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Toxicidade de nanopartículas de óxido de ferro (γ-Fe_2O_3) funcionalizadas com citrato ao longo do desenvolvimento inicial do zebrafish (*Danio rerio*)

Orientador: Dr. Thiago Lopes Rocha
Coorientador: Dr. Bruno Basto Gonçalves

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Toxicidade de nanopartículas de óxido de ferro (γ-Fe_2O_3) funcionalizadas com citrato ao longo do desenvolvimento inicial do zebrafish (*Danio rerio*)

Dissertação de Mestrado apresentada ao Programa de Pós-Graduação em Genética e Biologia Molecular (PGBM) da Universidade Federal de Goiás (UFG) como pré-requisito para obtenção do título de mestre em Genética e Biologia Molecular.

Orientador: Dr. Thiago Lopes Rocha

Coorientador: Dr. Bruno Basto Gonçalves

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Ata nº 77 da sessão de Defesa de Dissertação de **Aryelle Canedo Pereira**, que confere o título de Mestre(a) em **Genética e Biologia Molecular**, na área de concentração em **Genômica funcional, estrutural e proteômica**.

Ao/s **vinte e quatro dias do mês de janeiro de 2020**, a partir da(s) **09h00**, na **Sala de Leitura do IPTSP**, realizou-se a sessão pública de Defesa de Dissertação intitulada "Embriotoxicidade de nanopartículas de óxido de ferro (γ-Fe2O3) funcionalizadas com citrato em zebrafish (Danio rerio)". Os trabalhos foram instalados pelo(a) Orientador(a), Professor(a) Doutor(a) **Thiago Lopes Rocha - IPTSP/UFG** com a participação dos demais membros da Banca Examinadora: Professor(a) Doutor(a) **Gisele Augusto Rodrigues de Oliveira- FF/ UFG**, membro titular externo; Professor(a) Doutor(a) Leonardo Pereira Franchi, membro titular externo. Durante a arguição os membros da banca fizeram sugestão de alteração do título do trabalho. A Banca Examinadora reuniu-se em sessão secreta a fim de concluir o julgamento da Dissertação, tendo sido(a) o(a) candidato(a) **aprovada** pelos seus membros. Proclamados os resultados pelo(a) Professor(a) Doutor(a) **Thiago Lopes Rocha**, Presidente da Banca Examinadora, foram encerrados os trabalhos e, para constar, lavrou-se a presente ata que é assinada pelos Membros da Banca Examinadora, ao(s) **vinte e quatro dias do mês de janeiro de 2020**.

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Lista de abreviaturas

Ag NPs: Nanopartículas de prata

ANE: Alterações nucleares em eritrócitos

Au NPs: Nanopartículas de ouro

CAT: Catalase

Cds: Sulfeto de cádmio

CeO_2 NPs: Nanopartículas de óxido de cério

CNTs: Nanotubos de carbono

$CoFe_2O_4$ NPs: Nanopartículas de ferrita de cobalto

CS NPs: Nanopartículas de quitosana

CuO NPs: Nanopartículas de óxido de cobre

DLS: *Dynamic Light Scattering*

EC_{10}: *Effect Concentration 10 %*

EC_{50}: *Effect Concentration 50 %*

ELS: Espalhamento de luz eletroforético

EROS: Espécies Reativas de Oxigênio

Fe_3O_4: magnetita

FET: *Fish embryo test*

GSH: Glutationa reduzida

FTIR: Espectroscopia no infravermelho em pastilhas de KBr

ISO: *International Organization for Standardization*

LC_{50}: *Median lethal concentration*

MDA: Malondialdeído

MgO NPs: Nanopartículas de óxido de magnésio

MoA: *Mechanism of action*

Ni NPs: Nanopartículas de níquel

NMs: Nanomateriais

NPs: Nanopartículas

NPMs: Nanopartículas magnéticas

NOFs: Nanopartículas de Óxido de Ferro

NOM: Matéria orgânica natural

OECD: *Organisation for Economic Cooperation and Development*

PEG: Polietileno glicol

PS NPs: Nanopartículas de poliestireno

PG: Pristine graphene

Pt NPs: Nanopartículas de platina

QDs: *quantum dots*

REACH: *Registration, Evaluation, Authorisation, and Restriction of Chemicals*

RES-NCTD: Nanopartículas de Resveratrol-Norcantharidin

Rp: *Reactional pattern*

ROS: *Reactive oxygen species*

SiO_2 NPs: Nanopartículas de dióxido de silício

SnO_2 NPs: Nanopartículas de óxido de estanho

SOD: Superóxido dismutase

TEM: Microscopia eletrônica de transmissão

TiO_2 NPs: Nanopartículas de dióxido de titânio

UV- Vis: Espectroscopia ultravioleta-visível

XDR: Difração de raios-X

ZET: *Zebrafish embryotoxicity test*

ZnO NPs: Nanopartículas de óxido de zinco

γ-Fe_2O_3 NPs: Nanopartículas de óxido de ferro de maghemita

α-Fe_2O_3: Hematita

γ-Fe_2O_3: maghemita

Resumo

As nanopartículas de óxido de ferro (NOFs) estão sendo cada vez mais utilizadas em aplicações médicas, ambientais e tecnológicas. Contudo, com a sua crescente produção e aplicação surgem preocupações sobre sua liberação no meio ambiente e impacto na saúde humana e ambiental. Nesse sentido, o *zebrafish* (*Danio rerio*) veem se mostrando um excelente sistema modelo para análise e classificação da toxicidade dos nanomaterias. Assim, o objetivo geral do presente estudo foi avaliar a toxicidade das NOFs (γ-Fe_2O_3 NPs) funcionalizadas com citrato ao longo do desenvolvimento inicial do *zebrafish*, comparando a toxicidade com os íons de ferro, após exposição estática e semi-estática. Para tanto, os embriões de *zebrafish* foram expostos em modo de exposição estática (sem renovação do meio) e semi-estática (renovação do meio a cada 24 h) às γ-Fe_2O_3 NPs funcionalizadas com citrato e ao cloreto de ferro em diferentes concentrações ambientalmente relevantes de ferro (0,3, 0,6, 1,25, 2,5, 5,0 e 10 mg L^{-1}) por 144 h, juntamento com o grupo controle mantido em água reconstituída em placas de 24 poços. Os resultados mostraram que as γ-Fe_2O_3 NPs foram acumuladas principalmente no córion dos embriões e no sistema digestivo e no fígado das larvas de *zebrafish*. As γ-Fe_2O_3 NPs apresentam baixa toxicidade em comparação com os íons de ferro, indicando que a toxicidade *in vivo* de ambas as formas de ferro é mediada por diferentes mecanismos de ação. Entretanto, as NPs causaram alterações significativas após exposição semi-estática causando diminuição da frequência cardíaca, induziram acúmulo de sangue e formação de edema do pericárdio nos embriões do *zebrafish*, indicando seu efeito cardiotóxico. Em relação ao método de exposição, foi observado que ambas as formas de ferro induziram alta embriotoxicidade em condições de exposição semi-estática em comparação com a exposição estática, indicando que a toxicidade depende da frequência de exposição. Este foi o primeiro estudo sobre o impacto da condição de exposição na toxicidade das γ-Fe_2O_3 NPs no desenvolvimento do *zebrafish*.

Palavras-chave: Nanoecotoxicologia, nanomateriais, embriotoxicidade, cardiotoxicidade, biomarcadores.

Abstract

Iron oxide nanoparticles (NOFs) are being increasingly used in medical, environmental and technological applications. However, with its growing production and application, concerns arise about its release into the environment and its impact on human and environmental health. In this sense, the zebrafish (*Danio rerio*) is proving to be an excellent model system for the analysis and classification of nanomaterial toxicity. Thus, the general objective of the present study was to evaluate the toxicity of NOFs (γ-Fe_2O_3 NPs) functionalized with citrate throughout the initial development of zebrafish, comparing toxicity with iron ions, after static and semi-static exposure. For this purpose, the zebrafish embryos were exposed in static (without medium renewal) and semi-static (medium renewal every 24 h) exposure modes to γ-Fe_2O_3 NPs functionalized with citrate and iron chloride in different concentrations environmentally relevant iron (0.3, 0.6, 1.25, 2.5, 5.0 and 10 mg L^{-1}) for 144 h, together with the control group maintained in water reconstituted in 24-well plates. The results showed that γ-Fe_2O_3 NPs were accumulated mainly in the chorion of the embryos and in the digestive system and liver of zebrafish larvae. Γ-Fe_2O_3 NPs have low toxicity compared to iron ions, indicating that the *in vivo* toxicity of both forms of iron is mediated by different mechanisms of action. However, NPs caused significant changes after semi-static exposure causing a decrease in heart rate, induced blood accumulation and formation of pericardial edema in zebrafish embryos, indicating its cardiotoxic effect. Regarding the exposure method, it was observed that both forms of iron induced high embryotoxicity under semi-static exposure conditions compared to static exposure, indicating that the toxicity depends on the frequency of exposure. This was the first study on the impact of the exposure condition on the toxicity of γ-Fe_2O_3 NPs on the development of zebrafish.

Key words: Nanoecotoxicology, nanomaterials, embryo, cardiotoxicity, biomarkers.

CAPÍTULO I

Introdução geral

1. INTRODUÇÃO

1.1. Nanotecnologia e nanomateriais

Nos últimos anos, devido ao contínuo crescimento da área da nanotecnologia, a produção global de produtos comerciais contento nanomateriais (NMs) cresceu exponencialmente (Kabir et al., 2018; Rodriguez et al., 2019). A quantidade e a variedade de NMs liberados no meio ambiente durante a fabricação, transporte, uso e descarte têm aumentado. Estima-se que sejam liberados no meio ambiente mais de 10.000 toneladas/ ano (Salieri et al., 2018) (Fig. 1). Estima-se ainda que até 2050, a concentração de NMs em águas doces e marinhas, sedimentos e solos seja 1.000 vezes maior, resultando em inevitável exposição humana e ambiental (Sun et al., 2017; Giese et al., 2018).

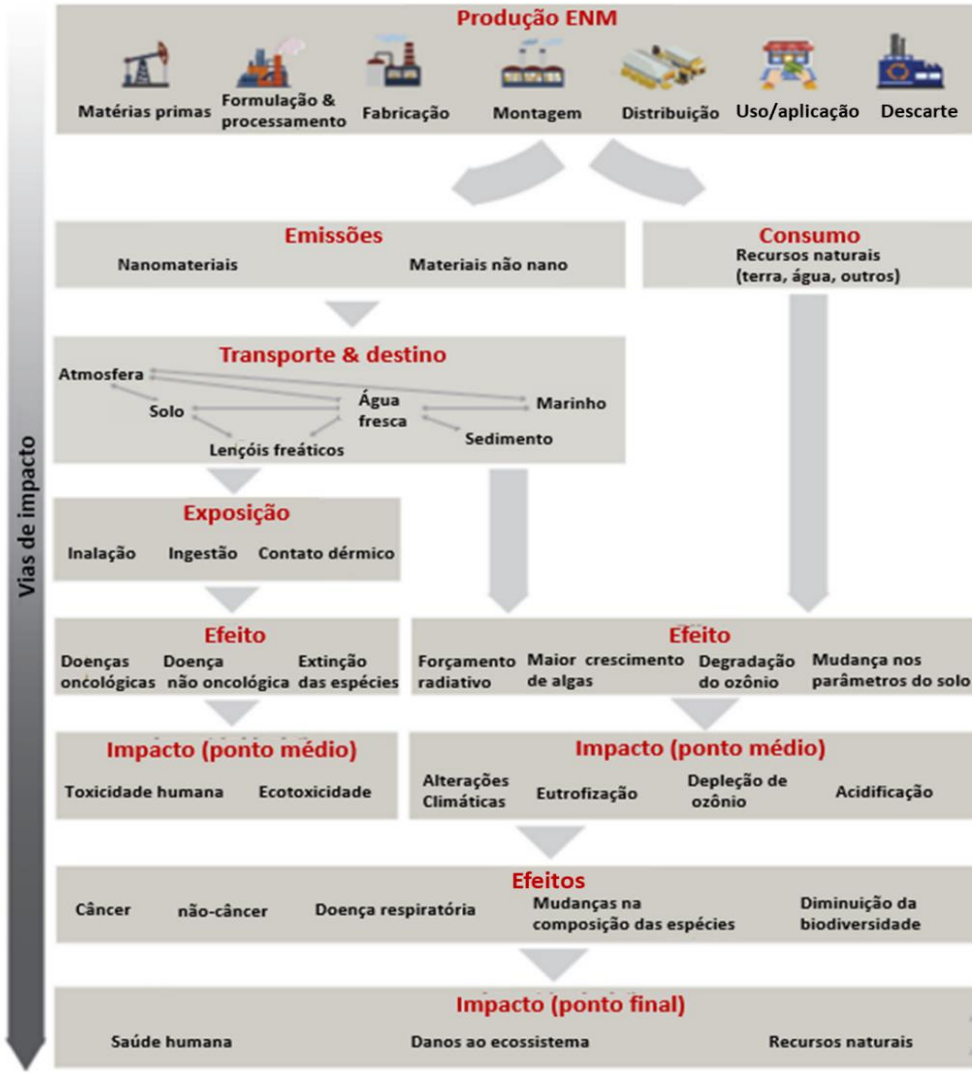


Figura 1. Potencial liberação e exposição dos nanomateriais durante diferentes estágios do ciclo de vida. Fonte: adaptado de Cucurachi e Rocha, 2019. ENM (Engenharia de nanomateriais).

A nanotecnologia é a área da ciência que engloba a produção, caracterização e a aplicação de estruturas controlando sua forma e tamanho em escala nanométrica (Prajitha et al., 2019). Os NMs são definidos como materiais de origem natural, acidental ou sintético que contenham partículas, aglomeradas ou não, no qual 50 % das partículas possuem uma ou mais dimensões externas com tamanho entre 1 - 100 nm (Comissão Européia, 2011). Além disso, as nanopartículas (NPs) são estruturas que possuem as três dimensões na escala de 1 a 100 nm que apresentam a área superficial específica inversamente ao seu tamanho. A redução do tamanho garante uma mudança nas propriedades eletromagnéticas, térmicas, mecânicas e ópticas das partículas (Khan et al., 2017).

Devido a uma maior área superficial, tamanho e formas específicas, os NMs apresentam propriedades físico-químicas diferentes das propriedades das substâncias a granel ou de materiais com maiores dimensões (Khan et al., 2017). Considerando suas propriedades únicas, os NMs vêem sendo usados na engenharia, eletrônica, cosméticos, alimentos, biotecnologia, farmácia, medicina, meio ambiente, dentre outros (Dolez, 2015; Gajanan e Tijare, 2018; Zhu et al., 2019). Dentre suas aplicações, destacam-se o uso dos NMs como sistema de entrega de fármacos (Chen et al., 2019), tratamento de câncer por hipertermia magnética (Daboin et al., 2019), protetores solares (Zaccariello et al., 2019), dispositivos eletrônicos (Zhiwei et al., 2019), tintas (Bellotti et al., 2015), nanorremediação (Corsi et al., 2018), e outros.

Os NMs variam em relação ao tamanho, forma, superfície e composição química, e podem ser classificados de acordo com a sua dimensão em nanoplacas (1 Dimensão), nanofios, nanotubos, nanobastões, etc., (2 Dimensões) e NPs formadas por cristais de tamanho nanométrico (3 Dimensões). Os NMs podem também ser classificados segundo a sua composição química em orgânicos e inorgânicos (Prajitha et al., 2019) (Fig. 2). Os NMs inorgânicos podem ser classificados em NMs metálicos, NMs de óxidos metálicos, SiO_2 NMs e os quantum dots (QDs) (Dolez, 2015; Saleh e Gupta, 2016). As nanopartículas magnéticas (NPMs), possuem inúmeras aplicações na área médica e ambiental, principalmente por causa das suas propriedades magnéticas (Chen et al., 2018; Falcaro et al., 2016). Dentre as NPMs, destacam-se as NPs de óxido de ferro (NOFs) devido as suas vantagens como facilidade de síntese, magnetismo, cristalinidade, capacidade de adsorção, biocompatibilidade e baixo custo (Gericke e Pinches, 2006; Xu et al., 2012; Babić-Stojić et al., 2017).

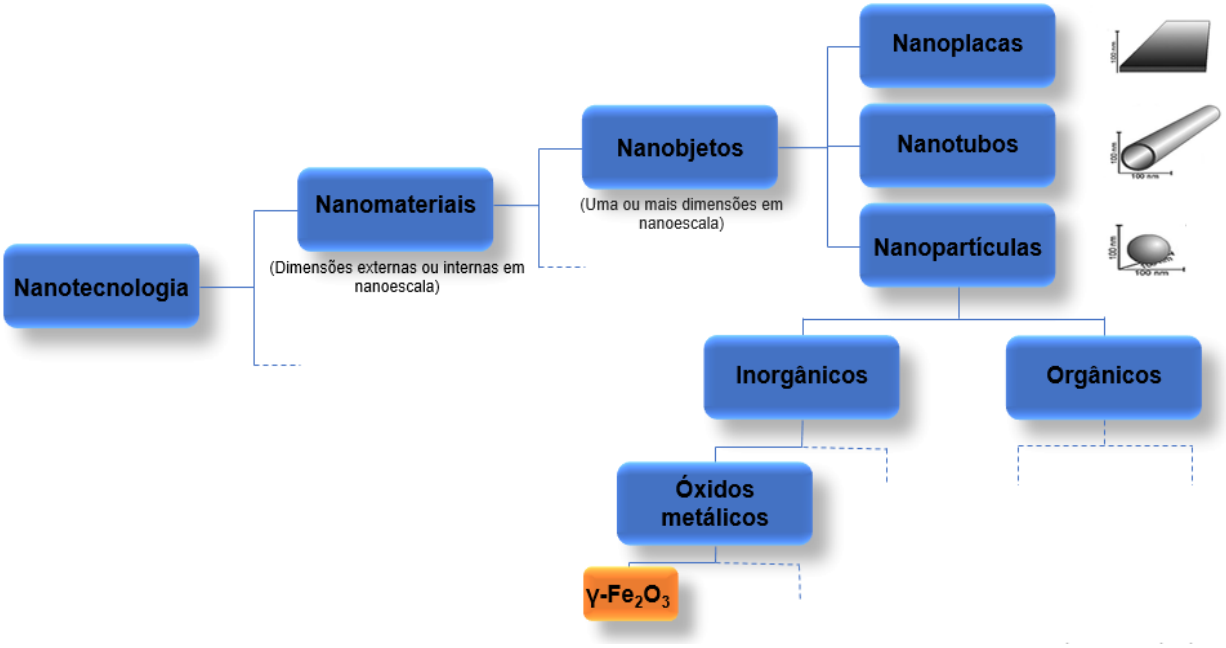


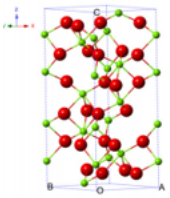
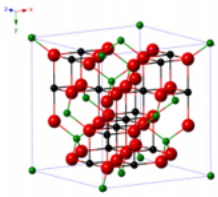
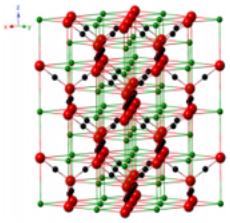
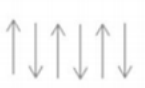
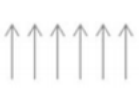
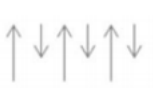
Figura 2. Classificação dos nanomateriais (NMs) de acordo com suas dimensões e constituição química, evidenciando as nanopartículas de óxido de ferro de maghemita (γ-Fe_2O_3 NPs) que são objeto de interesse no presente estudo. (Fonte: a autora).

1.2 Nanopartículas de óxido de ferro

As NOFs podem ser de vários tipos, no qual destacam-se a magnetita (Fe_3O_4), hematita (α-Fe_2O_3) e maghemita (γ-Fe_2O_3) (Su, 2016) (Tabela 1). A γ-Fe_2O_3 NP é um óxido de ferro que possui a estrutura cúbica tipo espinélio invertido e propriedades magnéticas semelhantes às Fe_3O_4 NPs e a mesma estequiometria da α-Fe_2O_3 NPs, ou seja, deficiente em Fe^{3+}, pois não há cátions suficientes para o preenchimento de todos os sítios de coordenação octaédricos. As γ-Fe_2O_3 NPs podem ser formadas através da oxidação da Fe_3O_4 NPs (Chen et al., 2018).

As γ-Fe_2O_3 NPs apresentam propriedade superparamagnética, alta área superficial, tamanho reduzido, alta cristalização, capacidade de adsorção e biocompatibilidade (Xu et al., 2012; Vikram et al., 2017). As γ-Fe_2O_3 NPs têm se mostrando muito promissoras em aplicações na área médica, tais como tratamento de câncer por hipertermia magnética (Giustini e Andrew, 2010), ressonância magnética (Fatima e Kim, 2018), biossensor (Miller et al., 2002), sistema de entrega de fármacos ("*drug delivery*") (Laurent et al., 2014), além de aplicações ambientais, como a nanorremediação (Hjorth et al., 2017) e o tratamento de água e esgoto (Xu et al., 2012).

Tabela 1. Propriedades físicas e magnéticas dos diferentes tipos de nanopartículas de óxido de ferro.

Propriedade	Hematita	Magnetita	Maghemita
Fórmula	α-Fe_2O_3	Fe_3O_4	γ-Fe_2O_3
Sistema Cristalino			
	Trigonal	Cúbico	Cúbico ou tetragonal
Estrutura	Corundum	Espinélio invertido	Cúbica de espinélio
Densidade (g/cm³)	5,26	5,18	4,87
Ponto de fusão (°C)	1350	1583-1597	——
Tipo de magnetismo			
	Antiferromagnético	Ferromagnético	Ferrimagnético

Fonte: adaptado de Teja e Koh (2009); Wu et al., (2015).

A aplicação das γ-Fe_2O_3 NPs depende da sua preparação e estabilidade em diferentes ambientes. O desempenho dessas NPs mostrou-se melhor quando o seu tamanho permanecia entre 10 - 20 nm (Chen et al., 2018). Nesse sentido, ao longo dos anos buscou-se desenvolver γ-Fe_2O_3 NPs com suas formas, tamanhos, composição, estruturas e funcionalização controladas. Adicionalmente, as γ-Fe_2O_3 NPs são facilmente oxidadas em ambientes com presença de oxigênio, fazendo com que elas percam sua capacidade magnética. Para proteger essas NPs de sofrerem oxidação, elas podem ser revestidas com uma camada protetora orgânica ou inorgânica, que além de protegê-las, pode garantir funções especiais às NPs (Gupta et al., 2007). Além disso, elas podem ser funcionalizadas, ou seja, são adicionados grupos funcionais na superfície das NPs, aumentando a estabilidade e a biocompatibidade, evitando a sua aglomeração e garantindo certas funções dependendo do tipo da funcionalização e aplicação, tais como, carreamento de drogas, adsorção com íons específicos ou metais pesados (Lu et al., 2007; Kirillov et al., 2014). Nesse trabalho, as γ-Fe_2O_3 NPs foram funcionalizadas com citrato, para garantir maior estabilidade, baixa toxicidade, tamanho reduzido e biocompatibilidade (Ruizmoreno et al., 2013; Kirillov et al., 2014).

1.3 Funcionalização das Nanopartículas de óxido de ferro com citrato

O citrato de sódio tribásico, cuja forma molecular é $Na_3C_6H_5O_7$ (Fig. 3), tem sido usado na funcionalização de diferentes NOFs (Farimani et al., 2013; Andreas et al., 2012; Kirillov et al., 2014). As NFOs funcionalizadas com citrato possuem aplicações especialmente na área médica, como agente de contraste (Srivastada et al., 2011; Saraswathy et al., 2014), sistema de entrega de fármacos no tratamento de câncer (Nigam et al., 2011) e no tratamento de células cancerígenas por hipertermia (Cheraghipour et al. 2012). Na área ambiental, existem estudos com as γ-Fe_2O_3 NPs funcionalizadas com citrato para dessalinização da água em um sistema de osmose reversa (Na et al. 2014) e nanorremediação (Hjorth et al., 2017).

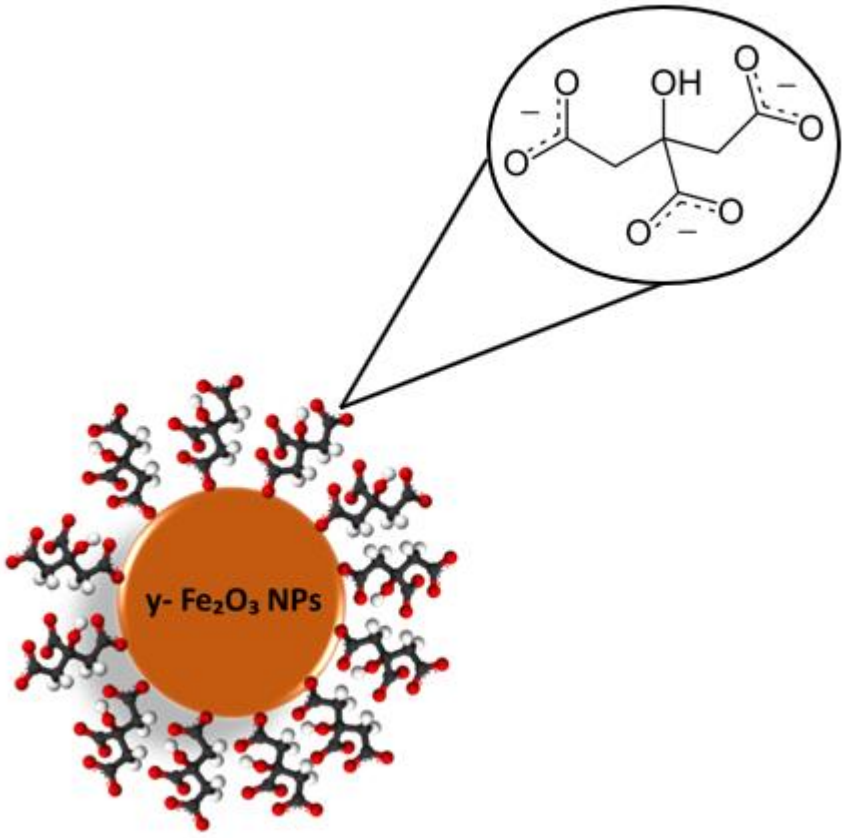


Figura 3. Nanopartículas de óxido de ferro de maghemita (γ-Fe_2O_3 NPs) funcionalizadas com citrato, evidenciando a fórmula estrutural do citrato. Fonte: a autora.

Alguns estudos mostraram que as γ-Fe_2O_3 NPs funcionalizadas com citrato apresentam uma diminuição do tamanho, o que promove maior estabilidade das NPs, além de baixa toxicidade e biocompatibilidade para mamíferos (Ruizmoreno et al., 2013;

Kirillov et al., 2014). A interação do citrato com as γ-Fe_2O_3 NPs acontece pelo grupo carboxilato com o ferro, garantindo que as NPs possuam carga superficial negativa. Além disso, o citrato também garante maior estabilidade as γ-Fe_2O_3 NPs, devido ao íon citrato gerar repulsão estérica por causa do tamanho das cadeias do citrato e repulsão eletrostática entre as NPs por causa da carga negativa do grupos carboxilatos (Nigam et al., 2011; Ruizmoreno et al., 2013).

1.4 Comportamento e destino das Nanopartículas de óxido de ferro no ambiente aquático

Apesar da funcionalização com o citrato garantir maior estabilidade as γ-Fe_2O_3 NPs, sabe-se que ao serem liberadas no meio ambiente, essas NPs podem sofrer transformações alterando seu comportamento e destino (Lei et al., 2018). Quando liberadas no meio aquático, as NOFs interagem umas com as outras e com o meio envolvente, podendo se sedimentar e depositar nos rios, sofrer homoagregação e heteroagregação com macromoléculas, tal como a máteria orgânica natural (NOM), dissolução de íons, reações redox, além de poder sofrer degradação da sua funcionalização alterando a estabilidade dessas NOFs (Moore, 2006; Patil et al., 2016; Lei et al., 2018) (Fig. 4).

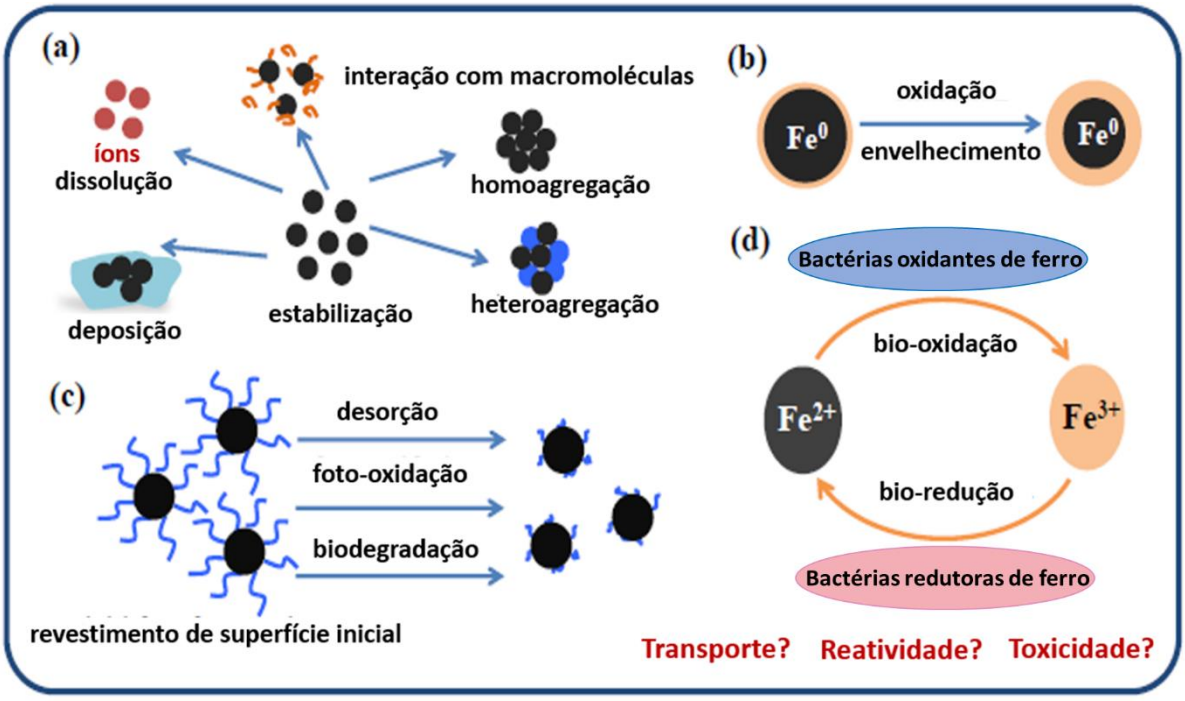


Figura 4. Possíveis transformações das Nanopartículas de óxido de ferro no ambiente aquático. Fonte: adaptado de Lei et al., 2018.

As possíveis transformações que as NOFs podem sofrer ao serem liberadas no ambiente aquático, dependem das suas propriedades nano-específicas, tais como tamanho, forma, composição química, carga superficial, revestimento e funcionalização (Rocha et al., 2015; Lei et al., 2018). Além das condições do ambiente, como pH, temperatura, força iônica, composição e concentração de NOM que desempenham um papel importante no comportamento das NPs (Chen et al., 2011). Identificar todas essas diferentes condições é essencial para prever o comportamento e destino das NOFs, no ambiente aquático. Assim, é possível avaliar quais são os potenciais riscos que a liberação das NOFs pode causar no meio ambiente, bem como a toxicidade causada nos diferentes tipos de organismos vivos (Deng et al., 2017).

Outro fator importante a ser considerado é que, após a emissão dessas NOFs no ambiente aquático, elas podem interagir com outros poluentes que estão presentes no ambiente, levando a ocorrência do efeito de misturas e causando uma co-exposição nos organismos (Deng et al., 2017; Naasz et al., 2018). O Efeito Cavalo de Tróia ("Trojan horse effect" é bastante conhecido e veem sendo descrito como um dos efeitos da interação dos NMs com outros poluentes. Segundo esse efeito, as NOFs poderiam facilitar a entrada de outros poluentes nos organismos vivos e em suas células, levando subsequentemente a uma alteração da toxicidade (Deng et al., 2017; Naasz et al., 2018).

Naasz et al. (2018) observaram que de 151 trabalhos que avaliaram a co-exposição de NMs com poluentes, o principal efeito foi o de Cavalo de Tróia, além de outros diferentes tipos de efeitos como enriquecimento da superfície, retenção, inertismo e coalismo (Fig. 5). Podemos observar na Figura 5, a classificação dos efeitos causados após a co-exposição a NMs com outros poluentes (Naasz et al., 2018). Em (1) Cavalo de Tróia (+): ocorre um aumento da toxicidade devido ao aumento da absorção química. (2) Cavalo de Tróia (-): Apesar de ocorrer o aumento da absorção química, a substância química não se torna biodisponível, resultando em baixa toxicidade. (3) Enriquecimento de superfície (modificação da disponibilidade): Quando um NM causa um enriquecimento local da concentração química ao entorno do organismo, ocorre um aumento da toxicidade. (4) Retenção: Ocorre a redução da toxicidade devido uma menor disponibilidade do produto químico. (5) Inertismo/interação passiva: Só irá ocorrer um aumento na toxicidade se o produto químico é co-transportado com o NM. (6) Coalismo/interação física: Só irá ocorrer um aumento da toxicidade se a absorção de um

produto químico for facilitada devido aos danos físicos causados no organismo pelo NM (Naasz et al., 2018).

