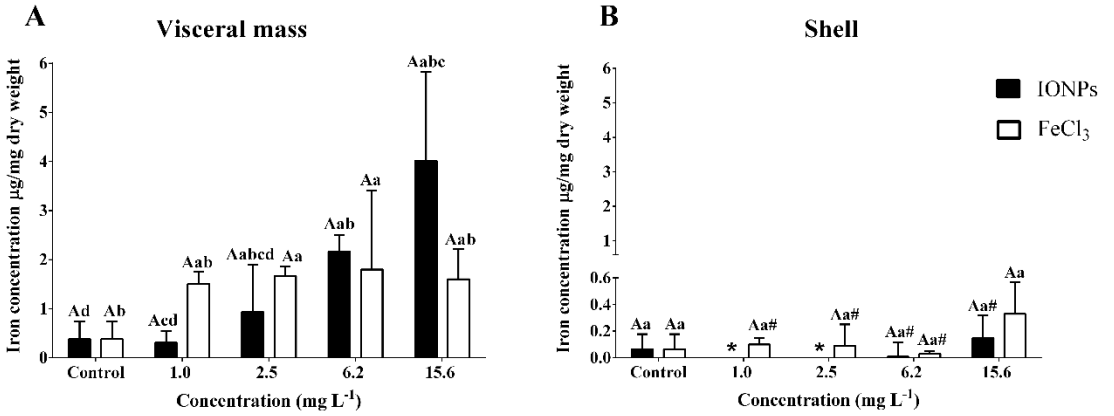

Figure 3. Iron concentration (µg mg-1 dry weight) in the whole soft tissues and shell of the freshwater snail *Biomphalaria glabrata* from control group and after exposure to IONP and FeCl3 for 28 days. Different uppercase letters indicate statistical difference ($p < 0.05$) between the same concentrations of IONPs and $FeCl_3$. Diferentt lowercase letters indicate statistical difference ($p < 0.05$) between the concentration gradient of the same experimental group. (#) Significative difference between same concentration of different tissues ($p < 0.05$). (*) Below of detection level (< 0.01 µg/mL – limit detection).

Iron concentration in visceral mass of snails exposed to IONPs at 6.2 and 15.6 mg L^{-1} was higher (5.7- and 7.4-fold; $p = 0.01$ and 0.04, respectively) compared to the control group and increased in a concentration-dependent pattern ($y = -0.1004 + 0.8575x$; $R^2 = 0.607$, $p < 0.01$, $F = 20.1$). On the other hand, the bioaccumulation in the snails exposed to iron ions at 2.5 and 6.2 mg L^{-1} was higher compared to unexposed snails (4.3 and 4.7 times; $p = 0.04$ and 0.05 respectively), but this bioaccumulation did not have a concentration-dependent increase. The sediment foraging habit in the snail may have contributed to the bioaccumulation of both iron forms, since both iron forms formed precipitates at the bottom of the aquarium. Similarly, the metal bioaccumulation in the freshwater snail *Lymanea stagnalis* after dietary exposure to CuO NPs (7 nm) was higher when compared to waterborne exposure (Croteau *et al.*, 2014), such as observed for Ag

NPs (13 nm) (Croteau *et al.*, 2011b) and ZnO NPs (20 – 70 nm) (Croteau *et al.*, 2011) indicating that NP accumulation in snails is dependent on type of NPs, species and experimental conditions, especially route of exposure.

NPs are usually internalized in snail cells *via* endocytosis (Dombu *et al.*, 2010), receptor-mediated endocytosis (Decuzzi and Ferrari, 2007), or phagocytosis (Anselmo *et al.*, 2015), while iron ions enters the cell by conventional cation uptake facilitated by the clathrin receptor on the apical membrane (Le and Richardson, 2002). The high iron accumulation induced several cellular damages in the snails, such as reactive oxygen species (ROS) production, oxidative stress, lipid peroxidation (LPO), and consequently protein and nucleic acids degradation, and cell death (Laffon *et al.*, 2018; Le and Richardson, 2002). These toxic effects induced by NP exposure may lead to changes in the reproduction and development of snails, as observed for snails *B. glabrata* exposed to GLA-functionalized IONPs.

6.3.3. Mortality

During the exposure period (28 days), a gradual decrease in survival rate (SR) was observed in snails exposed to IONPs ($p < 0.05$; Fig. 4A), while similar SR was identified in the snails exposed to iron ions when compared to control group ($p < 0.05$; Fig. 4B). The snails exposed to IONPs at 15.6 mg L^{-1} for 28 days showed reduced SR (37 ± 12%) when compared to iron ions (20 ± 20 %) and the control groups (17 ± 5.8 and 23 ± 5.7 %) ($p < 0.01$). Conversely, no mortality was reported the snail *B. glabrata* exposed to meso-2,3-dimercaptosuccinic acid (DMSA)-functionalized IONPs (5.7 nm; 1.0 – 100 mg L^{-1}) for 4 weeks (Oliveira-Filho *et al.*, 2016), indicating that the GLA-functionalized IONPs induced high mortality in the *B. glabrata* when compared to DMSA-fuctionalized IONPs. The GLA is a biocompatible compound that has been used as an endosomolytic agent in multi-drug drug delivery systems because it improves solubility by providing a hydrophilic fraction that facilitates cell uptake (Pack *et al.*, 2004, 2000), probably facilitating the internalization and toxic effects of IONPs. Similar results have also been reported for other invertebrate species, such as increased mortality in *Daphnia magna* (Crustacea: Daphniidae) exposed to Mn conjugated to GLA (Sillanpaa *et al.*, 2003).

