Universidade Federal de Goiás

Instituto de Ciências Biológicas

Programa de Pós-Graduação em Biodiversidade Animal

Efeitos toxicológicos de nanopartículas de maghemita (γ-Fe_2O_3) em células e tecidos de *Poecilia reticulata*.

GABRIEL QUALHATO

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Programa de Pós-Graduação em Biodiversidade Animal

Efeitos toxicológicos de nanopartículas de maghemita (γ-Fe_2O_3) em células e tecidos de *Poecilia reticulata*.

O presente é apresentado para ser apreciado e avaliado pela banca examinadora de defesa de dissertação, em cumprimento a exigência do PPGBan para obtenção do título de mestre em Biodiversidade Animal pelo ICB – UFG.

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Goiânia, 2018

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Qualhato, Gabriel
Efeitos toxicológicos de nanopartículas de maghemita (?-Fe2O3) em células e tecidos de Poecilia reticulata. [manuscrito] / Gabriel Qualhato. - 2018.
109 f.: il.

Orientador: Profa. Dra. Simone Maria Teixeira de Sabóia-Morais; co-orientador Dr. Julio Roquete Cardoso.
Dissertação (Mestrado) - Universidade Federal de Goiás, Instituto de Ciências Biológicas (ICB), Programa de Pós-Graduação em Biodiversidade Animal, Goiânia, 2018.
Bibliografia.
Inclui lista de figuras, lista de tabelas.

1. Nanopartículas. 2. Ensaio cometa. 3. P. reticulata; . 4. Histopatologia . 5. Melanomacrófagos. I. Teixeira de Sabóia-Morais, Simone Maria , orient. II. Título.

CDU 57

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ATA DA SESSÃO PÚBLICA DE DEFESA DE DISSERTAÇÃO DE Nº 16

Aos vinte e oito dias do mês de fevereiro de dois mil e dezoito (28/02/2018), às nove horas (09h), na sala de vídeo conferência (CIAR-UFG-Centro de Eventos), reuniram-se os componentes da banca examinadora: **Profa. Dra. Simone Maria Teixeira de Sabóia-Morais, ICB/UFG; Prof. Dr. Thiago Lopes Rocha - IPTSP/UFG; e Profa. Dra. Cláudia Bueno dos Reis Martinez – DCIF/UEL**; para, em sessão pública presidida pelo primeiro examinador citado, procederem à avaliação da defesa de dissertação intitulada: **"Efeitos toxicológicos de nanopartículas de maghemita (γ-Fe_2O_3) em células e tecidos de *Poecilia reticulata*"**, em nível de mestrado, área de concentração em Biodiversidade Animal, de autoria de **Gabriel Qualhato**, discente do Programa de Pós-Graduação em Biodiversidade Animal da Universidade Federal de Goiás. A sessão foi aberta pelo presidente, que fez a apresentação formal dos membros da banca. A palavra, a seguir, foi concedida à(o) autor(a) da dissertação que, em cerca de 30 minutos, procedeu à apresentação de seu trabalho. Terminada a apresentação, cada membro da banca arguiu à(o) examinada(o), tendo-se adotado o sistema de diálogo sequencial. Terminada a fase de arguição, procedeu-se à avaliação da dissertação. Tendo-se em vista o que consta na Resolução nº 1403 de 10 de junho de 2016, do Conselho de Ensino, Pesquisa, Extensão e Cultura (CEPEC), que regulamenta o Programa de Pós-Graduação Biodiversidade Animal, a dissertação foi aprovada, considerando-se integralmente cumprido este requisito para fins de obtenção do título de Mestre em Biodiversidade Animal pela Universidade Federal de Goiás. A conclusão do curso dar-se-á quando da entrega da versão definitiva da dissertação na secretaria do programa, com as devidas correções sugeridas pela banca

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Simone Mª T. de Sabóia-Morais

Profa. Dra. Simone Maria Teixeira de Sabóia-Morais
Presidente da Banca
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Thiago Lopes Rocha

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Agradecimentos

Primeiramente agradeço a Deus, pois colocar suas orientações em primeiro lugar na minha vida me ajudou a conseguir atingir meus objetivos sem perder o meu foco das coisas que realmente importam, Seu amor e o amor de minha família. Agradeço o apoio dos meus queridos pais Roberto e Madalena Qualhato, por sempre me apoiarem nos meus objetivos e por serem sempre um exemplo de fé e perseverança para mim.

Agradeço a equipe do LCC, onde neste período se tornou a minha segunda casa, lembrado especialmente dos estudantes Lucas Guimarães, Victoria Costa, João Marcos, Raquel Silva, Felipe Cirqueira, Cândido Carvalho Rodrigues, Iago Madureira, Nicholas Trigueiro, Paulo Roberto Fleury e Bruno Gonçalves pela companhia e trabalho em equipe. A professora Daniela de Melo e Silva e sua equipe pelo apoio e aprendizado que me deram durante meus experimentos.

Agradeço também meus colegas de mestrado Jessica Custodio, Lorena Mesquita e Alex Lucas Hanusch por tudo que aprendemos e enfrentamos juntos sempre apoiando um ao outro.

A minha querida orientadora Profª Drª Simone Maria Teixeira de Sabóia-Morais que além de me orientar neste trabalho e em outros aspectos científicos me ajudou a lidar e entender melhor a universidade, sempre com muita paciência e carinho. Não esquecendo meu companheiro para todas as horas Prof. Dr. Thiago Lopes Rocha, sem sua ajuda, suas ideias, experiência, orientações e correções eu não teria chegado aqui.

Nunca me esquecerei do apoio que recebi dos professores da Anatomia animal Prof. Alberto Corrêa Mendonça, Prof. Marcelo Seixo de Brito e Silva, Prof.ª Viviane Souza Cruz e Prof. Júlio Roquete Cardoso. Sem a ajuda, compreensão e apoio de todos não seria possível a realização deste trabalho.

Sou muito grato a minha querida esposa Djully Caroline, que por toda essa jornada sempre me apoiou em todos os momentos, compreendendo minhas ausências e loucuras, me apoiando em todas as minhas atividades, sempre comigo, sempre sendo a minha melhor amiga, o meu amor e minha companhia para todas as momentos felizes e dificuldades. Nunca conseguirei demonstrar a minha gratidão por

tudo que você representa para mim, mas posso sempre demonstra o amor que sinto por você.

Por fim agradeço a Fundação de Amparo à Pesquisa do Estado de Goiás (FAPEG), pelo apoio financeiro por meio da bolsa de Mestrado (Edital 03-2016), e agradeço ao programa de Pós-Graduação em biodiversidade Animal pela oportunidade de formação e qualificação.

Sumário

Resumo

O risco ambiental dos nanomateriais projetados e utilizados para nanoremediação se mostram hoje de grande interesse econômico e ambiental, mas seus efeitos ecotóxicológicos para os organismos aquáticos ainda permanecem obscuros. Neste presente estudo, as nanopartículas de oxido de ferro funcionalizadas com citrato (maghemita) (NOFs) foram sintetizadas e seus efeitos genotóxicos, mutagênicos, e histopatológicos foram investigados em fêmeas de *Poecilia reticulata*. Os peixes foram expostos a maghemita em concentrações de ferro ambientalmente relevantes (0,3 $mg.L^{-1}$) durante 21 dias e os animais foram coletados no início do experimento e após 3, 7, 14 e 21 dias de exposição. A genotoxicidade e a mutagenicidade foram avaliadas por meio do dano causado ao DNA (Ensaio cometa), o teste do micronúcleo (MN) e anormalidades nucleares de eritrócitos (ANE) avaliaram o potencial mutagênico. Os fígados foram dissecados, fixados, desidratados em etanol, imersos em xilol, embebidos em parafina, e seccionados com 5 µm de espessura, os cortes corados em H.E. e Tricrômico de Mallory foram analisados e obtidos dados histomorfométricos. Os resultados mostraram efeitos genotóxicos e mutagênicos diferenciais das NOFs em *P. reticulata* de acordo com o tempo de exposição. As NOFs induziram danos ao DNA após exposição aguda (3 e 7 dias) e de longo prazo (14 e 21 dias), enquanto os efeitos mutagênicos foram observados somente para a exposição prolongada. O dano no DNA e a frequência total de ANE aumentaram linearmente ao longo do tempo de exposição, indicando uma maior taxa de indução de efeitos clastogênicos e aneugênicos em eritrócitos de *P. reticulata* após exposição prolongada a NOFs. Os guppies expostos também

mostraram aumento do número, área e perímetro dos MMC quando comparados ao grupo controle, especialmente após 7 dias de exposição. Os resultados mostraram um aumento na frequência de alterações histopatológicas em peixes após os 7 dias de exposição as NOFs, tais como esteatose micro- e macro-vesicular, aumento dos MMC, exsudatos e focos hemorrágicos. A exposição aguda (3 e 7 dias) e a longo prazo (14 e 21 dias) de *P. reticulata* a NOFs induziu índices histopatológicos elevados associados a distúrbios circulatórios e respostas inflamatórias. Os resultados indicaram que os tecidos sanguíneo e hepático dos guppies são excelentes órgão alvo para estudar a ecotoxicidade das NOFs, confirmando que o *P. reticulata* é uma espécie biomonitora indicada para estudos sobre a ecotoxicidade das NOFs.

Palavras-chave: nanopartículas; Biomarcadores; Ensaio cometa; *Guppy*; melanomacrófagos.

Abstract

The environmental risk of nanomaterials (NMs) designed and used in nanoremediation process is of emerging concern, but their ecotoxilogical effects to aquatic organism remains unclear. In this present study, the citrate-coated (maghemite) nanoparticles (IONPs) were synthesized and its genotoxic, mutagenic, the melanomacrophage centers (MMC) response and hepatic histopathological biomarkers were investigated in female guppy. Fish were exposed to IONPs at environmentally relevant iron concentration (0.3 mg L^{-1}) during 21 days and the animals were collected at the beginning of the experiment and after 3, 7, 14 and 21 days of exposure. The genotoxicity and mutagenicity were evaluated in terms of DNA damage (comet assay), micronucleus (MN) test, erythrocyte nuclear abnormalities (ENA) frequency and the liver were dissected and fixed dehydrated through increasing ethanol gradient, immersed in xylene PA, embedded in paraffin, performed of liver sections (5 µm thick) (3 sections per animal) in the microtome, stained by Mallory's Trichrome and H. E. and analyzed. Results showed differential genotoxic and mutagenic effects of IONPs in the *P. reticulata* according to exposure time. The IONP induced DNA damage in *P. reticulata* after acute (3 and 7 days) and long-term exposure (14 and 21 days), while the mutagenic effects were observed only after long-term exposure. The DNA damage and the total ENA frequency increase linearly over the exposure time, indicating a higher induction rate of clastogenic and aneugenic effects in *P. reticulata* erythrocytes after long-term exposure to IONPs. Guppies exposed to IONPs showed increasing in the number, area and perimeter of MMC when compared to the unexposed ones, especially after 7 days of exposure. The

results showed an increase in the frequency of histopathological changes in fish after the 7 days of exposure to IONPs, such micro- and macro-vesicular steatosis, melanomacrophage aggregates, exudate and haemorrhagic foci. The acute (3 and 7 days) and long-term (14 and 21 days) exposure of *P. reticulata* to IONPs induced high histopathological indexes associated with circulatory disorders and inflammatory responses. Results indicated that blood system and liver tissue of *P. reticulata* are excellent target organs to studies the ecotoxicity of IONPs. Confirming that *P. reticulata* is a biomonitor specie indicated for studies of ecotoxicity of IONPs.