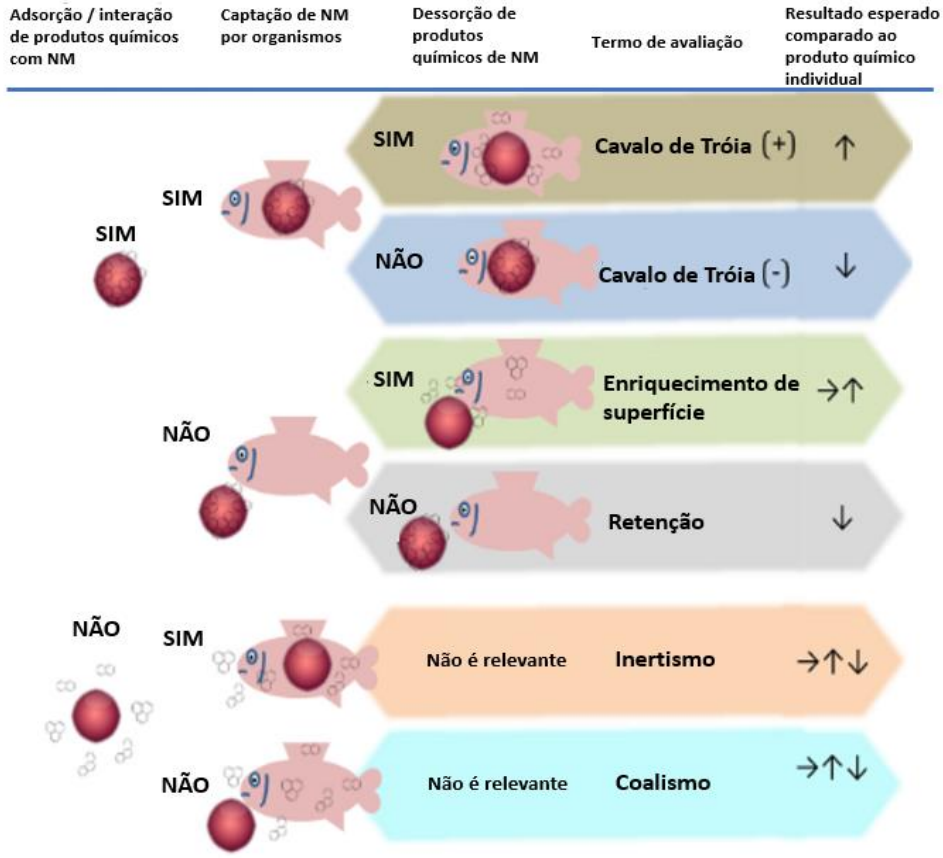


Figura 5. Processo de classificação dos efeitos causados após a co-exposição aos nanomateriais com outros poluentes. As setas indicam: ↑ toxicidade aumentada esperada, ↓ diminuição da toxicidade esperada, → toxicidade nao modificada esperada. Fonte: adaptado de Naasz et al., 2018.

1.5 Bioacumulação e transferência trófica das nanopartículas de óxido de ferro

A liberação das NOFs no ambiente aquático levanta outra problematização, em relação a bioacumulação nos organismos vivos. Sabe-se que essas NPs podem se acumular em diferentes orgãos e tecidos dos organismos, induzindo diferentes tipos de mecanismos de toxicidade como estresse oxidativo, dano ao DNA, dentre outros (Jiang et al., 2019). Apesar de se conhecer muito sobre a bioacumulação de NMs nos organismos aquáticos, sabe-se pouco sobre o processo de biomagnificação, ou seja, transferência dos NMs ao longo dos níveis tróficos (Deng et al., 2017). Até o momento não existem resultados consistêntes se o processo de biomagnificação acontece com os NMs, sendo proposto como ponto de partida que fatores como a transformação dos NMs ao serem liberados no meio aquático, os mecanismos de absorção e acumulação em presas, o

destino interno e a localização na presa, além da fisiologia digestiva do predador poderiam interferir no efeito de biomagnificação (Tangaa et al., 2016) (Fig. 6).

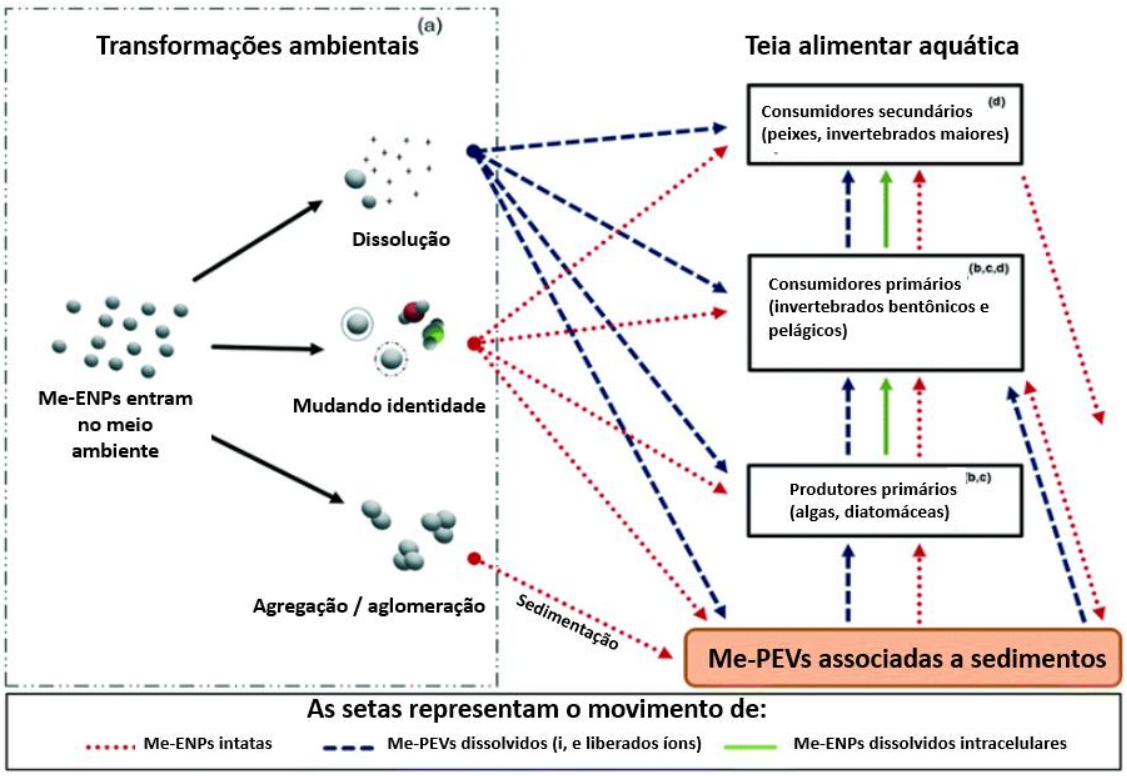


Figura 6. Transformação das nanopartículas metálicas (Me-ENPs) no meio ambiente e bioacumulação ao longo dos níveis tróficos. Fonte: adaptado de Tangaa et al., 2016.

Os produtores e os consumidores primários são os mais estudados, em relação a absorção, bioacumulação e os efeitos nano-específicos. Por outro lado, os outros níveis tróficos ainda permanecem poucos estudados (Tangaa et al., 2016; Zhao et al., 2017). É necessário mais estudos que examinem as características específicas das espécies em níveis tróficos inferiores e superiores incluindo as rotas de captação, características de acumulação e distribuição subcelular (Bhuvaneshwari et al., 2017). O destino e comportamento das NPs dentro dos organismos, onde essas NPs residem após a absorção e bioacumulação, como a fisiologia digestiva do predador influencia na captação dessas NPs. Conhecer todos esses parâmetros irá auxiliar na compreenssão dos efeitos tóxicos que essas NPs poderiam causar não só no organismo exposto, mas também ao longo de sua cadeia alimentar (Lammel et al., 2019).

1.4 Mecanismos de ação e toxicidade das Nanopartículas de óxido de ferro

A toxicidade das γ-Fe_2O_3 NPs em comparação com outras NOFs veem se mostrando menor, podendo observar que em ensaios de bioluminescência com bactérias as γ-Fe_2O_3 apresentaram menor toxicidade em relação as ZnO NPs, CuO NPs, Co_3O_4 NPs, Cr_2O_3 NPs e NiO NPs (Wang et al., 2016). No entanto, alguns estudos indicaram seu potencial ecotoxicológico em diferentes níveis tróficos (Skjolding, 2015; Tangaa et al., 2016; Baker, 2017), especialmente para os peixes. As γ-Fe_2O_3 NPs sem funcionalização (30 nm; 0,1 - 100 mg L^{-1}) induziram em embriões de *zebrafish* (*Danio rerio*) o surgimento de edema do pericárdio, acúmulo de sangue e alterações na curvatura da coluna durante 168 h de exposição (Zhu et al., 2012). Recentemente, também foi observado que as γ-Fe_2O_3 NPs funcionalizadas com citrato (3,97 nm; 0,3 mg L^{-1}) induziram efeitos genotóxicos (dano no DNA), mutagênicos (alterações nucleares em eritrócitos) e alterações histopatológicas (esteatose micro e macrovesicular, agregados de melanomacrófagos, exsudato e focos hemorrágicos) no fígado do guppy (*Poecilia reticulata*) durante 21 dias de exposição (Qualhato et al., 2017, 2018). Posteriormente, as NOFs obtidas através de diferentes métodos de síntese (coprecipitação e síntese verde) foram comparadas em *zebrafish*. Os resultados indicaram que as NOFs obtidas por coprecipitação (15,58 nm; 79,04 e 278,67 ppm) causaram danos aos eritrócitos e bioacumulação, enquanto a atividade da Na^+/ K^+ -ATPase foi inibida com o aumento da concentração durante as 96 h de exposição, quando comparadas em relação as NOFs obtidas pelo método de síntese verde (21-34 nm; 79,04 e 278,67 ppm), as quais não induziram efeitos tóxicos no *zebrafish* (Suganya et al., 2018).

Apesar dos estudos indicarem os efeitos tóxicos das NOFs em peixes, ainda não se sabe se a toxicidade é causada por propriedades nanoespecíficas, ou pelos íons de ferro liberados após a dissolução oxidativa ou uma combinação de ambos (Wang et al., 2016). O ferro é um metal parcialmente solúvel e alguns trabalhos veem sugerindo que a toxicidade das NOFs se origina pelos íons liberados ao invés das partículas (Keenan et al., 2009; Phenrat et al., 2009; Chen et al., 2013). Entretanto, deve-se levar em consideração o método de síntese, tamanho da partícula e revestimento superficial, visto que podem interferir na toxicidade das NOFs (Lei et al., 2018).

Até o presente momento, a produção de espécies reativas de oxigênio (EROs) induzindo o estresse oxidativo é o mecanismo tóxico predominante associado as NOFs em nível celular (Liu et al., 2013). As NOFs podem gerar EROs em meio extracelular e intracelular e a presença de EROs no meio extracelular ativaria um sistema intracelular

que causaria extresse oxidativo como resposta. As NOFs geram a produção de EROs *via* reação de Fenton e reação de Haber-Weiss, para essas reações ocorrerem depende dos íons de ferro liberados pelas NOFs (Wang et al., 2013; Lei et al., 2018) (Fig. 7). O aumento de EROs induz danos oxidativos, que podem ser evidenciados com o aumento de malondialdeído (MDA), e alterações da catalase (CAT), glutationa (GSH) e da superóxido dismutase (SOD) (Chen et al., 2012).

Outros potenciais mecanismos tóxicos das NOFs são consequência do seu pequeno tamanho, o que levaria à sua captura pela membrana plasmática da célula, desencadeando a geração de EROs no meio intracelular, podendo afetar a função de organelas como a mitocôndria, induzindo a morte celular. Essa facilidade das NOFs em serem capturadas pode levar a bioacumulação em diversos orgãos como cérebro, coração, trato gastrointestinal, dentre outros (Chen et al., 2012; Liu et al., 2013; Lei et al., 2018). Além disso, as NOFs também tem uma forte afinidade pela membrana celular e podem causar ruptura da membrana ou pertubação nos canais de transporte iônicos, induzindo efeitos negativos nas células, tais como geração de EROs resultando em estresse oxidativo e citotoxicidade (Auffan et al., 2008) (Fig. 7).

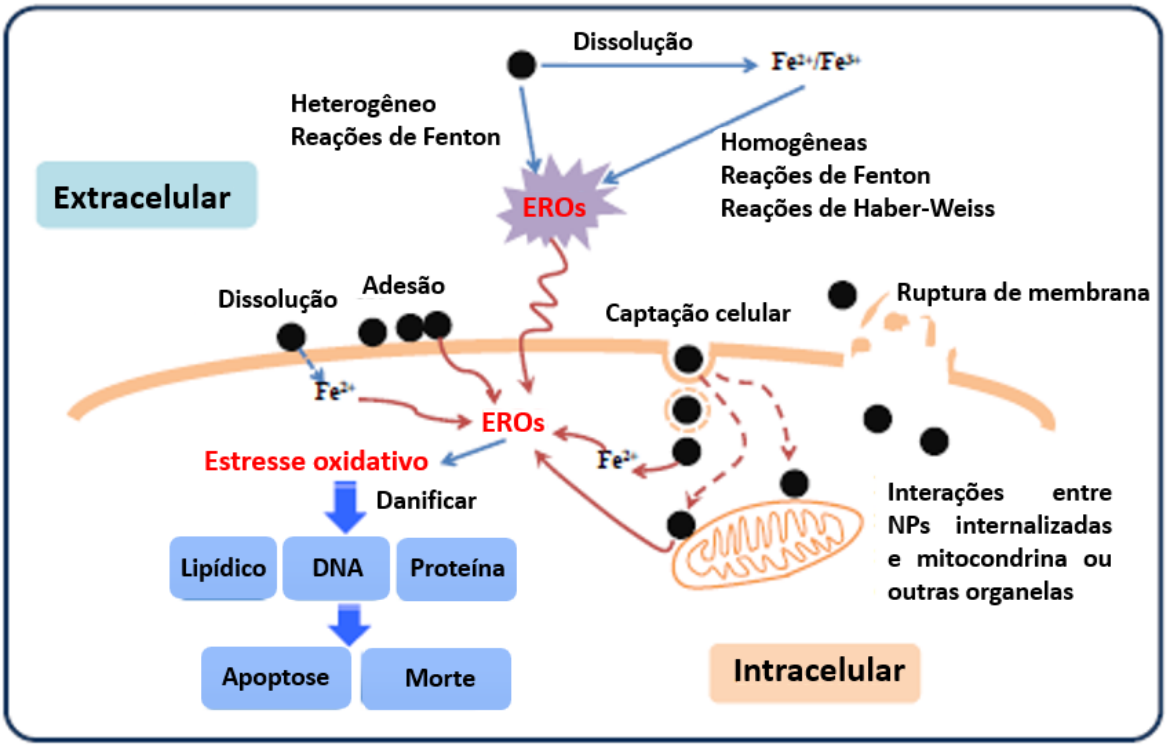


Figura 7. Possíveis mecanismos de toxicidade induzidos pelas nanopartículas de óxido de ferro (NOFs). EROs: Espécies Reativas de Oxigênio. Fonte: adaptado de Lei et al., 2018.

Com a crescente produção e liberação das Fe_2O_3 NPs no meio ambiente, torna-se essencial mais estudos sobre o comportamento dessas NPs e seus potenciais efeitos tóxicos ao entrarem em contato com os organismos vivos (Zoo e Zhao 2017; Liu et al., 2018), tal como os peixes.

1.6 *Zebrafish*

O *zebrafish* (*D. rerio*), conhecido popularmente como paulistinha no Brasil, é um peixe teleósteo da família Cyprinidae. Possui cerca de cinco centímetros na idade adulta. É nativo do sudoeste da Ásia onde é encontrado em rios de água doce, rasos e calmos. Em condições de biotério, o *zebrafish* pode ser facilmente mantido e distribuído em aquários com sistema de recirculação, sob condições de temperatura controlada a 26 ^{o}C, pH 7 ± 0,5 e fotoperíodo de 14 horas claro e 10 horas escuro (Lawrence, 2007).

O *zebrafish* possui dimorfismo sexual, sendo possível na fase adulta fazer a distinção entre machos e fêmeas. Os machos são mais retilíneos, apresentando coloração amarelada na porção ventral e nadadeiras quando estão prontos para reproduzir (Fig 8A). As fêmeas, em contrapartida, são mais prateadas e seu ventre é maior e abaulado, devido à grande quantidade de ovos que carrega (Fig 8B) (Robins et al., 1991; Valdesalici et al., 2003).

Figura 8. Macho (A) e fêmea (B) adulto do *zebrafish* (*Danio rerio*). Exemplares adultos (10 meses) obtidos do Setor de peixes do biotério do Instituto de Patologia Tropical e Saúde Pública (IPTSP) da Universidade Federal de Goiás (UFG). Fonte: (Braga et al., 2019).

Atualmente o *zebrafish* tem sido utilizado em pesquisas de diferentes áreas como genética, biologia molecular, embriologia, metabolismo, oncologia, neurociência, toxicologia e ecotoxicologia (George et al., 2011; Muth-Kohne, 2012; Lin et al., 2013; Chakraborty et al., 2016; Haque e Ward, 2018; Saleem e Kannan, 2018). O aumento nos estudos com esse animal é devido às inúmeras vantagens que essa espécie possui, tais como alta taxa reprodutiva, rápido desenvolvimento atingindo a fase adulta com aproximadamente três meses (Fig. 9), seus embriões são transparentes permitindo acompanhar todo o seu desenvolvimento embrionário (Kimmel et al., 1995; Giannaccini et al., 2014), além de apresentarem 70 % de similaridade gênica com os seres humanos, ou seja, possuem 26 mil genes ortólogos em comum com a espécie humana (Hill et al., 2005; Howe et al., 2013).

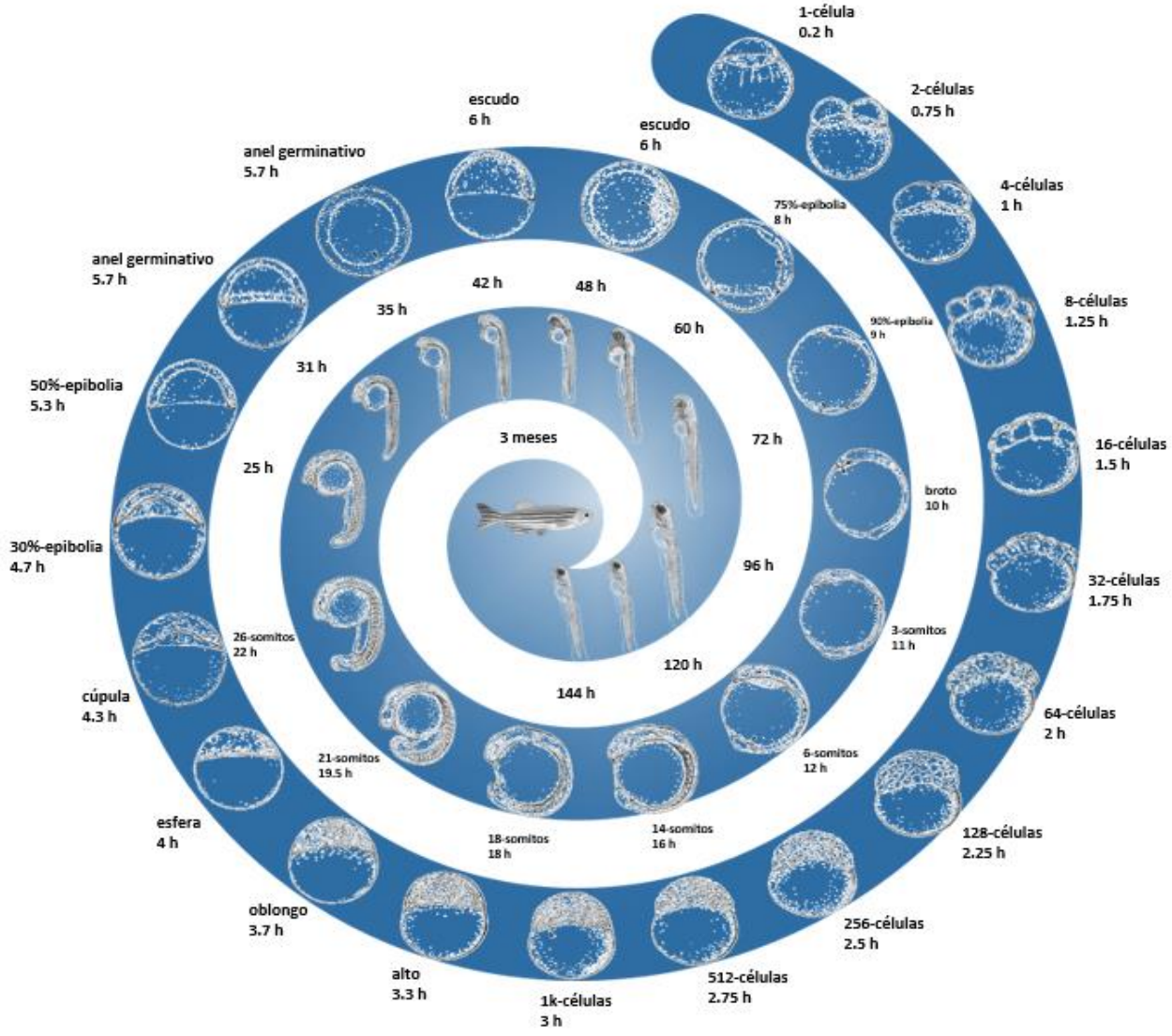


Figura 9. Fases do desenvolvimento do *zebrafish* desde o estágio embrionário de uma célula (zigoto) até a fase adulta. Fonte: a autora. O desenvolvimento do zebrafish foi classificado de acordo com Kimmel et al., 1995

Logo após o genoma do *zebrafish* ser sequenciado (Howe et al., 2013), as pesquisas com esse modelo animal aumentaram. O alto grau de similaridade com os genomas de humanos e camundongos permitiram que genes homólogos aos seres humanos encontrados no *zebrafish* fossem objeto de inúmeras pesquisas (Haffter et al., 1996; Wienholds et al., 2003; Amsterdam et al., 2004; Amsterdam and Hopkins, 2006). Além disso, esta espécie possui similaridade funcional de quase todos os órgãos e tecidos com os encontrados em humanos. Assim, é possível realizar inúmeras pesquisas de diversas doenças humanas, bem como estudos comportamentais, genéticos, toxicológicos e testar novos agentes terapêuticos (Kato et al., 2004; Haramis et al., 2006; Yu et al., 2006).

O desenvolvimento do *zebrafish* é rápido, entre 48 a 72 horas pós-fertilização (hpf) o embrião passa para o estágio larval e se torna adulto aos 3 meses de vida (Fig 9). Esses atributos são aproveitados para estudar alterações ao longo de diferentes períodos do desenvolvimento (Driever et al., 1996; Haffter et al., 1996). Sabe-se que existem aproximadamente 1400 genes que estão envolvidos com o desenvolvimento embrionário dos peixes, e 50 desses genes são essenciais para o desenvolvimento de alguns órgãos ou tipos de células, e desses genes 99 % tem homologia com genes humanos (Amsterdam et al., 2004). Essas informações garantem uma facilidade na aplicação de métodos e estratégias de avaliação genética e embriológica com esse modelo animal (Sun et al., 2004). Além disso, devido à transparência dos embriões e seu desenvolvimento ser externo ao corpo da fêmea, podem ser induzidos malformações em órgãos internos e acompanhar todo o seu desenvolvimento (Lieschke and Currie, 2007). Suas características tornam o *zebrafish* um excelente modelo animal para realização de pesquisas (Lammer et al., 2009; Truong et al., 2011; Beekhuijen et al., 2015; Sobanska et al., 2018), tal como a análise da toxicidade de NMs ao longo do desenvolvimento dos embriões de *zebrafish*.

1.7 Teste de embriotoxicidade com o zebrafish (ZET)

Visando contribuir com o conceito de 3R (substituição, redução e refinamento) do uso de animais, o teste de embriotoxicidade com *zebrafish* (ZET) é uma excelente alternativa, pois é um teste barato, rápido, permite o uso de um maior número amostral quando comparados com os testes em ratos e camundongos, além de substituir o uso de

adultos por embriões (Pereira et al., 2019). Além disso, o teste requer pouca quantidade de solução para exposição e oferece a vantagem de detectar efeitos dos compostos durante todo o período de desenvolvimento do embrião, devido à transparência dos embriões e seu rápido desenvolvimento, no qual as 120 h pós fertilização todos os principais órgãos, bem como os órgãos sensoriais, estão bem desenvolvidos sendo comparáveis com um embrião humano com três meses de idade (Kimmel et al., 1995; Parng et al., 2002; Giannaccini et al., 2014). Outra vantagem é que em nível genômico, 76 % dos genes humanos têm ortólogos em *zebrafish*, em comparação com 80 % em galinha e 84 % em camundongos, respectivamente (Howe et al., 2013). A anatomia e fisiologia do zebrafish de órgãos e parâmetros fisiológicos de interesse na toxicidade são bem descritas e compartilham homologia com mamíferos (Sieber et al., 2019).

De acordo com a diretriz do teste 236 da Organização para a Cooperação e Desenvolvimento Econômico (OECD) (OECD, 2013), os embriões no estágio de blástula (≤ 3 hpf) são expostos a pelo menos cinco concentrações de uma substância durante um período de 96 h. A cada 24 h, são analisados parâmetros específicos de toxicidade como a frequência de coagulação dos embriões, ausência de formação dos somitos, ausência de descolamento da cauda (Lammer et al., 2009) ausência de batimentos cardíacos, dentre outros (Babić et al., 2017; Krzykwa et al., 2018) (Fig. 10).

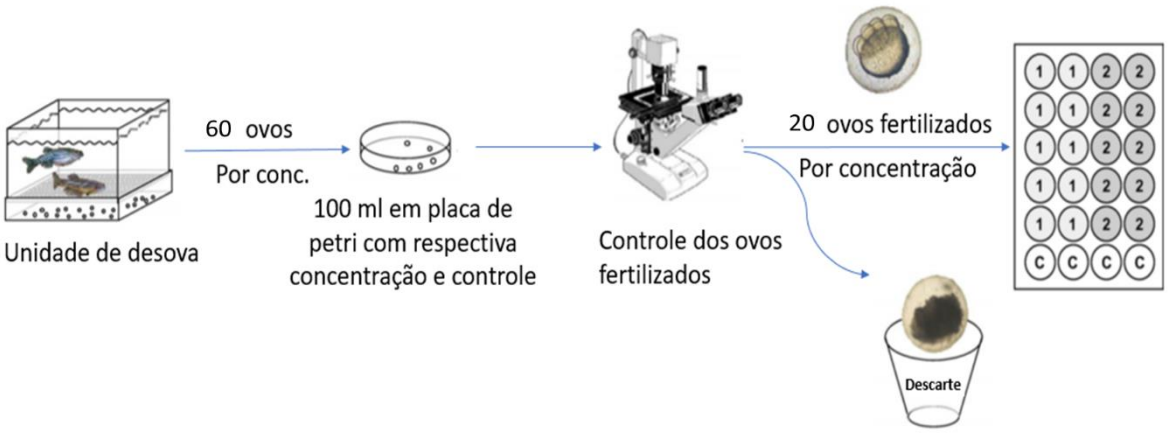


Figura 10. Esquema do teste de embriotoxicidade com o *zebrafish* (ZET). Após a reprodução, os embriões são selecionados em viáveis e inviáveis. A seguir, os embriões viáveis são expostos ao composto testado em microplacas de 24 poços. Fonte: adaptado de Lammer et al. (2009).

Atualmente novos parâmetros para avaliar efeitos subletais estão sendo incorporados ao protocolo do ZET como movimentos espontâneos, frequência cardíaca, parâmetros associados ao crescimento, análises do desenvolvimento neurológico e

cardiovascular (Babić et al., 2017; Krzykwa et al., 2018) (Fig. 11). Além disso, estudos prévios, recomendaram um tempo de exposição de 144 h, visto que com um maior tempo de exposição é possível observar um maior número de alterações durante o teste (Truong et al., 2011; Giannaccini et al., 2014; Beekhuijzen et al., 2015). Apesar da inclusão de novos parâmetros o protocolo do ZET ainda apresenta falhas, como por exemplo o estabelecimento do número de embriões correto para cada tipo de substâncias testada, visto que existem substâncias que se enquadram e outras não no número estabelecido pela OECD 236, ausência de especificação da frequência de exposição em estática, semi-estática e fluxo continuo dependendo do tipo de substância testada, o teste também apresenta limitações em testar a toxicidade de substâncias lipofílicas, visto que o teste acontece em meio E3, além disso o teste não incorpora testes toxicológicos com NMs e o modelo também apresenta limitações, pois não abrange todas as rotas de exposição (Giannaccini et al., 2014; Pereira et al., 2019; Sieber et al., 2019)

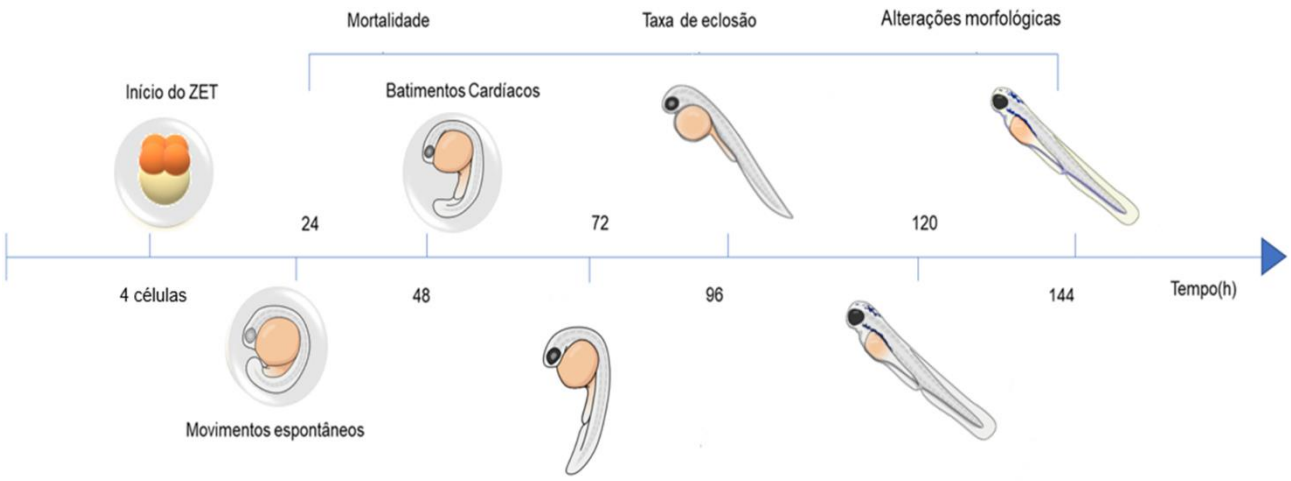


Figura 11. Análise dos múltiplos biomarcadores ao longo do ZET. (Fonte: a autora).

Apesar do guia da OECD 236 não incorporar testes toxicológicos com NMs, inúmeros trabalhos veem mostrando que o ZET é uma excelente alternativa para avaliar a toxicidade dos NMs (Fako and Furgeson, 2009; Griffitt et al., 2013; Choi et al., 2016; Chakraborty et al., 2016; Della Torre et al., 2018; Haque et al., 2018; Hou et al., 2018). Além disso, existem poucos estudos com as γ-Fe_2O_3 NPs e o ZET, sendo necessário mais

estudos sobre a toxicidade das NOFs ao longo do desenvolvimento inicial dos peixes (Pereira et al., 2019) (Fig. 12).

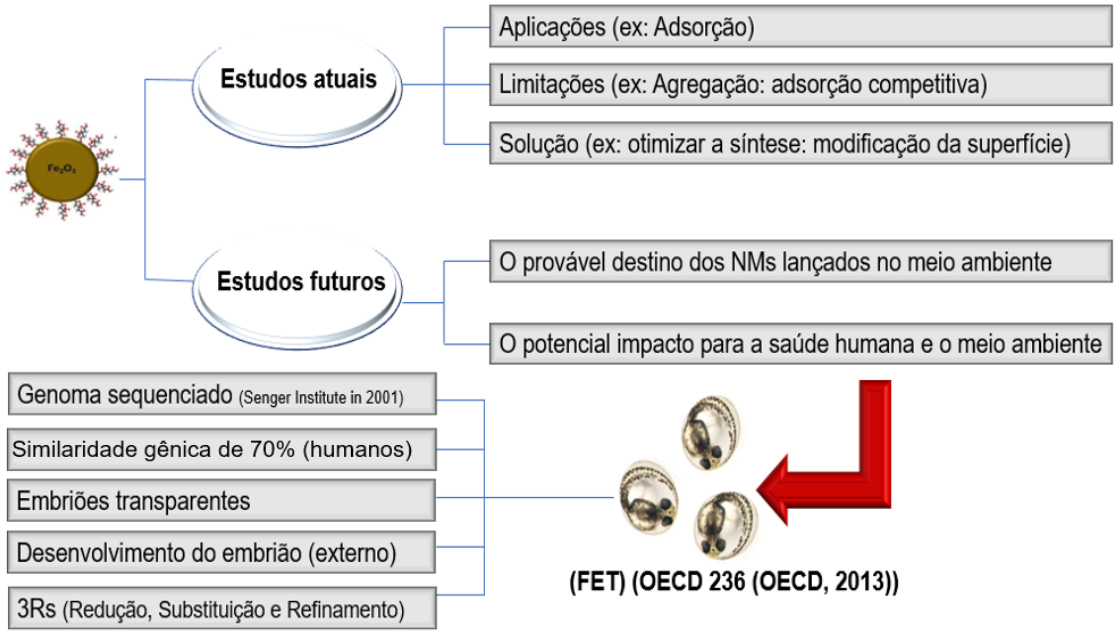


Figura 12. Esquema geral evidenciando a necessidade de mais estudos avaliando a toxicidade das γ-Fe_2O_3 NPs, e as principais características que tornam o *zebrafish* o modelo ideal para avaliar a toxicidades das γ-Fe_2O_3 NPs. Fonte: a autora.