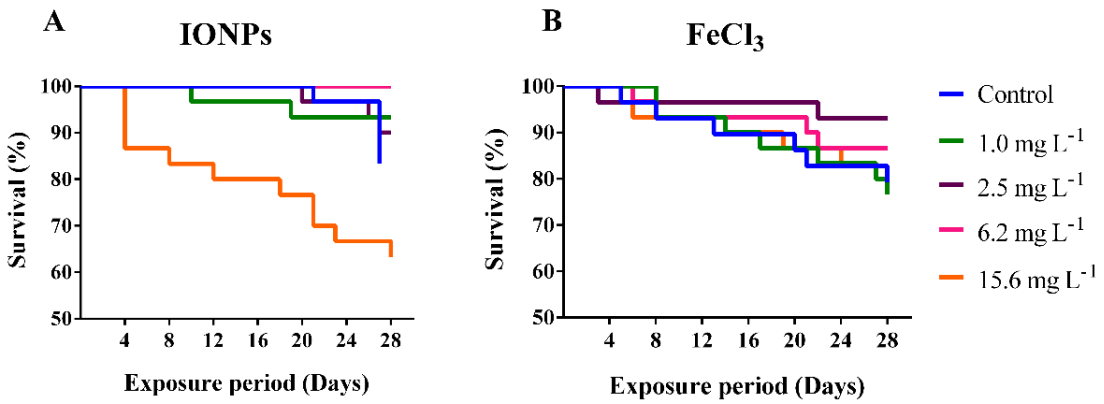

Figure 4. Survival rate (%) of adult snails *Biomphalaria glabrata* from control group and after exposure to IONPs and $FeCl_3$ during 28 days.

The mortality in snails exposed to IONPs was associated with its high accumulation in snail tissues (Fig. 3), which may induce ROS production, oxidative stress and oxidative damage, such as reported for other NMs (Ali *et al.*, 2016, 2012; Bao *et al.*, 2018; Ma *et al.*, 2017, 2016). Similarly, the land snail *Cornu aspersum* exposed to IONPs (20 nm; 0.05 and 1 mg L^{-1}; 20 d) (Sidiropoulou *et al.*, 2018) and to IONPs (26 nm; 1-3 mg g^{-1}; 42 d) (Besnaci *et al.*, 2019) showed ROS production, oxidative stress, increased antioxidant enzymes, oxidative damage (i.e. LPO and protein carbonylation), increased level of ubiquitin conjugates, increased caspase activity, and DNA damage (Besnaci *et al.*, 2019; Sidiropoulou *et al.*, 2018), confirming the toxic effects of IONPs on aquatic and land snails.

6.3.4. Somatic biomarkers

Snail exposed to both iron forms showed similar biometric parameters (shell diameter, body weight, GR and IGS) when compared to those unexposed ($p > 0.05$; Table 2). These results indicated that the IONPs did not interfere with the somatic or reproductive parameters of snails. On the other hand, previous studies showed that several metal-based NPs decreased the growth rate and the energetic metabolism (Ali *et al.*, 2015; Bernot and Brandenburg, 2013; Pang *et al.*, 2013, 2012; Ramskov *et al.*, 2015), indicanting that the effects of NPs to somatic parameters are dependent on type of NPs, experimental design and snail species.

Table 2. Biometric parameters [shell diameter (SD), total weight (W), growth rate (GR), gonadosomatic index (GSI) and condition index (CI)] of freshwater snails *Biomphalaria glabrata* from control group and after exposure to $FeCl_3$ and IONPs during 28 days. Results are presented as mean and standard deviation of total surviving snails after 28 days. *No significative difference was observed for all parameters.

Groups	IONPs					$FeCl_3$				
	SD (mm)	W (g)	GR (mm)	GSI	IC	SD (mm)	W (g)	GR (mm)	GSI	IC
Control	12.1 ± 0.9	0.29 ± 0.07	0.02 ± 0.02	0.03 ± 0.02	2.44 ± 0.4	11.7 ± 1.2	0.26 ± 0.08	0.05 ± 0.01	0.04 ± 0.02	2.15 ± 0.4
1 mg L^{-1}	12.6 ± 0.8	0.31 ± 0.06	0.03 ± 0.03	0.04 ± 0.01	2.44 ± 0.4	11.8 ± 1.6	0.26 ± 0.1	0.07 ± 0.02	0.05 ± 0.02	2.12 ± 0.5
2.5 mg L^{-1}	12.5 ± 1.1	0.32 ± 0.08	0.04 ± 0.02	0.04 ± 0.02	2.53 ± 0.5	11.6 ± 1.0	0.29 ± 0.06	0.07 ± 0.02	0.05 ± 0.05	2.19 ± 0.4
6.2 mg L^{-1}	11.9 ± 1.3	0.27 ± 0.09	0.04 ± 0.02	0.04 ± 0.02	2.28 ± 0.5	11.6 ± 1.3	0.26 ± 0.07	0.09 ± 0.02	0.04 ± 0.02	2.15 ± 0.4
15.6 mg L^{-1}	13.1 ± 0.9	0.36 ± 0.1	0.04 ± 0.03	0.05 ± 0.03	2.71 ± 0.6	11.4 ± 0.7	0.22 ± 0.04	0.05 ± 0.01	0.04 ± 0.02	1.88 ± 0.3

6.3.5. Behavioral impairment

Behavioral alterations in snails are excellent biomarkers for toxic stressors present in water, in addition to potential indicators of substances with a molluscicidal effect (Bernot *et al.*, 2005; Lürling, 2012; O. S. Pieri and Jurberg, 1981). In addition, changes in behavior can affect snail survival and reproduction strategies, contributing to the reduction or maintenance of the population, for example leading to greater susceptibility to predators (Justice and Bernot, 2014; Young *et al.*, 2017) or survival in an unfavorable environment (Mizrahi *et al.*, 2012; Poznańska *et al.*, 2015).

Snails exposed to both iron forms at 15.6 mg L^{-1} showed an increase in the total behavioral changes (TBC) after 7 days compared to the control group ($p < 0.05$; table 3). IONPs induced an increase in ACT (- 5 fold) compared to control in the first week of exposure. However, similar behavior (TBC) was observed in the IONP-exposed snails compared to control group over the remaining weeks ($p > 0.05$). Furthermore, $FeCl_3$ at 15.6 mg L^{-1} induced an increase in TBC after the second and fourth week of exposure compared to the control group (- 3.3 fold and - 5.2 fold, respectively ; $p < 0.05$; table 3).