Keywords: Nanoparticles; Biomarkes; Comet assay; Guppy; melanomacrophages.

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NPs – Nanopartículas;

NOFs – Nanopartículas de óxido de ferro;

TEM – Transmission Electron Microscopy;

IONPs – Iron oxide nanoparticles;

DLS – Dynamic Light Scattering;

ELS – Electrophoretic Light Scattering;

XRD – X-ray Diffraction;

FTIR – Infrared spectrum;

UV-Vis – Ultraviolet–visible spectroscopy;

ENA – Erythrocyte nuclear alterations

OTM – Olive tail moment;

TL – Tail length;

PCA – Principal component analysis

MMC – Melanomacrophage centers

Ih – Hepatic histopathological index;

Ij1 – circulatory disturbance index;

Ij2 – inflammatory response index;

Ij3 – regressive alteration index;

Ij4 – progressive alteration;

NMs – Nanomateriais.

Capítulo 1

Nanotecnologia e suas potencialidades tecnológicas e ecotoxicológicas

Este capitulo é a revisão bibliográfica que embasou justificativa, delineamento e escrita dos trabalhos realizados.

Introdução

Nanotecnologia de acordo com PAS 71 (Publicy Available Specification) é a ciência que envolve manipulações e processos utilizando-se de materiais e produtos que estejam na escala nanométrica, que varia entre 1 e 100 nanômetros, e uma nanopartícula (NP) tem de obrigatoriamente ter uma das suas dimensões dentro desta mesma escala (BSI, 2011) (Fig.1).

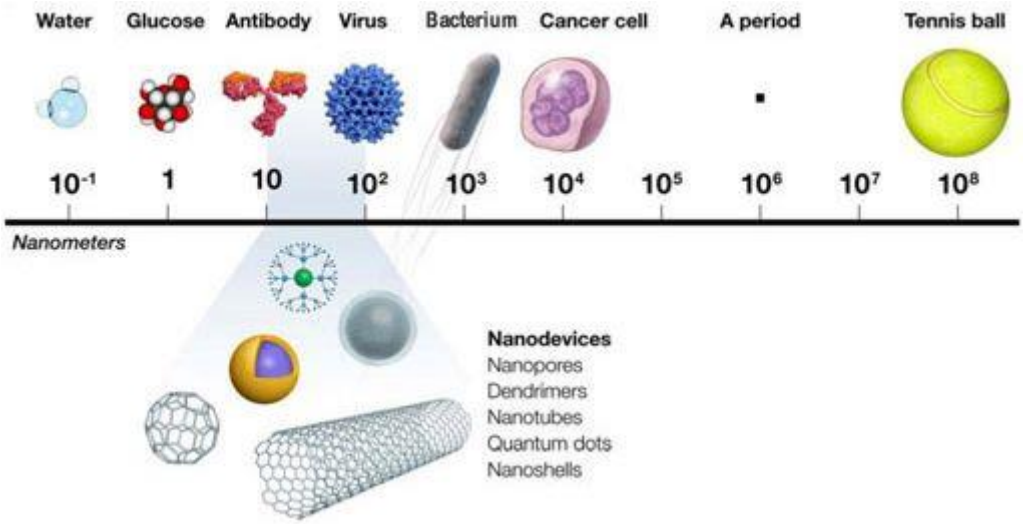


Figura 1 - Escala nanométrica. Notar a que nanoparticulas e nanomaterias estão localizados entre 1 e 100 nm.(http://nanowow.blogspot.com.br/2009/04/)

Portanto, para se obter NPs de acordo com os princípios preconizados pela PAS 71 (BSI, 2011) houve necessidade de se manipular e estabelecer características para as mesmas, o que levou ao surgimento de novas propriedades físicas e químicas, devido aos efeitos quânticos de tamanho e fenômenos de superfícies, ou seja, um material com seu tamanho reduzido à escala nanométrica pode apresentar propriedades eletrônicas, mecânicas e térmicas diferentes de quando estava em seu estado microscópico (sólido estendido ou “bulk”) (Kucheryavy et al., 2013).

Para além disto, outra consequência da redução do tamanho dos materiais é o aumento da área superficial, graças ao aumento significativo da quantidade de átomos superficiais quando comparados com o volume total da partícula, alterando assim, sua reatividade química (Cao, 2004). O aproveitamento dessas propriedades em aplicações tecnológicas forma a base da nanotecnologia de materiais e não há dúvida que esta área como nova fronteira oferece um promissor futuro de grandes avanços, os quais possibilitarão a melhora da qualidade de vida da população humana (Quina, 2004).

Devido as propriedades únicas, tais materiais atraem o interesse de inúmeros grupos de pesquisa em todo o mundo (Fig. 2), sendo possível mapear o crescente aumento dos locais que trabalham com nanotecnologia, isto devido ao potencial de aplicação destas nos mais variados setores industriais e ao impacto que seus resultados podem dar ao desenvolvimento tecnológico e econômico. Neste contexto, existe uma infinidade de áreas onde a nanotecnologia pode oferecer uma contribuição significativa, algumas das quais, inclusive, já possuem produtos sendo comercializados, como a indústria cosmética, farmacêutica e alimentícia (agronegócios) (El Naschie, 2006).

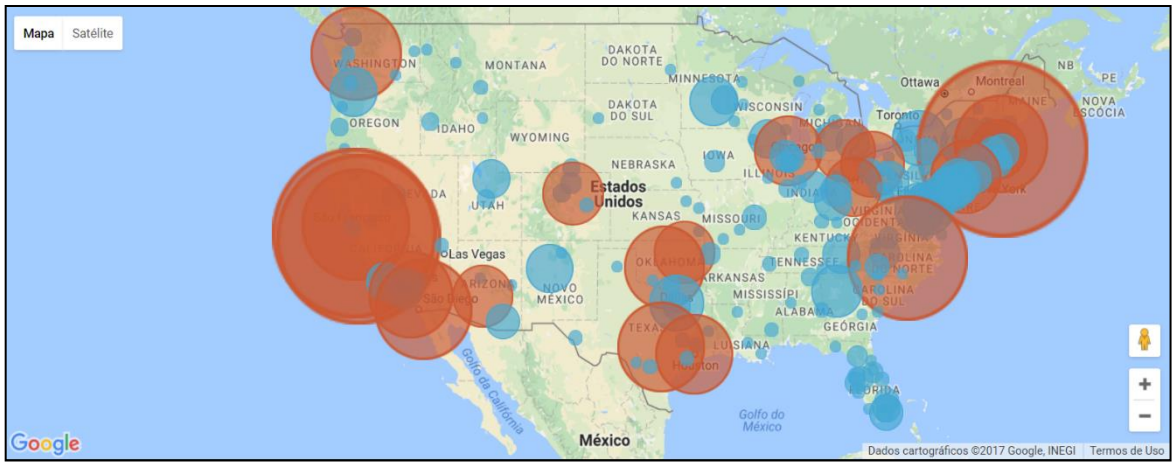


Figura 2 – Localização geográfica de empresas, universidades, laboratórios governamentais e organizações que trabalham com pesquisa, produção ou comercialização de nanotecnologia em todo os Estados Unidos (Nanotechproject, 2017).

Dentre as áreas que possuem interesse em NMs e suas aplicações estão a indústria farmacêutica, sobretudo na área cosmética, a indústria têxtil e eletrônica, além da sua crescente aplicação na engenharia e nas ciências médicas (Jain e Richey *et al.*, 2008).

Estudos recentes demonstram que as NPs ao serem aplicadas na área médica permitem que os elementos de contraste para diagnósticos de imagem, ressonância magnética e tomografias sejam refinados com maior contraste e precisão de imagem. Devido a isto, sugere-se que as NPs possam compor objetos de análise com tal refinamento que originem uma nova compreensão da área médica, sendo plausível supor que em breve existirá nova área médica, a nanomedicina. Uma vez que para além dos exames de imagens, há crescente uso desta nova tecnologia em tratamentos de câncer e até mesmo em carreamento e entrega de medicamentos (Ai *et al.*, 2011; Kucheryavy *et al.,* 2013). Devido a estas e muitas outras aplicabilidades, há crescente interesse em estudos com estes materiais, haja vista o aumento do número de patentes, publicações e incremento da produção científica verificados nas últimas duas décadas (Figuras 3 e 4).

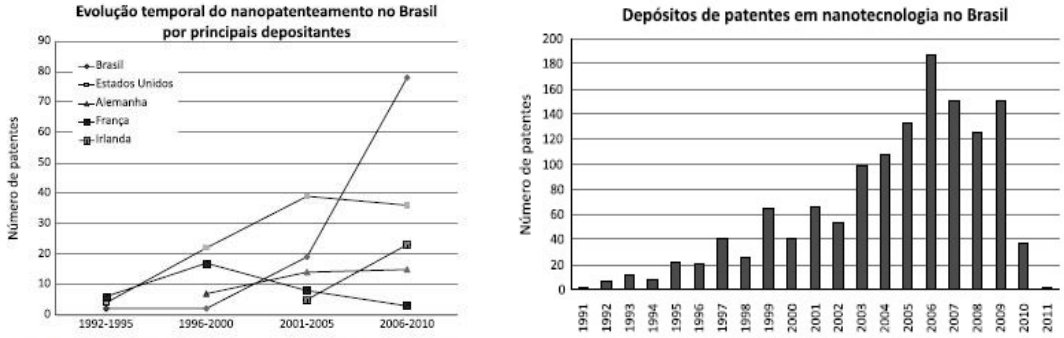


Figura 3 - Evolução ao longo do tempo do número de patentes e publicações envolvendo nanopartículas e nanotecnologia no Brasil e no Mundo (Sant'Anna et al, 2013).