2. JUSTIFICATIVA

As nanopartículas de óxido de ferro (NOFs) são usadas em inúmeras aplicações na área médica e ambiental, devido principalmente às suas propriedades magnéticas (Xu et al., 2012; Vikram et al., 2017). Apesar das inúmeras vantagens e benefícios decorrentes de suas propriedades físico-químicas, elas podem causar riscos para a saúde humana e ambiental (Chen et al., 2012; Liu et al., 2013; Lei et al., 2018). Dentre essas NOFs, as maghemitas (γ-Fe_2O_3) revestidas com citrato são bastante estudadas em relação a suas inúmeras aplicações (Nigam et al., 2011; Srivastada et al., 2011; Cheraghipour et al. 2012; Saraswathy et al., 2014; Na et al. 2014). No entanto, ainda são escassos os estudos que avaliam o comportamento dessas NPs ao serem liberadas no meio ambiente e os possíveis efeitos tóxicos decorrentes da exposição quando em contato com os organismos.

Em estudos de ecotoxicidade, os peixes são os principais organismos usados, devido a sua importância na cadeia alimentar (Lammer et al., 2009; Giannaccini et al., 2014; Beekhuijzen et al., 2015). Dentre as espécies utilizadas, o *zebrafish* (*D. rerio*) é um dos principais modelos alternativos usados, principalmente devido as suas inúmeras vantagens como rápido desenvolvimento, embriões transparentes, baixo custo e principalmente por apresentar seu genoma sequenciado, apresentando alta similaridade gênica com os seres humanos, permitindo que os resultados sejam extrapolados para os seres humanos (Lammer et al., 2009; George et al., 2011; Muth-Kohne, 2012; Lin et al., 2013; Chakraborty et al., 2016; Haque e Ward, 2018; Saleem and Kannan, 2018).

Nesse sentido, o nosso grupo de pesquisa nos últimos anos realiza testes de toxicidade com as NOFs, afim de gerar mais dados sobre os mecanismos de ação e toxicidade das γ-Fe_2O_3 NPs. Assim, o presente estudo busca avaliar os efeitos embriotóxicos das γ-Fe_2O_3 NPs funcionalizadas com citrato em embriões de *zebrafish*, através de uma abordagem de múltiplos biomarcadores (taxa de mortalidade, taxa de eclosão, taxa de movimentos espontâneos, taxa de batimentos cardíacos, frequências das alterações morfológicas, parâmetros biométricos).

3. OBJETIVOS

3.1 Objetivo geral

Avaliar a toxicidade das NPs de óxido de ferro (γ-Fe_2O_3) funcionalizadas com citrato sobre o desenvolvimento inicial do *zebrafish* (*Danio rerio*).

3.2 Objetivos específicos

i. Realizar uma revisão sistemática sobre o uso do teste de embriotoxicidade com o *zebrafish* (ZET) na avaliação da toxicidade dos nanomateriais.

ii. Comparar os potenciais efeitos embriotóxicos em *zebrafish* durante a exposição a γ-Fe_2O_3 NPs funcionalizadas com citrato em relação ao cloreto de ferro.

iii. Avaliar se a exposição às γ-Fe_2O_3 NPs funcionalizadas com citrato ou ao cloreto de ferro induz efeitos cardiotóxicos e neurotóxicos nos embriões de *zebrafish*

iv. Analisar as alterações morfométricas nas larvas de *zebrafish* após a exposição a γ-Fe_2O_3 NPs funcionalizadas com citrato e com o cloreto de ferro.

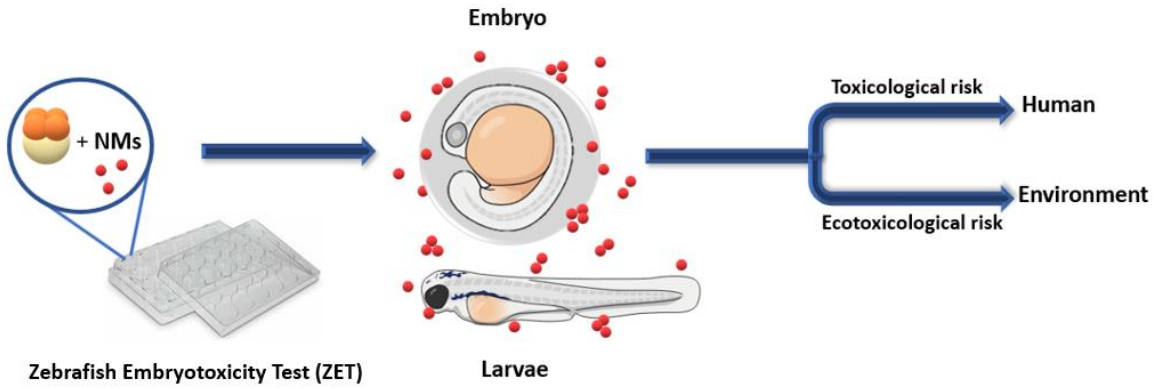
v. Comparar os tipos de exposição estática e semi-estática em relação aos efeitos causados nos embriões de *zebrafish* durante os dois diferentes tipos de exposição.

vi. Contribuir para o aumento das informações sobre a toxicidade das γ-Fe_2O_3 NPs, afim de prevenir que essas NPs atinjam níveis prejudiciais ao meio ambiente e causem prejuízos para a saúde animal, humana e ambiental.

CAPÍTULO II

Artigo I (revisão)

The zebrafish embryotoxicity test (ZET) for nanotoxicity assessment: from morphological to molecular approach



Highlights

- Systematic review on the use of the ZET test in the assessment of NM toxicity.
- ZET has been shown to be an excellent test to assess the toxicity of NMs.
- Most of the studies were with inorganic NMs (90 %) and only 10 % were with organic NMs.
- Inorganic NMs caused larger numbers of changes in zebrafish embryos.
- Further studies on the toxicity of NMs under environmental conditions are suggested.

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The zebrafish embryotoxicity test (ZET) for nanotoxicity assessment: from morphological to molecular approach

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ABSTRACT

Nanotechnology and use of nanomaterials (NMs) improve life quality, economic growth and environmental health. However, the increasing production and use of NMs in commercial products has led to concerns about their potential toxicity on human and environment health, as well as its toxicological classification and regulation. In this context, there is an urgent need to standardize and validate procedures for nanotoxicity testing. Since the zebrafish embryotoxicity test (ZET) has been indicated as a suitable approach for the toxicity assessment of traditional and emergent pollutants, the aim of this review is to summarize the existing literature on embryotoxic and teratogenic effects of NMs on zebrafish. In addition, morphological changes in zebrafish embryos induced by NMs were classified in four reaction models, allowing classification of the mode of action and toxicity of different types of NM. Revised data showed that the interaction and bioaccumulation of NMs on zebrafish embryos were associated to several toxic effects, while the detoxification process was limited. In general, NMs induced delayed hatching, circulatory changes, pigmentation and tegumentary alterations, musculoskeletal disorders and yolk sac alterations on zebrafish embryos. Recommendations for nanotoxicological tests are given, including guidance for future research. This review reinforces the use of the ZET as a suitable approach to assess the health risks of NM exposure.

A capsule: A critical review about the use of the zebrafish embryotoxicity test (ZET) on nanotoxicity assessments.

Key words: *Danio rerio*; nanoecotoxicity; teratogenicity; nanoparticles.

1. Introduction

The development of nanotechnology allowed the use of nanomaterials (NMs) in several products, and consequently their release in more than 10.000 tons per year. By 2050 a significant increase in the NM concentration is estimated in fresh and marine waters, sediments and soils (Giese et al., 2018). Despite the increasing production and use of NMs, their toxic effects on the aquatic environment and human health remain unclear (Kahn et al., 2017; Kobayashi et al., 2017). Furthermore, there is a lack of toxicological and ecotoxicological data for commercial NM-enable products, as well an increased concern regarding their toxicological classification and regulation (Bundschuh et al., 2018).

Aquatic toxicity testing is stipulated for environmental hazard and risk assessment by regulatory frameworks [e.g. Organisation for Economic Co-operation and Development (OECD) and International Organization for Standardization (ISO)], being the fish embryo toxicity (FET) test (OECD 236) one of the examples indicated in the regulatory context of Registration, Evaluation, Authorisation, and Restriction of Chemicals (REACH) (Busquet et al., 2014). The most commonly fish species used for FET is the zebrafish *Danio rerio*. Zebrafish have many characteristics that make it favourable to serve as model organisms in nanotoxicity tests, such as small size, easy to keep in laboratory conditions, high egg production and rapid development. Furthermore, zebrafish´s genetic material is similar to humans, which ensures a similarity between developmental processes, cell signalling, cell structure, anatomy and physiology with vertebrate species (Hill et al., 2005; Howe et al., 2013; Bambino and Chu et al., 2017). These characteristics offer zebrafish testing various advantages when compared with other vertebrate models, as for example rodents. Even though the toxicity of a variety of compounds is comparable between zebrafish and rodents, the use of zebrafish as important model systems is increasing as to reduce reliance on rodent testing from a financial (e.g. high costs and time spent in animal breeding and maintenance), ethical (e.g. embryonic manipulations) and biological (e.g. increased sensitivity) point of view (Chakraborty et al., 2016; Haque et al., 2018).

The literature provides an increasing number of studies about the use of adult zebrafish as model systems in nanotoxicology (Harper et al., 2011; Griffitt et al., 2013; Bugel et al., 2014; Chakraborty et al., 2016; Haque et al., 2018; Hou et al., 2018). However, most of these studies do not account for the differential toxicity during early

developmental stages, the use of molecular and genetics technologies associated to embryotoxicity tests, as well as the classification of morphological alterations on zebrafish embryos to support both environmental risk assessment and hazard classification. In this context, to better understand the effect of NMs on environmental and human health, recent studies have used zebrafish embryos as a more sensitive life stage for nanotoxicity assessment (George et al., 2011; Lin et al., 2013; Chakraborty et al., 2016; Shaw et al., 2016; Haque and Ward et al., 2018). The zebrafish embryotoxicity test (ZET) has been indicated as an excellent model to evaluate the toxicity of chemicals (Lammer et al., 2009; Beekhuijen et.al., 2015; Sobanska et al., 2018), such as NMs (Hanque and Ward, 2018), and has been shown to have a robust correlation with the Acute Toxicity Test in adults (Lammer et al., 2009). However, the ZET was not initially designed to assess the toxicity of NMs, generating concerns regarding the validity and accuracy of its results. Thus, it is essential to determine the parameters for the execution and determination of nanotoxicity using the ZET as a model. Accordingly, the aim of the present review was to summarize the embryotoxic and teratogenic effects induced by the different NM types using ZET as a model. Test conditions, such as exposure time, exposure medium and exposure chambers were considered, as well as types of NMs, physicochemical properties and concentrations used. Hatching rate, teratogenicity, LC_{50} (Median lethal concentration), EC_{10} (Effect Concentration 10 %) and EC_{50} (Effect Concentration 50 %) were also taken into consideration. In addition, changes in zebrafish development stages induced by exposure to NMs were classified into reactional models, as a means to characterize the mechanisms of action (MoA) and toxicity of NMs.

2. Methodology

A literature review was performed in February to December 2018 using the Web of Science, Science Direct and PubMed database, in which papers published between 2007 and 2019 were considered. The keywords “embryotoxicity”, “embryo” and “zebrafish” were combined with “nanoparticle” or “nanomaterial”, in both singular and plural forms, to retrieve data records in the database. Technical reports, academic theses or abstracts in scientific events were not included. A total of 86 papers were compiled in terms of year of publication, type of NMs, physical and chemical properties, experimental design (i.e. exposure time, concentration and exposure system) and endpoints used. The morphological alterations on zebrafish embryos and larvae induced by NMs were

classified into four reactional pattern (Rp): circulatory changes (Rp_1), pigmentation and tegumentary changes (RP_2), musculoskeletal disorders (Rp_3) and yolk sac alterations (RP_4), such as described in the Table 1.

Table 1. Reaction models of morphological changes in zebrafish induced by nanomaterials during the zebrafish embryotoxicity test (ZET).

Reaction pattern (Rp)	Alteration
Circulatory changes	Pericardial edema
	Heart malformation
	Bradycardia
	Hyperemia
	Body arch edema
	Anormal circulation or vasculature
	Blood accumulation
Pigmentation and tegumentary changes	Changes of pigmentation of the head
	Changes of pigmentation of the eyes
	Changes of pigmentation of the tail
	Body ulceration
Musculoskeletal disorders	Scoliosis
	Rachischisis
	Notochord malformations
	Spinal curvature
	Defects in the somites
	Tail flexure
	Decaying tail tissue
	Growth retardation
	Reduction of locomotor activity
	Craniofacial
	Axial
	Head malformation
	Absence or irregular size of eyes
	Reduced area of sub-intestinal vessels
	Swimming bladder deformity
	Pectoral fin malformatios
	Deformities mouth
	Changes in the sacculi/otoliths
Yolk sac alterations	Yolk sac edema
	Yolk deformity
	Bubble-like formations on the yolk sac

3. The use of ZET in nanotoxicological research

The first paper about the toxic effects of NMs on zebrafish embryos was published in 2007 (Cheng et al., 2007), which described the toxicity of carbon nanotubes (CNTs) during 96 h of exposure. The CNT accumulation in the zebrafish chorion was associated to delayed hatching at 120 mg L^{-1}. After this, the annual production of papers about the use of ZET on nanotoxicological research showed an increasing trend, especially after 2013 (Fig. 1). The revised data showed that this growth was directly linked to the increase in knowledge about the molecular biology of *D. rerio*. The sequencing of the zebrafish genome, initiated by the Sanger Institute in 2001 (Howe et al., 2013) has enabled increased research on zebrafish genes similar to those of humans and other vertebrates (Rubinstein, 2003; Kelkar et al., 2014). The development of OMIC techniques has led to an increase in the application of the ZET in nanotoxicological research, especially after 2000 (Dooley and Zon, 2000; Rubinstein, 2003; Moro et al., 2007; Deng et al., 2009; Meyer et al., 2018). Furthermore, in 2013 the OECD recognized the use of the FET as an official guideline to assess the effects of chemicals (OECD, 2013).

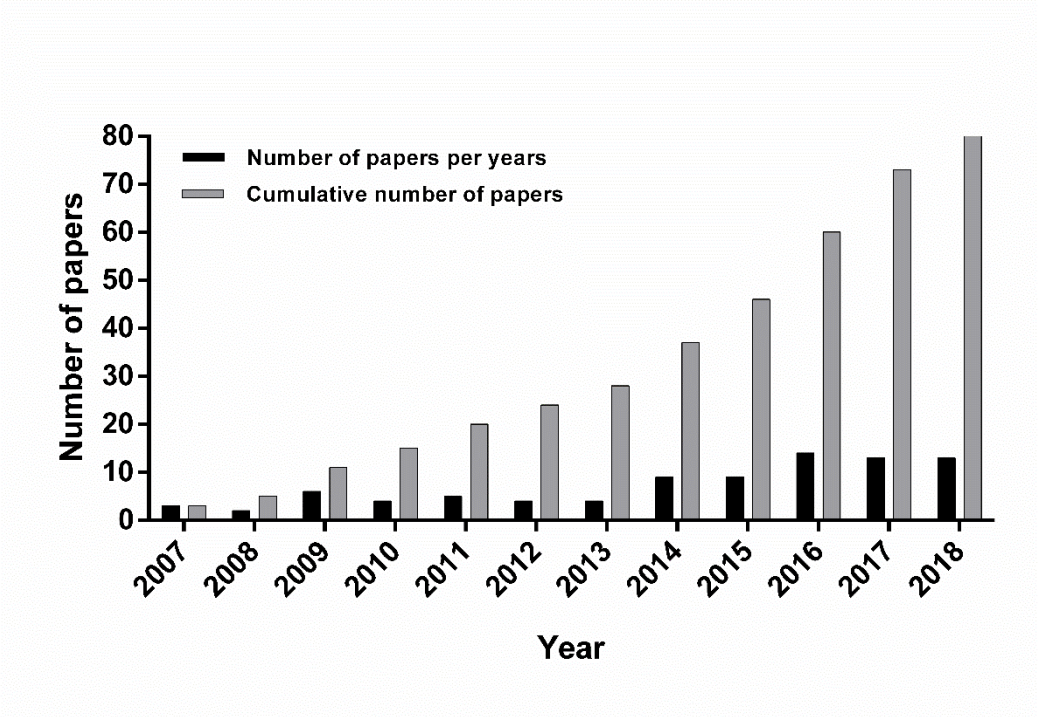

Figure 1. Timeline of the number (black) and cumulative number (white) of papers published *per* year about the zebrafish embryotoxicity test (ZET) applied in nanotoxicity assessment.

According to OECD test guideline 236 (OECD, 2013), zebrafish embryos at the blastula stage (≤ 3 hpf) are exposed to five increasing concentration of the test chemical and control during 96 h. Every 24 h, the toxicity is recorded in terms of coagulation, lack of somite formation, lack of detachment of the tail-bud from the yolk sac, and lack of heartbeat. At the end of exposure (96 h), the LC_{50} is estimated, the frequency (%) of endpoints recorded, jointly with physical-chemical properties of the exposure medium. To validate the test, some criteria are needed, such as fertilisation rate of all eggs collected ≥ 70 %, water temperature (26 ± 1 ºC) and dissolved oxygen concentration (≥ 80 %) maintained constants, survival rate and hatching rate of negative control ≥ 90 and ≥ 80 %, respectively after 96 h of exposure. However, the initial ZET protocol does not include endpoints about the sublethal effects. In this context, several modifications on ZET have been proposed, such as spontaneous contraction frequency and heart rate, sublethal endpoints associated to growth, neurodevelopment, cardiovascular development and functions (Babić et al., 2017; Krzykwa et al., 2018), and transgenic fish with fluorescent proteins and toxicogenomic approaches (Li et al., 2018).

In addition to including the 3 Rs (Reduction, Replacement and Refinement), the revised data indicated that the ZET has several advantages in nanotoxicity assessment, such as good reproduction captivity (production of a large number of embryos in a single reproduction), external fertilization, low cost, the need for small amounts of NMs, reduced exposure time, optically transparent embryos which facilitates the visualization of their development, short life cycle and rapid embryonic development, rapid phenotype discovery, genetic tractability, and cost-effective and ethically acceptable animal models for NM screening (Lammer et al., 2009; Jong et al., 2011; Giannaccini et al., 2014; Beekhuizen et al., 2015) In addition, the revised data showed that the ZET allows the evaluation of chronic responses, teratogenicity, cardiotoxicity, genotoxicity, muscle and bone disorder, phenotypic screens to identify gene function, ototoxicity, developmental genetics, neurobehavioral toxicity, organ specific toxicity (i.e. hepatotoxicity and nephrotoxicity), reproductive toxicity, endocrine disruption, oxidative stress and environmental risk assessment (Fig. 2).

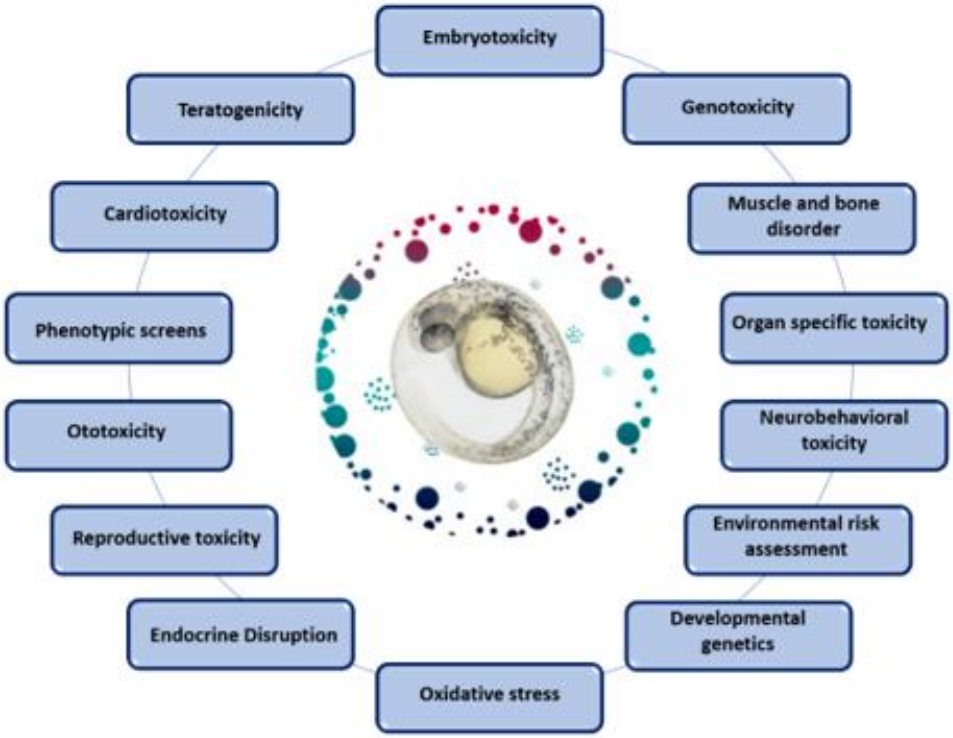

Figure 2. Approaches of the zebrafish embryotoxicity test (ZET) for nanotoxicity assessment.

Initially, nanotoxicological studies mainly addressed morphological aspects, hatching delay and mortality evaluation throughout the early developmental stages of zebrafish (Asharani et al., 2008; Bar-llan et al., 2009). However, the advancement of molecular biology allowed the mapping of mammalian homologous using zebrafish genes for the identification of molecular biomarkers. This allowed a better understanding of alterations in gene expression and biological responses induced by NM exposure. Among the molecular techniques applied to nanotoxicological studies, the following stand out: electrophoresis, RT-PCR (Barilan et al., 2011; Zhao et al., 2013; Wang et al., 2014; Massarsky et al., 2014; Miao et al., 2015; Gao et al., 2015, Cui et al., 2016; Zhao et al., 2016; Du et al., 2016; Ramachandran et al., 2017; Duan et al., 2017; Nikapitiya et al., 2018; Li et al., 2018), enzyme linked immunonosorbent assay - ELISA (Zhao et al., 2013), inductively coupled plasma mass spectrometry - ICP-MS (Muth-Kohne et al., 2013; Zhang et al., 2018) , RNA-Seq, qRT-PCR, whole-mount *in situ* hybridization - WISH (Cheng et al., 2007; Cui et al., 2016; Zhang et al., 2018), intracellular reactive oxygen species (ROS) Assay (Wang et al., 2014; Faria et al., 2014; Fang et al., 2014; Ganesan et al., 2015; Ahmad et al., 2015; Yuan et al., 2016; Duan et al., 2016; Thit et al.,

2017; Zhang et al., 2018; Li et al., 2018), western-blot (Wang et al., 2014; Duan et al., 2017), radioimmunoassay (Du et al., 2016), reporter genes and cloning (Barilan et al., 2011)

Advances in molecular biology and genetics have provided an understanding of the initial responses and mechanisms of action of NMs in zebrafish, especially in adults (Griffitt et al., 2013; Chakraborty et al., 2016; Haque et al., 2018; Hou et al., 2018), while the association between genotypic and phenotypic data from early developmental stages is scarce. In general, the revised data showed that NMs, as well as its dissolved ions, induced ROS formation, oxidative stress and damages to lipids, proteins and DNA in exposed zebrafish, which were related to apoptosis (Chen et al., 2017; Lei et al., 2018). The recent advances in the development of OMICs technologies associated to ZET, such as genomics, transcriptomics, proteomics and metabolomics, have provided rapid nanotoxicity screens with zebrafish embryos (Fako and Furgeson, 2009; Choi et al., 2016; Della Torre et al., 2018) (Fig. 3). In this context, the use of OMICs technologies to assess the ecotoxicological and health impacts induced by NMs is an emergent research in the environmental OMICs.

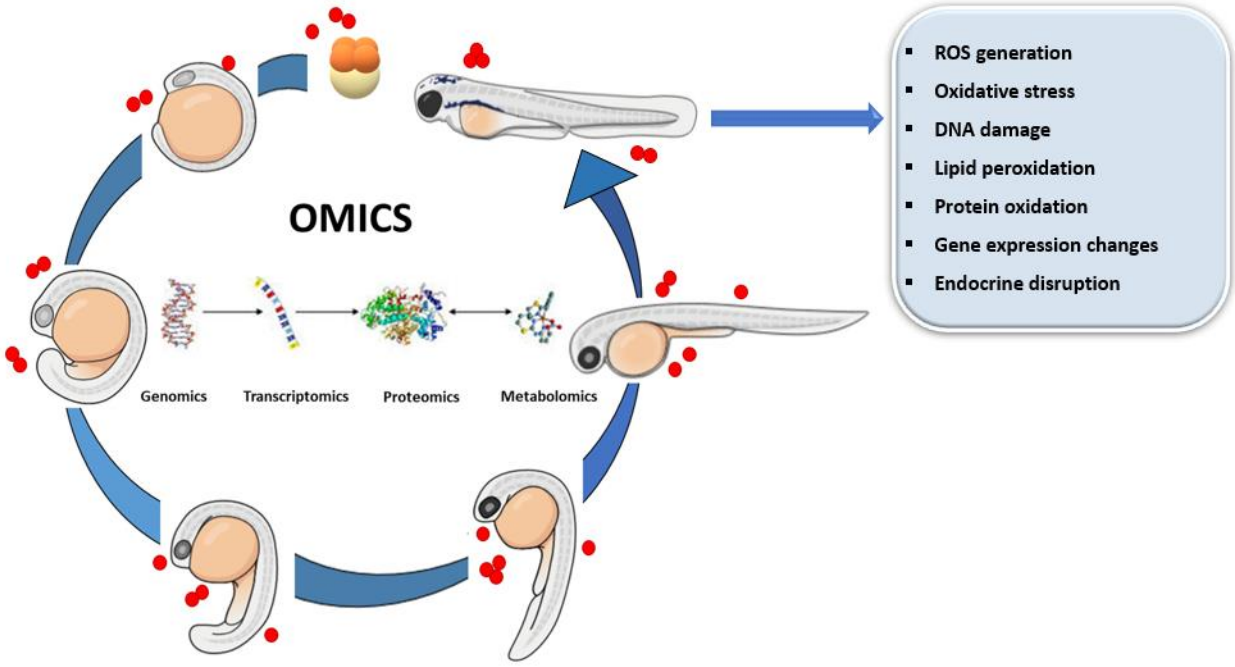

Figure 3. The use of OMICs technologies (genomic, transcriptome, proteomic and metabolomic) in the zebrafish embryotoxicity test (ZET).

4. Types of NMs assessed through the ZET

The ZET has been in majority applied to study the ecotoxicity of inorganic NMs (88 %) in contrast to organic NMs (12 %) (Fig. 4). Among the inorganic NMs, the most studied were metal oxides (33 %) and metal NPs (30 %), followed by SiO_2 NPs (19 %) and quantum dots - QDs (6 %) (Fig. 4). Metal and metal oxide NPs have been studied most frequently due to its numerous applications in the food industry (Singh et al., 2017), pharmacy (Mody et al., 2010), biomedicine (Salata, 2004), development of biomaterials (Hamouda, 2012), groundwater and soil remediation, among others (Rajan, 2011). Similar nanoecotoxicological data was reported for other fish species (Kashiwada, 2006), bivalves (Rocha et al., 2015, 2017), microcrustaceans (Castro et al., 2018) and algae (Becaro et al., 2015), indicating that studies about the ecotoxicity of organic NMs on aquatic organisms are still needed.

Among the inorganic NMs studied, Ag NPs (22 %) and SiO_2 NPs (19 %) were the commonly used for the ZET (Fig. 4). Ag NPs have aroused interest of the scientific community due to their suitable technological properties, such as high conductivity, high catalytic degree, high surface area, and antimicrobial and anti-inflammatory activity (Tian et al., 2007). Ag NPs are also widely used in commercial products used in biomedicine, such as tissues, implants, prostheses, surgical instruments, catheters, bandages and hydrogels (Xu et al., 2012). SiO_2 NPs have also been extensively studied, since these particles are used in the biomedical area due to their specific surface characteristics, porosity and functionality. These NPs are used as drug delivery systems, acting as contrast agents in the detection and separation of biomolecules (Bitar et al., 2012).

TiO_2 NPs (12 %) and ZnO NPs (11 %) represent the second group of metal oxide NPs most studied (Fig. 4). Because TiO_2 NPs are more efficient in UVB and ZnO in the UVA range, both NPs are commonly combined for the manufacture of sunscreens that guarantee greater UV protection (Smijs and Pavel, 2011; Lu et al., 2015). ZnO NPs also have antimicrobial activity, being used in the production of paints, fabrics and sprays (Padmavathy and Vijayaraghavan, 2008), while TiO_2 NPs are widely used in the removal of micropollutants in water treatment (Mahmoud et al., 2017).

From the remaining metal and metal oxide NPs, Au NPs (7 %), CuO NPs (5 %) and QDs (6 %) represent the least studied. The same can be said for other types of NMs, which account for only 18 % of the published studies (Fig. 4). Although the OECD acknowledges aluminium oxide NPs, dendrimers and nanoclays on the priority list of

manufactured NMs for the assessment of toxicology and risk to human and environmental health (OCED, 2010), there is currently not data available about its toxicity using the ZET (Fig. 4; Table 2), reflecting a significant gap of knowledge regarding this priority NMs. Recently, a new class of NMs, denominated hybrid NMs, has been drawing attention due to their emergent and improved properties (Silva et al., 2018). These NMs are synthetic particles with organic and inorganic components linked together by covalent or noncovalent bonds (Liu et al., 2017), which have several applications in environmental and health areas (Khan et al., 2019). However, due to the recent production and use of these hybrid NMs, their toxicological properties remain unclear. From the literature review performed, only Silva et al. (2018) addressed the toxicity of hybrid nanoparticles in zebrafish embryos. In this study, TiO_2-MWCNT (224.07 and 167.93 nm; 96h) showed a lower toxicity in comparison to TiO_2 NPs (25 nm; 30 – 110 mg L^{-1}) and MWCNT (1 – 25 nm; 30 – 110 mg L^{-1}). Given the current lack of published studies for this type of nanohybrid materials, additional studies about their toxicity are needed, especially considering their heterogeneity in terms of compositions and surface modifications.

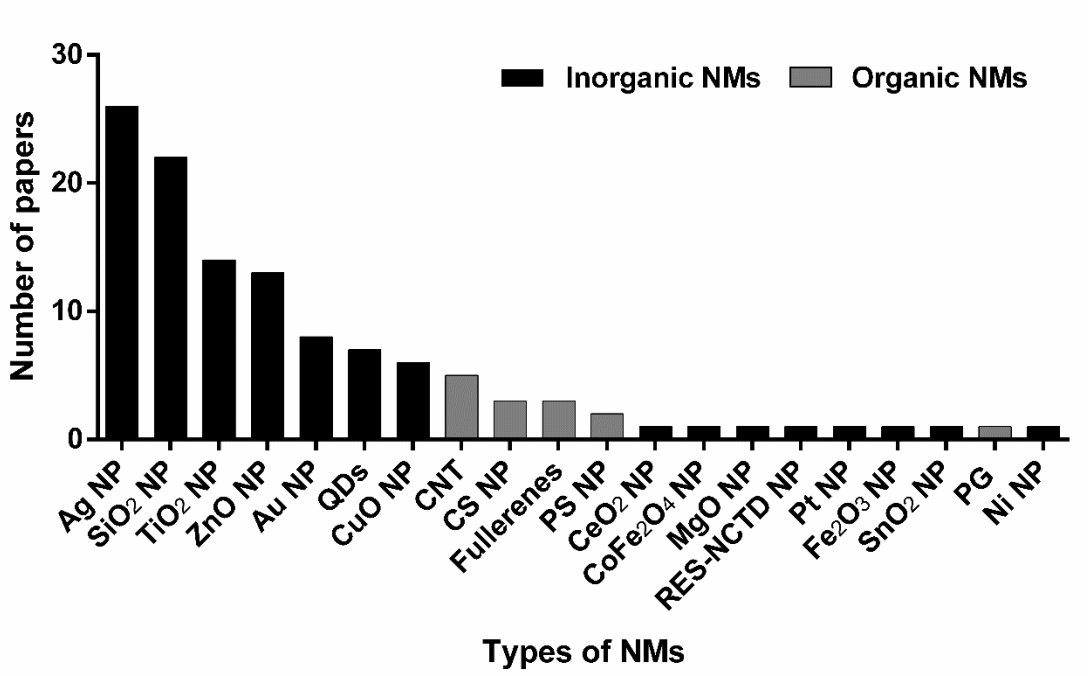

Figure 4. Number of papers published *per* year about the type of nanoparticle (organic and inorganic) analyzed by zebrafish embryotoxicity test (ZET) until May, 2018. QDs (Quantum Dots); CNT (Carbon Nanotubes); Ps NP (Polystyrene Nanoparticles); RES-NCTD NP (Amphiphilic Nanoparticles of Resveratrol-Norcantharidin) PG (pristine graphene).