Table 3. Behaviour alterations (%) in adult freshwater snails *Biomphalaria glabrata* from control group and after exposure to IONPs and $FeCl_3$ during 28 days. SWS: swim on the water surface; SR: shell reclusion; WA: water avoiding; L: lethargy; DS: Detach from the shell. Different uppercase letters indicate statistical difference ($p < 0.05$) between the same concentrations of IONPs and $FeCl_3$. Different lowercase letters indicate statistical difference ($p < 0.05$) between the concentration gradient of the same experimental group respectively. Superscript numbers indicate a significant difference of the same concentration over the weeks. The numbers 1, 2, 3 and 4 refer to the respective weeks.

	IONPs (mg L^{-1})					$FeCl_3$ (mg L^{-1})				
					1st Week					
Behaviour	**NC**	**1**	**2.5**	**6.2**	**15.6**	**NC**	**1**	**2.5**	**6.2**	**15.6**
SWS	0.5 ± 1.2	1.4 ± 3.8	0.5 ± 1.2	3.3 ± 5.5	3.9 ± 6.0	0.9 ± 1.6	0.53 ± 1.4		0.9 ± 1.6	0.9 ± 2.5
SR	0.5 ± 1.2	1.4 ± 3.8	0.9 ± 2.5	0.5 ± 1.2	7.7 ± 9.4	1.5 ± 3.9	2.0 ± 4.0	1.5 ± 3.4	3.3 ± 6.4	0.5 ± 1.2
WA	2.0 ± 3.2	1.4 ± 1.8	0.5 ± 1.2		2.5 ± 4.3					0.5 ± 1.2
L				0.5 ± 1.2						
Total	2.9 ± 4.2$^{Ab\ 3}$	4.3 ± 6.9 ^{Ab}	1.9 ± 4.1$^{Ab\ 3,4}$	4.3 ± 6.9^{Ab}	14.2 ± 12.0^{Aa}	2.4 ± 5.3 ^{Aa}	2.5 ± 4.4 $^{Aa\ 4}$	1.5 ± 9.3 $^{Aa\ 4}$	4.3 ± 6.7 ^{Aa}	2.0 ± 3.8 $^{Ba\ 2,3,4}$
					2nd Week					
SWS	1.4 ± 2.9	2.4 ± 4.4	0.9 ± 2.5	1.4 ± 3.8	1.4 ± 1.8			1.2 ± 3.1		7.4 ± 8.3
SR	0.5 ± 1.2	2.4 ± 3.5	1.4 ± 2.9	0.9 ± 2.5	3.0 ± 5.7	3.0 ± 4.6	1.7 ± 4.4	2.4 ± 4.1	1.0 ± 2.6	1.8 ± 4.7
WA	5.2 ± 6.0	0.5 ± 1.4	2.9 ± 3.4							
L					0.5 ± 1.3					0.9 ± 2.5
DS					0.6 ± 1.6					
Total	7.1 ± 6.1^{Aa}	5.4 ± 5.3 ^{Aa}	5.2 ± 6.2 ^{Aa}	2.4 ± 4.2 $^{Aa\ 4}$	5.6 ± 8.8 $^{Aa\ 4}$	3.0 ± 4.6 ^{Ab}	1.7 ± 4.4 $^{Ab\ 4}$	3.6 ± 4.4 $^{Ab\ 4}$	1.0 ± 2.6 $^{Ab\ 3}$	10.2 ± 9.7 $^{Aa\ 1}$
					3rd Week					
SWS	5.7 ± 7.8	1.5 ± 3.1	3.8 ± 6.8	3.8 ± 5.66	2.9 ± 5.0	1.1 ± 3.0	1.8 ± 3.2	3.4 ± 5.6	3.6 ± 6.0	9.9 ± 11.7
SR	4.9 ± 7.7	1.4 ± 2.9	5.8 ± 7.0	1.9 ± 4.14	3.2 ± 8.5	6.5 ± 7.0	2.8 ± 5.2	2.6 ± 4.9	2.0 ± 5.3	1.5 ± 3.9
WA	3.8 ± 5.7	3.0 ± 5.8	4.4 ± 6.1	2.9 ± 4.4	2.5 ± 4.0	1.6 ± 3.3	1.4 ± 4.4			3.6 ± 4.4
L		0.5 ± 1.4								
Total	14.4 ± 8.5$^{Aa\ 1}$	6.5 ± 7.5 ^{Aa}	14 ± 10.5 $^{Aa\ 1}$	8.6 ± 7.3 ^{Aa}	8.7 ± 12.3 ^{Aa}	9.3 ± 9.7 ^{Aa}	6.2 ± 8.8 ^{Aa}	6.0 ± 7.7 ^{Aa}	5.6 ± 8.5 $^{Aa\ 2}$	15.0 ± 12 $^{Aa\ 1}$
					4th Week					
SWS	1.1 ± 1.8	2.1 ± 5.4	7 ± 7.5	3.3 ± 8.9	3 ± 7.9	0.7 ± 1.8	3 ± 5.6	4.5 ± 7.3	3.8 ± 6.3	3 ± 5.4
SR	2.3 ± 5.0	2.1 ± 3.6	1.5 ± 3.0	3.8 ± 5.9	6.2 ± 10.3	3.1 ± 5.9	3 ± 6.3	2.3 ± 6.1	0.5 ± 1.4	16.8 ± 19.6
WA	5.7 ± 5.8	4.8 ± 7.7	7.3 ± 9.1	4.3 ± 5.6	5.9 ± 5.9	2.5 ± 5.4	6.5 ± 8.5	4.6 ± 6.4	1.1 ± 3.0	7.1 ± 10.9
L	0.5 ± 1.2		0.5 ± 1.4		0.8 ± 2.1					
DS					1 ± 2.5					
Total	9.5 ± 10.8 ^{Aa}	8.9 ± 10.6 $^{Aa\ 1}$	16.3 ± 12.8 $^{Aa\ 2}$	11.4 ± 10.5 $^{Aa\ 4}$	16.8 ± 17.1 ^{Aa}	6.2 ± 11 ^{Ab}	12.5 ± 15.0$^{Aab\ 1,2}$	11.4 ± 11.3 $^{Aab\ 1,2}$	5.5 ± 7.6 ^{Ab}	26.9 ± 17.5 $^{Aa\ 1}$

After exposure to both iron forms for 28 days, the most frequent behavioral alterations observed were swim on the water surface (SWS), shell reclusion (SR), water avoiding (WA) and lethargy (L) (Table 3), confirming its potential neurotoxic effects to freshwater snails. Physicochemical changes in the aquatic environment caused by NMs and heavy metals can often interrupt or alter the ion channel receptors form the gastropod neuronal membranes and lead them to reduce the ability to assess predation risk (Bernot and Brandenburg, 2013; Justice and Bernot, 2014; López-Doval *et al.*, 2019).