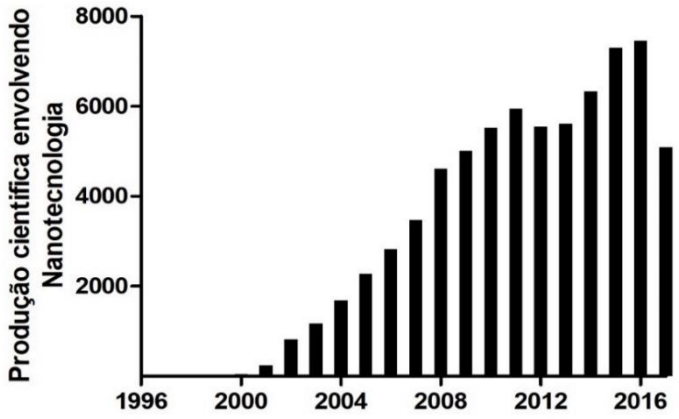


Figura 4 - Produção cientifica que cita a palavra "nanotechnology" como palavra-chave (Fonte:NCBI, Bando de dados da PUBMED)

Estes estudos indicam outras características das NPs, bem como o seu potencial associativo e de agregação qualificando-as para aplicações a diversos fins. Dentre estes em particular a nanoremediação, a qual traria benefícios enormes para mitigação de problemas de impactos ambientais, sendo esta a mais promissora área emergente da nanotecnologia com emprego na preservação de recursos naturais pela diminuição de efeitos da ação antrópica. Entretanto, para que tais métodos sejam eficazes, isto implicará na aplicação de nanomateriais reativos para transformação e desintoxicação de poluentes (Rajan, 2011).

Para tanto, estes NMs devem possuir propriedades que permitam redução química e catálise associativa aos poluentes presentes no ambiente a ser tratado. Tal procedimento consiste em aplicar NMs a águas ou solos contaminados para a redução ou decantação dos contaminantes, neutralizando ou facilitando a sua remoção (Karn et al., 2009). Devido a sua diminuta dimensão e a associação às NPs a inúmeros materiais , estas podem permear espaços muito pequenos na profundidade de camadas de materiais e permanecer suspensas nas águas subterrâneas, permitindo que as NPs penetrem muito mais do que partículas de maiores estruturas moleculares, desta maneira as NPs conseguem ampla distribuição e ação sobre partículas em solução, elementos em camada de tensão superficial como óleos ou parcialmente adsorvidos ao solo (Patil et al., 2015).

As nanopartículas de óxido de ferro (NOFs) e suas aplicações.

Nanopartículas de óxido de ferro (NOFs) se destacam dentre as demais devido as suas características exclusivas, tais como notável potencial para aplicações em diversas vertentes tecnológicas, pois se caracterizam por terem um tamanho na faixa de 3-20 nm em seus domínios magnéticos (partículas abaixo de um tamanho de partícula crítico (<20 nm), o que confere a elas um único domínio magnético) por isso, possuem alta potencialidade magnética e alto potencial associativo com tecidos biológicos e outros compostos químicos e nanoparticulados. As NOFs podem ser funcionalizadas com diferentes moléculas tais como ácido cítrico, DMSO, DMSA entre outros, podendo assim ser aplicadas na nanomedicina e na nanoremediação (Gupta e Gupta, 2005).

NOFs com tamanho e forma controlados são de grande interesse para a ciência e aplicações tecnológicas. Entre as NPs magnéticas, as ferritas, das quais se destaca a maghemita (γ-Fe^2O^3) constituem um grupo de materiais de alta permeabilidade e magnetização de saturação (Kim et al., 2012). Sendo assim as NOFs são relevantes para aplicação científica. No entanto, é essencial se avaliar criteriosamente seus usos nos sistemas biológicos (Silva et al., 2016).

No que tange a sua aplicabilidade, é descrito que na nanomedicina as NOFs são utilizadas para fabricar nanocompostos magnéticos, preparar fluidos magnéticos e carrear medicamentos. E suas propriedades também permitem transpor a barreira endotelial e se acumular especificamente nas células-alvo (Fig. 5). Tais características podem ser otimizadas por meio do recobrimento das NOFs com materiais biologicamente ativos. Para tanto, são necessários o controle de seus parâmetros físicos, tais como o tamanho da partícula, a susceptibilidade magnética da solução e o conhecimento do seu comportamento no organismo (Bietenbeck et al., 2016).

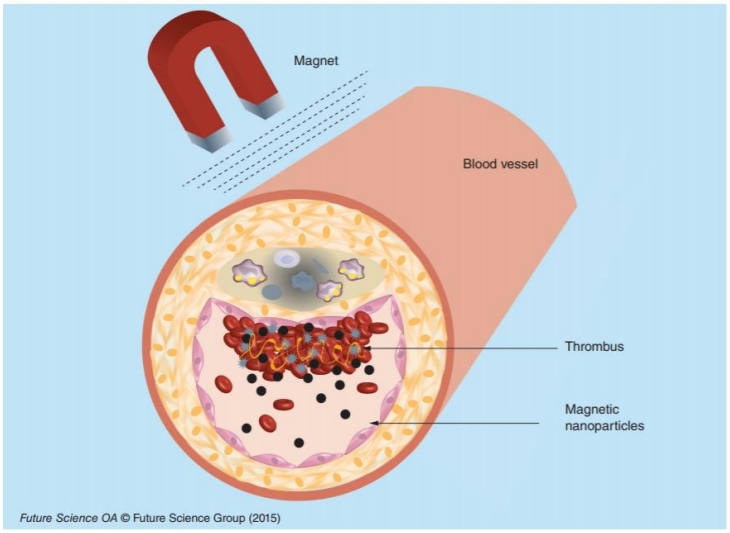


Figura 4 - Nanopartículas magnéticas de óxidos de ferro carregados com drogas trombolíticas são concentrados por um ímã externo para o tratamento de trombos intravenosos (Varna et al., 2015).

As NOFs são empregadas para a nanoremediação por terem potencial mais amplo, sobretudo no que se refere ao uso na contenção e imobilização de poluentes. Isto se deve a alta área de superfície associada à sua alta reatividade, o que as capazes de transformar ou degradar contaminantes em solos e água (Fig. 6). Por possuir potencial de redução de baixo padrão, sua dimensão favorece seu potencial de penetração em solos, sendo um dos compostos de notável eficiência no transporte através da matriz subterrânea e de lençóis freáticos (Tosco et al., 2014).

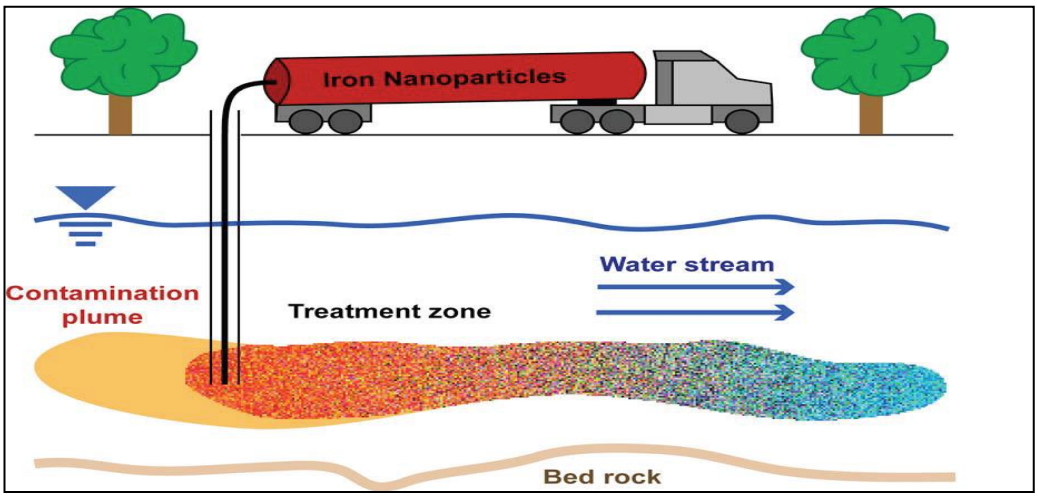


Figura 3- Esquema simplificado de aplicação de NOFs para remediação de águas subterrâneas contaminadas. A NOF é injetada na área contaminada pode até ser recuperadas posteriormente por tratamento magnético (Baumann, 2014).

Este potencial de agregação e imobilização se estende a outros metais pesados e até mesmo compostos clorados, pesticidas organoclorados, bifenilpoliclorados entre outros (Karn et al., 2009) (Fig. 7).

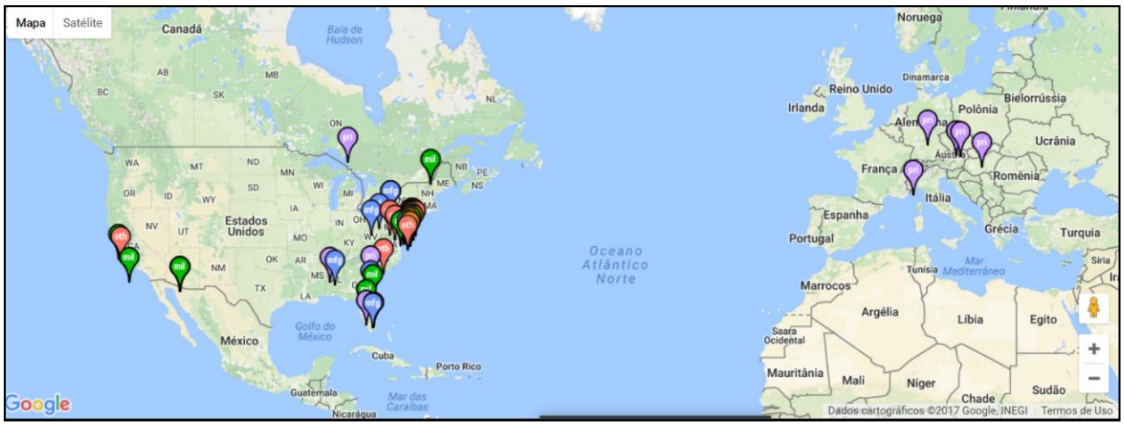


Figura 5- Localização geográfica de sítios contaminados utilizando alguma forma de nanoremediação utilizada em países da américa e Europa. Os pontos incluem campos de petróleo, fábricas, instalações militares, propriedades privadas e residências (Nanotechproject, 2017).

Potencial ecotóxico de nanopartículas de óxido de ferro (NOFs)

Mesmo com inúmeros estudos, pouco se sabe sobre o potencial impacto destas neste meio, bem como se desconhecem seus efeitos sobre os organismos aquáticos. Por isso, a preocupação com relação a sua toxicidade está relacionada ao fato das NOFs serem produzidas e utilizadas nos produtos comercias em larga escala, e isto amplia o risco de alcançarem os diferentes compartimentos ambientais (atmosfera, águas e solo) e se tornarem biodisponíveis (Paschoalino et al., 2010).