Table 2. Overview of reported toxicity of nanomaterials in the zebrafish using the zebrafish embryotoxicity test (ZET).

Nanomaterials			Exposure conditions				Accumulation[c]	EC_{10} (mg L^{-1})	EC_{50} (mg L^{-1})[d]	LC_{50} (mg L^{-1})	Hatching rate	Effects [e]	Ref.
Type	**Capping layer [a]**	**Size (nm)**	**Concentration (mg L^{-1})**	**Time (hpf)**	**Exposure chambers [b]**	**Medium**							
Fullerenes	–	–	100, 200, 250 and 1000 ppb	96	96 well MP	–	–	–	–	200 ppb	↓	PE, YSE, TM	Usenko et al., 2007
	Uncoated	100	1,5 and 50	96	24 well MP	E3	–	–	–	1,5	↓	PE	Zhu et al., 2007
	–	35.6 ± 10.9 nm	0.5 and 20	96	Petri plates (4 mL)	–	GIT	–	–	–	–	No malformatios	Della Torres et al., 2018
CS NPs	–	181.2	5, 10, 20 and 30 μg mL^{-1}	5 d	6 well MP	–	–	–	–	–	↓	CS, PE, H	Nikapitiya et al., 2018
	–	84-86	100, 150, 200, 250, 300 350 and 400	120	96 well MP	EM	–	–	–	270	↓	AM, PE, SWIM	Wang et al., 2016
	Tm	251-15	5, 10, 20, 30, 40 and 50	96	6 well MP	Fish culture medium	–	–	–	25.06	–	SWIM, CS	Yuan et al., 2016
CNTs	PEG	20-40	0.01, 0.1 and 1	96	96 well MP	–	CHO	–	–	–	↓	AG, DLA	Cordeiro et al., 2018
	–	10-20	1, 5, 10, 50, 100	96	24 well MP	–	CHO	–	–	–	↓	AG	Liu et al., 2014
	BSA	19.9± 8.25	2ng	96	–	–	BR, NO, HE	–	–	–	–	–	Cheng et al., 2009
	–	30-40	2.5, 5, 10, 20, 30, 40, 50, 60, 70, 100, 200, 300 μg mL^{-1}	72	24 well MP	E3	–	–	–	–	↓	YSE, PE, BAH, BDC, NCM	Asharani et al., 2008
	–	11	20, 40, 60, 120, 240 and 360	96	Petri plates (6 mL)	–	CHO	–	–	–	↓	–	Cheng et al., 2007
RES-NCTD	–	231,96 -18,68	10, 25 and 50 ppm	7 d	–	–	STO, INTV	–	–	–	–	–	Yang et al., 2016
PS NPs	–	25, 50, 250 and 700	25 nm: 25; 50 nm: 25; 250 nm: 5 700 nm: 5	120	24 well MP	–	25 and 50nm - eyes 250 and 750 nm - GIT	–	–	–	↓	–	Pomeren et al., 2017
PS NPs	–	34,5-10,8	0,1, 1 and 10 ppm	120	glass containers	ASW	YO, GIT, PA, BR, GAL, HE	–	–	–	–	FFF	Pitt et al., 2018
PG	–	170-390	1, 5, 10, 15, 20, 25, 30, 35, 40, 45, and 50 μg/L	96	24 well MP	–	–	–	–	–	↓	PE, SPC, YSE, HM, EYE, CIRC, TM	Manjunatha et al., 2018
Ag NPs	CT –	10-40	0, 0.2, 0.4, 0.75 and 1	96	*	–	–	–	–	–	–	YSE, HTM	Cui et al., 2016

Cont.

Uncoated	20	0.01, 0.1, 0.5, 1 and 10	21 d	–	–	LV, GIT	–	–	–	–	–	Cambier et al., 2018
CT and PVP	15	0.1, 0.2, 0.5, 0.8, and 1 μg/mL	96	24 well MP	Deionized water	–	–	–	–	↓	PE, SPC, PFM, TM	Qiaoshu et al., 2017
COO-	20	0.25, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.75, 6.5, 8.5 and 10	96	–	–	–	–	–	1.19	↓	PE, YSE, TM, SPC	Cáceres-Vélez et al., 2018
PVP	10-125	0.5, 1 and 10	120	6 well MP	E3	CHO	–	–	0.11	–	–	Boyle and Goss, 2018
–	30 ±16 nm	13.6, 21.6, 42.4, 64, 128 μgL^{-1}	72	12 well MP	–	–	–	–	–	–	TM, NCM, SOMI	Sarkar et al., 2018
–	14-50	0.5 and 100	72	Petri plates (25 mL)	–	–		0.12	0.14 0.14	–	PE, YSE, CS	Gao et al., 2015
–	100	0.5, 5, 10 and 25	120	6 well MP	E3	YO−	–	8.8e61 mg Ag/L	1.7	–	TM, YND, PE	Gupta et al., 2016
–	5-10	10, 100, 1, 2, 5, 10, 20, 30, 40 and 50 $\mu g\ mL^{-1}$	96	24 well MP	–	FPE	–	–	23.63 $\mu g\ mL^{-1}$	–	HM, YSE, PFA, BH, DCH, DM	Iniyan et al., 2017
–	20-10	1000 and 100	72	Petri plates (20 mL)	–	–	–	–	–	–	–	Serrano et al.,2014
–	8.39 ± 0.98	0.03, 0.16, 0.31, 0.78 and 1.55 $\mu g/mL^{-1}$	5 d	Petri plates 14 mL)	–	–	–	–	1.18 $\mu g\ mL^{1}$	↓	FCE, YND, CS, PE	Massarsky et al., 2013
SP	10-20	0.5 and 0.05 μg / mL	4 d	Petri plates (14 mL)	E3	–	–	–	–	↓	–	Massarsky et al., 2014
PVP and PEI	5.08 ± 2.03	0.01, 0.025, 0.05, 0.075 and 0.1	120	24 well MP	–	–	–	–	50 $\mu g\ L^{-1}$	↓	YSE, PE, FCE, SPC	Orbea et al., 2017
		0.3, 1, 3, 10 and 100 μM	5 d	96 well MP	–	CHO	–	–	–	↓	AG	Powers et al., 2010
CT and PVP	10-50 10	3, 10 and 30 μM	5 d	96 well MP	–	–	–	–	–	No effect	SWIM, AG	Powers et al., 2011
–	10	30, 60, 120 and 240 nM	120	*	–	–	–	–	50 nM	–	TM, CS, YND	Yoo et al., 2016
–	20	0.5, 0.66, 0.87, 1.15, 1.5, 4, 8 and 16	48	96 well MP	–	LI, ERY	–	1.09	1.26		AG, TM, HM, PE	Muth kohne et al., 2013
CT and PVP	20-110	0.8, 4, 20, 10 and 50	120	96 well MP	–	EMB	–	–	–	–	YSE	Kim and Tanguay, 2014
–	5-20	5, 10, 25, 50 and 100 $\mu g\ mL^{-1}$	72	24 well MP	–	BR, HE, YO, BEMB	–	–	50 $\mu g\ ml^{-1}$	↓	NCM, PE, BD, BAH, DTT	Asharani et al., 2008
–	20-50	0.4, 0.6, 0.7, and 0.8 mmol L^{-1}	96	.24 well MP	E3	–	–	–	Nanospheres: 0.0415	↓	SC, AG	Abramenko et al., 2018

Cont.

										Nanoplates: 0.0169			
Ag NPs and Au NPs	–	2-20 and 5-50	5, 10, 20, 40, 60, 80 and 100 μg mL^{-1}	96	24 well MP	–	–	–	–	–	No effect	YSE	Ramachandran et al., 2017
Ag NPs and Au NPs	–	3, 10, 50 and 100	250, 25, 2.5 and 0.25 μM	120	–	–	–	–	–	93.31 μM 125.66; cAg50: 126.96; cAg100: 137.26 μM	–	AgNPs: YND, HM, CIRC, BAH, TM, BYS, DTT, PE, CS, YSE; AuNPs: No malformation	Bar-lian et al., 2009
Ag NPs and TiO_2 NPs	PVP	61-70 and 15-25	10, 25, 50, 75 and 100 μg mL^{-1}	72	24 well MP	–	–	–	–	–	↓ ↓	↓ Heart rate	Pavagadhi et al., 2014
Ag NPs, Au NPs and Pt NPs	–	15-35, 5-35, and 3-10	10, 25, 50, 75 and 100	72	Petri plates (60 mL)	–	EMB	–	–	–	Ag-NP and Pt-NP: ↓	Ag-NP PE, HTM, EYE, CIRC Au-NP: no effect Pt-CS	Asharani et al., 2011
Ag NPs, Au NPs, CdS, ZnO NPs and SiO_2 NPs	Ag: MAL Au: CIT ZnO: ECOP90	24, 44, 96 4.4,13.5, 40.4 3.5-4 20, 70 27 15, 30, 70	0.001, 0.01, 0.1, 1.5; 0.1, 1, 10, 50, 100 0.01, 0.1, 1.5, 10 0.01, 0,1, 1, 5, 10 0.1, 1, 10, 50, 100	120	24 well MP	–	–	–	–	0.529 3.94 1.973 24.655 24.61 34.717 7.036 4.289 5.538 83.329	Ag- Died Au- Died after hatching CdS - delay ZnO - delay SiO2- delay (0.1, 1) and died (10, 50, 100)	Ag: YSE, EYE, PE, TM, SPC CdS- YSE, PE, FFF, SCP; ZnO: YSE, EYE, PE, SPC, FCE SiO_2: YSE, PE, SPC	Lacave et al., 2016
Au NPs		5-25	0.325, 0.65, 0.97, 1.3, 1.62, 1.95, 2.27 and 2.6	4 d	*	E3	–	–	–	–	↓	No malformatios	Ganeshkumar et al., 2012
	–	11.6 ± 0.9 nm	0.025, 0.05, 0.10, 0.20, 0.40, 0.60, 0.80, 1.0, 1.2 nM	120h	24 well MP	–	–	–	–	–	–	PE, YSE, CS, TM, HM	Browing et al., 2009
	–	25 e 40 nm	5.0 nM	168	–	–	BR, EYES	–	–	–	–	No malformatios	Wang et al., 2009
	MES or MEEE	1.5	10 μg mL^{-1} 50 μg mL^{-1} 50 μg mL^{-1}	120	96 well MP	–	–	–	–	–	–	Behavioral abnormality	Truong et al., 2012

Cont.

$CoFe_2O_4$ NPs	SA	40.1	10, 62.5, 125, 250 and 500 μM	96	–	E3	–	–	–	–	↓	EYE, TM, SPC, YSE, DLA, BAH	Ahmad et al., 2015
CuO NPs	–	10-20	0, 30, 60 and 121 ppb	96	96 well MP	–	LC	–	–	1.34 μM,	–	–	Chen et al., 2011
	–	6	0.1, 0.5, 2, 10, 50 and 200 μM	120	96 well MP	–	–	–	–	≈30 μM,	↓	–	Thit et al.,2017
	–	40-60	0, 0.15, 0.25, 0.5, 1	96	*	E3	–	–	–	–	–	FCE, SPC, YSE, AF, HY, HM, EYE, SWIM, SIVs	Zhang et al., 2017
	–	69 ± 18	0.01, 0.05 and 0.1	96	24 well MP	E3	–	–	–	–	↓	YSE, CS, NCM	Bai et al., 2010
	–	30 ± 9 and 40 ± 2	50, 125, 250 and 500	72	–	–	–	–	–	175	↓	PE, NCM	Kumari et al., 2017
	–	50	0, 5, 10, 20, 40, 60, 80, 100 and 120 ppm	48	24 well MP	E3	EMB	–	–	64 ppm	↓	AM, HM, SO HTM, YSE, AG, SC, TM, CS, RA, PE	Ganesan et al., 2015
Fe_2O_3 NPs	Uncoated	30	100, 50, 10, 5, 1, 0.5 and 0.1	168	24 well MP	Fish culture medium	–	–	10 and 36.06	53.35	↓	PE, BA, BU	Zhu et al., 2012
MgO NPs	–	20	50, 100, 200 and 400	144	24 well MP	–	–	–	174	428	↓	PE, AG, EYE, CS, YND, CFM, PIG, YSE	Ghobadian et al., 2015
Ni NPs	–	30, 60 and 100	10 - 1000	120	6 well MP	E3	GIT	189	–	221	–	HM	Ispas et al., 2009
QDs	Carboxyl		1, 4 and 8 nM	5 d	*	–	M, GI, F, I, GO, CH, OP, ASL, CHO, B	–	–	–	No effect	PE, YSE, TM	Chen et al., 2017
	PLL	382 ± 8	0, 0.2, 200 μM	120	96 well MP	–	EMB	–	–	–	–	PE, YSE, TM, CS, BU	King-Heiden et al., 2009
	Graphene	2-5	0, 12.5, 25, 50, 100, and 200 μg mL^{-1}	96	96 well MP	E3	–	–	–	–	↓	PE, YSE, FCE, CS, DLA	Guo et al., 2015
	CdTe	3,5	1, 25, 50, 100, 300, 200, 400 nM	120	–	–	–	–	–	185,9 nm	↓	PE, YSE, PIG, EYE, CS, TM	Zhang et al., 2012
SiO_2 NPs	–	40	0, 50, 100 and 200	96	96 well MP	E3	CHO, EMB		59, 40.2 and 28.2	–	↓	PE, CS, BDC	Chao et al., 2017

Cont.

	A	50	1, 10, 100	132	96 well MP	–	EYES	–	–	10 ng	–	HO, CY, AN, PE, YSE	Nelson et al., 2010
	–	300	3 and 0,01	72	6 well MP	–	–	–	–	–	–	BDC	Duan et al., 2016
	–	107	1, 3, 6, 12	72	*	–	–	–	–	–	–	PE, BDC, CIRC, SIVs	Duan et al., 2017
	G	570	0, 0.1, 0.3, 0.5 and 1	120	*	E3	–	–	–	–	–	DLA, BAH	Dumitrescu et al., 2017
	–	40	50, 100 and 200	96	96 well MP	E3	CHO	–	–	–	↓	PE, CS	Chao et al., 2018
	–	20, 50 and 80	12.5, 25, 50, 100 and 200	120	–	–	CHO	–	–	–	Premature hatch rate	PE, YSE, AG, CS, OY	Pham et al., 2016
	[Ru (bpy) 3] Cl_2	~ 60	0.0025 and 200	96	24 well MP	–	CHO	–	–	–	↓	No malformation	Fent et al., 2010
SiO_2 NPs, CdSe NPs, AgNPs and ZnO NPs	AUE, UA, CT, P	3-6, 703 ± 13, 6–35, 3–9	1, 10, 100	120	6 well MP	–	–	–	–	–	↓	CdSe: CS	Ong et al., 2013
TiO_2 NPs	–	27,73 ± 0.98	0.1	6 d	Becker (500 ml)	–	–	–	–	–	No effect	↑malformation	Fang et al., 2014
	–	9.83 ± 0.55	0.1	6 d	Glass containers (600 mL)	–	–	–	–	–	↓	HTM, PE, AM	Miao et al., 2015
	–	21	0, 0.01,10 and 1000 mg mL^{1}	120	24 well MP	–	–	0.073	107.2	–	↓	–	Samaee et al.,2015
	–		0, 10, 20, 60, 120 mg L^{-1}	96	–	–	–		60	–	↓	–	Shih et al.,2016
	–	7.04	0.1	7 d	Glass containers (500 mL)	–	–	–	–	–	No effect	No malformation	Wang et al., 2014
	C		0, 1, 10, 100, 500, 1000 μg ml^{-1}	120	24 well MP	–	–	–	–	300 μg mL^{-1}	–	AG, CFM, PE, TM	Bar-llan et al., 2012
	–	33.4 ± 1.9	0, 0.1, 1, 10 μg mL^{-1}	96	96 well MP	–	–	–	–	–	↓	PE, FCE, DLA	Hu et al., 2017
	–	434 ± 15	1	120	Becker (200 ml)	–	–	–	–	–	No effect	PE, CS, BD	Li et al., 2018
TiO2-MWCNT	–	20-50	30.0, 100.0, 130.0, and 110.0	96	24 well MP	E3	–	–	–	–	↓	No malformation	Silva et al., 2018
TiO_2 NPs and ZnO NP	–	19 ± 4	0, 1.5, 3, 6, 12, 24	120	96 well MP	–	–	–	1.3 and 7.3ZnO NP(total) + TiO2 NPs 3	7.1 and 9.5	↓	–	Hua et al., 2016

Cont.

TiO_2 NPs SnO_2 NPs ZnO NPs CeO_2 NPs	Uncoated	0.3-10	50, 10, 2, 0.4 and 0.08	120	96 well MP	–	–	–	0.5 and 3.51	3.5 and 9.1	–	NCM, YSE, CS, SC, EYE, PE, OTM, SOMI, PFM, PIG, CMS, SWIM, NCM	Welmas et al., 2015
ZnO NPs	–	50-70	0.1, 0.5, 1, 5 and 10	144	24 well MP	–	CHO	–	–	–	↓	No malformation	Chen et al., 2014
	–	40	50	14 d	–	–	–	–	–	–	–	SWIM, PE, CS, TM, YSE	Du et al., 2016
	CTS and PEG	16	1, 5, 10, 25, 50 and 100	144	6 well MP	E3	CHO	–	–	–	↓	–	Girigoswami et al., 2015
	–	10-30	10, 20, 50 and 100 ppm	96	Petri plates (3 mL)	–	–	–	–	–	↓	YSE, PE, CS, AM	Kteeba et al., 2017
	–	100	1, 5, 10, 20, 50 and 100	144	24 well MP	Fish culture medium	–	–	–	–	↓	H, PE, TM, CS	Zhao et al., 2013
	–	30, 40, 60	10, 30, 60, 90 and 120	96	Glass (2000 mL)	–	–	–	–	–	↓	PE, H, YSE, CS, TM, SWIM	Zhao et al., 2016
	–	20–30	0.01, 0.1, 1 and 10	96	96 well MP	–	–	–	–	–	–	TM, YSE, PE	Choi et al., 2016
	–	27, 32, 202	2, 4, 8, 16, and 32	120	96 well MP	–	–	–	2.2	9.6	↓	TM, YSE, PE	Hua et al., 2014

[a] Tm (Tween 80), PEG (Polyethylene glycol), CT (Citrate), SP (Sodium polyacrylate), PVP (Poly-N-vinyl-2-pyrrolidone), PEI (Polyethyleneimine), Maltose (MAL), ZnO- Ecodis P90 (ECOP90) MES (Negatively charged 2 mercaptoethanesulfonic acid), MEEE (Neutral 2-(2- mercaptoethox) ethoxy) binders of ethanol), SA (Secondary Amines), G (Glycine), AUE (Undecanoic Acid in Ethane), UA (Undecylenic acid), C (Carbon), CTS (Chitosan), Au-Sodium azide (CIT), P (Polymer), PG (pristine graphene), A (Amine), PLL (poly-L-lysine)

[b] MP (Microplate), *(does not specify which microplate)

[c] CHO (Chorion), STO (Stomach), INTV (Intestinal Villi), GIT (Gastrointestinal Tract), YO (Yolk), FPE (Fluid in the Pericardium), LI (Lumen of the intestine), ERY (Erythrocytes), EMB (Embryos), BR (Brain), HE (Heart), BEMB (Blood of Embryos), LC (Liver Cell), GO (Genital Openings), CH (Cheek), OP (Operculum), ASL (Abdominal Skin of Larvae), GI (Gill), M (Mouth), F (Fins), I (Intestine), LV (Liver), GAL (gallbladder)

[d] Ht (hatching), Mf (malformation), PA (Pancreas)

[e] AM (Axial Malformation), AG (Atrophic Growth), AF (Fin Abnormality), AN (anophthalmia), BAH (Blood Accumulation Heart Region), BA (Body Arch), BDC (Bradycardia), BD (Body Degradation), BYS (Bubble-like Formations on the Yolk Sac), BU (Body ulceration),CS (Curvature of the Spine), CMS (Abnormal Circulation or Vasculature), CFM (Craniofacial Malformations), CIRC (Abnormal Circulation or Vasculature), CY (cyclopia) , DM (Deformities Mouth), DCH (Deformities of Chamber), DLA (Disturbed Locomotive Activity), DTT (Decaying Tail Tissue),EYE (Eye Malformations Such as Large or Small Eyes), FFF (Flap Flexing Fold), FCE (Tail Flexure), GB (Gallbladder), H (Hyperemia), HTM (Heart Malformation), HM (Head Malformation), HY (Hypoplasia), HO (holoprosencephaly), NCM (Notochord malformations), OTM (Otic vesicle malformations), OY (0), PE (Pericardia Edema), PFA (Pericardial fluid is accumulated), PFM (Pectoral fin malformations), PIG (Abnormal pigmentation - hypo or hyper pigmentation), RA (Rachischisis), SWIM (Abnormal swim bladder development), SC (Scoliosis), SOMI (Abnormal somite), SO (Sacculi or Otolith), SPC (Spinal Cord), SIVs (Reduced area of sub-intestinal vessels), TM (Malformations Tail), TU (Tissue ulceration),YSE (Yolk sac edema), YND (Nondepleted)

5. Experimental design

Existing OECD guidelines on toxic testing of chemicals are not always suitable for NMs assessment. Thus, standard ecotoxicity testing with NMs often require modifications in experimental design (e.g. exposure media modification) to address specific NMs behaviour, which can have tremendous and unpredictable impacts on the results obtained. However, the modifications incorporated in these studies are not always clearly stated, and in conjunction with varying conditions, NMs types, size and surface functionalization, make comparisons between studies very difficult (Petersen et al., 2015).

Revised data showed a lack of standardization in the experimental protocols applied for nanotoxicity assessment using the ZET (Table 2; Fig. 5), which difficult the consistency in interpreting and comparing results and drawing conclusions. Several factors can interfere in toxic assessment of NMs using the ZET, such as exposure time, exposure chamber, exposure medium, temperature, pH, concentrations, exposure method (static and semi-static) and use of solvent, as previously reported for other pollutants (Lammer et al., 2009; Beekhuijen et al., 2015; Truong et al., 2011). For this reason, the development of standards protocols is required for the use of the ZET in a nanotoxicological context to maximize test consistency.

The exposure time in the ZET varies greatly in the revised studies (Table 2), from 48 (3 %) to 336 h (1 %), with the majority of the works (33 %) using 96 h (Fig. 5A). Zebrafish has a rapid development, and the early stages of embryonic development are completed within the first 24 hours post-fertilization (hpf), while the larvae is formed after 120 hpf (Kimmel et al., 1995; Giannaccini et al., 2014). In this context, an exposure length between 120 and 144 hpf is indicated as optimal and covers the development stages as organogenesis, yolk consumption and swimming behaviour.

The selection of exposure chambers can also impact the ecotoxicological outcome of testing with NMs. Increasing the consistency of the exposure chamber dimensions (material, size, aspect ratio, internal surface area) is known to reduce differences in the rate of MN agglomeration, settling, dissolution, or sorption, however, a single type of test vessel may not always be suitable for all types of MNs (Petersen et al., 2015). Regarding the revised data, 36 % of the studies used 24-well microplates with one embryo in each well, such as was recommended by Lammer et al. (2009) and Beekhuijzen et al. (2015).

Many studies used 96-wells microplates (24 %) and 6-wells microplates (12 %), while 16 % used petri dishes (25 mL) containing the embryos (10 to 20) or beakers (200 to 500 mL) containing a large number of embryos (≈400) (Fig. 5B). However, these exposure chambers are not recommended, because unviable embryos can affect viable embryos if kept together (Beekhuijzen et.al., 2015).

Another modification in ecotoxicity testing using NMs is related to the composition of test medium. The FET OECD guideline 236 allows for a flexibility in the selection of dilution water, as long as properly characterized and incorporated in the test as negative controls and internal plate controls (OECD 2013). However, for MNs toxicity testing, this flexibility can difficult comparison between test results, especially for studies that use the same basic test method (Petersen et al., 2015). Regarding the exposure medium reported in literature (Table 2, Fig. 5C), 65 % of the studies did not mention which medium was used, while the E3 medium was used by only 30 % of the studies, as recommended by the OECD guideline 236 (OECD, 2013). Few studies (5 %) have used other media types, such as dechlorinated water. It is widely recognized that NMs can interact with different components of the exposure media, such as proteins, metal ions, lipoproteins and coagulation factors (Saptarshi et al., 2013), making the choice of a suitable exposure medium one of the critical points to consider when conducting this type of studies.

The physico-chemical properties of the medium (e.g. temperature, pH, oxygen, etc.) are of importance to the toxic assessment in the ZET. According to OECD test guideline 236, water temperature should be maintained at 26 ± 1 °C in test chambers at any time during the test for it to be considered valid (OECD, 2013). However, according to the published studies, the temperature used varied between 25 and 30 °C, with 59 % of studies conducted a temperature of 28 ± 1 °C (Fig. 5D). Beekhuijen et al. (2015) showed that an increase in temperature causes an accelerated development of the zebrafish embryos, in which temperatures higher than 28 °C have been associated with an increase in the number of malformations. An increase in temperature could also induce evaporation of the test solutions, causing interference in the maintenance of the nominal test concentrations during the exposure period. Another important factor to consider is the pH of the exposure medium, since pH and ionic strength combined with NMs characteristics (such as area and surface charge), considerably affect NMs behaviour in the exposure medium and consequently its toxic potential (Clement et al. al., 2017). In addition, reducing the ionic strength or adjusting the pH of the dilution water may reduce

the rate of aggregation and deposition for many MNs but may be physiologically stressful for the zebrafish (Petersen et al., 2015). Published studies describe a pH range of 6.5 to 7.5, which is in accordance with the range stated in OECD test guideline 236 (OECD, 2013). The impact of modifications in the test medium when using the ZET further highlights the need for standardization of this test for nano-toxicological evaluations.

The principles of the FET test are based on four apical observations recorded as indicators of lethality during the exposure period, after which an LC_{50} is calculated (OECD, 2013). For this reason, at least 5 concentrations by a constant factor not exceeding 2.2 should be tested to obtain a reliable dose-response curve, especially for data associated with lethality (OECD, 2013). Nonetheless, the revised data showed that 53 % of the studies used less than 5 concentrations of NMs (Fig. 5E). Another potential modification to standard ZET test procedures is the frequency on which test media should be changed during a test with NMs. Three types of exposure method have been employed in the ZET studies published: static, semi-static and flow (Fig. 5F). The choice of the exposure method depends on the stability of the concentrations tested during exposure, which for NMs has been a highly discussed parameter in terms of experimental design (e.g. Handy et al., 2012). The primary objective of frequent media changes is to ensure that exposure and nominal test concentrations are maintained by increasing stability of NMs, as several NMs are known to change particle size/shape through aggregation, dissolve or sediment within short periods of time. The most used exposure method in literature was the static one (56 %), i.e. without renewal of the medium, and only 44 % of the studies renewed the medium every 24 h, 41 % of which renewed the whole medium and 3 % renewed only half (1 mL). In short acute tests, replacing the test media is optional, however this should need to be done more frequently when using NMs in comparison with traditional chemicals. Nonetheless, this can be overcome if a thorough characterization of the NMs is performed in the test medium beforehand to confirm alterations in particle stability, in addition to a proper chemical characterization of test media.

As for the use of solvents for example, 99.9 % de ethanol (Manjunatha et al., 2018), DMSO (Whemas et al., 2015; Li et al., 2018; Tian et al., 2019), aquatic toxicology does not recommend the use of solvents due to potentially secondary toxic effects towards the target organism, but when used a proper positive control should be included in the test (Beekhuijen et al., 2015). The final solvent concentration in the stock solution should not exceed 100 $\mu l\ L^{-1}$ and should be the same in all test vessels (OECD, 2013). Several MNs

are not stable in aqueous media without the addition of dispersants/stabilizing agents (e.g. citrate) or surface coatings (e.g. polyethylene glycol (PEG) or polyvinylpyrrolidone (PVP). When commercial MNs are synthesized with these additional characteristics, they should be considered an integral part of the MN, as they will vary in state in behaviour, depending largely on the testing media. Therefore, additional control experiments should be conducted to elucidate the impact (stimulatory or inhibitory) of the dispersant or capping agent on the overall results (OECD, 2013; Petersen et al., 2015).

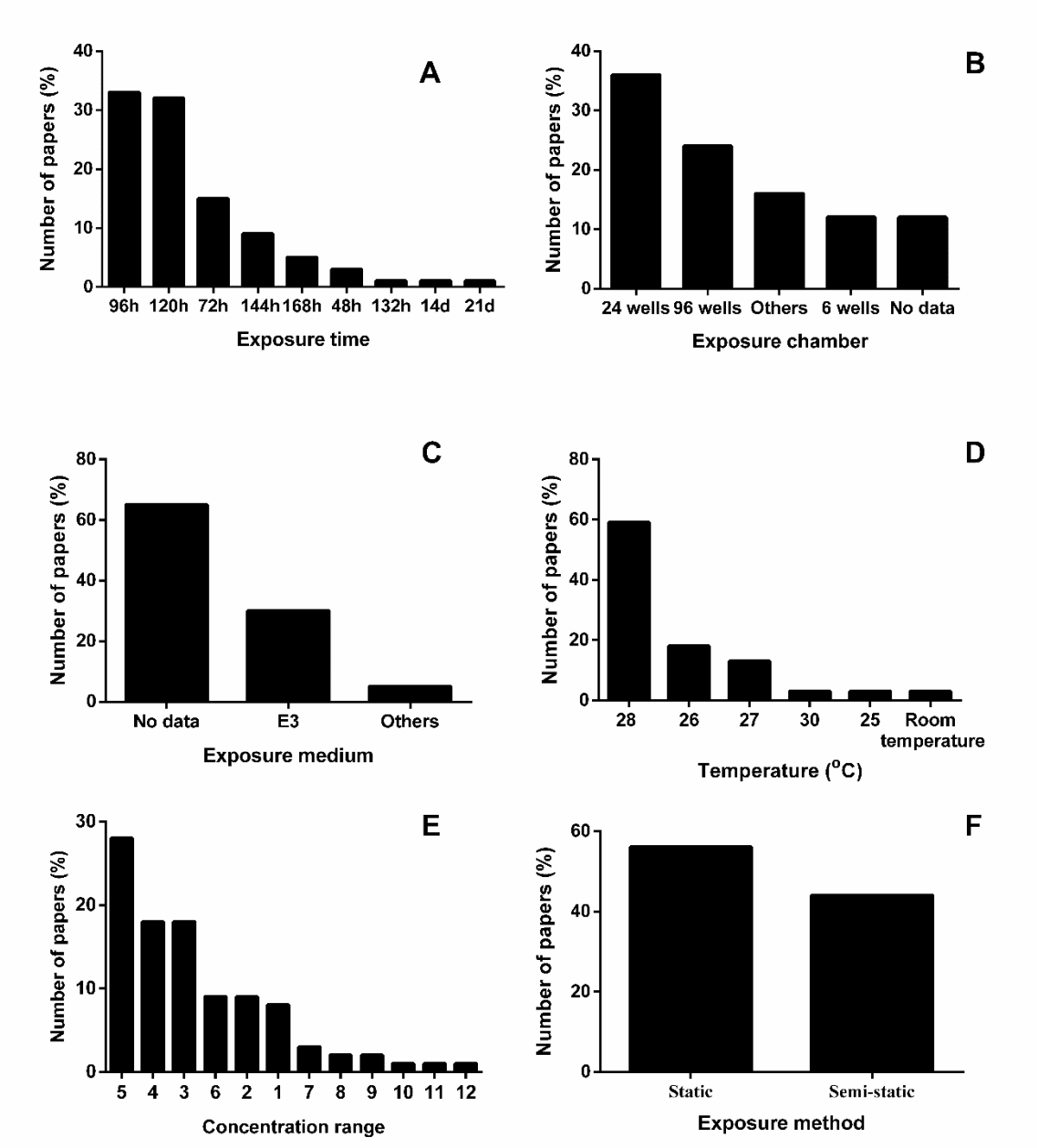

Figure 5. Experimental design of papers published about the zebrafish embryotoxicity test (ZET) applied in nanotoxicity assessment. A) Exposure time. B) Exposure chamber. C) Exposure medium. D) Concentration ranges.

6. Interaction of NMs with chorion, uptake and accumulation

The NM accumulation in zebrafish embryos and larvae is shown in Fig. 6 and Table 3. Revised data indicated that NMs accumulate in zebrafish embryos mainly in the region of the chorion, with accumulation being dependent on NM type and size. After uptake, NM can be transported to different organs, mainly the gastrointestinal system, heart, brain, yolk and liver (Asharani et al., 2008; Chen et al., 2017; Pitt et al., 2018). On the other hand, in zebrafish larvae NMs were observed mainly in the gastrointestinal tract, indicating that absorption, bioaccumulation and distribution of NMs in tissues is dependent on the development stage of zebrafish. The zebrafish chorion is a barrier that covers the embryo up to 48 - 72 h and has pores (diameter = 500 – 700 nm) that are important for the transport of oxygen, nutrients and excretion (Rawson et al., 2001). However, these pores can facilitate the entry of NMs that diffuse through the chorion membrane and may be toxic to embryo development during the period of organogenesis (Cheng et al., 2007). To date, little is known about the interaction of NMs with the chorion, and how this structure interacts and affects the absorption, accumulation and distribution of NMs in the embryos (Table 3).