The behavioural alterations induced by both iron forms, such as SWS and WA, have been reported for several compounds with potential molluscicidal activity (Lake-Thompson and Hofmann, 2019; Sarquis *et al.*, 1997; Schüder *et al.*, 2004), and may lead to increased predation risk. On the other hand, the SR behaviour consists of a defensive response of the snail by decreasing the surface area of exposure with the medium. However this behaviour can compromise the energy balance, the aptitude for reproduction, feeding and breathing activities and lead to death after long time (Bernot *et al.*, 2005; Pieri and Jurberg, 1981). In addition, the lethargy is a response to neuromuscular changes caused by metals, which can interfere with Ca^{2+} capture and muscle contractions, also leading to a higher risk of predation and to compromise in the snail's survival (Dobranskyte *et al.*, 2006, 2004; Niyogi *et al.*, 2014; Pyatt *et al.*, 2002).

Interestingly, after exposure to IONPs at 15.6 mg L^{-1} for 2 and 3 weeks, the frequency of the snail detached from the shell increased in comparation to the control (0.6 and 1 %, respectively). This behaviour is rare and characterized by the disengagement of the animal from its shell and maintenance of locomotion and feeding activities, followed by death after 48 h (Fig. 5). IONPs can interfere in the electrostatic current of the organic matrix of the mantle, formed by various acidic mucopolysaccharides, which help in the capture, stabilization and biomineralization of Ca^{2+} (Bielefeld *et al.*, 1993; Marxen *et al.*, 2003; Marxen and Becker, 2000) or even that they interfere with the Na^{+}/ K^{+} pump leading to an increase in membrane permeability to ions (mainly Ca^{2+}) involved in the intensification of muscle activity followed by contraction as well as in anti-helminthic drugs.

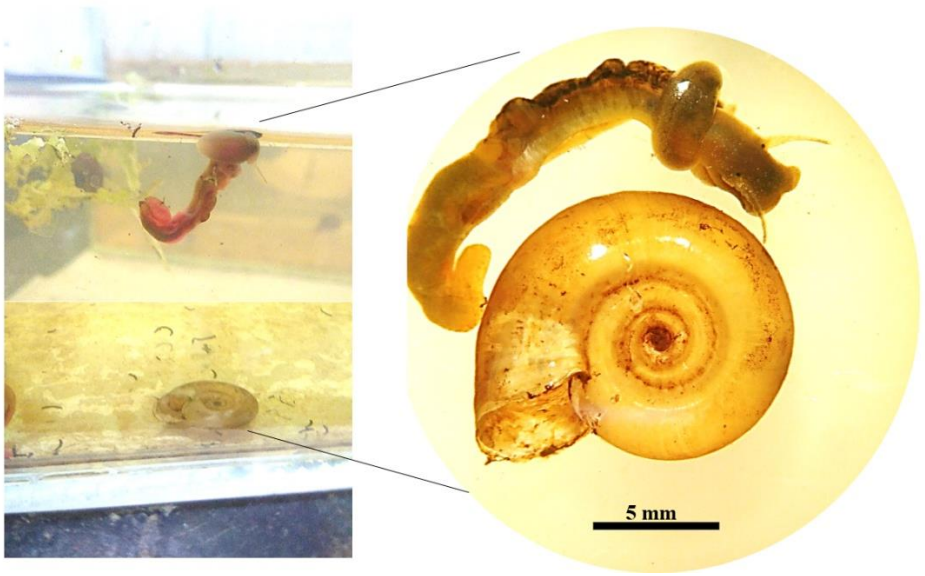

Figure 5. Snail detached from the shell after 12 days of exposure to IONPs at 15.6 mg L^{-1}. Snail still alive after 24h, swimming under the glass aquarium wall.

6.3.6. Fecundity

The fecundity parameters of snails during chronic exposure to IONPs and $FeCl_3$ are in Fig. 6. After 28 days of exposure, both iron forms reduced the number of egg-clutches *per* snails when compared to control groups, with high effects of iron ions compared to IONPs. Snails exposed to IONPs at 1.0, 2.5, 6.2 and 15.6 mg L^{-1} showed decreased number of egg-clutches compared to control group (1.2-, 1.1-, 1.4- and 1.2 – fold) (Fig. 6A), whereas those exposed to $FeCl_3$ at the same concentrations presented reduced the number of egg-clutches in 1.3-, 1.5-, 2.8-fold, respectively (Fig. 6C). On the other hand, only the $FeCl_3$ at 2.5, 6.2 and 15.6 mg L^{-1} reduced the number of eggs per egg-clutches when compared to control groups (1.6, 1.4 and 1.8-fold ; $p < 0.05$; Fig. 6D). Conversely, no effect of DMSA-functionalized IONPs (5.7 nm) at 1.0, 10 and 100 mg L^{-1} was observed in the fecundity of *B. glabrata* after chronic exposure (28 days) (Oliveira-filho *et al.*, 2016), confirming that the reproductive toxicity of IONPs is dependent on its functionalization. In addition, previous studies using the snail *Potamopyrgus antipodarum* and other metal-based nanoparticles, such as CuO NPs (7 – 40 nm; 240 μg g^{-1}; 9 wk) (Ramskov *et al.*, 2014) and Ag NPs PVP-coated (54.5nm; 0.10-1000 μg L^{-1}; 28 d) (Völker *et al.*, 2014), demonstrated that the reproductive toxicity induced by NPs also was dependent on exposure time, NP morphology and presence of other poluttants.