Os estudos relatam que a absorção de metais é mediada por transportadores de íons na membrana celular (Bury e Handy, 2010). Enquanto que a maior parte das NOFs é internalizada por endocitose, ou por meio de proteínas de membrana associadas (Panyam et al., 2003).

As NPs de maneira geral, dentre elas as NOFs adentram a célula principalmente por endocitose. Estudos mostram que partículas com 50 nm são mais rapidamente internalizadas quando comparadas as partículas com tamanhos menores que 14 nm ou maiores que 500 nm (Chithrani et al., 2006). Devido as suas características de fácil absorção para as células ainda não foi possível dimensionar os riscos que tais partículas podem causar ao organismo e ao ambiente.

O aumento da produção e uso de NOFs conduzirá inevitavelmente à sua liberação e permanência nas águas residuais e no ambiente aquático, em que estas NPs são consideradas contaminantes emergentes (Hall et al., 2009; Valdiglesias et al., 2016).

Embora na literatura tenha sido indicado baixa ou nenhuma toxicidade das NOFs em modelos de mamíferos (Petters et al., 2014), vários estudos demostraram que este nanomaterial tem potencial cumulativo e efeitos tóxicos sobre as espécies de peixes (Ates *et al.*, 2013; Remya *et al.*, 2015; Zhang *et al.*, 2015; Zhu *et al.*, 2012; Qualhato *et al.*, 2017). No entanto, a genotoxicidade e a mutagenicidade nos organismos aquáticos após a exposição aguda e a longo prazo a NOFs permanecem obscuras.

Para se avaliar adequadamente os sistemas não biológicos e sua funcionalidade as NOFs deveriam ser testadas quanto a sua associação em organismos vivos. Isto porque será possível avaliar os mecanismos de defesa celular, a ativação de várias vias celulares e bioquímicas, entre outros parâmetros. Uma vez que as análises em modelos biológicos são de grande relevância para se avaliar os impactos destes poluentes e suas implicações na saúde e bem-estar dos animais, entre eles o homem (Gouveia, 1999).

Desta maneira, os testes biológicos são importantes para verificar os possíveis efeitos toxicológicos da NOFs em determinado organismo, podendo ser ele o alvo da ação destas NPs. Com o crescente aumento do uso dessas NOFs em diversos campos, entre eles as áreas agrícolas e biológicas, as investigações de nanotoxicologia ambiental também precisam ser ampliadas para a avaliação de risco do uso desses contaminantes emergentes a fim de garantir segurança e saúde ambiental (Quandt *et al.*, 2014).

Modelo de estudo – *Poecilia reticulata*

Segundo estudos que relacionam biomonitores como diferentes biomarcadores (genéticos, bioquímicos, etológicos, histológicos, morfológicos e enzimáticos), estes são necessários para o biomonitoramento de um corpo hídrico, bem como, são empregados para se estabelecer a relação entre o grau da resposta dos biomarcadores as condições de exposição e a condição de saúde ambiental (Harayashiki *et al.*, 2013). Dentre os biomonitores indicados pela Organização para a Cooperação e Desenvolvimento Económico (OECD), consta da listagem o guppy, *Poecilia reticulata* (Peters,1859), pertencente ao grupo dos teleósteos e a família Poeciliidae.

A família Poeciliidae é compreendida por espécies de peixes que em sua maioria, possuem pequeno porte e são encontradas em regiões de climas tropicais, dentre essas está a espécie *P. reticulata* (popularmente conhecida como “guppy” ou guaru). Estes animais são originários da América Central (Caribe), embora também sejam nativos da Venezuela, Suriname, norte do

Brasil, Trinidad e Tobago, Barbados, México e Guiana (Fig. 8) (Araújo *et al.*, 2009).

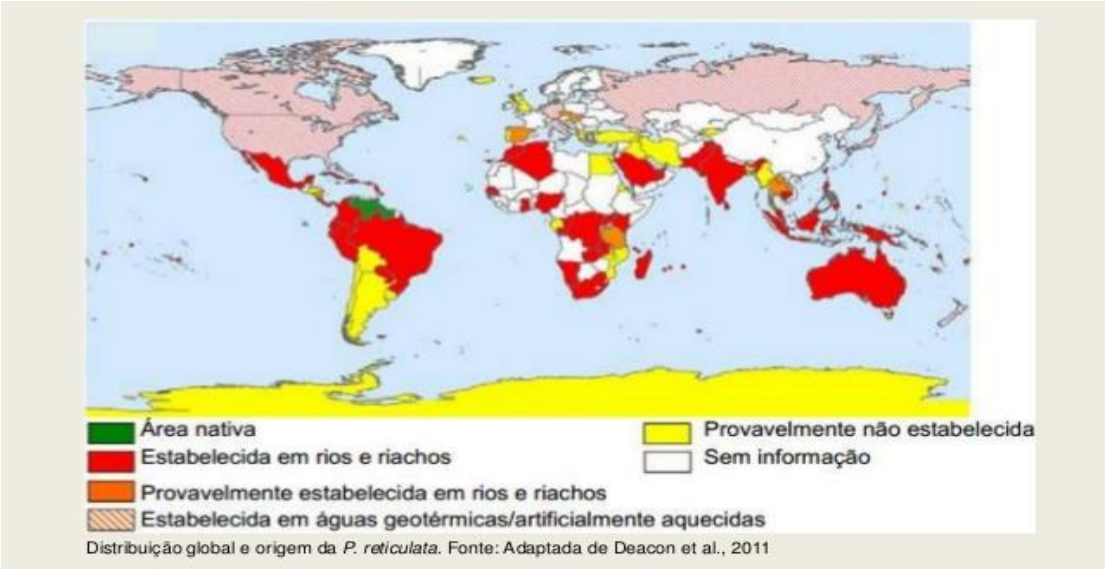


Figura 6 - Mapa indicando a localização geografica do P. reticulata ao redor do mundo.

Os guarus foram introduzidos em muitas regiões do mundo como uma importante espécie para o controle biológico atuando na predação de larvas de mosquito, além do interesse em torna-lo um peixe ornamental, com isso, ocorreu a sua ampla distribuição geográfica e ele passou a ser caracterizado como uma espécie cosmopolita (Montag *et al.*, 2011).

Esta espécie é caracterizada por apresentar dimorfismo sexual, possuir corpo coberto por escamas, nadadeira anal curta com 10 raios ou menos, para os machos essa nadadeira é modificada em uma estrutura para cópula denominada de gonopódio. Indivíduos machos possuem ainda manchas esféricas negras (ocelos) e regiões de coloração variada na metade posterior do corpo e na nadadeira dorsal (Fig. 9A). As fêmeas apresentam coloração castanho claro uniforme, ventre acinzentado, nadadeiras hialinas e, quando

adultas, seu comprimento total é maior quando comparadas aos machos (Fig. 9 B) (Casatti *et al.*, 2004).

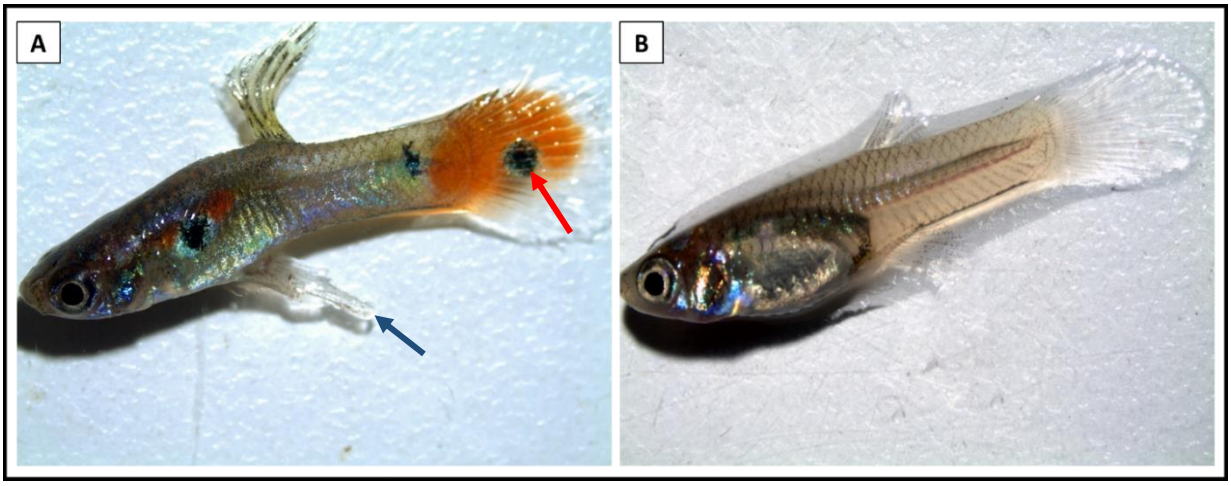


Figura 7. Dimorfismo sexual entre espécimes de *Poecilia reticulata.* (A) Espécime macho onde podemos visualizar o Gonopódio (seta azul) e o ocelo (seta vermelha) (19,1 ± 1,5 mm) (B) Espécime fêmea (22,7 ± 5,0 mm).

O *P. reticulata* é um peixe ovovivíparo com desenvolvimento interno em torno de 25 a 30 dias de duração, entretanto, a variação desse período depende da temperatura da água e da dieta que é destinada as fêmeas. De modo geral, as características ecológicas relacionadas com a variada dieta dos guarus possibilitam a sua sobrevivência em ambientes que sofreram alterações antrópicas (Montag *et al.*, 2011).

Os guarus respondem às alterações ambientais, sendo capaz de suportar condições que muitas outras espécies não suportariam, porque são eficientes para ativar diferentes vias de proteção celular e tecidual. Assim, muitas espécies da família Poeciliidae, principalmente *P. reticulata* são usadas como indicadoras da integridade dos habitats aquáticos por meio do estudo de suas características bioquímicas, fisiológicas, anatômicas, histológicas e etológicas (Montag *et al.*, 2011).

Modelos indicados para os testes toxicológicos e para os processos de classificação de risco ambiental devem ser de importância significativa na cadeia trófica aquática (Villarroel *et al.*, 2003). Nesse contexto, a espécie é indicada em normas de estudos ecotoxicológicos pela Associação Americana de Saúde Pública (APHA, 1989) e pela OECD (1992). Além do mais a espécie já foi utilizada para caracterizar o impacto ambiental de vários contaminantes, bem como utilizado na avaliação genotóxica e mutagênica (De Souza Filho *et al.*, 2013; dos Santos *et al.*, 2016; Escarrone *et al.*, 2016). Mas, poucos estudos vêm sendo feitos nesta espécie para testar uma provável toxidade de NOFs no ambiente.