Fent et al. (2010) described the interaction of fluorescent silica NPs (FS NPs) with the chorion, determining its absorption capacity and biodistribution in zebrafish embryos. Large FS NPs (60 nm – 200 nm) did not cross the chorion and did not induced malformations during zebrafish development. In addition, these NPs did not interfere in gas exchange processes of embryos that are essential for their development. Similar results were seen for CNTs (11 nm; 360 mg L^{-1}), in which the chorion also prevented the passage and toxicity of these NMs, and consequently no alterations in the gas exchanges was observed during 96 h of exposure (Cheng et al., 2007). In opposite, Ag NPs (5 – 20 nm) crossed the zebrafish chorion and accumulated in the brain, heart, yolk and blood of embryos, leading to the several morphological changes, such as pericardia edema, deformities mouth, notochord malformations, decaying tail tissue and blood accumulation heart region (Asharani et al., 2008). Similar results have also been reported in other studies (Asharani et al., 2011; Chen et al., 2017; Pitt et al., 2018).

In environmentally relevant exposure conditions, the formation of NM aggregates in aqueous suspensions increase the hydrodynamic diameters of NMs and may reduce its uptake by zebrafish chorion (Chao et al., 2018; Cheng et al., 2007).

Furthermore, the NM interaction with others macromolecules present in the test medium also changes its interaction and uptake by zebrafish embryos. The NM interaction with natural organic matter (NOM) can reduced its toxicity on zebrafish embryos, such as reported by Kteeba et al. (2017) using ZnO NPs (10 – 30 nm) and NOM isolated from Milwaukee-WI, Yukon-AK and Suwannee River-GA rivers. The NOM was able to mitigate toxic effects induced by ZnO NPs, resulting in reduced delays in hatch rate, mortality, and malformations.

After hatching (72 hpf), the zebrafish embryo loses its protective barrier (the chorion) and is susceptible to NM exposure by other routes during the larval period (Fig. 6). Pomeren et al. (2017) investigated the different uptake routes (via chorion, dermal and oral exposure) of polystyrene (PS) NPs (25, 50, 250 and 700 nm) in three phases of zebrafish development in order to investigate the influence of size in NP route of exposure and whether the uptake route determines the target organ to be reached. The three stages consisted of: the first stage when the embryo is still protected by the chorion, the second stage when the embryo's mouth is still closed and the third stage which the embryo is fully formed, and the functions of absorption and excretion are functioning. In this study, during the period of 24 hpf, the NPs were adsorbed by the chorion, and only after hatching (72 hpf), uptake of small NP (25 and 50 nm) were detected in the embryos through the oral and dermal routes, following by distribution in the body and accumulation in the eye of the larvae, while the larger NPs (250 and 700 nm) were found in the digestive tract and absorbed by the epidermis.

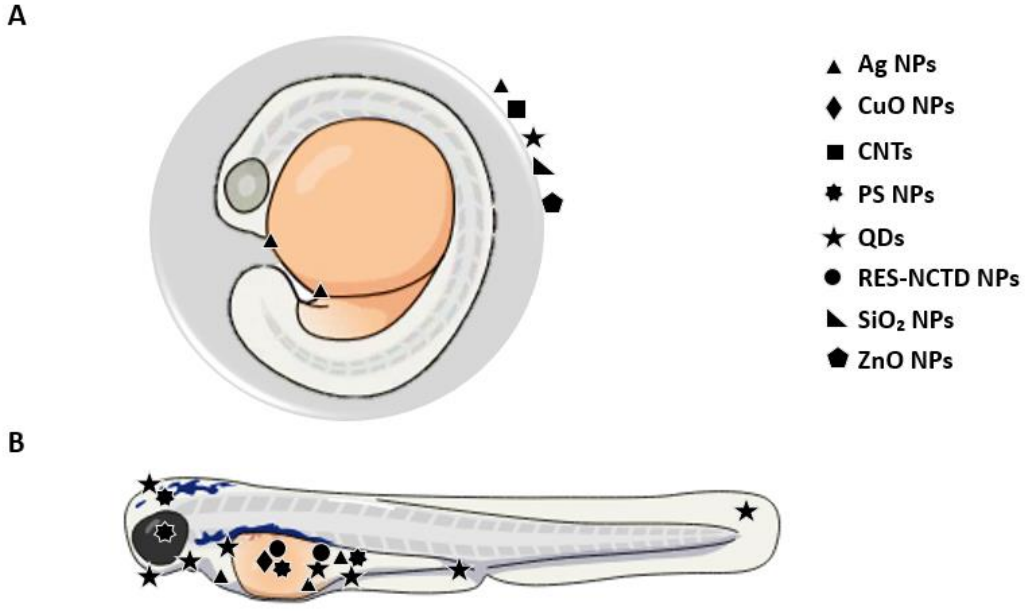

Figure 6. General scheme of the accumulation of nanomaterials in the zebrafish embryo (A) and larvae (B).

Table 3. Number of papers published related to accumulation of nanomaterials in the zebrafish using the zebrafish embryotoxicity test (ZET).

Accumulated region	Number of articles	Types NMs
Abdominal skin of larvae	1	QDs
Blood of embryos	2	Ag NPs
Brain	4	Ag NPs, PS NPs, QDs, Au NPs
Chorion	12	CNTs, Ag NPs, ZnO, SiO_2 NPs, QDs
Cheek	1	QDs
Embryos	5	Ag NPs, CuO NPs, SiO_2 NPs, QDs
Eye	2	PS NPs, Au NPs
Fins	1	QDs
Gallbladder	1	PS NPs
Gastrointestinal tract	8	RES-NTCDs NPs, PS NPs, Ag NPs, QDs, Ni NPs
Gill	1	QDs
Genital openings	1	QDs
Heart	3	Ag NPs, PS NPs
Liver	3	Ag NPs, CuO NPs
Mouth	1	QDs
Operculum	1	QDs
Pancreas	1	PS NPs
Stomach	1	RES-NTCDs NPs
Yolk	3	Ag NPs, PS NPs

7. Mortality

The LC_{50} values reported in the literature indicate that inorganic NMs present high toxicity compared to organic ones (Table 2). Among the inorganic NMs, lower LC_{50} values were reported for CuO NPs (0.00139 mg L^{-1}; Chen et al., 2011), PVP-functionalized Ag NPs (0.11 mg L^{-1}; Boley and Goss,2018), TiO_2 NPs (3.5 mg L^{-1}; Welmas et al., 2015), Ecodis-P-90 functionalized ZnO NPs (4.289 mg L^{-1}; Lacave et al.,

2016), QDs CdS (7.036 mg L^{-1}; Lacave et al., 2016), Au NPs (24.61 mg L^{-1}; Lacave et al., 2016), Fe_2O_3 NPs (53.35 mg L^{-1}; Zhu et al., 2012), SiO_2 (83.329 mg L^{-1}; Lacave et al., 2016), MgO NPs (428 mg L^{-1}; Ghobadian et al., 2015). Theses LC_{50} values are above the concentrations reported in environmental water samples, which are in the order of ng L^{-1} (Gottschalk et al., 2009, 2013), indicating low effects of NMs on mortality at environmentally relevant concentrations. Furthermore, two studies estimated the LC_{50} for organic NMs, which showed that the Tween 80 -functionalized CS NPs has high toxicity (25.06 mg L^{-1}) (Yuan et al., 2016) when compared to uncoated CS NPs (270 mg L^{-1}) (Wang et al., 2016), confirming the role of the NM functionalization on nanotoxicity.

8. Morphological alterations on zebrafish embryos induced by NMs

A total of 33 distinct morphological alterations induced by NM exposure were reported in the revised studies (Table 4). To facilitate the comparison and discussion of the nanotoxicological data, the zebrafish alterations induced by NMs were grouped in the following four reaction patter (Rp): Rp_1 (circulatory changes), Rp_2 (pigmentation and tegumentary changes), Rp_3 (musculoskeletal disorders) and Rp_4 (yolk sac alterations) (Table 4).

Among the teratogenic effects observed in the zebrafish exposed to NMs, inorganic NMs induced mainly pericardial edema (18 %), followed by spinal curvature (14 %), flexure tail (10 %), edema of the yolk sac (9 %), absence or irregular eye size (7 %), swimming bladder deformity (4 %), notochord malformations (4 %), growth retardation (4%), abnormal circulation or vasculature (4 %) and other malformations represent (26 %). In accordance, inorganic NMs induced mainly musculoskeletal disorders (Rp_3), circulatory changes (Rp_1) and yolk sac alterations (Rp_4) when compared to pigmentation and tegumentary changes (Rp_2). As for organic NMs, there is no general trends in data regarding morphological alterations due a lack of studies using this type of NMs (Table 4). A summary of the embryotoxicity of both types of NMs in terms of morphological alterations are summarized in the sections below.

8.1 Embryotoxicity of inorganic NMs

8.1.1 Ag NPs

Ag NPs induced a high number of morphological alterations in the zebrafish embryos (Table 4), especially those of Rp_1 and Rp_3, such as heart edema (19 %), yolk edema (13 %), spinal curvature (13 %) and tail flexure (11 %). In addition, the mouth deformities (2 %) and bubble-like formations in the yolk sac (2 %) were found only after exposure to Ag NPs. The citrate-functionalized Ag NPs (15 – 50 nm; 0.2 to 1 mg L^{-1}; 96 h) induced yolk edema and heart malformation on zebrafish embryos (Cui et al., 2016). Similar effects were reported in embryos exposed to citrate- and PVP-functionalized Ag NPs (2 – 110 nm, 0.8 to 50 mg L^{-1}) for 120 h (Kim and Tanguay, 2014). However, the citrate-functionalized Ag NPs (10 nm; 3 – 30 µM; 120 h) increased the frequency of abnormal swim bladder development and atrophic growth (Powers et al., 2011), while the uncoated Ag NPs (5 – 20 nm; 5 – 100 µg L^{-1}; 72 h) induced the notochord malformations, heart oedema, body degradation, blood accumulation heart region and decaying tail tissue (Asharani et al., 2008). These revised data indicated that the embryotoxic and teratogenic effects of Ag NMs are dependent on size and functional groups.

8.1.2. SiO_2 NPs

SiO_2 NPs induced five types of malformations during embryo development (Table 4), which belong to Rp_1, Rp_2 and Rp_3, mainly pericardial edema (28 %), bradycardia (14 %), spinal curvature (14 %), yolk sac edema (14 %), abnormal circulation or vasculature (9 %). The heart edema is caused by the swollen atrium and ventricle, and this abnormal accumulation of fluid in the pericardial cavity generates intrapericardial pressure. When cardiac function is completely blocked, the formation of yolk edema was observed (Chao et al., 2017).

SiO_2 NPs (300 nm; 3 mg L^{-1}) induced bradycardia in zebrafish embryos after 72 hpf (Duan et al., 2016). Similarly, embryos exposed to SiO_2 NPs (107 nm; 1 to 12 mg L^{-1}) for 72 h showed bradycardia, pericardial edema, abnormal vascular circulation and reduction of the area of sub-intestinal vesicles (Duan et al., 2017). SiO_2 NPs (20 – 80 nm; 12.5 to 200 mg L^{-1}) after 120 h of exposure induced pericardial edema, yolk sac edema, decreased growth, changes of the spine curvature and deformities in the yolk (Phan et al., 2016). These results confirm that exposure to SiO_2 NPs induced mainly circulatory changes (Rp_1) in zebrafish embryos.

8.1.3 ZnO NPs

ZnO NPs induced morphological changes, especially those of Rp_1 and Rp_3, such as pericardial edema (19 %), yolk sac edema (17 %), spinal curvature (14 %) and tail flexure (14 %) (Table 4). The chitosan and PEG-functionalized ZnO NPs (16 nm, 1 to 100 mg L^{-1}) did not induce any type of malformation after 144 h of exposure (Girigoswami et al., 2015). On the other hand, the embryo exposed to uncoated ZnO NPs (100 nm, 1 to 100 mg L^{-1}) for 144 h induced pericardial edema, hyperemia, curvature of the vertebral column and malformation of the axial region of the head (Zhao et al., 2013). Similar toxicity of uncoated ZnO NPs was reported by Du et al. (2016) and Zhao et al., 2016, indicating that the NP functionalization with chitosan or PEG decreased the toxicity of NMs during the early developmental stages of the zebrafish, as well as confirms the role of functionalization in the nanotoxicological potential.

8.1.4 TiO_2 NPs

The TiO_2 NP toxicity to zebrafish embryo was associated with morphological alterations in the category Rp_1, Rp_2, Rp_3 and Rp_4, mainly pericardium edema (25 %), absence or irregular eyes (10 %) and notochord malformation (10 %) (Table 4). TiO_2 NPs functionalized with organically coated; 99.5% trace metal basis, (15 – 25 nm; 10 to 100 μg mL^{-1}) showed low toxicity when compared to PVP-functionalized NPs (61 – 70 n; 10 to 100 μg mL^{-1}) after 72 h of exposure (Pavagadhi et al., 2014). The embryos exposed to TiO_2 NPs (33.4 ± 1.9 nm; 0.1 to 10 μg mL^{-1}) for 96 h induced pericardial edema, fluid accumulation in the pericardium and decreased locomotor activity (Hu et al. 2017). Similar results were observed in embryos exposed to TiO_2 NPs (9.83 nm; 0.1 mg L^{-1}) for 144 h (Miao et al., 2015), confirming the TiO_2 NP embryotoxicity.

8.1.5 Au NPs

Despite the reduced number of studies, the AuNPs induced few morphological changes in zebrafish embryos, such as yolk sac edema (32 %), pericardium edema (17 %), curvature of the spine (17 %), tail flexion (17 %), head malformation (17 %), which belongs to category Rp_1, Rp_3 and Rp_4. The citrate-functionalized Au NPs (4.4, 13.5, 40.4

nm, 0.1 to 100 mg L^{-1}) were the only NPs that did not induce any type of malformation in the embryos when compared to CdS, ZnO NPs and SiO_2 NPs (Lacave et al., 2016). Similar results were found by Asharani et al. (2011), which showed low embryotoxicity of Au NPs (15 – 35 nm; 10 to 100 mg L^{-1}) when compared to Ag NPs and Pt NPs. Only Ramachandran et al. (2017) indicated that Au NPs without functionalization (5 - 50 nm; 5 to 100 μg mL^{-1}) induced morphological changes in the yolk sac of embryos. Thus, the revised results indicate that Au NPs present low toxicity to the early stages of zebrafish development.

8.1.6 CuO NPs

The CuO NPs showed to be extremely toxic to the embryos, leading to serious teratogenic effects. Alterations of Rp_1, Rp_2 and Rp_3 were observed after CuO NP exposure, among them yolk edema (13 %), curvature of the spine (13 %), tail flexion (9 %), head malformation (9 %) and pericardium edema (9 %). The notochord malformations (4 %) and malformation of the sacrum and otolith (4 %) were found only after exposure to CuO NPs (Zhang et al., 2017) (Table 4). The exposure of zebrafish embryos to CuO NPs (40 – 60 nm; 0.15 to 1 mg L^{-1}) for 96 h induced several morphological alterations, including tail flexure, spinal curvature, yolk sac edema, malformation of the head, irregular absence and size of the eyes, swimming bladder deformity and reduction of the area of sub-intestinal vesicles (Zhang et al., 2017). Similar results were found after the exposure to CuO NPs (50 nm; 5 to 120 ppm) for 48 h (Ganesan, 2015), confirming that CuO NPs induce several teratogenic effects on zebrafish.

8.1.7 QDs

The zebrafish embryos exposed to QDs showed changes in Rp_1, Rp_3 and Rp_4, mainly pericardial edema (21 %), spinal curvature (21 %), yolk sac edema (21 %) and tail flexion (17 %) (Table 4). The carboxyl-QDs (340-390 nm; 1, 4 and 8 nM; 120 h) can accumulate in various regions of the embryo, and may penetrate the epithelium or be ingested through the mouth and gills and reach internal organs through the cardiovascular system (Chen et al., 2017). Furthermore, the embryos exposed to CdS QDs (3.5 – 4 nm; 0.01 to 10 mg L^{-1}) for 120 h showed pericardial edema, yolk sac edema, spinal curvature,

yolk deformity (Lacave et al., 2016). Graphene QDs (2 – 5 nm; 12.5 to 200 μg mL^{-1}) induced pericardial edema, spinal curvature, yolk sac edema and tail flexion after 96 h of exposure (Guo et al. 2015).

8.1. Embryotoxicity of organic NMs

Organic NMs have few studies when compared to inorganic ones. The carbon nanotubes (CNTs) (4 %), fullerenes (2.5 %) and NPs of chitosan (CS NPs) (2.5 %) were the most studied organic NMs when compared with PS NPs (1.5 %). Amphiphilic Nanoparticles of Resveratrol-Norcantharidin (RES-NCTD) (1 %) and pristine graphene (PG) (1 %) (Table 4). The CNTs induced a low rate of morphological alterations in the zebrafish embryos, indicating low toxicity. There were few changes in Rp_1, Rp_3 and Rp_4 categories, such as reduction of growth (28 %), pericardium edema (14 %), abnormal circulation (14 %), notochord malformation (14 %), reduction of locomotor activity (14 %) and yolk sac edema (14 %). The PEG-coated CNTs (20 – 40 nm, 0.01 to 1 mg L^{-1}) reduced growth and the locomotor activity after 96 h of exposure (Cordeiro et al., 2018). Similarly, the CNTs exposure (10 – 20 nm; 1 to 100 mg L^{-1}; 96 h) reduced the zebrafish embryo growth (Liu et al., 2014), confirming that the CNTs interfere in the growth and behaviour of zebrafish.

CS NPs induced changes in zebrafish in categories Rp_1 and Rp_3, such as pericardial edema (25 %), spinal curvature (25 %), swimming bladder deformity (25 %), hyperemia (12.5 %) and head malformation (12.5 %). The CS NPs (84 – 86 nm; 100 to 400 mg L^{-1}) induced pericardium edema, axial head malformation and swimming bladder deformity of zebrafish embryos after 120 h (Wang et al., 2016). CS NPs (181.2 nm; 5 up to 30 μg mL^{-1}) also induced similar alterations, such as pericardial edema, hyperemia and curvature of the spinal column after 120 h exposure (Nikapitiya et al., 2018). Although the high concentrations of CS NPs are toxic to the embryos leading to the appearance of malformations, these NPs at 5 μg mL^{-1} did not cause effects during embryo development and are able to increase the larvae resistance to *Aeromonas hydrophila*, because it has strong immunomodulatory activities, which promote immune defence functions *in vivo*. In this sense, the toxicity of these NPs depending on their physic and chemical properties, indicating their potential biotechnological applications. In relation to fullerenes, they induced changes in the categories Rp_1, Rp_2 and Rp_3, such as pericardial edema (50 %), tail flexure (25 %) and yolk sac edema (25 %). The PG also caused alterations in the same

categories of fullerenes, with pericardial edema (17 %), blood accumulation (17 %), spinal curvature (17 %), head malformation (17 %), absence and eye size (17 %) and yolk of the yolk sac (17 %) (Table 4).

PS NPs (34.5 - 10.8 nm, 0.1 to 10 ppm) did not induce embryo alterations, although they accumulated in the gastrointestinal tract, gallbladder, liver, pancreas, heart and brain after 24 h of exposure (Pitt et al., 2018), confirming their systemic distribution and accumulation. Similarly, Yan et al. (2016) observed that the accumulation of RES-NCTD NPs in the stomach and intestine were not associated with morphological alterations, indicating that future studies are necessary to understand the cellular and molecular responses of zebrafish embryos exposed to organic NMs, as well as their effects on gastrointestinal microbiome.

Table 4. Morphological changes in zebrafish induced by nanomaterials using the zebrafish embryotoxicity test (ZET).

Morphological malformations		Types of NPs																			
		SiO_2 NPs	Ag NPs	ZnO NPs	TiO_2 NPs	Au NPs	Cu_2O NPs	QDs	CS NPs	PS NPs	SnO_2 NPs	$COFe_2O_4$ NPs	MgO NPs	RES-NCTD NPs	Pt NPs	Fe_2O_3 NPs	CeO2 NPs	CNTs	PG	Ni NPs	Fullerenes
Circulatory changes	Pericardial edema	5	12	8	4	1	2	5	2		1		1			1	1	1	1		2
	Heart malformation		2		1		1														
	Bradycardia	3																			
	Hyperemia			2					1												
	Blood accumulation	1	2									1									
	Anormal circulation or vasculature	2	2	1	1						1						1	1	1		
	Body arch edema															1					
Pigmentation and tegumentary changes	Changes of pigmentation of the head																				
	Changes of pigmentation of the eyes			1	1			1			1		1				1				
	Changes of pigmentation of the tail																				
	Body ulceration							1								1					
Musculoskeletal disorders	Scoliosis		1	1	1		1				1						1				
	Rachischisis						1														
	Notochord malformations		2	2	2		2				1						2	1			
	Spinal curvature	3	8	6	1	1	3	5	2		1	1	1		1		1		1		
	Defects in the somites		1	1	1						1						1				
	Tail flexure		7	6	2	1	2	4				1									1
	Decaying tail tissue		2																		
	Growth retardation	1	4		1		1						1					2			
	Reduction of locomotor activity	1			1			1				1						1			
	Craniofacial	1			1								1								
	Axial			1	1		1		1												
	Head malformation		3			1	2												1	1	
	Absence or irregular size of eyes		2	3	2		1	1			1	1	1				2		1		
	Reduced area of sub-intestinal vessels	1					1														
	Swimming bladder deformity		1	3	1		1		2		1						1				
	Pectoral fin malformatios			1	1						1						1				
	Deformities mouth		1																		
	Changes in the sacculi/otoliths						1														
Yolk sac alterations	Yolk sac edema	3	8	7	1	2	3	5			1	1	1				1	1	1		1
	Yolk deformity	1	4					1					1								
	Bubble-like formations on the yolk sac		1																		

9. Effect of NMs on hatching rate

Several studies showed that the Ag NPs reduced the hatching rate of zebrafish embryos (Table 2). The exposure to Ag NPs (8.39 ± 0.98 nm; 0.03 – 1.55 μg mL $^{-1}$) for 48 h decreased the hatching rate (80 %) (Massarsky et al., 2013), while the Ag NPs (10 – 20 nm; 0.5 and 0.05 μg mL^{-1}) inhibited 40 – 50 % after 56 h of exposure. Similar results were reported by Asharani et al. (20080, Powers et al. (2010) and Orbea et al. (2017).

SiO_2 NPs (40 nm; 50 – 200 mg L^{-1}) (Chao et al., 2017) reduced 39.6 % of the hatching rate of zebrafish, respectively. However, the SiO_2 NPs (20, 50 e 80 nm, 12.5 – 200 mg L^{-1}) accelerated the hatching rate (Pham et al., 2016). The premature hatching of zebrafish embryos can be explained due to the blockage of the pores of the corion that causes a hypoxic condition and hinders the excretion of metabolites. These conditions may facilitate the release of enzymes that facilitate chorion rupture (Silva et al., 2018).

ZnO NPs functionalized with chitosan and PEG (16 nm; 1 – 100 mg L^{-1}) for 76 h (Girigoswami et al., 2015) and ZnO NPs (100 nm; 1 – 100 mg L^{-1}) for (Zhao et al., 2013) reduced 62 and 25.72 % of the hatching rate, respectively. The effects of ZnO NPs on hatching rate also were reported by Chen et al. (2014), Hua et al. (2014), Zhao et al. (2016) and Kteeba et al. (2017). It was also observed that ZnO NP (20 nm; 1-100 mg L^{-1}) in the highest concentration did not hatch embryos causing embryos to die inside the chorion (Ong et al., 2013). The delay in hatching rate may be caused by interference in the expression of genes related to the hatching process. Hgg1 (*Cathepsin L*, *ctslb*), a well-established incubator enzyme gene, is expressed abundantly during the hatching process. This gene acts as a transcriptional factor in the intracellular environment and in the extracellular environment in the migration of cancer cells, matrix degradation and cell digestion. Thus, changes in hatching rate interfere with gene expression (Zhang et al., 2018).

Inhibition of hatching of zebrafish embryos have also been reported for different types of inorganic and organic NMs, such as TiO_2 NPs (21 nm; 0.01 – 1000 mg L $^{-1}$) (Samare et al., 2015), TiO_2 NPs (33.4 ± 1.9 nm; 0.1 – 10 μg L^{-1}) (Hu et al., 2017), TiO_2 NPs (9.83 ± 0.55 nm; 0.1 mg L^{-1}) (Miao et al., 2015), CuO NPs (6 nm; 0.1 – 200 μM) (Thit et al.,2017), CuO NPs (50 nm; 5 – 120 ppm) (Ganesan et al., 2015), graphene-functionalized QDs (2 – 5 nm; 12.5 – 200 μg mL^{-1}) (Chen et al., 2017), PEG-functionalized CNTs (20 – 40 nm; 0.01 – 1 mg L^{-1}) (Cordeiro et al., 2018), CNT (11 nm; 20 – 360 mg L^{-1}) (Cheng et al., 2007), CNT (10 – 20 nm; 1 – 100 mg L^{-1}) (Tong et al., 2014), PS NPs (25 – 700 nm; 5 –

25 mg L^{-1}) (Pomeron et al., 2017), $COFe_2O_4$ NPs (40.1 nm; 10 – 500 μM) (Ahmad et al., 2015), MgO NPs (20 nm; 50 – 400 mg L^{-1}) (Ghobadian et al., 2015), Au NPs (5 – 25 nm; 0.32 – 2.6 mg L^{-1}) (Ganeshkumar et al., 2012), Fe_2O_3 NPs (30 nm; 0.1 – 100 mg L^{-1}) (Zhu et al., 2012) and Au NPs (5-25 nm; 0.325 – 2.6 mg L^{-1}).

The effect of NMs on hatching success of zebrafish depend on their surface composition, concentration and exposure period (Table 2), because no effects were reported for TiO_2 NPs (27.73 ± 0.98 nm; 0.1 mg L^{-1}) (Fang et al., 2014), CdSe carboxyl-functionalized whit carboxyl (340 – 390 nm; 1 – 8 nM) (Chen et al., 2017), CeO_2 NPs (0.3-10 nm; 0.08 – 50 mg L^{-1}) (Welmas et al., 2015), SnO_2 NPs (0.3-10 nm; 0.08 – 50 mg L^{-1}) (Welmas et al., 2015), Pt NPs (3 – 10 nm; 10 – 100 mg L^{-1}) (Asharani et al., 2011) and RES-NCTD NPs (231,96-18,68 nm; 10 – 50 mg L^{-1}) (Yan et al., 2016).

10. Interactive effects of NMs with other pollutants

Although some NMs alone did not induce toxicity in organisms, several studies indicate that the NM interaction with other pollutants may potentiate its toxic effects, alerting about the potential risks of releasing these NMs into the environment (Fang et al., 2014; Li et al. 2018). After interaction with other molecules, the NMs may undergo changes in their properties, such as ion dissolution, aggregation state and redox reactions (Bundschuh et al., 2018; Lei et al., 2018). Due their physicochemical properties, NMs can act as carriers of other molecules to the cells, including contaminants ("Trojan horse effect" (Limbach et al., 2007), inducing changes in their bioavailability and toxicity. The co-exposure of NMs can induce additive, synergistic or antagonistic responses in different types of organisms (Hartmann and Baun, 2010), such as observed in the early developmental stages of zebrafish (Table 2).

The co-exposure of TiO_2 NPs (434 ± 15 nm; 1 mg L^{-1}) and the pesticide cypermethrin (0.4 to 10 μg L^{-1}) for 120 h increased the bioaccumulation of cypermethrin and induced several morphological alterations in the zebrafish embryos, such as pericardial edema, body curvature, decrease in body length, besides inducing neurotoxicity due to the reduction of neurotransmitters (i.e. serotonin, dopamine and γ-aminobutyric acid - GABA) that caused a reduction of locomotor activity (Li et al. 2018), indicating that TiO_2 NPs may potentiate the effects of cypermethrin on zebrafish embryos.

The co-exposure of TiO_2 NPs (7.04 nm; 0.1 mg L^{-1}) with the flame retardant polybrominated diphenyl ethers (BDE-209) (0.38 mg L^{-1}) induced increasing in the BDE-209 bioaccumulation, changes in the gene and protein expression of thyroid hormones and reduced the locomotor behavior of the zebrafish larvae, potentiating the effect of endocrine thyroid disorders and developmental neurotoxicity in the zebrafish embryos (Wang et al., 2014). Similarly, the co-exposure of TiO_2 NPs (27.73 ± 0.98 nm; 0.1 mg L^{-1}) with the insecticide and herbicide pentachlorophenol (0, 3, 10, and 30 μg L^{-1}) for 144 h increased the reactive oxygen species (ROS) production, DNA damage and morphological alterations on zebrafish embryos (Fang et al., 2014).

The interactive effects of ZnO NPs (40 nm; 50 mg L^{-1}) and the fluorosurfactant perfluorooctane sulfonate (0.2 to 0.8 mg L^{-1}) induced thyroid dysfunction in zebrafish by increasing the triiodothyronine (T3) and changes in the expression of thyroglobulin (TG), transthyretin (TTR) and thyroid receptors, as well as reduced growth and increased the embryo malformations, such as pericardial edema, yolk sac edema, spinal curvature and swimming bladder deformity (Du et al., 2016). A literature overview showed that more studies about the interactive effects of NMs with other pollutants during the early developmental stages of zebrafish are need, especially in environmental relevant conditions.

11. Conclusions and future perspectives

NMs are being produced and used on a large scale. However, with the increased release of these NMs into the environment, new toxicological studies are needed. Zebrafish is emerging as an important *in vivo* model system for testing NM toxicity, and offers various advantages that can connect developmental, disease and toxicological endpoints. This wide recognition is partly due to their low cost and easy maintenance in laboratory conditions, as well as rapid development. In addition, specific physiological impacts can be assessed at multiple stages of development and be linked to several routes of exposure (including direct exposure in water), which is highly relevant for environmental nanotoxicological applications. Overall, the toxicity studies compiled in this review identified a range of parameters regarding exposure conditions (concentration, route, exposure time, etc.) and physiochemical properties of different NMs (size, shape, surface charge/chemistry) that impacted their potential toxicity towards zebrafish

embryos. The toxicity level of these NMs was assessed in zebrafish embryos by observing a wide range of morphological, physiological and molecular effects, that included uptake and accumulation, mortality, malformations and functional defects, as well other sub-lethal effects as oxidative damage and alterations in gene expression.

Regardless of the strong evidence provided by literature, some drawbacks on the use of this model species can limit its robustness, such as lack of standardized protocols and experimental designs far from realistic conditions. For this reason, a specific protocol should be created for the use of ZET in nanotoxicity assessment, in which modifications linked to specific NMs physicochemical properties need to be included. In addition, studies are required in which the behavior of NMs under relevant environmental conditions are considered, as to understand and link their transformation in the environment to the observed impacts in organisms. A range of high-throughput techniques can also be used to complement the ZET assay and help to further understand the potential toxicity of NMs and uncover nano-specific MoA in zebrafish embryos. For this effect, various advanced technologies (e.g. OMIC techniques) and zebrafish transgenic lines are available nowadays for nanotoxicity evaluation, which have the possibility of uncovering the still debated nanomaterial toxicity, as well as discover new biomarkers. In addition, techniques that provide dynamic imaging of embryos, including NMs distribution *in vivo*, could also provide additional information in terms of absorption, distribution, metabolism and excretion (toxicokinetics) of different NMs inside embryos. In this sense, the ZET in combination with high-content approaches can be expected to continue to contribute to novel insights into the toxicity of NMs and improve the protection of human and environmental health. To summarize, there are several key aspects that need to be considered when evaluating the toxicity of NMs using the ZET:

a) Interlaboratory comparation of studies for standardization and validation of protocols;
b) Transformation and fate of NMs in complex environment matrices;
c) Assessment of NMs toxicity in more environmentally relevant conditions;
d) Interactive effects of NMs with other contaminants (Trojan horse effect);
e) Interaction of NMs with biological fluids (i.e., protein corona formation);
f) Toxicokinetics of NMs on zebrafish embryo and larvae and the role of chorion on nanotoxicity;

g) Development of new methods for characterization of the interaction and cell/tissue distribution of NMs on zebrafish embryo and larvae;

h) Embryotoxicity induced by multigenerational exposure to NMs;

i) Assessment of the toxicity of organic NMs, NMs in consumer products and their lifecycle transformation products/transformations, as well new classes of NM as hybrid NMs;

j) Encourage the use of the ZET for the registration, evaluation, classification of NMs.