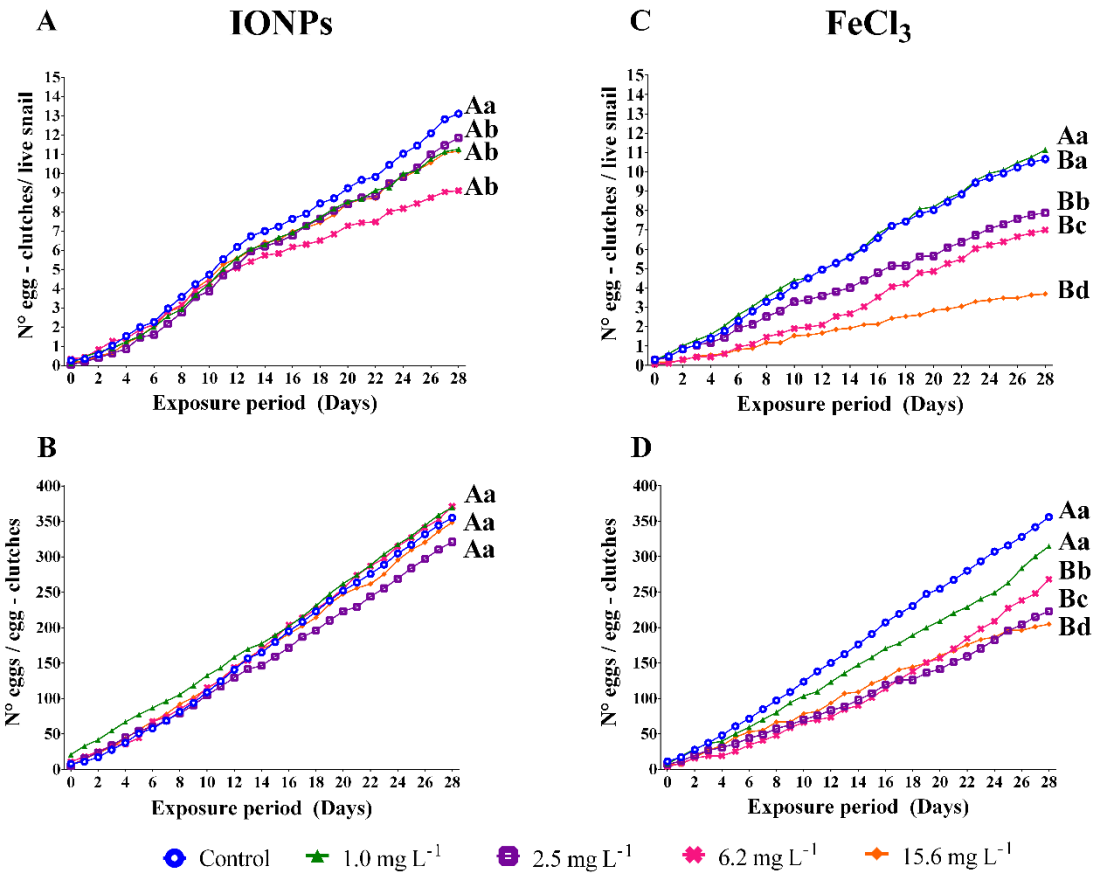

Figure 6. Production of egg-masses (A and C) and number of eggs per egg masses (B and D) of the freshwater snail *Biomphalaria glabrata* from control group and after exposure to IONPs (A and B) and $FeCl_3$ (C and D) during 28 days. Different uppercase and lowercase letters indicate statistical difference ($p < 0.05$) between the same concentrations of IONPs and $FeCl_3$, and between the concentration gradient of the same experimental group, respectively.

6.3.7. Developmental toxicity in the first generation (fertility)

6.3.7.1 Embryotoxicity and hatching rate

The embryo mortality and hatching rate in the first generation of snails after parental exposure to both iron forms are in Fig. 7 A-B. Overall results showed differential embryotoxicity of both iron forms in a time exposure and concentration dependent patterns. IONPs induced high mortality and hatching delay in the *B. glabrata* when compared to iron ions. After 8, 16 and 24 days of exposure, the IONPs at 15.6 mg L^{-1} increased the embryo mortality (5.4, 3.7, e 3.2-fold, respectively) compared to the control group ($p < 0.05$; Fig .7A). Similarly, IONPs at 15.6 mg L^{-1} reduced the hatching rate

compared to control after 8 (0.6-fold), 16 (0.7-fold) and 24 (68-fold) days of exposure ($p < 0.05$; Fig. 7A). On the other hand, the iron ions at 15.6 mg L^{-1} induced significant embryo mortality and hatching delay only after 20 days of exposure ($p < 0.05$; Fig 7B).