Os bioensaios que utilizam *P. reticulata* como modelo biológico demonstram que os dados poderão ser usados como ferramenta primordial para dar subsidio à medidas mitigadoras (Araújo *et al.*, 2006), tendo a possibilidade de delineamento de bioensaios em ambientes laboratoriais para testes de toxidade de substâncias cujo potencial tóxico é pouco conhecido, como as NOFs.

Frente a esse cenário de incertezas sobre ação de NOFs pode-se destacar a importância dos peixes como organismos modelos (bioindicadores) para detecção de efeitos tóxicos oriundos da exposição aguda à NMs de ferro. E a aplicação deste modelo para elucidar o potencial tóxico da NOFs maghemita sobre os animais e o ambiente aquático.

Objetivos

Objetivos gerais

Analisar os efeitos tóxicos (mutagênicos, genotóxicos, citotóxicos, histopatológicos) das NOFs em fêmeas de Poecilia reticulata de potenciais biomarcadores de ecotoxicidade das NOFs em vertebrados aquáticos.

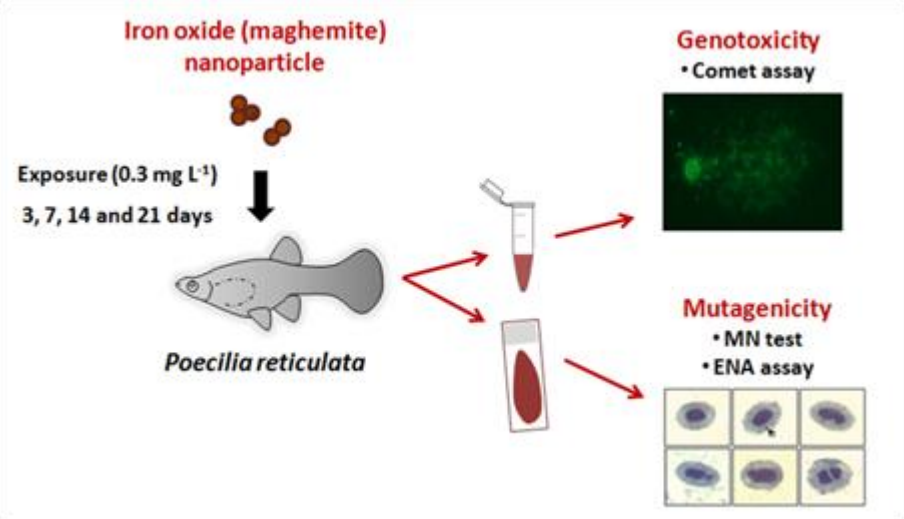
Objetivos específicos

- Síntetizar nanopartículas de óxido de ferro funcionalizadas com citrato.
- Caracterizar nanopartículas de óxido de ferro funcionalizadas com citrato.
- Analisar a genotoxidade e mutagênese em eritrócitos.
- Investigar a resposta da MMC à exposição NOFs.
- Analisar as alterações histopatológicas induzidas pelas NOFs por meio da abordagem qualitativa, quantitativa (resposta CMM) e semiquantitativa baseada em índices histopatológicos hepáticos.

Capítulo 2

Avaliação genotóxica e mutagênica da nanopartícula de óxido de ferro (maghemite-γ-Fe^2O^3) no guppy *Poecilia reticulata*.

Este capítulo trata do artigo já publicado na revista “Chemosphere” (Fator: 4.506; A1) no mês de Maio de 2017, contendo os resultados obtidos no presente estudo.



Genotoxic and mutagenic assessment of iron oxide (maghemite-γ-Fe_2O_3) nanoparticle in the guppy *Poecilia reticulata*

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ABSTRACT

The environmental risk of nanomaterials (NMs) designed and used in nanoremediation process is of emerging concern, but their ecotoxic effects to aquatic organism remains unclear. In this study, the citrate-coated (maghemite) nanoparticles (IONPs) were synthesized and its genotoxic and mutagenic effects were investigated in the female guppy *Poecilia reticulata*. Fish were exposed to IONPs at environmentally relevant iron concentration (0.3 mg L^{-1}) during 21 days and the animals were collected at the beginning of the experiment and after 3, 7, 14 and 21 days of exposure. The genotoxicity and mutagenicity were evaluated in terms of DNA damage (comet assay), micronucleus (MN) test and erythrocyte nuclear abnormalities (ENA) frequency. Results showed differential genotoxic and mutagenic effects of IONPs in the *P. reticulata* according to exposure time. The IONP induced DNA damage in *P. reticulata* after acute (3 and 7 days) and long-term exposure (14 and 21 days), while the mutagenic effects were observed only after long-term exposure. The DNA damage and the total ENA frequency increase linearly over the exposure time, indicating a higher induction rate of clastogenic and aneugenic effects in *P. reticulata* erythrocytes after long-term exposure to IONPs. Results indicated that the *P. reticulata* erythrocytes are target of ecotoxicity of IONPs.

Keywords: Nanoparticles; nanoremediation; bioaccumulation; DNA damage; MN test; guppy.

1. Introduction

The iron oxide nanoparticles (IONPs) have received increasing attention due to its ability to absorb and remove contaminants in groundwater and wastewater treatment, process known as nanoremediation (Sanchez-Galan et al., 1999; Fu et al., 2014; Adeleye et al., 2016), as well as its use in the nanomedicine, drug delivery, biosensors and electronic (Magro et al., 2012; Kolosnjaj-Tabi et al., 2015; Marcus et al., 2016). The use of IONPs in environmental remediation is mainly due to its nano-specific properties, such as higher surface area, higher reactivity, magnetic properties, low cost and eases to produce, as well as the low toxicity of iron ions (Fu et al., 2014; Adeleye et al., 2016; Gil-Díaz et al., 2016). The ability of the IONPs to trap contaminants has been described for inorganic and organic contaminants, such as Cu(II) (Chang et al., 2005), Pb(II) and Ni(II) (Tran et al., 2010) and rhodamine B (Wang et al., 2010).

The increased production and use of IONPs will inevitably lead to their release and permanence into the wastewater and aquatic environment, wherein these NPs are considered as emerging contaminants (Moore et al., 2006; Valdiglesias et al., 2016). Although the literature indicated none or low toxicity of the IONPs to mammal models (Petters et al., 2014), several studies showed that this engineered nanomaterial (ENM) induces metal accumulation and toxic effects on fish species (Zhu et al., 2012; Remya et al., 2015; Zhang et al., 2015; Ates et al., 2016). Fe_2O_3 (80 – 90 nm) and Fe_3O_4 (140 – 160 nm) NPs were uptake by gastrointestinal tract of *Danio rerio* after 28 days of exposure at 4 and 10 mg L^{-1} (Zhang et al., 2015), while α- Fe_2O_3 (30 nm; ≥ 10 mg L^{-1}) decreased the

embryo-hatching after 168 h of exposure (Zhu et al., 2012). *Labeo rohita* exposed to Fe_2O_3 (100 x 200 nm; 500 mg L^{-1}; 25 days) showed changes in haematological parameters, ionregulatory and gill Na^+/K^+-ATPase activity (Remya et al., 2015), indicating toxic effect of the IONP on fish physiology. On the other hand, *Oreochromis niloticus* exposed to α-Fe_2O_3 (20 – 90 nm) or γ-Fe_2O_3 (40 – 120 nm) at 0.1, 0.5 and 1 mg L^{-1} for 60 days showed no changes in haematological parameters and respiratory burst, while these NPs increased the glutamic oxaloacetic transaminase (GOT), glutamic pyruvic transaminase (GPT), lactate dehydrogenase (LDH) and myeloperoxidase (MPO) (Ates et al., 2016), indicating that the ecotoxicity of the IONP to fish species is exposure time and concentration dependent. However, the genotoxicity and mutagenicity in aquatic organisms after acute and long-term exposure to IONPs remain unclear.

In terms of biomarkers of genotoxicity, the comet assay is a suitable technique for the identification of DNA strand breaks induced by ENMs in fish erythrocytes (Bolognesi and Hayashi, 2011), while the micronucleus (MN) and erythrocyte nuclear abnormalities (ENA) assay has been indicated to measure mutagenic effects and chromosomal damage on fish after exposure to ENMs (Augusto and Tiago, 2008; Bolognesi and Hayashi, 2011). Therefore, the combination of both techniques may allow a more realistic analysis of genotoxic and mutagenic effects of ENMs on fish species, such as reported for other biomonitor species (Rocha et al., 2014; 2017).

Accordingly, the aim of this work was to synthesize the IONP (γ-Fe_2O_3 NPs), characterize their environmental behavior in aquatic systems and analyze their genotoxic and mutagenic effects in the female guppy *Poecilia reticulata* during long-term exposure (21 days). The biomonitor *P. reticulata* is

recommended by the American Public Health Association (APHA) (APHA, 1989) and by the Organization for Economic Cooperation and Development (OECD) (OECD, 1992) and used to characterize the environmental impact of several contaminants, as well as used in genotoxic and mutagenic assessment (Souza Filho et al., 2013; Rocha et al., 2015; Santos et al., 2017). However, but little is known about the mode of action (MoA), genotoxicity and mutagenicity of ENMs in the guppy *P. reticulata*. To the best of our knowledge, this is the first study of genotoxicity and mutagenicity in fish species exposed to IONPs.

2. Materials and Methods

2.1. Synthesis and characterization of IONPs

Citrate functionalized maghemite (γ-Fe_2O_3) NPs were prepared based on method described by Unal et al. (2010) and Zia et al. (2016) with modifications. 100 mL of a solution of Fe (II) sulfate ($FeSO_4 \cdot 7H_2O$) and Fe (III) chloride ($FeCl_3 \cdot 6H_2O$) (with the molar ratio 1:2, respectively) were filtered through 0.22-µm syringe filter (Kasvi, Brazil) to remove precipitates resulting of iron ions hydrolysis. To this solution, under strong mechanical agitation, was added 30 mL of 0.2 M sodium citrate ($Na_3C_6H_5O_7.2H_2O$) solution, followed by the addition of 50 mL of sodium hydroxide (NaOH) 5 $molL^{-1}$ solution. A black precipitated was immediately formed, and the suspension was mechanically stirred during 20 min in order to complete the iron ions s hydrolysis reaction. The magnetite suspension obtained was sonicated with a sonication probe (Q500 Sonicator, Qsonica) for 5 minutes in a amplitude 40 %. With the aim of to promote the oxidation of IONPs the suspension was heated at the boiling point (~ 96 ºC under oxygen bubbling for one hour, and stocked in glass bottle for 48 hours for total oxidation. The

reddish suspension obtained was dialyzed against demineralized water until the conductivity of the water became constant. The pH of the suspension was adjusted to 7 using NaOH and HCl diluted solutions, and after that, it was labeled as stock suspension. The total iron content of the stock suspension was measured by atomic absorption spectrophometry using a Spctrophotometer PerkinElmer AAnalyst 200.