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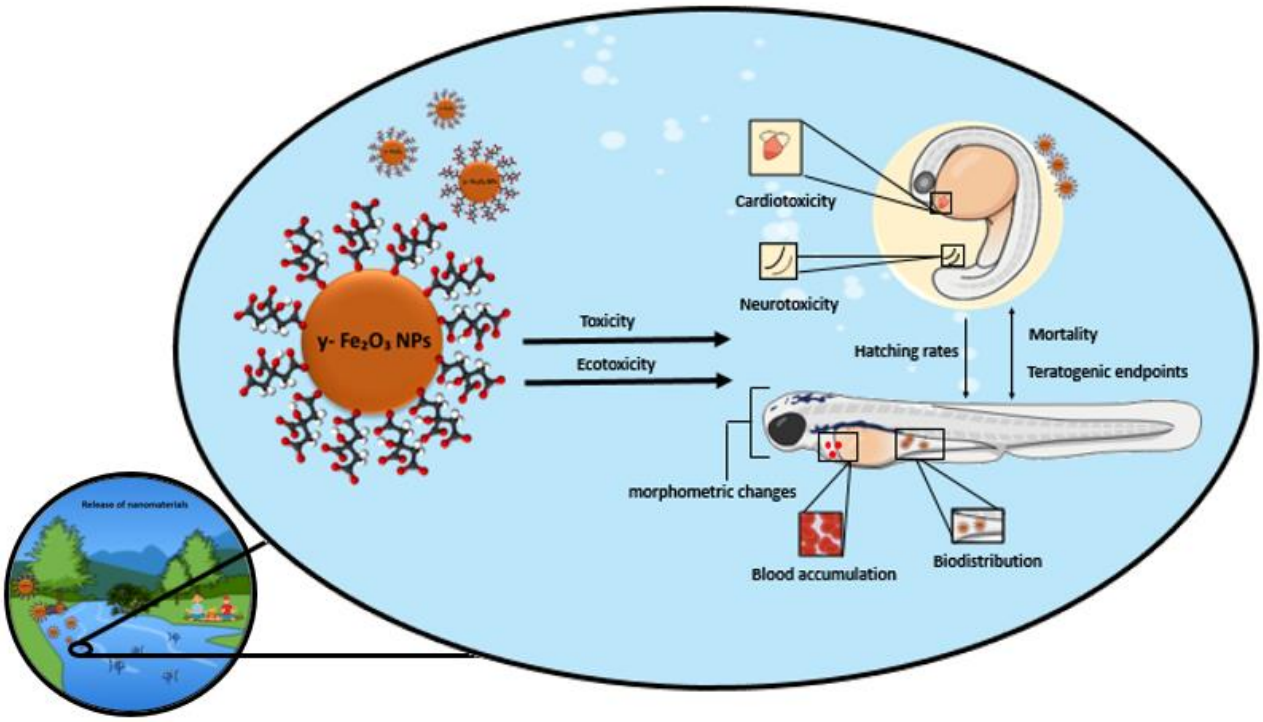
CAPÍTULO III

Artigo 2

Comparative developmental toxicity of iron oxide nanoparticles (γ-Fe_2O_3) and ferric chloride to zebrafish (*Danio rerio*) after static and semi-static exposure



Highlights

- Differential developmental toxicity of γ-Fe_2O_3 NPs and iron ions in zebrafish.
- Embryotoxicity of NPs depends on the condition type (static and semi-static).
- Semi-static exposure induced high embryotoxicity than static exposure.
- γ-Fe_2O_3 NPs induced several cardiotoxic effects on zebrafish embryos.
- Zebrafish embryotoxicity test (ZET) in the nanotoxicology assessment.

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Comparative developmental toxicity of iron oxide nanoparticles (γ-Fe_2O_3) and ferric chloride to zebrafish (*Danio rerio*) after static and semi-static exposure

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ABSTRACT

Iron oxide nanoparticles (IONPs) are used in several medical and environmental applications, but their mechanism of action and hazardous effects to early developmental stages of fish remain unknown. Thus, the present study aimed to assess the developmental toxicity of citrate-functionalized IONPs (γ-Fe_2O_3 NPs), in comparison with its dissolved counterpart, in zebrafish (*Danio rerio*) after static and semi-static exposure. Embryos were exposed to environmental concentrations of both iron forms (0.3, 0.6, 1.25, 2.5, 5 and 10 mg L^{-1}) during 144 h, jointly with negative control group. The interaction and distribution of both Fe forms on the external chorion and larvae surface were measured, following by multiple biomarker assessment (mortality, hatching rate, neurotoxicity, cardiotoxicity, morphological alterations and 12 morphometrics parameters). Results showed that IONPs were mainly accumulated on the zebrafish chorion, and in the digestive system and liver of the larvae. Although the IONPs induced low embryotoxicity compared to iron ions in both exposure conditions, these nanomaterials induced sublethal effects, mainly cardiotoxic effects (reduced heartbeat, blood accumulation in the heart and pericardial edema). The semi-static exposure to both iron forms induced high embryotoxicity compared to static exposure, indicating that the nanotoxicity to early developmental stages of fish depends on the exposure system. This is the first study concerning the role of the exposure condition on the developmental toxicity of IONPs on fish species.

Keywords: nanoecotoxicology, nanomaterials, cardiotoxicity, biomarkers, zebrafish embryotoxicity test (ZET).

1. Introduction

Nanomaterials (NMs) have been produced and incorporated in commercial products on a large scale, which consequently increases their release into the aquatic environment (Giese et al., 2018). These emerging pollutants have nano-specific properties that can induced hazardous effects to the environment and human health (Zoo and Zhao, 2017). Among these NMs, iron oxide nanoparticles (IONPs) have been used in several medical and environmental applications, such as cancer treatment by hyperthermia (Giustini et al., 2010), contrast agents (Fatima and Kim, 2018), drug delivery (Laurent et al., 2014), nanoremediation (Hjorth et al., 2017) and water treatment (Xu et al., 2012). The main types of IONPs with biological and environmental applications are magnetite (Fe_3O_4 NPs), hematite (α-Fe_2O_3 NPs) and maghemite (γ-Fe_2O_3 NPs) (Su, 2016), especially due to their physicochemical properties, such as high surface area, small size, optimum crystallization, magnetism, adsorption capacity and biocompatibility (Xu et al., 2012; Vikram et al., 2017; Babic-Stojic et al., 2018).

Despite the numerous advantages of IONPs, previous studies have indicated its environmental transformation and hazardous impact to aquatic organisms and human health (Zoo and Zhao, 2017; Qualhato et al., 2017, 2018). After their release into the aquatic environment, IONPs can undergo physical, chemical and biological transformations, including homoaggregation, heteroaggregation with natural organic matter (NOM), oxidative dissolution, aging through oxidation-reduction reactions, bio-reduction and adsorption with other pollutants (Trojan horse effect) (Moore, 2006; Patil et al., 2016; Lei et al., 2018). Regarding its potential ecotoxicological impact, the environmental concentration (0.3 mg L^{-1}) of citrate-functionalized γ-Fe_2O_3 NPs (3.97 nm) induced genotoxic (DNA damage) and mutagenic effects (micronucleus and erythrocytic nuclear alterations), and hepatic alterations (i.e., micro and macrovesicular steatosis, melanomacrophage aggregates, exudate and hemorrhagic foci) in guppy (*Poecilia reticulata*) during 21 days of exposure (Qualhato et al., 2017, 2018). In addition, the exposure of zebrafish embryos to uncoated γ-Fe_2O_3 NPs (30 nm; 0.1 - 100 mg L^{-1}) for 168 h induced pericardial edema, tissue ulceration and spinal curvature (Zhu et al., 2012). The IONP toxicity to fish also depends on the synthesis method. IONPs obtained by coprecipitation (15.58 nm; 79.04 and 278.67 ppm) induced bioaccumulation and increased erythrocyte damage in adult zebrafish during 96 h of exposure, while those

obtained by the green synthesis method (21.34 nm; 79.04 and 278.67 ppm) inhibited the Na^+/K^+ -ATPase activity in a concentration dependent pattern (Suganya et al., 2018). On the other hand, the role of the exposure condition on the embryotoxicity of IONPs on fish species remain unknow.

The zebrafish embryotoxicity test (ZET) has been indicated as a suitable approach to assess the NM toxicity on human and environmental health (Pereira et al., 2019), as well as a preclinical *in vivo* screening model for nanomedicines (Siebel et al., 2019). According to the guideline nº 236 of the Organization for Economic Cooperation and Development (OECD) (OECD, 2013), the semi-static exposure system is recommended for volatile substance and materials with high precipitation rate. On the other hand, few studies perform medium exchange during ZET for nanotoxicity assessment (Pereira et al., 2019). Thus, the lack of standardization of tests may cause interference with the toxicity results of NMs (Lammer et al., 2009; Beekhuijen et al., 2015; Truong et al., 2011).

Zebrafish (*D. rerio*) has been an excellent animal model for the analysis of multiple biomarkers of NM toxicity (Fako and Furgeson, 2009; Harper et al., 2011; Lin et al., 2013; Bugel et al., 2014; Chakraborty et al., 2016; Haque et al., 2018; Pereira et al., 2019), especially due to its numerous advantages, such as easy maintenance in the laboratory conditions, small size, high egg production, embryos are transparent which allows to follow their whole development, and fast development when compared to other vertebrate models (Lammer et al., 2009; Giannaccini et al., 2014; Beekhuijen et.al., 2015; Sobanska et al., 2018). Information networks such as ZFIN (zfin.org), combined with the fact that embryos can be cryopreserved, ensure quick and easy access to transgenic zebrafish lines (Sieber et al., 2019). In addition, its genetic material is 70 % similar to humans (Howe et al., 2013), ensuring a similarity between developmental processes, cell signalling, cell structures, anatomy and physiology (Rubinstein, 2003; Hill et al., 2005; Kelkar et al., 2014).

In this context, the present study aimed to assess the developmental toxicity of citrate-functionalized γ-Fe_2O_3 NPs and its dissolved counterpart on zebrafish, using two aquatic exposure systems (static and semi-static exposure). The ZET was performed during 144 h and the interaction and distribution of both Fe forms on the external chorion (embryo) and larvae surface were measured, following by multiple biomarker assessment: mortality rate, hatching rate, neurotoxicity (spontaneous movement rate),

cardiotoxicity (heart beats rate), teratogenesis (morphological alterations), following by measured of 12 morphometrics parameters. To the best of our knowledge, the present study is the first report on the role of the exposure condition (static and semi-static aquatic systems) on toxicity of IONPs during the early developmental stage of fish. Assumingly, studies such as the present one broadens the possibility of using the ZET on analysis and classification of the toxicity and environmental impact of NMs.

2. Materials and methods

2.1 Synthesis and characterization of IONPs

Citrate-functionalized maghemite (γ-Fe_2O_3 NPs) were synthesized according to Unal et al. (2010) and Zia et al. (2016) with some modifications, such as described by Qualhato et al. (2017). After synthesis, NPs were characterized in terms of morphology and individual diameter (D_{TEM}) by Transmission Electron Microscopy (TEM), while the hydrodynamic diameter (D_h) and surface charge (zeta potential) were analyzed by dynamic light scattering (DLS) and electrophoretic light scattering (ELS), respectively. The IONPs were also characterized by X-ray diffraction (XDR), KBr tablet infrared spectroscopy (IR-KBR) and ultraviolet-visible (UV-vis) spectroscopy, as previously described by Qualhato et al. (2017, 2018).

2.2. Zebrafish

Adult wild-type zebrafish with 6 - 24 months (total length = 3 ± 5 cm) were obtained and maintained in the zebrafish facilities of the Institute of Tropical Pathology and Public Health (IPTSP) from Federal University of Goiás (UFG), using in polycarbonate aquariums (11.5 cm x 34.5 cm x 15.5 cm) (Alesco®) with constant water circulation (3 L), under controlled conditions of water temperature (26 ± 1 ° C), pH (7.0 ± 0.5), dissolved oxygen (> 80 %), and photoperiod (light: dark cycle 14:10 h), such as recommended by Dammski et al. (2011). Animals were fed daily with commercial food (Cardume® 36 %; VB alimentos) and *Artemia salina* nauplii. The maintenance of adult animals and the execution of ZET followed the regulations of OECD Guide 236, (OECD,

2013) and were approved by the Institutional Committee of Animal Use and Care from UFG (nº 094/17).

2.3. Zebrafish embryotoxicity test (ZET)

The ZET was performed according to OECD Guideline nº 236 (OECD, 2013), Lammer et al. (2009) and Pereira et al. (2019). After reproduction induction in breeding tanks (Alesco®) using males (n = 12) and females (n = 4) at rate of 3:1, embryos were transferred to a Petri dish, washed with reconstituted water (ISO 1996) and the viable embryos (up to 4 hours post-fertilization – hpf; 4 to 128-cell stage) were selected using photomicroscope (Leica DM 750) according to Kimmel. et al. (1995) and Lammer et al. (2009).

For the bioassays, zebrafish embryos were distributed in 24-well microplates (Kasvi®), using 1 embryo *per* well containing 2 mL of each concentration of citrate functionalized γ-Fe_2O_3 NPs or ferric chloride (0.31, 0.62, 1.25, 2.5, 5.0 and 10 mg L^{-1}), jointly with the negative control group (reconstituted water) (Fig. 1). The iron concentrations used are environmentally relevant and are below the maximum allowable concentration in the aquatic environment (15 mg L^{-1}) according to CONAMA Resolution No 39/2008 (Brasil, 2008). The average predicted environmental concentration of IONPs found in surface water was 28 mg L^{-1} (Wang et al., 2016). The exposure was conducted in triplicate (3 microplates with 10 embryos *per* group; totalizing 30 embryos *per* experimental condition) using static (without exposure solution renewal) and semi-static conditions (exposure solution renewed every 24 h with redosing of concentrations of both iron forms) during 144 h under controlled photoperiod (14:10 h light/dark cycles) and temperature (27 ± 1 °C) in BioOxygen Demand (BOD) incubator (SOLAB SL-224/120, Brazil). For the test validation, the fertilization rate > 90 % and the negative control group survival > 90 % were considered (ISO, 1996, 2007; OECD, 2013; Lammer et al., 2009).

2.4. Interaction of IONPs to embryo chorion and larvae surface

The zebrafish embryos (n = 4) and larvae (n = 4) from control group and after exposure γ-Fe_2O_3 NPs (1.25 e 10 mg L^{-1}) and ferric chloride (1.25 mg L^{-1}) for 48 and 144 h of exposure, respectively, were immediately fixed by immersion in 10 % paraformaldehyde for 4 h, washed in 0.2 M PBS buffer at pH 7.2, dehydrated through increasing ethanol gradient (70 % and 100 %) and dried with liquid carbon dioxide (CO_2) in critical-point drier (Autosamdri® 815 A). Dried materials were sputtered-coated with gold in a Denton Vacuum Sputter Coater (Denton Vacuum, LLC, Moorestown, NJ, USA), and analyzed using a scanning electron microscope (SEM) (Jeol JSM-6610) coupled with energy dispersive X-ray spectroscopy (EDS) (Thermo scientific NSS Spectral Imaging).

2.5. Accumulation

After the exposure period (144 h) to citrate-functionalized γ-Fe_2O_3 NPs or ferric chloride, zebrafish larvae (n = 10 *per* experimental group) were fixed by immersion in 4 % buffered paraformaldehyde at pH 7.2 for 4 h, washed with 0.2 M PBS buffer at pH 7.2 and stored in 70 % ethanol at 4 ºC. Photographs of the lateral region of zebrafish larvae (0.31, 0.62, 1.25, 2.5, 5.0 and 10 mg L^{-1}) at 500× magnification were taken in the photomicroscope (Leica DM 750) coupled to the image capture system (Leica Microsystem LAS EZ). The accumulation area was determined using Image J software according to following equation: accumulation area = iron accumulation area in the intestinal system/ total area of the intestinal system of larvae.

2.6. Biomarker responses

The experimental design and multiple biomarker assessment during the ZET are in Figure 1. During the exposure period, embryos were analyzed daily (24, 48, 72, 96, 120 and 144 h) using a photomicroscope (Leica DM 750) associated with the LEICA ICC50 HD camera and LAS EZ software. A battery of biomarkers was analyzed, such as mortality and hatching rate (24 to 144 h), spontaneous movement rate (24 h), heart beat rate (48 h), morphological alterations frequency (24 to 144 h) and biometric parameters (144 h).

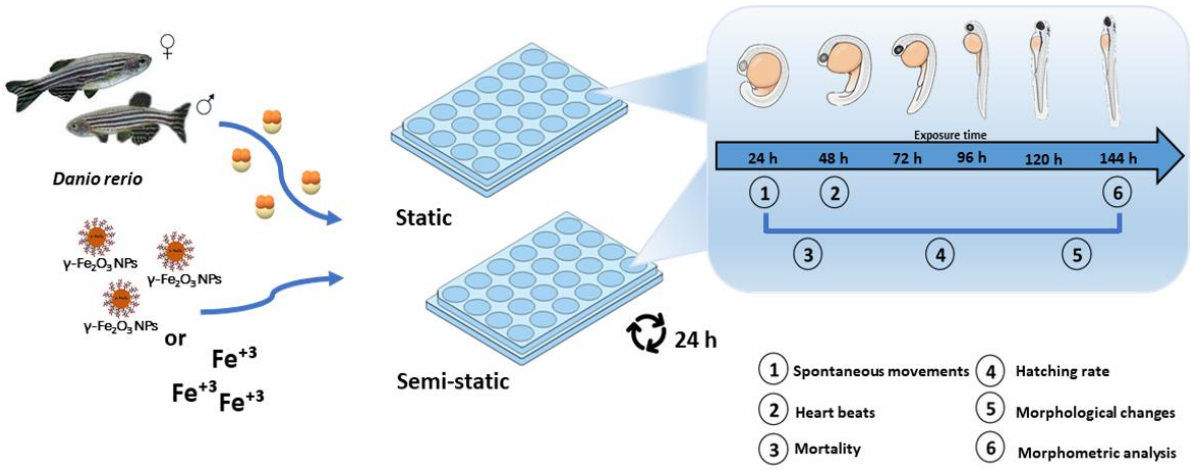

Figure 1. Experimental design and analysis of multiple biomarkers during the zebrafish embryotoxicity test (ZET). The developmental toxicity of citrate-functionalized γ-Fe_2O_3 NPs and iron ions was analyzed during 144 h under static (without exposure medium renewal) and semi-static conditions (exposure medium renewed every 24 h with redosing of concentrations of both iron forms).

The zebrafish embryos and larvae with no spontaneous movements, heartbeat or coagulated were as considered dead (Lammer et al., 2009). The mortality rate (%) was determined using the following equation: mortality rate (%) = (number of dead embryos / total number of embryos) *x* 100. The hatching rate (%) was calculated through the following equation: hatching rate (%) = (number of hatched embryos / total number of embryos) *x* 100. The morphological alterations were measured by determining the frequency of embryos or larvae with blood accumulation in the heart, deformation of the yolk sac, edema of the pericardium, spinal curvature, swimming bladder deformity and tail malformation, according to Lammer et al. (2009) and Beekhuijzen et al. (2015). The total morphological alteration frequency is the sum of all morphological changes. In addition, these morphological alterations were classified in to four reaction pattern (Rp): circulatory changes (Rp_1), pigmentation and integumentary changes (Rp_2), musculoskeletal disorders (Rp_3) and yolk sac alterations (Rp_4), as described by Pereira et al. (2019).

2.6.1. Neurotoxicity and cardiotoxicity

The neurotoxicity was determined through the rate of spontaneous movements *per* minute (n^o min^{-1}) in embryos (26 – 28 hpf) after 24 h of exposure (Babic et al., 2017). The cardiotoxicity was determined through the heart beat rate *per* minute (bpm) in embryos (50 – 52 hpf) after 48 h of exposure (Beekhuijzen et al., 2015). Both parameters

were analyzed in 30 embryos *per* experimental group using a photomicroscope (Leica DM 750) coupled to an image capture system (Leica Microsystem LAS EZ).

2.6.2. Morphometric analysis

At the end of the exposure period (144 h), the larvae were fixed by immersion in 4 % paraformaldehyde pH 7.2 for 4 hours, washed in 0.2 M PBS buffer pH 7.2 and stored in 70 % alcohol at 4 °C. Afterwards photographs of the lateral and dorsal region of zebrafish larvae (n = 10 *per* experimental group) were obtained using the photomicroscope (Leica DM 750) coupled to the image capture system (Leica Microsystem LAS EZ). Four categories of morphometric parameters (n = 12) were measured in the larvae using the Image J software, accordingly Malafaia et al. (2020): (*i*) sensory (eye area – AO, minimum interocular distance – DI and maximum interocular distance – DIM); (*ii*) physiological (heart area – AC, swimming bladder area – AB, and vitelline sac area – AV); (*iii*) structural / skeletal (head height – CA, head width – CL, head depth – CP, distance from mouth to anus – DAB); (*iv*) structural / muscular (angle between myosepts and distance between myosepts) (Fig. 2).

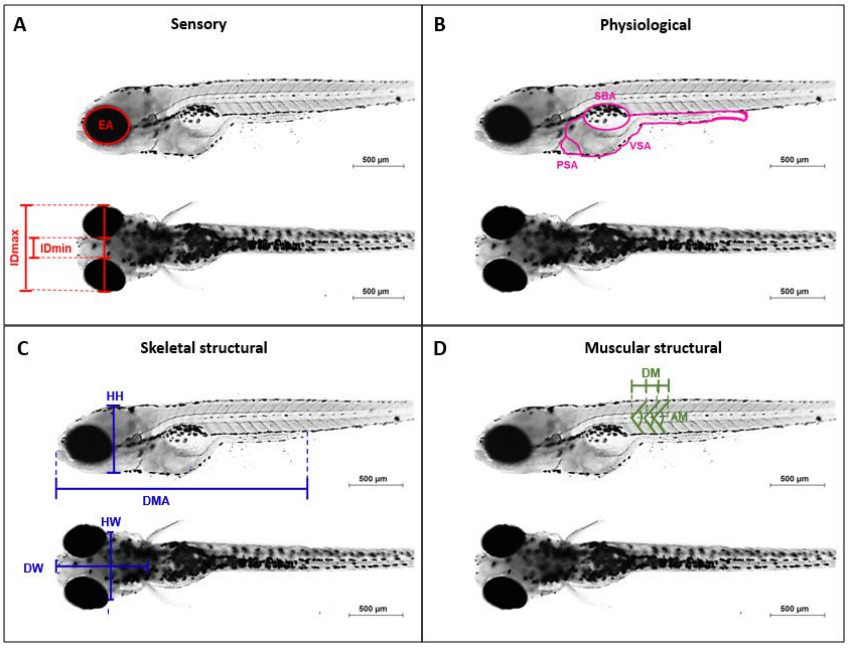

Figure 2. Morphometric parameters of zebrafish larvae measured after 144 h of exposure to citrate-functionalized γ-Fe_2O_3 NPs and iron ions. Theses parameters were classified in sensory (A), physiological (B), skeletal structural (C) and muscular structural parameters (D). EA: eye area (µm^2); SBA: swimming bladder area (µm^2); VSA: vitelline sac area (µm^2); PSA: pericardial sac area (µm^2); AM: angle between the myoseptides (degrees); HH: head height (µm); HW: head width (µm); DH: depth of the head (µm); DM: distance between myoseptides on the notochord line (µm); DMA: distance from the mouth to the anus (µm); IDmin: minimum interocular distance (µm); IDmax: maximum interocular distance (µm). (A – D) Representative photomicrographs of zebrafish larvae under lateral and dorsal view. Scale bar = 500 µm.

2.7. Statistical analysis

The graphs were organized using the Prism 7 GraphPad® software, while the statistical analyzes were performed using the R software. Normality and homoscedasticity were verified with the Shapiro-Wilk and Levene tests, respectively. Heart beat rate, spontaneous movement rate and morphometric parameters were analyzed by one-way ANOVA, following by Tukey post hoc tests. Mortality rate, hatching rate and teratogenic effects were analyzed using multiple factor ANOVA (Underwood, 1997). Results were considered when $p < 0.05$.

2. Results and discussion

3.1. IONP characterization

IONPs used in the present study were previously characterized by Qualhato et al. (2017, 2018). TEM results showed the citrate-functionalized γ-Fe_2O_3NPs with rounded shape, excellent crystallinity and a D_{TEM} of 3.97 ± 0.85 nm. The FTIR spectrum demonstrated two bands at 1594 cm^{-1} and 1380 cm^{-1}, confirming the functionalization of γ-Fe_2O_3 NPs by citrate. The citrate confers negative surface charge to IONPs in reconstituted water (-19.5 ± 6.5 mV) and Milli-Q water (-51.1 ± 7 mV). Higher hydrodynamic diameter was observed in reconstituted water (21.4 ± 0.39 nm) compared to Milli-Q water (14.11 ± 0.2 nm). Atomic absorption spectrophotometric analysis showed that the total iron content in the γ-Fe_2O_3 NPs stock dispersion was 6.8 mg mL^{-1}. The XRD analysis showed two broad picks (311 and 440) with low intensity, indicating cubic spinel phase and small particles (Teja et al., 2009; Wu et al., 2015). In addition, the XRD pattern along with the UV-Vis-Near IR allowed to characterize maghemite-type NPs. The UV-Vis-Near IR of the γ-Fe_2O_3 NPs suspension showed the characteristic absorption near the wavelength region attributed to the presence of Fe (III) ions in a tetrahedral site, confirming the transformation of magnetite to maghemite NPs by oxidation. Overall results showed that the zebrafish embryos and larvae were exposed to aggregates of maghemite with negative surface charge, such as reported in previous studies (Zhu et al., 2012).

3.2. Nanobiointeraction on embryo and larvae surface

SEM followed by EDS analysis showed the presence of γ-Fe_2O_3 NPs and iron adhered to the embryo chorion after 48 h of exposure (Fig. 3). Results indicated that the IONP interaction with the zebrafish embryo chorion may be mediated by three processes: (*i*) binding to the chorion surface; (*ii*) binding and blocking of chorion pore canals; (*iii*) passing through the chorion pore canals (Fig. 3). Glycoproteins and carbohydrates present on the chorion surface may interact with IONPs, promoting their adhesion to the embryo surface, as well as reducing their capture and toxicity, such as observed with SiO_2 NPs (Chao et al., 2018), polystyrene NPs (Pomeren et al., 2017; Lee et al., 2019), and fluorescent silica NPs (Fent et al., 2010). However, further studies are needed on the mechanism of interaction of these molecules with NMs.

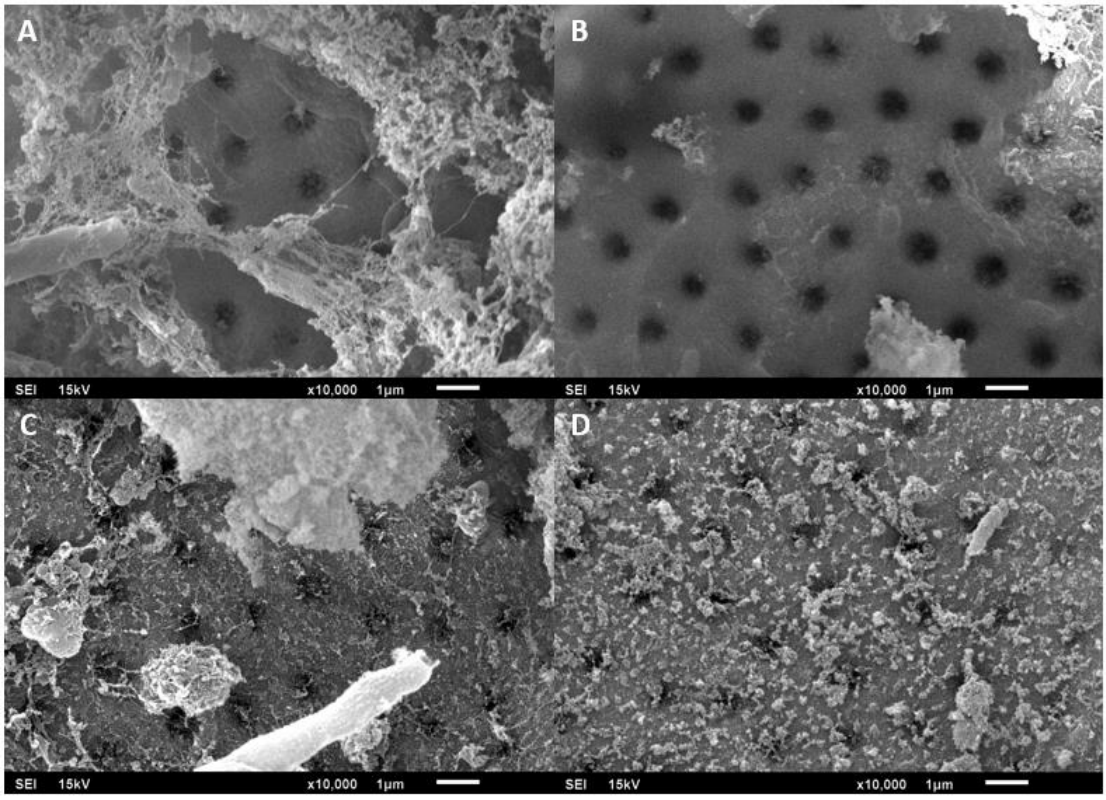

Figure 3. Scanning Electron Microscopy (SEM) of zebrafish embryo chorion from control group and after exposure to γ-Fe_2O_3 nanoparticle (IONPs) and iron ions for 48 h. (A) Control. (B) Iron ions at 1.25 mg L^{-1}. (C) IONP at 1.25 mg L^{-1}. (D) IONP at 10 mg L^{-1}. Scale bar = 1 µm.

Although the zebrafish chorion has been indicated as a selective barrier to the entry of NMs into zebrafish embryos (Cheng et al., 2007; Pomeren et al., 2017), the citrate-functionalized IONP aggregates have small diameter in reconstituted water (21.4 ± 0.39 nm) when compared to chorion pore size (500 - 700 nm) (Rawson et al., 2001), confirming that the γ-Fe_2O_3 NPs, being small in size compared to other NMs, are easier

to cross the chorion barrier (Cheng et al., 2007). Furthermore, the adherence and blocking of chorion pore canals by particles can reduce the gas exchange of the embryos, leading to pre-hatching hypoxia changes (Silva et al., 2018; Malafaia et al., 2019). In addition, high IONP aggregates adhered to the zebrafish chorion was observed after exposure to γ-Fe_2O_3 NPs at high concentration (10 mg L^{-1}) (67 %) compared to low concentration (1.25 mg L^{-1}) (20 %), indicating that the adherence was concentration dependent (Fig. 4).

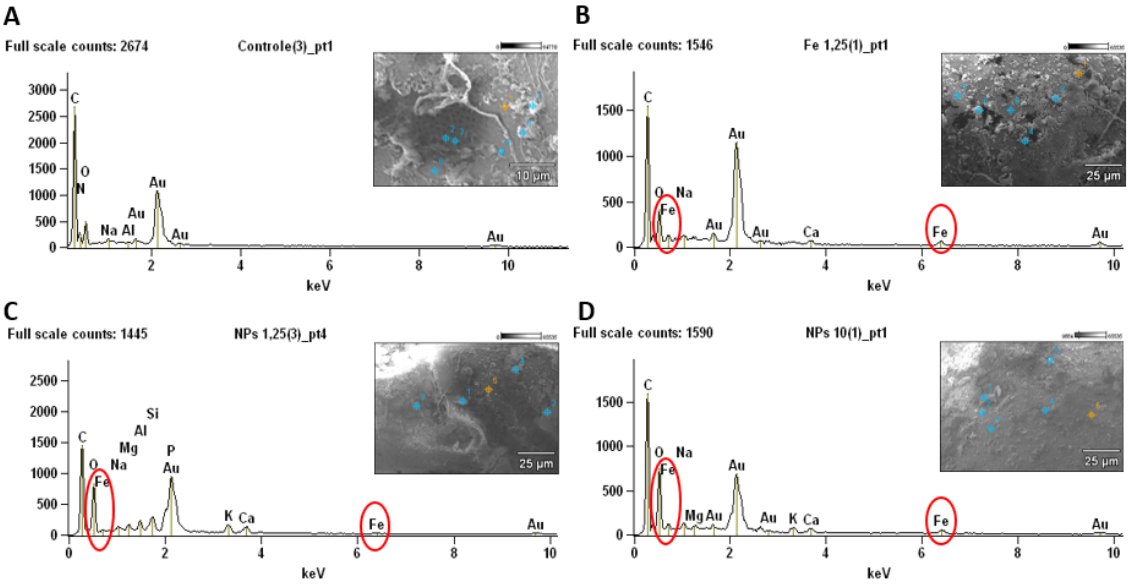

Figure 4. Energy Dispersive X-Ray Spectroscopy (EDS) and Scanning Electron Microscopy (SEM) of zebrafish embryos chorion from control group and after exposure to γ-Fe_2O_3 nanoparticle (IONPs) and iron ions for 48 h. (A) Control. (B) Iron ions at 1.25 mg L^{-1}. (C) IONP at 1.25 mg L^{-1}. (D) IONP at 10 mg L^{-1}. Scale bar = 25 µm.

After hatching (48 – 72 hpf), the zebrafish larvae can interact and capture IONPs through the digestive system and gills. In addition, adherence of IONPs with skin and eyes of the larvae was also confirmed by SEM followed by EDS (Fig. 5). Larvae exposed to γ-Fe_2O_3 NPs and ferric chloride for 144 h showed iron aggregates in the eye, heart, intestinal tract and tail regions. The zebrafish larvae exposed to γ-Fe_2O_3 NPs at 1.25 mg L^{-1} showed NP aggregates adhered to eye (40 %), tail (40 %), heart (20 %) and abdomen (10 %), while those exposed to γ-Fe_2O_3 NPs at 10 mg L^{-1} showed NP aggregates mainly deposited in the heart (20 %), eyes (10 %), abdomen (10 %) and tail (10 %) (Fig. 5). Similarly, the larvae exposed to iron ions at 1.25 mg L^{-1} showed iron mainly deposited in the eye (50 %), abdomen (40 %), heart (30 %) and tail (20 %) (Fig. 5), confirming the interaction and adhesion of both iron forms on the larvae surface.