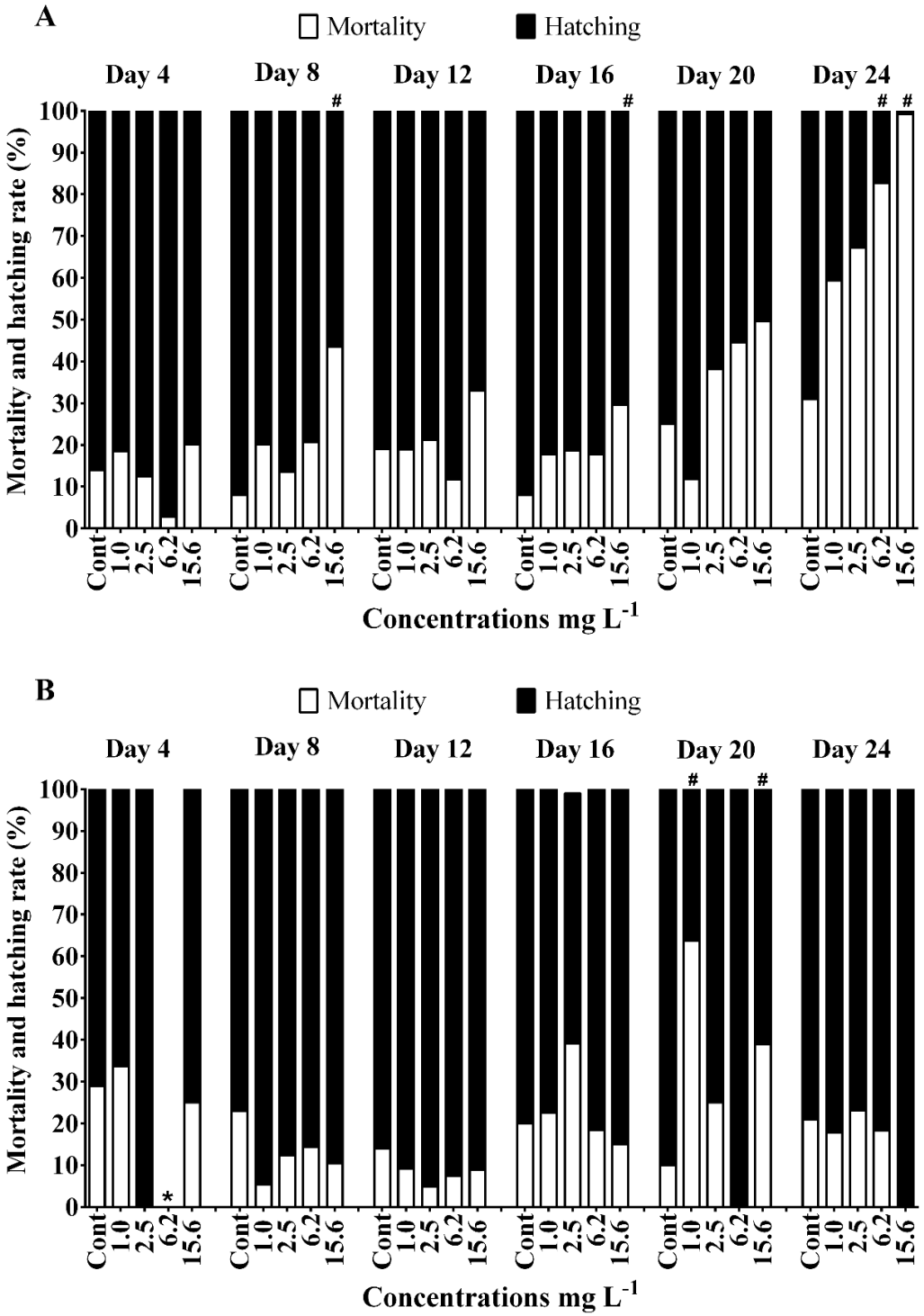

Figure 7. Mortality and hatching rate (%) of embryos from parental snails of the control group and after exposure to IONPs (A) and $FeCl_3$ (B) during 28 days. Cont – control, *Egg masses were not produced. # statistical difference ($p < 0.05$) from the control group

The embryotoxicity of metal-based NPs in snail species has been associated to ROS production, oxidative stress and oxidative damage, such as observed for CdTe NPs (3 nm; 1 -20 nM; 24 h) in *B. glabrata* (Vasconcelos - Lima *et al.*, 2019), Ag NPs (24 nm; 0.001- 100 μg L^{-1}; 96 h) (Bernot and Brandenburg, 2013) and α-alumina and γ-alumina NPs (20-80 nm; 0.005 - 0.5 g Kg^{-1} of dry sediment; 96 h) in *Physella acuta* (Musee *et al.*, 2010). The ROS production and the stress oxidative induced by IONPs also were reported for terrestrial snail such as *Corunu aspersum* (Besnaci *et al.*, 2019; Kaloyianni *et al.*, 2020; Sidiropoulou *et al.*, 2018) and other aquatic organisms such as bivalve and fish (Kádár *et al.*, 2010; Qualhato *et al.*, 2017; Villacis *et al.*, 2017), confirming their potential embryotoxic effect mediated by oxidative stress.

6.3.7.2 Developmental stages

The frequency (%) of the embryonic developmental stages after 144 hours post fertilization (predicted period of hatching) was evaluated in embryos from adult parental snails exposed to IONPs (Fig. 8A) and $FeCl_3$ (Fig. 8B) during 24 days. The exposure of the parental snails to IONPs at 15.6 mg L^{-1} for 8, 20 and 24 days reduced the frequency of embryos in hippo stage (1.1, 1.1- and 2.2-fold) when compared to control group ($p < 0.05$). Developmental delays were predominantly interrupted at the trocophore and veliger stages. On the other hand, similar frequency of hippo stage was observed snails exposed to iron ions or to IONPs at low concentration (1.0 to 6.2 mg L^{-1}) compared to control group ($p > 0.05$), confirming the high embryotoxicity of IONPs compared to its dissolved counterpart.

The snail developmental inhibition induced by direct egg-clutches exposure to metal-based and inorganic NPs has been reported to *P. acuta* exposed to Ag NPs (Gonçalves *et al.*, 2017), *Biomphalaria pfeifferi* exposed to curcumin-nisin polylactic acid NPs (Omobhude *et al.*, 2017), *Biomphalaria alexandrina* exposed to SiO_2 NPs (Attia *et al.*, 2017) and *B. glabrata* exposed to CdTe NPs (Vasconcelos - Lima *et al.*, 2019). However, this is the first study concerning the effects of parental exposure to IONPs on development in the first generation. The mechanism of action and toxicity of IONPs for aquatic organisms was related to oxidative stress by the production of ROS, genotoxicity, lipid peroxidation, proteolysis, protein carbonylation and consequently, apoptosis and necrosis (Kaloyianni *et al.*, 2020; Sidiropoulou *et al.*, 2018; Villacis *et al.*, 2017). Other studies have also associated the toxicity of IONPs and their ionic form to the differences

in the mechanisms of uptake, internalization, transference, bioavailability and biotransformation of iron inside the cell (Kadar *et al.*, 2010; Kádár *et al.*, 2010), which corroborates ours findings of the differential toxicity of IONPs and $FeCl_3$ using multiple biomarker responses.