2.1.2. Shape, size, surface charge and aggregation

Particle shape and individual size was characterized using Transmission Electron Microscopy (TEM), while surface charge (zeta potential) and hydrodynamic diameter (d_h) were analyzed by Dynamic Light Scattering (DLS) and Electrophoretic Light Scattering (ELS), respectively. For TEM analysis, a drop of the stock solution was deposited onto a mesh copper grid coated with a carbon layer and dry at room temperature. The images were obtained in a JEOL (JEM-2100) microscope, using image analysis software Scandium da Olympus Soft Imaging Solutions GmbH and ImageJ (National Institute of Health, USA). The d_h of IONPs was determined by DLS using a Malvern ZetaSizer, model Nano-ZS90. In these measurements, 12 mm square disposable polystyrene cells (DTS0012, Malvern, Inc.) were used. Zeta potential (ζ-potential) was determined using the same equipment in a disposable polycarbonate capillary cell (DTS1061, Malvern Inc.) at 25 ºC. For the analysis of the ζ-potential and d_h of IONPs in different aqueous media, IONPs (0.3 mg Fe L^{-1}) were suspended in Milli-Q water (18 MΩ/cm) and reconstituted water (ISO, 1986) at pH 7.0 and sonicated for 15 min.

2.1.3. X-ray Diffraction (XDR)

The synthesized IONPs were characterized by X-ray diffraction (XRD) using a diffractometer Shimadzu - XRD 600. This study was conducted with Cu Kα radiation. Samples of colloidal suspensions containing IONPs were dried for 18 hours at 60 °C and crushed to analysis. A continuous scan XRD data were collected at 2theta diffraction angles between 20° and 80° operating at 40 mA and 40 kV.

2.1.4. Infrared spectroscopy in KBr Pellet (IR-KBR)

The infrared spectrum (FTIR) of the citrate functionalized maghemite NPs were acquired by absorption technique in the infrared, Bruker Vertex 70. A aliquot of the colloidal suspensions containing IONPs were dried for 18 hours at 60 °C and the pellet were prepared with 1 % sample in KBr. Readings were taken in the spectral range of 400 - 4000 cm^{-1} with a resolution of 4 cm^{-1}, each spectrum being the result of the average of 64 scans.

2.1.5. Ultraviolet–visible spectroscopy (UV-Vis)

The stock IONP suspension was diluted with demineralized water and conditioned in quartz cells (1 mm optical path) and analyzed the optical absorption spectrum. The data was obtained in Perkin Elmer apparatus Lambda 1050. The absorption spectrum was obtained within an interval of 450 to 1850 nm.

2.2. Experimental design

Adult females of the *P. reticulata* were collected in the Water Treatment Station (Saneamento de Goiás – SANEAGO – 16° 37’ 59” S and 49° 15' 44" W, Goiânia, Goiás, Brazil) and acclimated during 30 days in static tanks (60 L) containing reconstituted water (ISO, 1986) at 27 ± 2 °C, pH (7.1 ± 2) and

photoperiod (14:12 h light/dark), such as recommended by (Rocha et al., 2015). Fishes were fed *ad libitum* three times per day with the commercial fish food Cardume 36 % (VB Alimentos Ltd).

After the acclimation period, 200 fish (total weight of 0.21 ± 0.09 g; total length of 2.63 ± 0.3 cm; standard length of 1.95 ± 0.3 cm) were placed in 50 L tanks filled with 30 L of reconstituted water (ISO, 1986) (2 fish L^{-1}) and exposed to 0.3 µg Fe L^{-1} of IONPs (citrate coated Fe_2O_3 NPs), jointly with a control group kept in clean reconstituted water in a triplicate design (3 tanks *per* treatment) for 21 days (exposure period), as recommended by the OECD guide (OECD, 1992). The IONP concentration used in this study is according to the iron concentration detected in the aquatic environment, such as reported by OECD (2011) and USEPA (2010). Furthermore, similar IONP concentrations were used in previous studies (Chen et al., 2013; Griffitt et al., 2013; Juganson et al., 2015; Zhang et al., 2015; Ates et al., 2016). Water was changed daily with redosing of the IONP concentration and fishes were fed with the same commercial fish food used in the acclimatization period.

Twenty fishes from each experimental condition were collected at the beginning of the experiment and after 3, 7, 14 and 21 days of exposure. Experiments were conducted in a static-renewal condition under 14h:10h light/dark cycles and abiotic parameters was analyzed daily by measuring temperature 27 ± 2 °C, pH (7.1 ± 2) and oxygen saturation (8.3 ± 0.5 mg L^{-1}) . No mortality was observed in the unexposed fish and those exposed to IONPs by the end of exposure period.

2.3. Genotoxicity

Genotoxicity was estimated using the comet assay (DNA damage) according to Singh et al. (1988) with modifications. Peripheral blood was obtained using the tail artery, centrifugated (1000 rpm; 1 min), diluted in agarose low melting ultra-pure (0.75 %) and spread in microscopic slides coated by agarose normal melting ultra pure (1.5 %). After soaking the slides in the lysis solution for 24 hours (1 % triton X-100, 10 % DMSO, 2.5 M NaCl, 100 mM Na_2EDTA and 10 mM Tris at pH 10) and the electrophoresis 1V cm^{-1} to 300 mA in the running solution (300 mM NaOH, 1 mM EDTA, pH > 13) is accomplished neutralization of the blades in Tris buffer (0.4 M Tris). The slides are fixed in 100 % ethanol and stored in the dark at room temperature. Slides are stained with SYBR® Green (S9430 Sigma-Aldrich) and analyzed in the fluorescence microscope Axio Imager 2 (Zeiss®) associated to ISIS® software (MetaSystems, Altlussheim, Germany). The Comet Imager® analysis system was used to score 100 randomly chosen cells for each slide (50 in each gel from each fish) at a total magnification of *x*400 (total of 1000 cells *per* experimental group). Cells were also categorized for grade of DNA damage using tail DNA % according to Almeida et al. (2011): zero or minimal 10 % tail, low damage 10 – 25 %, mid damage 25 – 50 %, high damage 50 – 75 %, and extreme damage > 75 %.

2.4. Mutagenicity

Mutagenicity was analyzed by MN test and ENA assay according to the protocol described by Carrasco et al. (1990) and Fenech et al. (2003) with modifications. Peripheral blood was obtained using the tail artery, centrifugated (1000 rpm; 1 min), diluted in 0.1 M PBS Buffer at pH 7.2, extended in microscopic slides and stained with hematology Stain (New Prov®). The ENA was measured

by determining the frequency of hemocytes with lobed nucleus (LN), binucleated nucleus (BN), segmented nucleus (SN), kidney-shaped nucleus (KN) and micronucleus (MN) according to Fenech et al. (2003) and Vignardi et al. (2015). The total ENA frequency is the sum of all nuclear abnormality frequency (LN + BN + SN + KN + MN).

2.5. Statistical analysis

Statistical analyses were carried out using the Statistica 7.0 software (Statsoft Inc., 2005, Tulsa, OK, USA). The results were compared using parametric tests (two-way ANOVA, followed by the Tukey's test) and/or non-parametric test (Kruskall-Wallis), depending on the distribution of the data and homogeneity of variance (Shapiro-Wilk and Levene's tests). Linear regression analyses were also applied to verify existing relationships between variables. Principal component analysis (PCA) was used to evaluate the relationship between DNA damage and nuclear alteration frequencies in unexposed fish and those exposed to IONP during the 21 days of exposure. Results were considered significant when $p < 0.05$.

3. Results and discussion

3.1. IONP characterization

The total iron content in the IONP stock suspension determined by atomic absorption spectrophotometry was 6.8 mg mL^{-1}. TEM results present crystalline and rounded IONP with an average individual diameter of 3.97 ± 0.85 nm (Fig. 10 A-B). The DLS and ELS analysis showed that the IONPs has higher d_h and high surface charge in reconstituted water (21.4 ± 0.39 nm and -19.5 ± 6.5 mV) compared to ultrapure water (14.11 ± 0.2 nm and -51.1 ± 7 mV) (Fig. 11 A-B),

indicating that the ionized citrate groups confer negative surface charge of IONPs. The zeta potential value observed in the IONPs dispersed in reconstituted water is attributed to presence of the cations Ca^{2+} and Mg^{2+} which are adsorbed on negative nanoparticles surface and partially neutralize the negative charge provided by the COO^- groups.

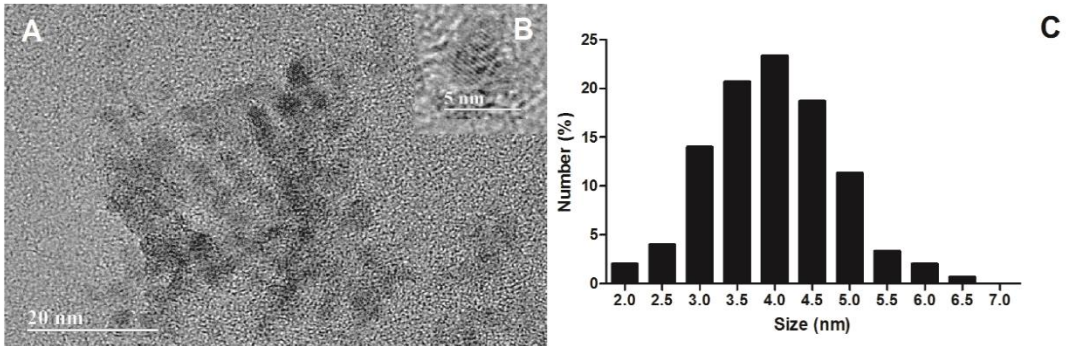

Figure 8 - Transmission Electron Microscopic (TEM) image of iron oxide (maghemite) nanoparticle (IONPs) (A and B) and the individual diameter histogram of IONPs obtained from TEM figures (C).

The XRD pattern presented in Fig. 11-C shows broad pick with low intensity characteristic of small particles. The picks relative to the plans 311 and 440 characteristics of the cubic spinel phase were indexed according to International Center for Diffraction Data (ICDD; PDF No. 39-1346). The absence of Fe (II) ions reveled by the UV-vis-Near IR added to features of the XRD pattern allows to characterize the IONPs as maghemite phase. In the FTIR spectrum (Fig 11-D) of the citrate functionalized IONPs the bands observed at 1594 cm^{-1} and 1380 cm^{-1} are ascribed to the asymmetric and symmetric stretching vibration of carboxylate, respectively. The presence of such bands confirms the efficient functionalization of the NPs by citrate ions (Morais et al., 2006). Fig. 11-E present the UV-Vis-NIR spectrum of the IONP suspension which present the characteristic absorption near the UV wavelength region ascribed as the

presence of Fe (III) ions in tetrahedral site (Sherman and Waite, 1985). The absence of the band at the near IR attributed to the temperature-dependent electron exchange between Fe(II) and Fe(III) ions, characteristic of the magnetite phase (Tang et al., 2003), revels that the precipitated magnetite NPs were completely converted to maghemite by oxidation.