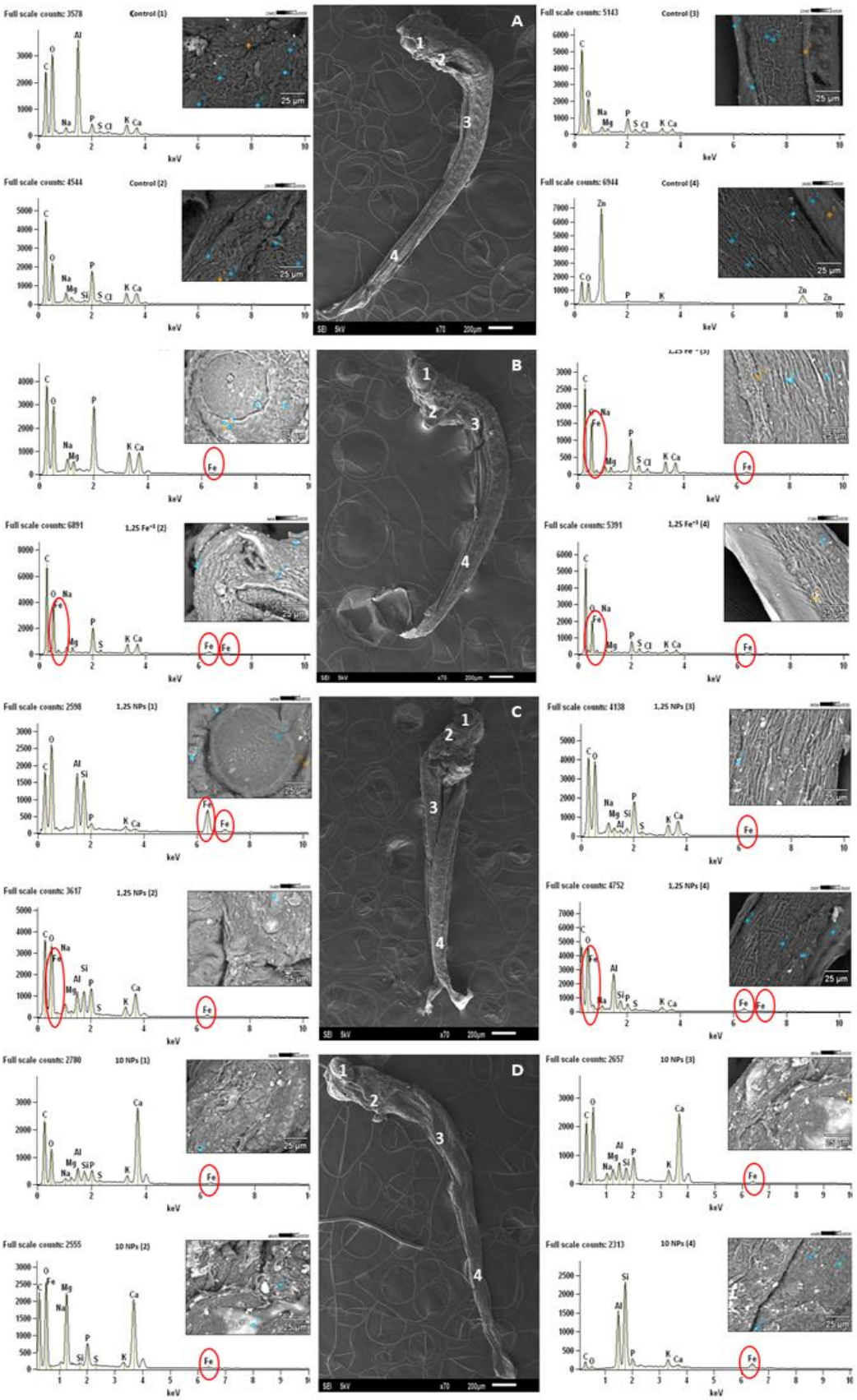

Figure 5. Scanning Electron Microscopy (SEM) and Energy Dispersive X-Ray Spectroscopy (EDS) of zebrafish larvae from control group and after exposure to γ-Fe_2O_3 nanoparticle (IONPs) and iron ions for 144 h. (A) Control. (B) Iron ions at 1.25 mg L^{-1}. (C) IONP at 1.25 mg L^{-1}. (D) IONP at 10 mg L^{-1}. Scale bar = 200 µm.

3.3 Accumulation

Both iron forms were accumulated mainly in the intestinal tract of zebrafish larvae in a concentration dependent pattern (Fig. 6). Larvae exposed to γ-Fe_2O_3 NPs at 10 mg L^{-1} showed higher accumulation area when compared to control group and other IONP and iron ion concentrations (Fig. 6G). Similarly, previous studies have reported accumulation of several NPs in the zebrafish intestinal tract, such as Ag NPs (Cambier et al., 2018), quantum dots (Cheng et al., 2017), PS NPs (Pitt et al., 2018) and fullerenes (Della Torre et al., 2018). The IONP accumulation in the intestinal tract was also reported for invertebrate species, such as *Ceriodaphnia dubia* (Hu et al., 2012) and *Daphnia magna* (Magro et al., 2018).

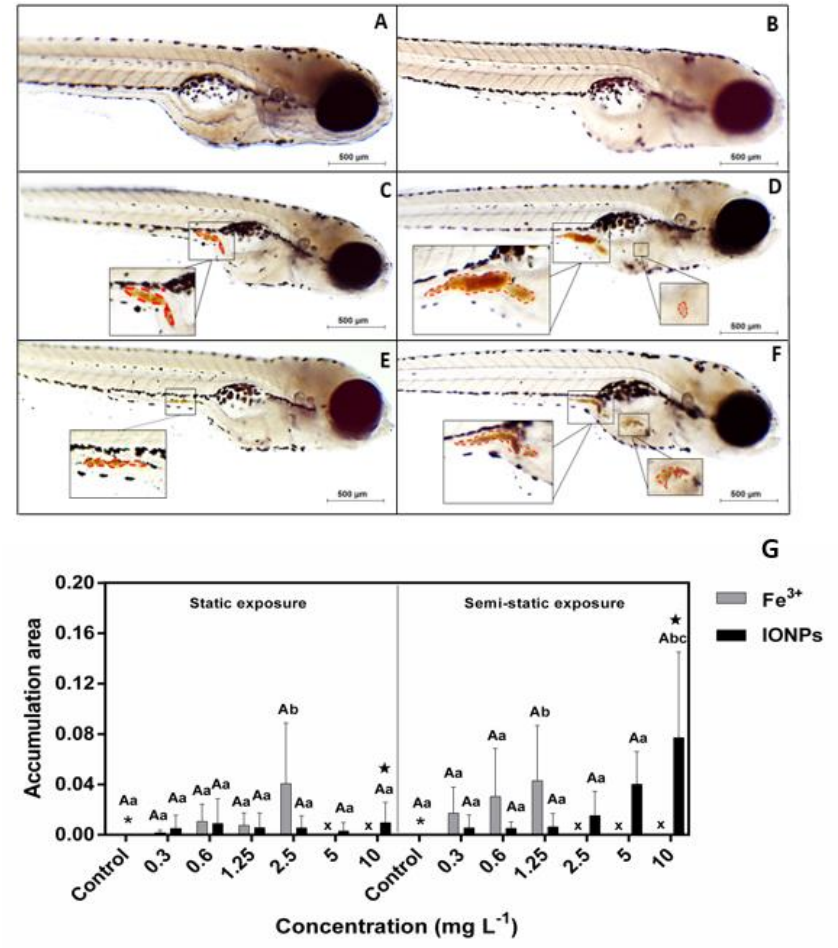

Figure 6. Photomicrographs of zebrafish larvae after exposure to γ-Fe_2O_3 nanoparticle (IONPs) and iron ions under static and semi-static conditions for 144 h, showing presence of both iron forms in the gastrointestinal tract. (A – B) Control. (C) Iron ion at 1.25 mg L^{-1} under static condition. (D) IONP at 10 mg L^{-1} under static exposure. (E) Iron ions at 1.25 mg L^{-1} under semi-static exposure. (F) IONP at 10 mg L^{-1} under semi-static exposure. Scale bar = 500 µm. (G) Accumulation area in the gastrointestinal tract in zebrafish larvae after 144 h exposure to γ-Fe_2O_3 NPs and iron ions. (*) No accumulation; (★) Difference between static and semi-static exposure. Capital letters indicate significant differences between NPs and ferrous chloride at the same concentration and different lower letters indicate significant differences in the same form of iron across concentrations. Results are expressed as mean from 10 larvae.

The intestinal tract has digestive, absorptive, secretory and protective functions (Bergin and Witzmann, 2013; Cheng et al., 2016). After ingestion, NPs can interact with the mucosa of the intestinal tract, as well as be absorbed, reach the bloodstream, and distribute to other organs. NPs can induce changes in physiological, metabolic and immunological functions (Bergin and Witzmann, 2013; Ates et al., 2013). As for example, TiO_2 NPs induced physiological changes in zebrafish larvae (Yang et al., 2017), CuO NPs promoted alterations in the zebrafish immune system (Aksakal and Ciltas., 2019) and polystyrene NPs caused metabolic changes in larvae of zebrafish (Brun et al., 2019).

After distribution to other organs, such as the liver and spleen, NOFs are captured by the mononuclear phagocytic system via endocytosis by Kupffer cells and spleen macrophages. After phagocytosis, NOFs can be degraded by Kupffer cell lysosomes and spleen macrophages, releasing iron ions that interfere with iron homeostasis (Patil et al., 2015). Iron ions released by NOF degradation can participate in the Fenton Reactions and produce hydroxyl radicals, which are extremely toxic to cells, causing oxidative stress, inducing damage to cell organelles, altering their cell functions, and causing cell death. Normally free iron ions are stored in proteins, such as ferritin. However, iron overload exceeds the body's ability to store iron ions, and these free iron ions lead to the reactive oxygen species (ROS) production (Voinov et al., 2011; Yang et al., 2015). Iron overload primarily affects the heart, which is one of the major organs that IONPs have affinity, inducing myocardial injury, which can cause decreased heart rate, bleeding, and other effects on the heart (Parkes et al., 1993; Lekawanvijit and Chattipakorn, 2009; Bostan et al., 2016), such as observed in the present study.

3.4. Zebrafish embryotoxicity test - ZET

3.4.1. Mortality and hatching rate

The exposure of zebrafish embryos to γ-Fe_2O_3 NPs induced low mortality when compared to iron ions. After 144 h of exposure, the mortality rate of embryos exposed to γ-Fe_2O_3 NPs at 0.3, 0.6, 1.25, 2.5, 5 and 10 mg L^{-1} was similar to the control group ($p > 0.05$; Fig. 7). Similarly, low concentrations of iron ions (0.3, 0.6, 1.25 and 2.5 mg L^{-1}) also induced similar mortality to the control group ($p > 0.05$; Fig. 7). However, high

mortality was observed after exposure to iron ions at 5.0 and 10 mg L^{-1} compared to control group ($p < 0.05$; Fig. 7). In addition, the embryotoxicity of iron ions was time and concentration dependent, such as indicated by Zhu et al. (2012).

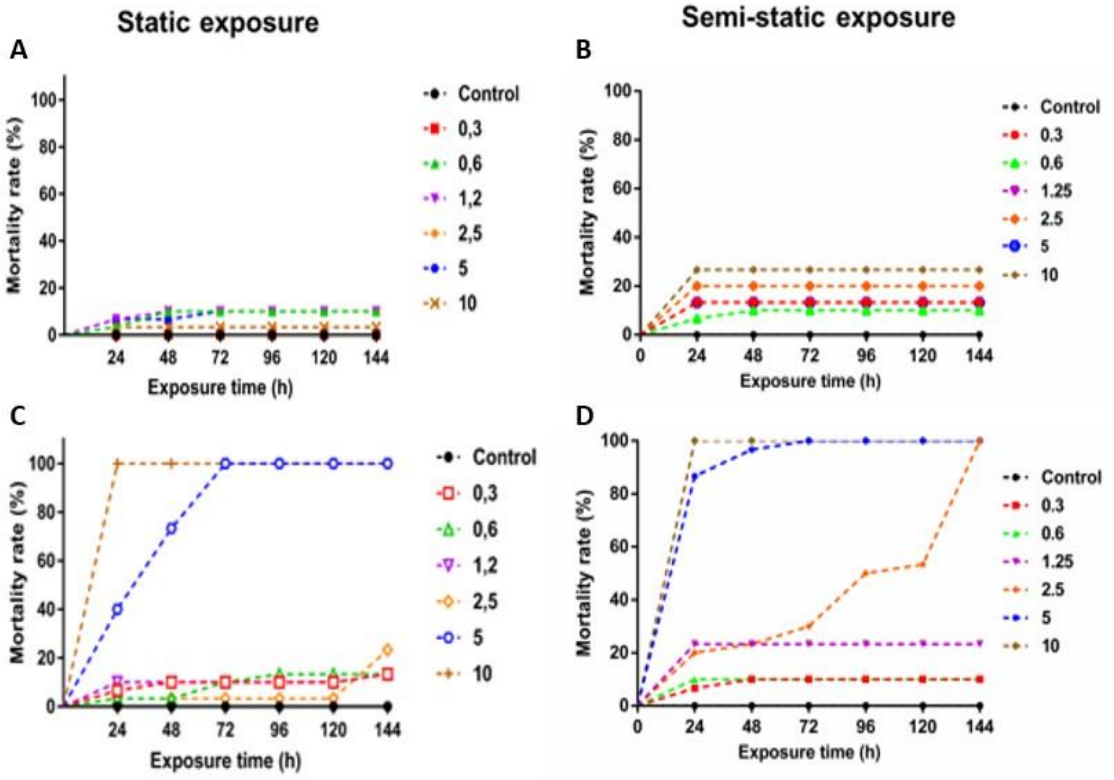

Figure 7. Mortality rate (%) of zebrafish embryos from control group and after exposure to γ-Fe_2O_3 nanoparticle (IONPs) and iron ions under static (A, C) and semi-static conditions (B, D) for 144 h. (A) Static exposure to IONPs. (B) Semi-static exposure to IONPs. (C) Static exposure to iron ions. (D) Semi-static exposure to iron ions. Results are expressed as mean of triplicates.

The present study demonstrated that the type of exposure also influences the nanotoxicity during the early developmental of fish species. OECD guideline nº 236 (OECD, 2013) does not specify medium renewal in NM exposures, indicating only that in cases of volatile or highly adsorbed substances, medium renewal should be performed. The results showed the importance of a specific guideline to perform tests with NMs, since the exposure conditions influence the embryotoxicity of NMs (Pereira et al., 2019).

The static and semi-static exposure to both iron forms did not influence the hatching rate of zebrafish embryos ($p > 0.05$; Fig. 8), indicating that γ-Fe_2O_3 NPs and iron ions did not alter the hatching mechanism of embryos. Similarly, no effect on hatching rate was observed on zebrafish exposed to Pt NPs (Asharani et al., 2011), CeO_2

NPs (Welmas et al., 2015), SnO_2 NPs (Welmas et al., 2015) and CdSe (Chen et al., 2017). In opposite, others metal-based NPs inhibited the zebrafish hatching, such as Ag NPs (Asharani et al., 2008; Powers et al., 2010; Massaesky et al., 2013; Orbea et al., 2017), SiO_2 NPs (Chao et al., 2017) and ZnO NPs (Ong et al., 2013; Chen et al., 2014; Hua et al., 2014; Zhao et al., 2014; Kteeba et al., 2017), confirming that the toxicity of NMs depends on their nano-specific properties (Cheng et al., 2007; Chao et al., 2018; Pereira et al., 2019).

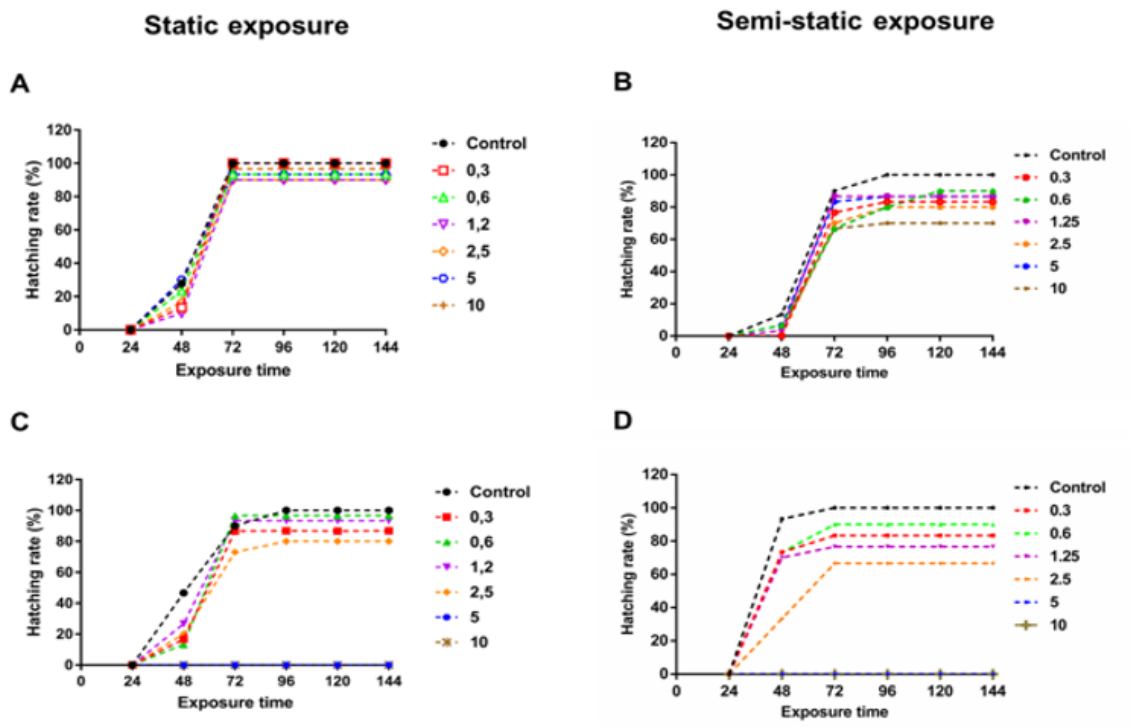

Figure 8. Hatching rate (%) of zebrafish from control group and after exposure to γ-Fe_2O_3 nanoparticle (IONPs) and iron ions under static (A, C) and semi-static conditions (B, D) for 144 h. (A) Static exposure to IONPs. (B) Semi-static exposure to IONPs. (C) Static exposure to iron ions. (D) Semi-static exposure to iron ions. Results are expressed as mean of triplicates.

The zebrafish chorion is a barrier that protects the embryo up to 48 - 72 hpf and has pores with a diameter ranging from 500 - 700 nm, which are important for the oxygen and nutrients transport, and embryo excretion (Rawson et al., 2011). However, pores may facilitate the entry of small NPs (Cheng et al., 2007). When diffused throughout the chorion, these NPs can accumulate and distribute in various embryo organs, causing toxic effects during embryonic development (Chao et al., 2018). The hatching rate in zebrafish involves the expression of specific genes that produce enzymes (i.e. Hgg1 (Cathepsin L, ctslb)) that degrade the chorion facilitating its disruption. NPs can interfere with the expression of the genes responsible for hatching, such as reported for Cu NPs (Zhang et

al., 2018), delaying the hatching of embryos (Zhang et al., 2018). Another condition that may interfere with embryo hatching rate is the accumulation of NPs around the chorion that would cause a hypoxic condition and make it difficult to excrete metabolites through the pores, leading to premature hatching of zebrafish embryos (Silva et al., 2018). Neuroactive substances can induce hypoactivity in zebrafish embryos, leading hatching inhibition, such as observed in embryos exposed to Si NPs (62.2 nm; 25 - 200 μg mL^{-1}, 96 h) (Duan et al., 2013).

3.4.2. Neurotoxicity

After 24 h of exposure, spontaneous movement rate of embryos exposed to IONPs in static and semi-static conditions was similar to the control group ($p > 0.05$; Fig. 9). However, the semi-static exposure to iron ions at 5.0 mg L^{-1} reduced the spontaneous movement rate when compared to low iron ions concentrations (0.3 and 0.6 mg L^{-1}) and control group ($p < 0.05$; Fig. 9). Changes in spontaneous embryo movements are indicators of neurotoxicity (Wang et al., 2013) and may be altered by expression of various genes involved in neural protein synthesis (Fan et al., 2010; Richendrfer and Creton, 2015) or induction of muscle alterations (Campos et al., 2015). However, no changes in the movement spontaneous rate were observed in the embryos exposed to IONPs, indicating that the γ-Fe_2O_3 NPs were no neurotoxic during the early developmental stages of the zebrafish.

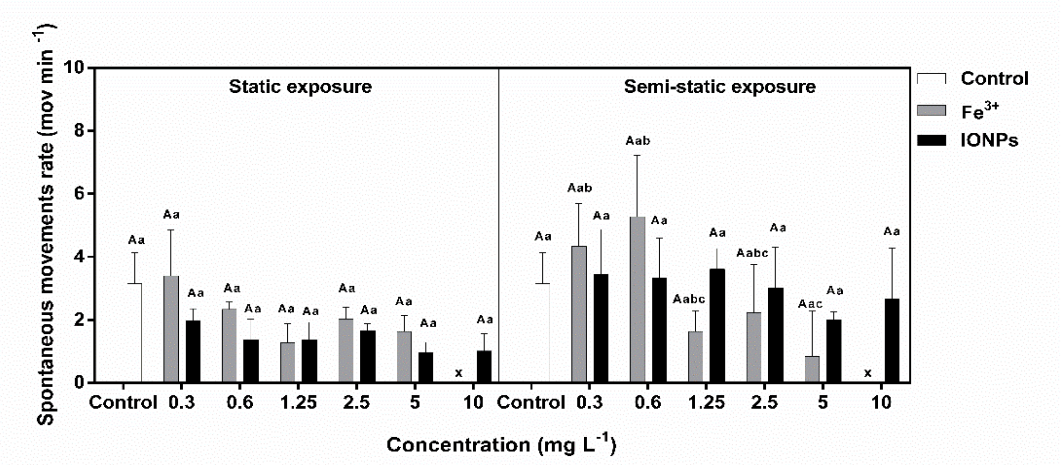

Figure 9. Spontaneous movements rate (mov min^{-1}) of zebrafish embryos from control group and after exposure to γ-Fe_2O_3 nanoparticle (IONPs) under static, iron ions under static, IONPs semi-static conditions and iron ions semi-static conditions for 24 h. "x" indicated mortality. Capital letters indicate significant differences between NPs and ferrous chloride at the same concentration and different lower letters indicate significant differences in the same form of iron across concentrations. Results are expressed as mean and standard deviation of triplicates.

Changes in spontaneous embryo movement are indicative of neurotoxicity (Wang et al., 2013) and were associated to alterations in the expression of various genes involved in neural protein synthesis (Fan et al., 2010; Richendrfer and Creton, 2015) or induction of muscle changes (Campos et al., 2015). Although the neurotoxic effects induced by IONPs in zebrafish has not been described, the ability of amino groups-functionalized γ-Fe_2O_3 NPs (30 nm; 25 - 200 μg mL^{-1}) to translocate directly from the olfactory nerve to the brain in rats has been reported, reaching the striatum and hippocampus regions, causing neurotoxic effects (Wu et al., 2013). The striatum and hippocampus are important brain structures related to the development of neurodegenerative diseases (Wu et al., 2013). Dopaminergic damage has also been reported in rat brains induced by γ-Fe_2O_3 NPs (10 - 30 nm; 2.5 - 40 μg mL^{-1}), revealing a mechanism of neural damage (Imam et al., 2015). In addition, γ-Fe_2O_3 NPs (50 nm; 5 mg kg^{-1} b.w.) exposure induced neurotoxicity and inflammation in rats, causing a decline in the enzymes acetylcholine, esterase, norepinephrine, serotonin, dopamine and antioxidants, increased lipid peroxidation, nitric oxide and p53 tumor suppressor gene, as well as alteration in gene expression of mTFA and PGC-1 α (Yousef et al., 2019).

3.4.3. Cardiotoxicity

Cardiotoxicity was the main toxic effect of both iron forms on the early development of zebrafish (Fig. 10A). After semi-static exposure for 48 h, both iron forms reduced the heart beat rate in zebrafish embryos compared to unexposed ones ($p < 0.05$; Fig. 10A), while no effect was observed after static exposure to γ-Fe_2O_3 NPs ($p > 0.05$; Fig. 10A). High frequency of embryos with bradycardia was observed after semi-static exposure to γ-Fe_2O_3 NPs, especially at 0.3 mg L^{-1} (66.6 ± 9.42 %), 0.6 mg L^{-1} (76.6 ± 20.54 %), 5 mg L^{-1} (60 ± 29.43 %) and 10 mg L^{-1} (80 ± 14.14 %) at static exposure and at 0.3 mg L^{-1} (46.66 ± 38.58 %), 0.6 mg L^{-1} (56.6 ± 26.24 %), 1.25 mg L^{-1} (30 ± 8.16 %) and 2.5 mg L^{-1} (33.3 ± 12.47 %) after static exposure (Fig 10B). Zebrafish embryos after static exposure to iron ions showed similar heart beat rate to unexposed ones ($p > 0.05$; Fig. 10A). However, significant alterations on heart beat rate were observed after semi-static exposure to iron ions at 0.3 mg L^{-1} ($p = 0.0018$), 0.6 mg L^{-1} ($p = 0.00062$), 1.25 mg L^{-1} ($p = 0.0269$) and 2.5 mg L^{-1} ($p = 0.0038$) (Fig. 10). The frequency of embryos with bradycardia increased after semi-static exposure to iron ions, mainly at 0.3 mg L^{-1} (73.33 ± 4.71 %), 0.6 mg L^{-1} (66.6 ± 28.67 %) and 2.5 mg L^{-1} (76.66 ± 9.42 %) when compared

to static exposure to iron ions at 0.3 mg L^{-1} (46.6 ± 37.7 %), 0.6 mg L^{-1} (33.3 ± 20.5%) and 2.5 mg L^{-1} (3.3 ± 4.71%) (Fig.10), confirming the cardiotoxic effects induced by both iron forms in zebrafish embryos.

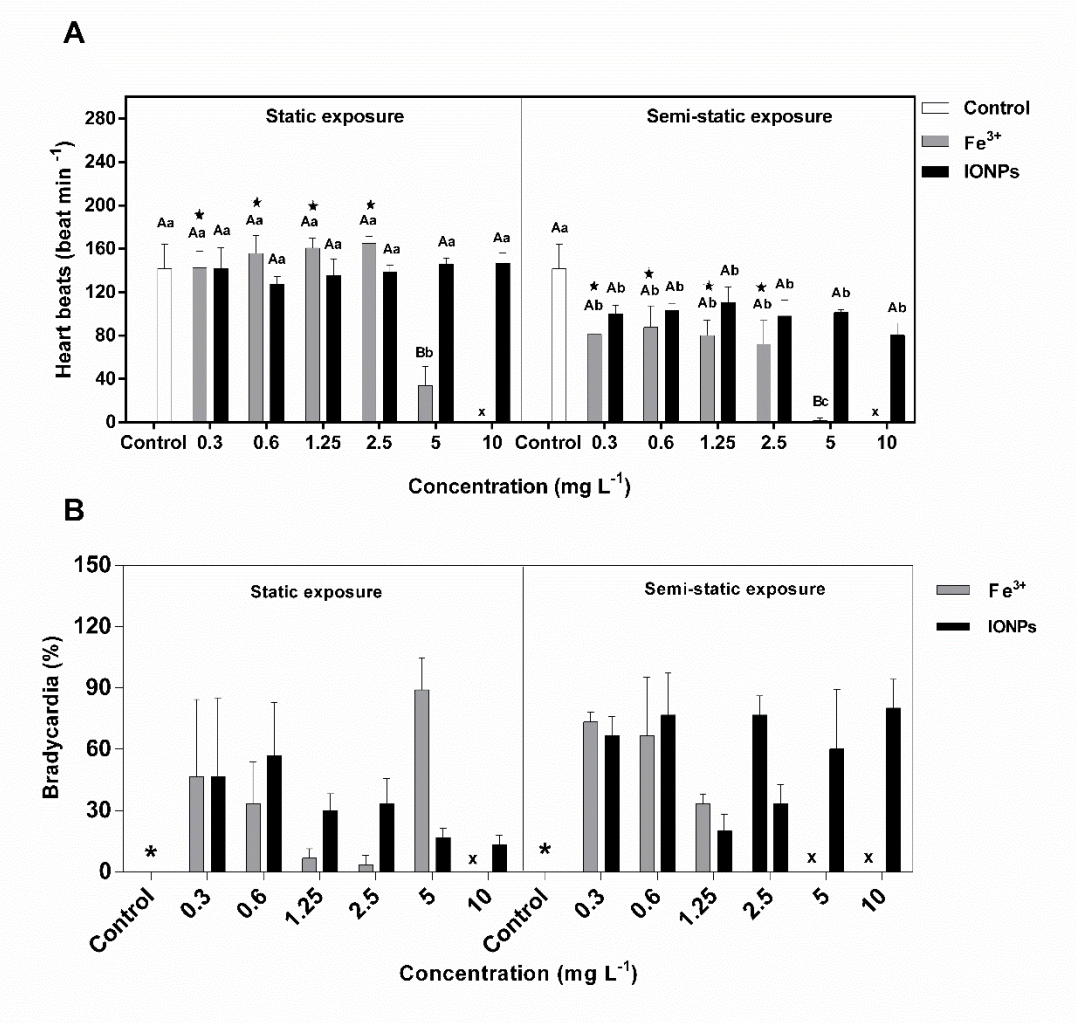

Figure 10. (A) Heart beat rate (beat min^{-1}) of zebrafish embryos from control group and after exposure to γ-Fe_2O_3 nanoparticle (IONPs) under static, iron ions under static, IONPs semi-static conditions and iron ions semi-static conditions for 48 h. "x" indicated mortality. (★) Difference between static and semi-static exposure. Capital letters indicate significant differences between NPs and ferrous chloride at the same concentration and different lower letters indicate significant differences in the same form of iron across concentrations. Results are expressed as mean and standard deviation of triplicates. (B) Frequency (%) of embryos with bradycardia after exposure to static and semi-static exposure to IONPs and iron ions. (*) without bradycardia, (x) dead.

Cardiotoxic effects induced by γ-Fe_2O_3 NPs in zebrafish embryos should be related to the release of iron ions, which can induce inflammation, lipid peroxidation (LPO) and oxidative stress, such as observed in BALB/c mice (Bostan et al., 2016). The heart is an organ sensitive to iron accumulation, which induces apoptosis and tissue degeneration, as well reduced heart beat rate (Parkes et al., 1993; Lekawanvijit and

Chattipakorn, 2009; Bostan et al., 2016). Dextran, pluronic and polyethylene glycol functionalized IONPs (10 - 30 nm; 100 - 500 μg mL^{-1}) accumulation in the heart has been previously reported in BALB/c mice (Feng et al., 2018). Ca^{2+} type L channels provide the main pathway for iron to enter cardiomyocytes, which leads to this iron accumulation (Oudit et al., 2003). The NP accumulation in the heart induced the production of highly toxic hydroxyl radicals through the Fenton-catalysed Haber-Weiss reaction. These free radicals damage the lipid-rich cardiomyocyte cell membrane through LPO, as well as changed Na^+ K^+-ATPase and 5'-nucleotidase. Oxidative stress can also induce cardiomyocyte cell death (Bartfay et al., 1999; Gujja et al., 2010; Kremastinos and Farmakis, 2011; Shen et al., 2015). The oxidative stress in zebrafish embryos also was reported after exposure to Ag NPs (8.39 ± 0.98 nm; 0.03 to 1.55 μg mL^{-1}) (Massarsky et al., 2013), indicating that the NM-induced cardiotoxicity was mediated by ROS production and oxidative stress.

Similarly, polyacrylic acid-functionalized γ-Fe_2O_3 NPs (6.8 nm) at 10 mg Kg^{-1} reduced the heart beat rate in BALB mice after intravenously injection (Inversen et al., 2013). In addition, other metal-based NPs have similar cardiotoxicity mechanisms (Cao et al., 2018). Polyvinyl acetate (PVA)-functionalized Ag NPs (5 - 35 nm; 10 - 100 μg mL^{-1}) decreased heart beat rate, pericardial edema and circulatory defects in zebrafish embryos (Asharani et al., 2011), as observed in the present study. Ag NPs also showed direct effects on ion channels, decreasing Na^+ and K^+ transport, reducing the recovery of Na^+ and K^+ channels from cardiomyocytes, causing a decrease in heart beat rate (Lin et al., 2017). Despite the widespread use of zebrafish as an animal model for cardiovascular disease studies (Chico et al., 2008; Hoage et al., 2012; Zakaria et al., 2018), the present study is a pioneer in the identification of the IONP-induced cardiotoxicity in zebrafish embryos. Thus, it is emphasized the need for further studies on the effects of γ-Fe_2O_3 NPs on the heart, mainly due to their numerous medical applications.

3.4.4. Morphological alterations and morphometric analysis

Citrate-functionalized γ-Fe_2O_3 NPs induced low frequency of morphological alterations on zebrafish embryo and larvae when compared to iron ions (Table 1, Fig. 11). Furthermore, several morphological alterations were observed in zebrafish embryos exposed to both iron forms in an exposure condition dependent pattern, wherein a larger

number of morphological alterations induced by both iron forms was observed after semi-static exposure when compared to static exposure (Table 1, Fig. 11), confirming the importance of establishing exposure patterns for nanotoxicological tests, such as ZET.