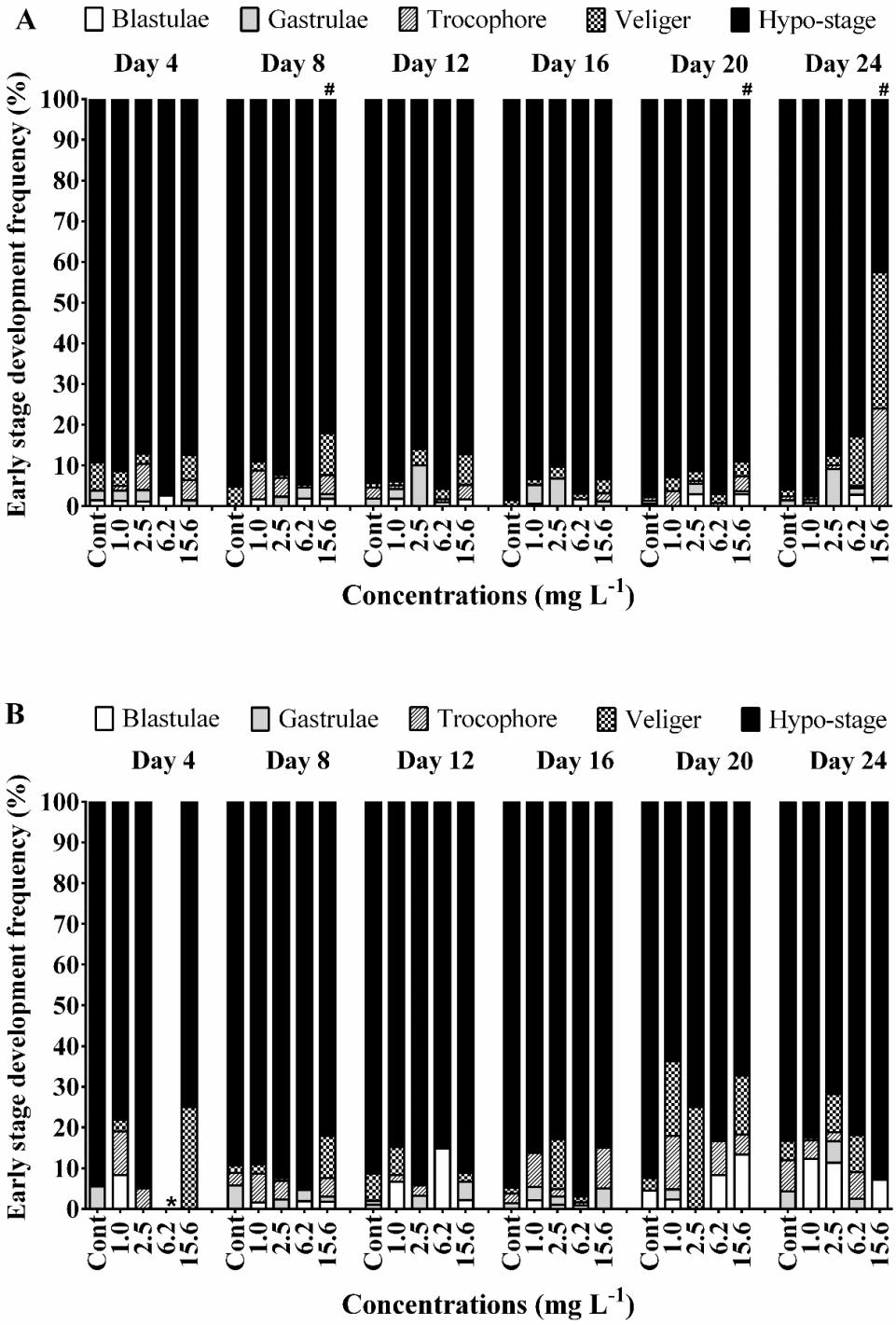

Figure 8. Frequency (%) of the early developmental stages of *Biomphalaria glabrata* from parental snails of the control group and after exposure to IONPs (A) and $FeCl_3$ (B) by their adult parents. Cont. –control. *Egg masses was not produced. # statistical difference ($p < 0.05$) from the control group.

6.4. Conclusion

The chronic exposure to both iron forms induced differential bioaccumulation, behavioral impairments and reproductive toxicity in the freshwater snail *B. glabrata*. IONPs induced high bioaccumulation, mortality rate and behavioral alterations when compared to its dissolved counterpart. The main behavioral impairments induced by IONPs were avoiding water, shell reclusion and swim on the water surface. Similarly, both iron forms reduced the fecundity and fertility, as well as the parental exposure induced developmental delays in the first generation. Overall results showed that the potential toxicity of gluconic-acid functionalized IONPs to freshwater snail *B. glabrata*, indicating their potential use for control of snail that acts as intermediate host of *Schistosoma mansoni*. New studies with non-target species, and covering specific functionalization to the snail should be explored, since the IONPs had advantages over other MNs due to the low cost of production and the possibility of withdrawing from the environment, thus being able to be applied in the control of schistosomiasis and minimizing environmental impacts.

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7. CONCLUSÕES E PERSPECTIVAS

Este foi o primeiro estudo a realizar uma bibliometria e uma revisão sistemática da literatura sobre a toxicidade dos nanomateriais (NMs) em gastrópodes aquáticos e terrestres, e também, o primeiro a avaliar comparativamente a toxicidade crônica das nanopartículas de óxido de ferro (NOFs) funcionalizadas com ácido glucônico (AGL) e sua contrapartida iônica ($FeCl_3$), no caramujo *Biomphalaria glabrata*.

Os resultados da revisão sistemática indicaram um total de 60 estudos, entre o período de 2010 (primeiro registro) a dezembro de 2019, com a aplicação de 18 tipos de NMs em 21 espécies de gastrópodes, terrestres e aquáticos, utilizados como sistema modelo para avaliar a toxicidade destes NMs. Todas as espécies de gastrópodes encontradas, foram associadas à sua importância epidemiológica, atuando como hospedeiros intermediários de parasitos (trematoides e nematoides) de importância médica e/ou veterinária, mesmo que embora os estudos não estivessem utilizando os NMs com a finalidade de controle do caramujo.