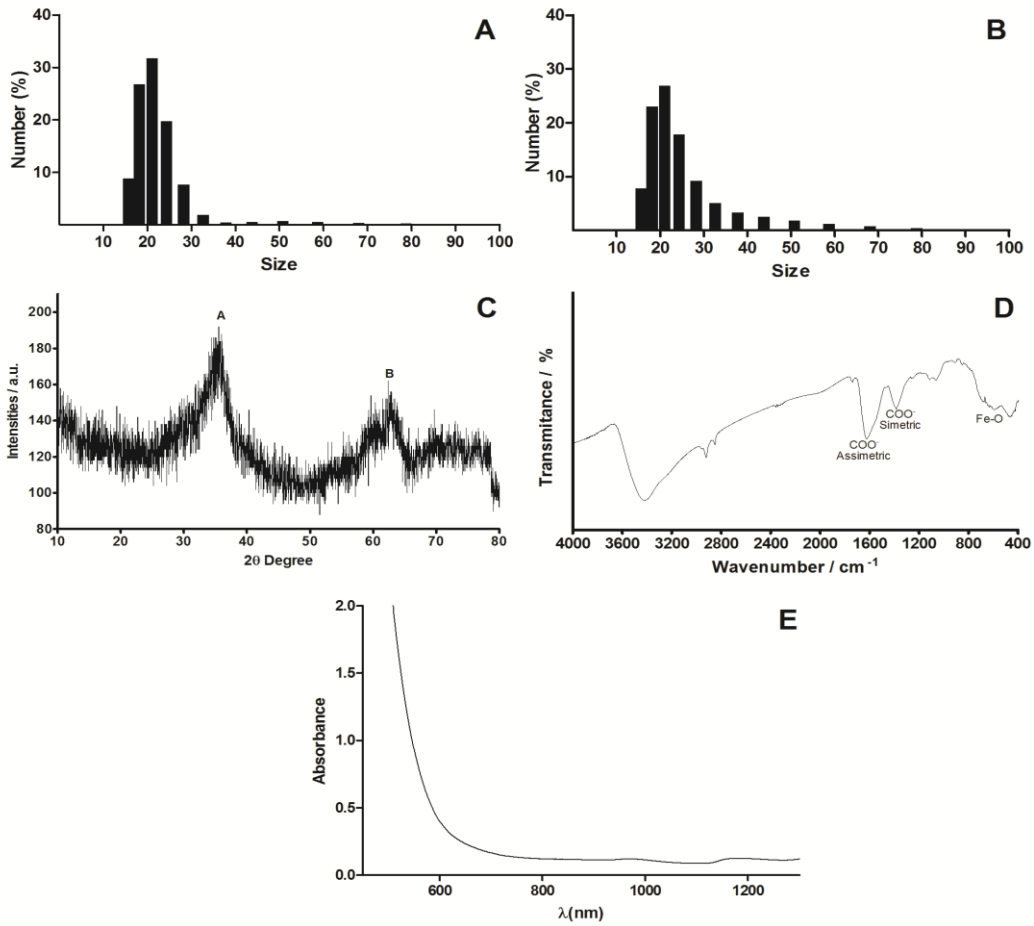

Figure 9 - Hydrodynamic diameter of iron oxide (maghemite) nanoparticle (IONPs) suspended in milli-Q water (A) and in reconstituted water (B). IONPs Powder XRD diffraction patter (C). FTIR spectrum of the citrate functionalized IONPs (D). Representative UV-Vis-NIR absorption spectrum of IONPs (E).

3.2. Genotoxicity

The DNA damage in peripheral erythrocytes of the *P. reticulata* was analyzed by comet assay and expressed as % of tail DNA (Figs. 12-13) and the

results of the olive tail moment (OTM) and comet tail length are in the supplementary material (Fig. S1 A-B). DNA damage of unexposed fish did not change over time and no significant changes were detected in any of the parameters during the course of the experiment ($p > 0.05$; Figs. 12-13).

However, early DNA damage was detected in fish exposed to IONPs for 3 (1.9-fold) and 7 (2.1-fold) days compared to those unexposed ($p < 0.05$; Figs. 12-

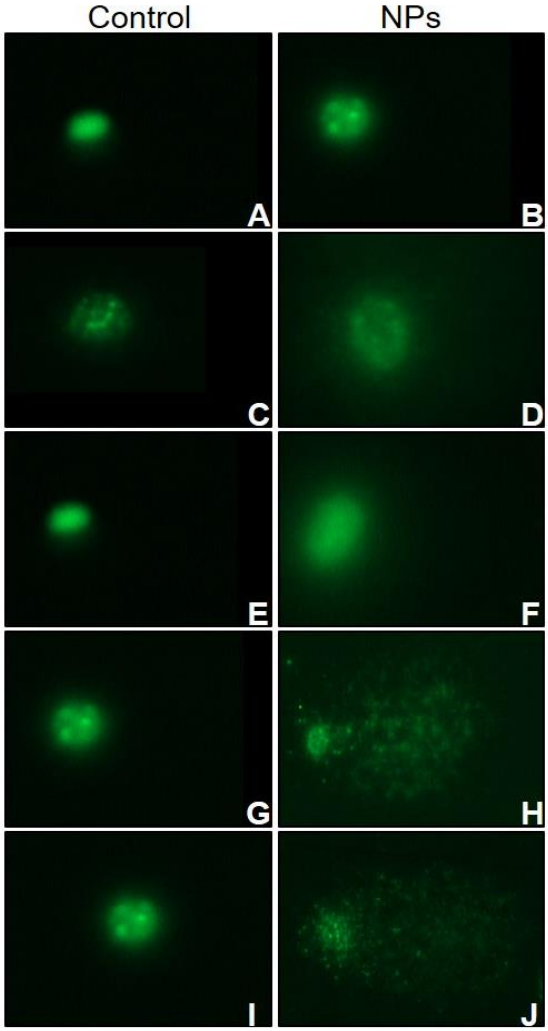

Figura 10- Representative comet assay image in erythrocytes of the guppy P. reticulata from control and exposed to IONPs at the beginning of the experiment (A-B) and after 3 (C-D), 7 (E-F), 14 (G-H) and 21 (I-J) days. Cells were stained with SYBR® Green and image recorded with an optical fluorescence microscope Axio Imager 2 (Zeiss®) associated to ISIS® software (MetaSystems, Altlussheim, Germany) using a total magnification of 400x.

13). On the other hand, higher DNA damage was observed in IONP-exposed fish after 14 (11.3-fold; $p < 0.05$) and 21 (13.7-fold; $p < 0.05$; Figs. 3-4) days compared

to unexposed fish, indicating that the genotoxic effects induced by IONP exposure are exposure time dependent (y = 1.5502x + 0.7339; r^2 = 0.90; p < 0.05), with higher DNA damage levels detected after long-term exposure when compared to acute exposure.

The % of DNA in the tail was also used to categorize the grade of damage in unexposed and IONP-exposed fish (Table 1). Erythrocytes from unexposed fish showed minimal or low damage along all exposure period (from 0 % to 14.81 %). On the other hand, a significant percentage of cells with mid and high damage was observed in INOP-exposed fish after 3 (mid: 4 %; high: 0.2 %) and 7 days (mid: 2 %; high: 0.8 %), while fish exposed to IONP for 14 and 21 days showed

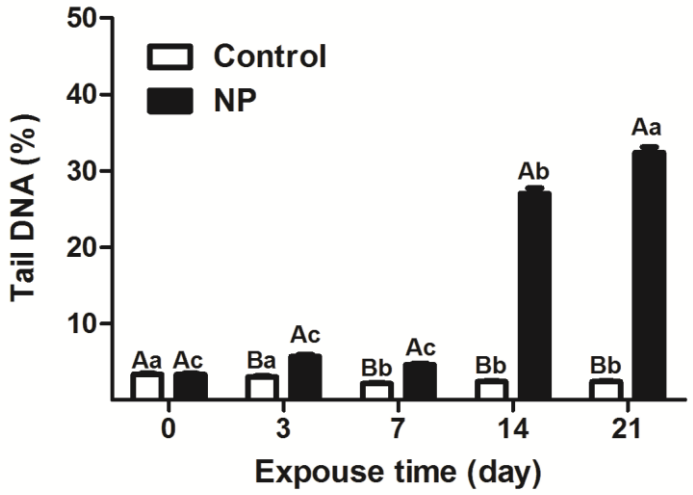

Figura 11 - DNA damage expressed as tail DNA % (mean ± SD) in *P. reticulata* from control and exposed to iron oxide (maghemite) nanoparticle (IONPs) during 21 days.

higher percentage of cell with high (14 d: 14.8 %; 21 d: 17.9 %) and extreme damage (14 d: 2.4 %; 21 d: 3.9 %) (Table 1), confirming that the IONPs are more genotoxic after long-time exposure.

	Time	DNA Damage (%)				
		0 a 10	10 a 25	25 a 50	50 a 75	75 a 100
Control	0	90.8	9.2	0	0	0
	3	93.1	6.9	0	0	0
	7	97.9	2.1	0	0	0
	14	96.7	3.3	0	0	0
	21	95.2	4.8	0	0	0
IONPs	0	90.8	9.2	0	0	0
	3	82.8	13	4	0.2	0
	7	89.8	7.3	2	0.8	0.1
	14	30.9	20.1	31.8	14.8	2.4
	21	14.9	28.6	34.7	17.9	3.9

Table 1- Frequency of erythrocytes (%) distributed by grade of DNA damage in *P. reticulata* from control and exposed to iron oxide (maghemite) nanoparticle (IONPs) for 21 days.