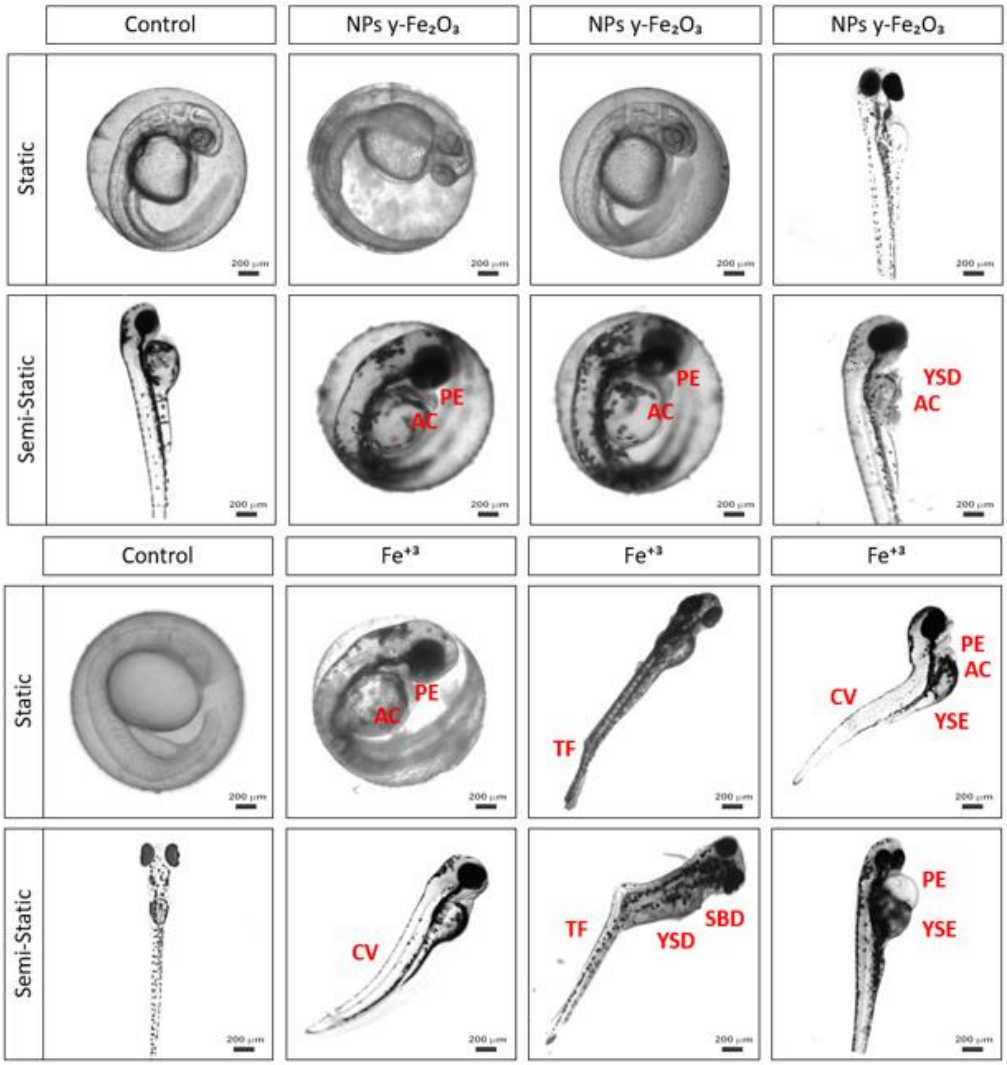

Figure 11. Photomicrographs of zebrafish embryos and larvae from control group and after exposure to γ-Fe_2O_3 nanoparticle (IONPs) under static (0.3-10 mg/L^{-1}), iron ions under static (0.3 - 2.5 mg/ L^{-1}), IONPs semi-static conditions (0.3 - 10 mg L^{-1}) and iron ions semi-static conditions (0.3 - 1.25 mg /L^{-1}) during 144 h. Blood accumulation (AC), pericardial edema (PE), tail flexion (TF) spinal curvature (CV), swim bladder deformity (SBD), yolk sac edema (YSE) and yolk sac deformity (YSD).

After static exposure, similar frequency of morphological alterations was observed in zebrafish exposed o γ-Fe_2O_3 NPs compared to unexposed ones. However, after semi-static exposure to γ-Fe_2O_3 NPs zebrafish showed pericardial edema, blood accumulation in the heart, spinal curvature, tail deformation, swim bladder deformation and vitelline sac edema (Table 1, Fig. 11). Interestingly, zebrafish embryos exposed to uncoated γ-Fe_2O_3 NPs (30 nm; 0.1 - 100 mg / L^{-1}) under static condition for 168 h showed

pericardial edema, tissue ulceration and spinal curvature in zebrafish embryos (Zhu et al., 2012), confirming that unfunctionalized IONPs, even under static conditions, may present higher oxidative dissolution following by ion release, inducing greater embryotoxicity in zebrafish. Similarly, previous studies indicated that the IONP toxicity was associated to NP dissolution and oxidative stress induced by released iron ions (Wang et al., 2013; Patil et al., 2015; Lei et al., 2018). On the other hand, results showed that the iron ions induced several teratogenic effects after both exposure conditions, such as pericardial edema, blood accumulation in the heart, tail deformation, spinal cord curvature, swim bladder deformity, and yolk sac edema, confirming their embryotoxicity after static and semi-static exposure conditions.

Table 1. Morphological alterations in zebrafish embryos after exposure to γ-Fe_2O_3 nanoparticle (IONPs) and iron ions under static and semi-static conditions during 144 h.

	Morphological alteration	Static exposure							Semi-static exposure						
		C	0.3	0.6	1.25	2.5	5	10	C	0.3	0.6	1.25	2.5	5	10
Iron chloride	**Edema of the pericardium**	0 ± 0	0.20 ± 0.35	0.20 ± 0.35	0.03 ± 0.06	0.13 ± 0.23	0 ± 0	0 ± 0	0 ± 0	0.03 ± 0.06	0.10 ± 0.17	0.10 ± 0.17	0 ± 0	0 ± 0	0 ± 0
	Spinal curvature	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0.03 ± 0.06	0 ± 0	0 ± 0	0 ± 0	0.03 ± 0.06	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	Blood accumulation	0 ± 0	0.07 ± 0.12	0 ± 0	0 ± 0	0.13 ± 0.23	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	Tail malformation	0 ± 0	0 ± 0	0 ± 0	0.03 ± 0.06	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0.03 ± 0.06	0.10 ± 0.17	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	Deformation of the yolk sac	0 ± 0	0 ± 0	0.03 ± 0.06	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0.07 ± 0.12	0 ± 0	0 ± 0	0 ± 0
	Swimming bladder deformity	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0.03 ± 0.06	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	Total	0 ± 0	0.27 ± 0.46	0.23 ± 0.40	0.07 ± 0.12	0.30 ± 0.52	0 ± 0	0 ± 0	0 ± 0	0.13 ± 0.23	0.20 ± 0.35	0.20 ± 0.35	0 ± 0	0 ± 0	0 ± 0
γ-Fe_2O_3 NPs	**Edema of the pericardium**	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0.10 ± 0.17	0.33 ± 0.58	0.27 ± 0.46	0.17 ± 0.29	0.43± 0.50	0.30 ± 0.52
	Spinal curvature	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0.03 ± 0.06	0.03 ± 0.06	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	Blood accumulation	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0.13 ± 0.23	0.37 ± 0.55	0.23 ± 0.40	0.13 ± 0.23	0.27 ± 0.46	0.30 ± 0.52
	Tail malformation	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0.03 ± 0.06	0.03 ± 0.06	0.03 ± 0.06	0 ± 0	0 ± 0	0 ± 0
	Deformation of the yolk sac	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	Swimming bladder deformity	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0		0.03 ± 0.06	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	Total	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0.33 ± 0.58	0.77 ± 0.40	0.53 ± 0.50	0.30 ± 0.52	0.67 ± 0.58	0.60 ± 0.53

Morphometric parameters of zebrafish larvae exposed to both iron forms for 144 h under static and semi-static conditions are shown in Table 2. An overview of these results indicates that the IONPs did not change the sensory, muscular structural and skeletal structural parameters after static and semi-static exposures. On the other hand, iron ions changed the physiological parameters after semi-static exposure, such as reduced the swimming bladder area at 1.25 mg L^{-1} ($p = 0.00015$), reduced the area of yolk sac ($p= 0.02659$), change skeletal parameters such as, reduced the head height at 0.3 mg L^{-1} ($p = 0.00548$). Furthermore, the iron ions at 2.5 mg L^{-1} reduced the distance from mouth to anus after static conditions ($p = 0.0365$; Table 2).

Quantitative analysis of morphometric parameters helps to eliminate errors of qualitative analysis, making possible to observe previously subtle morphological change (Hinz et al., 2012). The observed physiological and structural changes in zebrafish larvae after semi-static exposure to iron ions are indicative of somatic and systemic changes. Larva length and natural bladder area are usually the most affected parameters (Teixidó et al., 2018). The development of the swim bladder depends on blood circulation and therefore may have a side effect of changes in vascularization (Yue et al., 2015). Chemicals that affect heart rate, causing bradycardia, usually cause lack of swim bladder inflation (Bittner et al., 2018). As observed in this study, iron ions in semi-static conditions decreased the heart beat rate (Fig. 10A) and induced blood accumulation (Fig. 11), confirming the effect on bladder inflation.

Table 2. Morphometric parameters in zebrafish larvae after exposure to γ-Fe_2O_3 nanoparticle (IONPs) and iron ions under static and semi-static conditions for 144 h.

	Classification	Morphological alteration	Static exposure							Semi-static exposure						
			C	0.3	0.6	1.25	2.5	5	10	C	0.3	0.6	1.25	2.5	5	10
Iron chloride	**Sensory**	Eye area	928477.7 ± 72223.94	906142 ± 223317	954365 ± 165093	863159 ± 83406.8	877618 ± 102296	0 ± 0	0 ± 0	928477.6 ± 72223.9	995302± 162413	911095 ± 143018	915476 ± 155986	0 ± 0	0 ± 0	0 ± 0
		Minimal interocular distance	422 ± 69	359.3 ± 133.8	493.5 ± 126.4	514.4 ± 99.2	487.2 ± 118	0 ± 0	0 ± 0	422 ± 69.3	366.7 ± 79.9	430.5 ± 60.8	401.3 ± 100.9	0 ± 0	0 ± 0	0 ± 0
		Maximum interocular distance	2093.7 ± 126.5	1821.8 ± 327.6	2119 ± 139.7	2130.2 ± 145	1883 ± 159.5	0 ± 0	0 ± 0	2093.7 ± 126.5	2020.8 ± 156.6	2034.9 ± 147.5	2022.1 ± 198.5	0 ± 0	0 ± 0	0 ± 0
	Physiological	Heart area	188427 ± 28614	195529.5 ± 41633.4	237218 ± 82951.2	222422.9± 61730.7	200816.5 ± 45511.8	0 ± 0	0 ± 0	188427.7 ± 28614.5	215554.4 ± 33380.5	206793 ± 64721.8	219962.5 ± 34267.9	0 ± 0	0 ± 0	0 ± 0
		Swimming bladder area	640180 ± 75.9	597835.3 ± 37809.1	658182.1 ± 117408	567968.7 ± 57403.8	569173 ± 91607.2	0 ± 0	0 ± 0	640180.1 ± 75978.5	672322.6 ± 140231.1	646758.3 ± 177503	529785.1 ± 147722.3	0 ± 0	0 ± 0	0 ± 0
		Yolk sac area	1695404 ± 218.1	1637391.7 ± 222232	1585311 ± 354914	168945.5 ± 248819	1477585.2 ± 233675	0 ± 0	0 ± 0	1695404.7 ± 222232	1499574.4 ± 252191	1626380.7 ± 98973.5	1475146.6 ± 158187	0 ± 0	0 ± 0	0 ± 0
	Structural skeletal	Head height	1842 ± 90	1584.3 ± 257	1764.1 ± 139	1766.5 ± 35.8	1696.5 ± 77	0 ± 0	0 ± 0	1842 ± 90	1797.2 ± 127.7	1788.7 ± 95.9	1779.1 ± 91.6	0 ± 0	0 ± 0	0 ± 0
		Head width	1820.9 ± 137	1635.9 ± 258.9	1806 ± 101.6	1856.7 ± 149.4	1743.4 ± 80.7	0 ± 0	0 ± 0	1820.9 ± 136.8	1719.1 ± 123	1675.4 ± 210.7	1711.7 ± 149.6	0 ± 0	0 ± 0	0 ± 0
		Depth of head	2563.4 ± 78.6	2466.7 ± 112.7	2484.4 ± 126.1	2527.1 ± 88.3	2440.7 ± 168	0 ± 0	0 ± 0	2563.4 ± 78.6	2519.2 ± 83.8	2468.9 ± 127.5	2581.4 ± 222.1	0 ± 0	0 ± 0	0 ± 0
		Distance from mouth to anus	6905 ± 297	6851.5 ± 289.1	6717.2 ± 252.6	6696.4 ± 349.9	6384.2 ± 485.9	0 ± 0	0 ± 0	6905 ± 297.2	6959 ± 526.1	6947.8 ± 260.4	6980.6 ± 246	0 ± 0	0 ± 0	0 ± 0
	Sstructural muscle	Angle between the miosseptos	92.5 ± 7.0	90.5 ± 9.1	98 ± 7.6	100.6 ± 6.8	99.8 ± 9.6	0 ± 0	0 ± 0	92.5 ± 7	97.5 ± 5.6	97 ± 7.8	105 ± 6	0 ± 0	0 ± 0	0 ± 0
		Distance between the miosseptos	567 ± 31	607 ± 70	571.5 ± 42	607 ± 44	568 ± 58	0 ± 0	0 ± 0	567 ± 31	610 ± 47	622 ± 58	608 ± 43	0 ± 0	0 ± 0	0 ± 0
γ-Fe_2O_3 NPs	**Sensory**	Eye area	928477.7 ± 72223.94	917941.6 ± 42448.7	930007.6 ± 90592.6	1046487 ± 143005.9	995589.1 ± 145474.8	1035729 ± 1626671.2	931423.7 ± 1238281.7	928478 ± 72223.9	917814 ± 84459.7	955016 ± 140628	938661 ± 129280	898085 ± 1075800	1002319 ± 1342667	920462 ± 1876588
		Minimal interocular distance	422 ± 69	465.6 ± 79	483.1 ± 79	504.1 ± 123	500.5 ± 78	507.3 ± 73	410.6 ± 82.4	422 ± 69.3	471.7 ± 70.3	450.9 ± 83.8	404.5 ± 79.9	389.4 ± 93.6	488.1 ± 118.6	514.6 ± 136.8
		Maximum interocular distance	2093.7 ± 126.5	2089 ± 75.8	2234.8 ± 145	2107.4 ± 134.6	2055.8 ± 138.8	2119 ± 112.5	2093.7 ± 126.5	2093.7 ± 126.5	2072.4 ± 135.1	2059.1 ± 81.5	2048.2 ± 133.9	2007.3 ± 119.9	2014.9 ± 208.4	2101.4 ± 155.3
	Physiological	Heart area	188427 ± 28614	158889 ± 26572	237217 ± 82951	202134 ± 53150	180170 ± 33282	178616 ± 43352.5	178610.6 ± 29801.17	188428 ± 28614.6	206787 ± 31736	244648 ± 42913.3	239521 ± 44783.8	224096 ± 84574.2	241268 ± 67674.2	216498± 58599.5
		Swimming bladder area	640180 ± 75.9	647374 ± 130.4	663378 ± 120.9	599156 ± 112.3	612749 ± 135.3	663031 ± 130.4	704690 ± 96.67	188428 ± 28614.6	206787 ± 31736	244648 ± 42913.3	239521 ± 100.3	650461.1 ± 83.4	594829.6 ± 57.8	547941 ± 150.8
		Yolk sac area	1695404 ± 218.1	1390896.3 ± 176.332	1419835.2 ± 221.941	1497423 ± 291.25	1475718.9 ± 249.3	1439236.1 ±	1547211.4 ±	640180.1 ± 75.9	668128.8 ± 120.1	630211 ± 96.9	585397.6 ± 100.3	650461.1 ± 83.4	594829.6 ± 57.8	547941 ± 150.8

Cont.

							196577.4	180.55							
Structural skeletal	Head height	1842 ± 90	1779.6 ± 86.9	1770.6 ± 76.9	1819 ± 111.1	1806.5 ± 99.2	1784.9 ± 127.9	1854.8 ± 115.7	1842 ± 90	1823.4 ± 109.6	1838.6 ± 69.1	1860.7 ± 76.3	1830.1 ± 77.8	1806.9 ± 108.3	1776.5 ± 163.9
	Head width	1820.9 ± 137	1898.4 ± 119	1785.3 ± 87	1811.4 ± 136	1751.3 ± 88	1812.8 ± 98	1903.3 ± 86	1820.9 ± 137	1739.6 ± 107	1716.6 ± 92	1750.6 ± 66	1742.6 ± 63	1732.7 ± 142	1794.1 ± 98
	Depth of head	2563.4 ± 78.6	2512.5 ± 75.7	2530 ± 105.4	2568.8 ± 104.9	2571.4 ± 157.7	2588.3 ± 126.5	2466.7 ± 74.5	2563.4 ± 78.6	2518.2 ± 118	2446.6 ± 152.4	2485.7 ± 194.1	2520.6 ± 101.6	2440.4 ± 96	2447.1 ± 158.1
	Distance from mouth to anus	6905 ± 297	6634 ± 208	6574 ± 409	7039 ± 515	6694 ± 361	6941 ± 351	389 ± 390	6905 ± 297	6977 ± 263	6961.6 ± 355	6974.1 ± 253	6820.1 ± 239	7061.1 ± 112	6907 ± 261
Structural muscle	Angle between the miosseptos	92.5 ± 7.0	88.7 ± 6.4	86.7 ± 7.3	92.8 ± 6.5	94.2 ± 6.4	91.1 ± 6.7	91.3 ± 6.6	92.5 ± 7	93.6 ± 8.8	91.1 ± 6.8	95.1 ± 8.5	95.65 ± 6.7	87.8 ± 7.7	90.9 ± 6.3
	Distance between the miosseptos	567.2 ± 31	564.8 ± 33	589.7 ± 65	627.7 ± 62	568.6 ± 59	632.6 ± 35	601.4 ± 37	567.2 ± 31	639.3 ± 49	612.3 ± 68	570.8 ± 91	584.3 ± 78	611.8 ± 61	630.5 ± 63

3. Conclusion

The present study confirmed the importance of ZET for assess the toxic effects of NMs. The analysis of multiple biomarkers in the ZET, especially the sublethal parameters, allowed the description of mechanisms of action and toxicity of NMs. Results showed that the citrate-functionalized γ-Fe_2O_3 NPs induced low toxicity to zebrafish embryos and larvae when compared to their dissolved counterpart. Iron ions at the highest concentrations (2.5 to 10 mg L^{-1}) induced 100 % mortality in the zebrafish embryos, while the sublethal concentrations (0.3 to 1.25 mg L^{-1}) induced cardiotoxicity and severe morphological changes, such as pericardial edema, blood accumulation in the heart, yolk sac edema, swim bladder deformity, tail flexure, and curvature of the spine. However, γ-Fe_2O_3 NPs induced cardiotoxic effects in zebrafish embryos, such as bradycardia, pericardial edema, and accumulation of blood in the heart. Results observed in the present study with zebrafish were similar to those reported in other mammalian models (Inversen et al., 2013; Cao et al., 2018; Feng et al., 2018; Yousef et al., 2019). Exposure to high concentrations of NOFs induced their accumulation in the heart, myocardial damage and bleeding, and decreased heart rate, confirming that zebrafish is an appropriate model system for analysing the cardiotoxic potential of NMs.

The toxicity of both iron forms was concentration and exposure condition dependent. Under semi-static exposure conditions there was a potentiation of effects induced by citrate functionalized γ-Fe_2O_3 NPs when compared to static exposure. Given the pioneering nature of this study, observed results recommend further research about other types of exposure conditions, such as continuous flow or environmental relevant conditions (i.e., mesocosm or multi-exposure species). Results indicated the sub-lethal effects of citrate-functionalized γ-Fe_2O_3 NPs on aquatic organisms, specially cardiotoxicity.

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CAPÍTULO IV

Conclusão e Considerações Finais

1. Conclusão e considerações finais

O uso da nanotecnologia cresceu em grande escala, possuindo inúmeros tipos de NMs que veem sendo empregados em produtos na área médica, ambiental e tecnológica. Contudo, com o crescimento da utilização dos NMs surge também a preocupação quanto aos seus potenciais riscos para o meio ambiente e para a saúde humana. Dentre esses NMs, as γ-Fe_2O_3 NPs funcionalizadas com citrato foram objeto desse estudo, devido a sua alta aplicabilidade, propriedades físico-químicas de interesse e por existirem poucos estudos sobre a toxicidade dessas NPs. Além disso, o estudo propôs uma comparação entre a toxicidade das γ-Fe_2O_3 NPs e dos íons de ferro, afim de avaliar se os mecanismos de ação e toxicidade são semelhantes entre as γ-Fe_2O_3 NPs e os íons de ferro, visto que ainda existe uma dúvida sobre o comportamento das NPs e se a toxicidade é causada pela NPs (propriedades nanoespecíficas) ou pelos íons liberados após a dissolução oxidativa. Além disso, no ZET foi realizado uma comparação sobre o método de exposição estático e semi-estático, visto que, devido as propriedades magnéticas das γ-Fe_2O_3 NPs elas têm por tendência se aglomerar e depositar no fundo das microplacas. Pensando em dois ambientes diferentes, um no qual não há a liberação constante das γ-Fe_2O_3 NPs e outro no qual elas são constantemente liberadas, essa simulação durante o teste permite mostrar a diferença no comportamento dessas NPs em duas situações de exposição diferentes.

Partindo desses objetivos, o presente estudo realizou um levantamento bibliométrico e uma revisão sistemática da literatura sobre o uso do ZET na avaliação da toxicidade dos NMs. Os dados revisados demonstraram que o ZET é promissor na avaliação da toxicidade dos NMs, apesar que um protocolo específico deve ser desenvolvido para testes com NMs. Além disso, deve-se levar em consideração o comportamento desses NMs em condições ambientais durante o teste, visto que, esses NMs sofrem transformações ao serem liberados no meio ambiente. Nesse contexto, em relação as γ-Fe_2O_3 NPs existe somente um trabalho sobre a sua embriotoxicidade no *zebrafish*, o que levanta ainda mais a importância de estudos futuros com essas NPs.

Em relação ao teste ZET, pode-se observar que os íons de ferro se mostraram mais tóxicos aos embriões de *zebrafish* em relação as γ-Fe_2O_3 NPs funcionalizadas com citrato, induzindo uma alta mortalidade nas maiores concentrações, maior diminuição dos batimentos cardíacos dos embriões, além de maiores alterações morfológicas nos embriões de *zebrafish*. Apesar dos íons terem se mostrado mais tóxicos, as γ-Fe_2O_3 NPs

induziram alterações no batimento cardíaco (cardiotoxicidade) e causaram principalmente acúmulo de sangue na região do coração e formação de edema do pericárdio, sugerindo ação das γ-Fe_2O_3 NPs no sistema cardiovascular. O miocárdio é um dos tecidos mais sensíveis ao ferro, sendo que a sobrecarga crônica de ferro induz lesões no miocárdio, hemorragias, além de insuficiência cardíaca. Sabe-se que essas NOFs ao entrarem em contato com o organismo tem afinidade pelo coração, sendo o principal órgão que essas NPs se acumulam. O mecanismo de toxicidade das NOFs nos cardiomiócitos é mediada por formação de radicais hidroxila altamente tóxicos por meio da reação de Haber-Weiss catalisada por reação de Fenton (Fig 1). Esses radicais livres danificam a membrana celular dos cardiomiócitos por peroxidação lipídica. Estruturas localizadas na membrana celular como Na^+/K^+ ATPase e 5'-nucleotidase também são alteradas. Além dessas estruturas, a toxicidade das NOFs mediada por estresse oxidativo e formação de EROs também afeta outras organelas e funções celulares e pode induzir a morte celular dos cardiomiócitos (Fig 1).

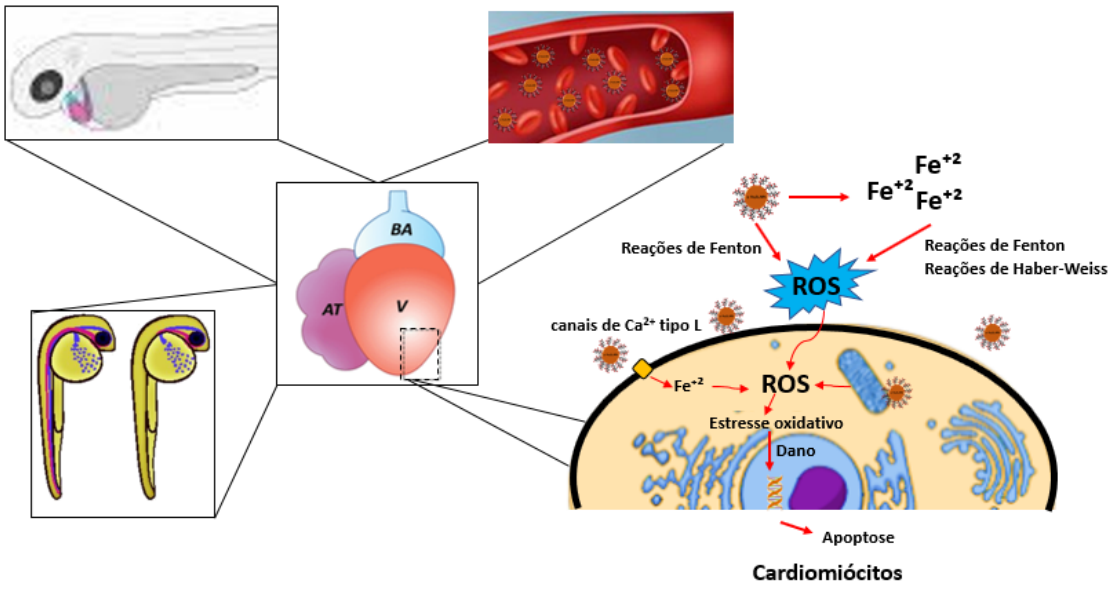

Figura 1. Esquema ilustrativo do mecanismo de ação e toxicidade das Nanopartículas de óxido de ferro nos cardiomiócitos do *zebrafish*. Fonte: a autora.

Em relação ao método de exposição estático e semi-estático foi possível observar que na exposição semi-estática houve uma potencialização dos efeitos das γ-Fe_2O_3 NPs funcionalizadas com citrato e dos íons de ferro. Após exposição semi-estática γ-Fe_2O_3 NPs apresentaram aumento da acumulação no sistema digestivo, diminuição dos batimentos cardíacos e alterações morfológicas em comparação com a exposição estática, a qual induziu apenas acumulação das IONPs no sistema digestivo. Cabe ressaltar a

importância de analisar os efeitos subletais durante o ZET, visto que como observado nesse estudo só foi possível identificar os efeitos tóxicos das IONPs analisando a cardiotoxicidade e neurotoxicidade nos embriões de *zebrafish*. Os íons de ferro apresentaram acumulação no sistema digestivo, aumento da taxa de mortalidade, diminuição dos batimentos cardíacos, alterações morfológicas e nos parâmetros morfométricos na exposição estática e após a exposição semi-estática o efeito nesses biomarcadores aumentaram, além de aumentar os movimentos espontâneos dos embriões (Quadro 1). Logo, é essencial que os estudos com NMs levem em consideração as condições de exposição, visto que, os efeitos variam de acordo com o tipo de exposição. Considerando que no ambiente podemos ter dois tipos de situações, na qual, esses NMs podem ser liberados constantemente no mesmo local, e outro que não existe uma liberação constante desses NMs no ambiente, torna-se essencial realizar a comparação entre esses dois tipos de exposição. Cabe ressaltar que ainda são necessários estudos futuros sobre a toxicidade das NPs em condições ambientalmente relevantes, tais como fluxo contínuo, mesocosmo e transferência trófica.

Quadro 1. Biomarcadores após exposição estática e semi-estática as γ-Fe_2O_3 NPs e ao cloreto de ferro. Sem efeito (Verde); com efeito (Vermelho); aumento (↑); diminuição (↓).

	Estático		Semi-estático	
Biomarcadores	**γ-Fe_2O_3 NPs**	**Fe^{+2}**	**γ-Fe_2O_3 NPs**	**Fe^{+2}**
Acumulação	↑	↑	↑	↑
Taxa de mortalidade		↑		↑
Taxa de eclosão				
Movimentos espontâneos				↑
Batimentos cardíacos		↓	↓	↓
Alterações Morfológicas		↑	↑	↑
Parâmetros morfométricos		↓		↓

Fonte: a autora.

Os resultados do presente estudo indicam que futuros estudos sobre a genotoxicidade, mutagenicidade e estresse oxidativo após a exposição as IONPs devem ser realizados para melhor compreensão dos efeitos causados por essas NPs dentro do organismo. Estudos sobre a toxicocinética das IONPs em larvas e embriões de *zebrafish* também contribuirão para entender seu comportamento no organismo. Em relação ao risco ambiental dessas NPs, estudos sobre o comportamento das IONPs em condições ambientais e sua interação com outros poluentes são os próximos passos que devem ser realizados.

Produção científica durante o mestrado

Artigo publicado

Pereira, A. C., Gomes, T., Machado, M. R. F., Rocha, T. L., 2019. The zebrafish embryotoxicity test (ZET) for nanotoxicity assessment: from morphological to molecular approach. **Environ Pollut.** https://doi.org/10.1016/j.envpol.2019.06.100

Artigo submetido

Pereira, A. C., Gonçalves, B. B., Brito, R. S., Vieira, L. G., Lima, E. C. O., Rocha, T. L., 2020. Comparative developmental toxicity of iron oxide nanoparticles (γ-Fe_2O_3) and ferric chloride to zebrafish (*Danio rerio*) after static and semi-static exposure. **Chemosphere.**

Apresentação de trabalho

Aryelle Canedo Pereira. Comparative embryotoxicity of iron oxide nanoparticle (γ-Fe_2O_3) and ferric chloride to zebrafish (*Danio rerio*). 2019. (Apresentação oral/poster, First International Conference of Nanoscience and Nanobiotecnology).

Aryelle Canedo Pereira. Comparative embryotoxicity of iron oxide nanoparticles nanoparticle (γ-Fe_2O_3) after static and semi-static exposure in zebrafish. 2019. (Danio rerio). (Apresentação de poster, Anais do VI Simpósio Zebrafish como Modelo Animal de Pesquisa).

Palestra

Aryelle Canedo Pereira. Comparative Embryotoxicity of iron oxide nanoparticle (γ-Fe_2O_3) and ferric chloride to zebrafish. 2018. (Palestra/ V Simpósio Zebrafish Como Modelo Animal de Pesquisa).

Minicurso

Aryelle Canedo Pereira. Zebrafish (*Danio rerio*) como sistema-modelo no biomonitoramento ambiental. 2019. (ministrado/ III Encontro de Biodiversidade Animal).

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Prêmio

Menção honrosa pelo trabalho intitulado: Comparative Embryotoxicity of iron oxide nanoparticle (γ-Fe_2O_3) and ferric chloride to zebrafish (*Danio rerio*). 2019. First International Conference of Nanoscience and Nanobiotecnology.

Trabalhos em Colaboração

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Malafaia, G., De Souza, A. M., Canedo, P. A., Gonçalves, S., Da Costa Araújo, A. P., Xavier, R. R., Lopes, R. T., 2019. Developmental toxicity in zebrafish exposed to polyethylene microplastics under static and semi-static aquatic systems. **Science of the total environment.** 10.1016/j.scitotenv.2019.134867

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Resumos

Renan Xavier Ribeiro. Toxicity assessment of Brazilian wastewater treatment plant effluents using zebrafish (*Danio rerio*) embryotoxicity test. 2018 (Poster/ V Simpósio Zebrafish Como Modelo Animal de Pesquisa)

Rafaella Silva Brito. Are titanium dioxide nanoparticles a new threat to fish health? A case study with early development of the zebrafish (*Danio rerio*). 2019 (Poster/ VI Simpósio Zebrafish como Modelo Animal para Pesquisa)

Andreza Martins Souza. Bioaccumulation and developmental toxicity of pristine polyethylene microplastics to zebrafish (Danio rerio). 2019 (Poster/VI Simpósio Zebrafish como Modelo Animal para Pesquisa)

Rafaella Silva Brito. Nanopartículas de dióxido de titânio: Um risco para a saúde ambiental? Estudo de caso com o zebrafish. 2019 (Poster/III Simpósio de Nanotecnologia e Saúde Ambiental)

Prêmio

1º lugar na categoria poster, apresentado na modalidade de iniciação científica. Toxicity assessment of Brazilian wastewater treatment plant effluents using zebrafish (*Danio rerio*) embryotoxicity test. V Simpósio Zebrafish Como Modelo Animal de Pesquisa.

1º lugar em apresentação de poster na categoria de Nanotoxicologia. Nanopartículas de dióxido de titânio: Um risco para a saúde ambiental? Estudo de caso com o zebrafish. III Simpósio de Nanotoxicologia e saúde ambiental.

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