Os NMs foram capazes de induzir a bioacumulação, estresse oxidativo, genotoxicidade, mutagenicidade, embriotoxicidade, toxicidade reprodutiva e transgeracional, imunotoxicidade, mortalidade e alterações comportamentais nas diferentes espécies de gastrópodes utilizadas. A partir destes efeitos, pudemos concluir que os NMs (embora pouco explorados com essa finalidade), apresentaram resultados promissores para sua utilização como agentes moluscicidas. Além disso, os caramujos foram considerados um sistema modelo adequado para avaliar os efeitos tóxicos dos NMs a partir de diferentes biomarcadores e formas de exposição.

Algumas lacunas na pesquisa para ampliar o conhecimento e a utilização de NMs como agentes moluscicidas foram indicadas como: (*i*) realizar estudos de campo e abordagens de concentrações ambientalmente relevantes; (*ii*) analisar os efeitos comparativos dos NMs entre espécies nativas de caramujos e aquelas que atuam como hospedeiros intermediários; (*iii*) avaliar a toxicocinética, especialmente a distribuição tecidual, metabolismo e processo de desintoxicação de NMs em caramujos e (*iv*) utilizar de tecnologias OMICs (como transcriptômica, proteômica e metabolômica) para ajudar a entender o mecanismo de ação e toxicidade dos NMs para caramujos. Além disso, (v) avaliar a toxicidade comparativa dos NMs durante todos os estágios de desenvolvimento dos caramujos e (*vi*) analisar a toxicidade dos NMs em caramujos infectados. Bem como;

(*vii*) verificar efeitos tóxicos NMs para os parasitos dentro dos caramujos e (*viii*) estudar a toxicidade dos NMs ao longo do ciclo de vida de parasitos, que tenham caramujos como hospedeiros intermediários, são aspectos importantes para o desenvolvimento de moluscicidas baseados em NMs.

Em relação a toxicidade crônica das NOFs funcionalizadas com AGL e sua contrapartida iônica ($FeCl_3$) (1,0 – 15,6 mg L^{-1}), nos caramujos *Biomphalaria glabrata*, efeitos diferenciais para as duas formas de ferro foram observados, e estão esquematicamente resumidos na Figura 18. Ambas as formas de Fe induziram a bioacumulação, alterações no comportamento e reduziram a fecundidade do caramujo *B. glabrata*, em termos da redução na produção de ovos e massas ovígeras por caramujo após exposição de 28 dias. Contudo, efeitos mais expressivos para a bioacumulação e as alterações no comportamento foi verificado para as NOFs, enquanto maior efeito sobre a toxicidade reprodutiva associada a iônica do Fe. Apenas as NOFs induziram a mortalidade dos caramujos adultos e a redução da fertilidade, em termos do aumento da taxa de mortalidade, redução da taxa de eclosão e aumento da frequência de atrasos de desenvolvimento dos embriões na maior concentração de exposição (15,6 mg L^{-1}). Efeitos sobre os biomarcadores somáticos (taxa de crescimento, índice de condição corporal e índice gonadossomático) não foram evidenciados para nenhuma das formas de ferro.

Biomarcadores	NOFs	$FeCl_3$
Bioacumulação	↑↑	↑↑
Mortalidade dos (adultos)	↑	x
Biomarcadores somáticos (TC, IC, IGS)	x	x
Alterações no comportamento	↑	↑↑
Fecundidade (massas/caramujos)	↓↓↓↓	↓↓↓
Fecundidade (ovos/massas)	x	↓↓↓
Taxa de mortalidade embriões	↑↑↑↑	↑↑
Taxa de eclosão	↓↓↓↓	↓↓
Taxa de atrasos no desenvolvimento	↑↑	x
Total de respostas aos biomarcadores	**n = 7**	**n = 6**
Resposta das concentrações ou tempo	**n = 18**	**n = 14**

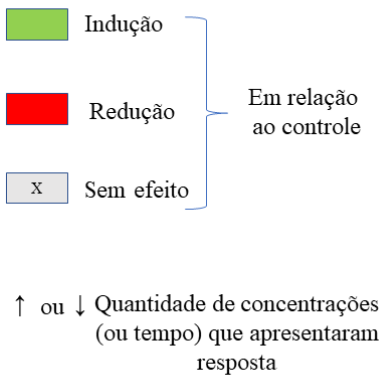

Figura 18. Resposta a diferentes biomarcadores no caramujo *Biomphalaria glabrata*, após exposição crônica (28 dias) as NOFs funcionalizadas com (ácido glucônico) AGL e ao $FeCl_3$. TC (taxa de crescimento), IC (índice de condição corporal), IGS (índice gonadossomático).

Estudos sobre alterações no metabolismo energético e alterações histopatológicas nas gônadas e glândula digestiva do caramujo *B. glabrata* desta exposição as NOFs funcionalizadas com AGL e ao $FeCl_3$, estão sendo realizados para melhor se compreender os mecanismos de ação e toxicidade do Fe no caramujo. Tendo em vista os efeitos de aumento da mortalidade na concentração de 15.6 mg L^{-1} nas NOFs e os efeitos da toxicidade reprodutiva associada ao ferro, este estudo traz como perspectiva a utilização das NOFs como agente moluscicida para o controle da esquistossomose, já que de acordo com os parâmetros estabelecidos pela OMS, a atividade moluscicida de um composto é reconhecida em concentrações abaixo de 100 mg L^{-1} (WHO, 1993). Futuras investigações, avaliando outras concentrações, tipos de funcionalização e diferentes estágios do desenvolvimento do caramujo, bem como caramujos infectados, são requeridos para se constatar a susceptibilidade do caramujo das NOFs, e para o avanço do conhecimento da utilização deste NM como agente moluscicida e/ou de controle da população de caramujos *B. glabrata* aliados ao controle da esquistossomose.

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