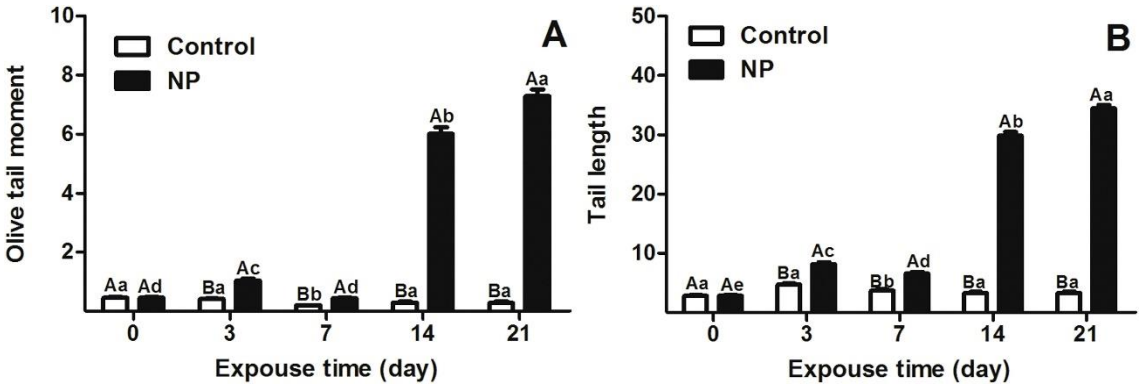

Figure S1 - Olive tail moment (OTM) (**A**) and tail length (**B**) (mean ± SD) in erythrocytes of the P. reticulata from control and exposed to iron oxide (maghemite) nanoparticle (IONPs) during 21 days.

The IONPs may affect the DNA structure by direct or indirect mechanisms. If the NPs that are present in the nucleus might directly interact with DNA organized in chromatin or chromosomes depending on the phase of cell cycle and for this reason a relations in NPS and DNA fragmentation is a viable possibility (Jiménez-Villarreal et al., 2017). Thereafter, they may interact in DNA bases and cause a direct damage both genotoxicity as mutagenic by direct form (Magdolenova et al., 2014). But if it interacts with other nuclear structures (nuclear proteins, organelles, membrane proteins) the DNA damage become a consequence of secondary lesions outside the nucleus. In this sense, the DNA damage can occur as DNA base modifications by oxidation, DNA single strand

breaks, DNA double strand breaks, cross links or structural DNA changes. Previous studies indicated that the exposure to IONPs induced reactive oxygen species (ROS) production, changes of the antioxidant enzyme activities and oxidative stress in different fish species (Li et al., 2009; Chen et al., 2013; Srikanth et al., 2014; Ates et al., 2016), indicating that the genotoxic effects observed in *P. reticulata* are mediated by oxidative stress. The system of repair to DNA damage can influenced on this results, in the first days this repair was active, but after 14 days this system suffer a overload, and the damage increase. Other studies showed that de IONPs, given the direct adherence, can aggregate to cell surface this iron overload could thus have toxic implications and an imbalance in homeostasis (Zhu et al., 2012). The ROS can be originated by this inflammatory process caused by the interactions of magnetical NPs with erythrocytes. As know the inflammation is a path to induce a formation of ROS, those inflammations when exceed the capacity of systems cell repair can damage to DNA (Rim et al., 2013).

3.3. Mutagenicity

The mutagenic effects induced by INOPs are exposure time dependent, wherein higher nuclear abnormalities were observed after long-term exposure when compared to acute exposure (Figs. 14-15). The ENA frequency in unexposed fish remains unchanged during the whole experiment and is according to ENA level previously reported for *P. reticulata* (Souza Filho et al., 2013). However, the total ENA frequency in IONP-exposed fish increase linearly until the end of the exposure period ($y = 3.3214x + 1.5476$; $r^2 = 0.94$; $p < 0.05$;

Fig. 15-A), indicating a higher induction rate of clastogenic and aneugenic effects in *P. reticulata* erythrocytes after long-term exposure to IONPs.

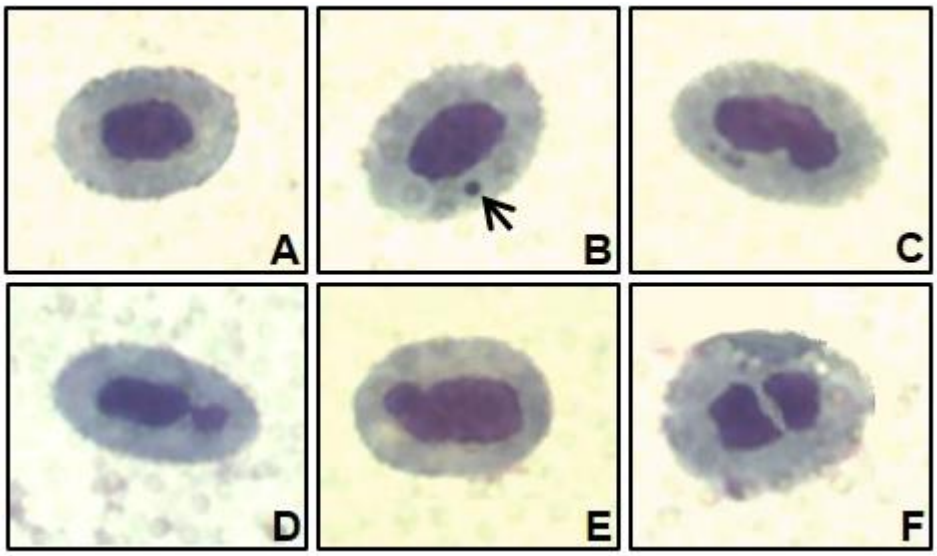

Figure 12 - Erythrocyte nuclear alterations (ENA) observed in *P. reticulata* after exposure to iron oxide (maghemite) nanoparticle (IONPs) for 21 days. (A) Normal nucleus. (B) Micronucleus (arrow). (C) Kidney-shaped nucleus. (D) Segmented nucleus. (E) Lobed nucleus. (F) Binucleated cell.

The fish exposed to IONPs showed a linear increase in the KN cell frequency during 14 days of exposed ($y = 0.9648x + 4.2166$; $r^2 = 0.81$; $p < 0.05$), reaching then a steady state after 21 days of exposure (Fig. 15-B). On the other hand, significant frequency of LN, SN and MN cells was only observed after exposure to IONPs for 14 (197, 64, 63, respectively) and 21 days (310, 110, 62, respectively) ($p < 0.05$; Fig. 15 C-E), while higher BN cell frequency was observed only after 21 days of exposure ($p < 0.05$; Fig 15-F). The formation of the KN, SN and MN in *P. reticulata* could be related to effects of IONPs on tubulin polymerization (Alexander et al., 2016; Sadiqul et al., 2016), no efficient capacity of the fish to expel damaged chromatin or altered chromosome fragments and/or associated to clastogenic effect of IONP on peripheral erythrocytes. On the other hand, MN are small chromatin present outside the main nucleus formed by

acentric chromosome fragment or by whole chromosome formed in abnormal anaphase (Fenech et al., 2011). In this sense, the results of the MN and BN frequency indicated that the IONPs can induce changes in the cytoskeleton (e.g. microtubules) during the mitosis of erythropoiesis of the *P. reticulata*. Longer-term effect of IONPs and magnetic particles can interfere in the actin and tubulin cytoskeleton resulting in alterations of cytoskeleton architecture, formation of non-specific adhesion and impair cell division, resulting in nuclear alteration (Soenen et al., 2010; Singh et al., 2010).

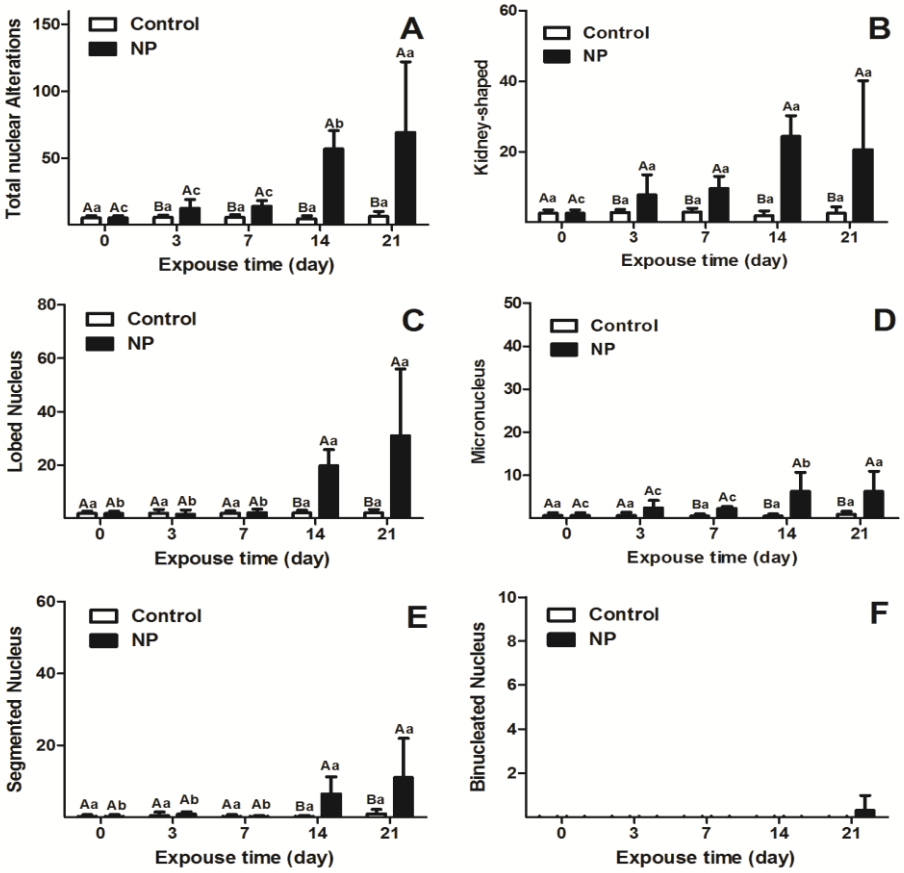

Figure 13 - Total erythrocyte nuclear alterations (A) and frequency of the erythrocytes with kidney-shaped nucleus (B), lobed nucleus (C), micronucleus (D), segmented nucleus (E) and binucleated cells (F) in P. reticulata from control and exposed to iron oxide (maghemite) nanoparticle (IONPs) for 21 days. Results was expressed as mean ± SD.

Linear correlation was observed between LN (y = 1.521x - 2.4693; r^2 = 0.92; p < 0.05), SN (y = 0.5421x - 1.1786; r^2 = 0.9046; p < 0.05) and MN (y = 0.281x + 1.0307; r^2 = 0.87; p < 0.05) and the exposure time, confirming the mutagenic effects of the IONP after long-term exposure. Although the mechanisms underlying the formation of ENAs in fish species are not fully known, several studies indicate that ENAs are induced in fish after exposure to different metal NPs, such as reported in *Oryzias latipes* exposed to Fe-oxide and Fe(II) NPs (30.1 nm; 1 to 100 mg L^{-1}; 7 and 14 days) (Chen et al., 2011), *O. niloticus* exposed to Fe_2O_3 NPs (30 – 40 nm; 0.1 - 1.0 mg L^{-1}; 30 and 60 days) (Ates et al., 2016) and to ZnO NPs (10 – 30 nm; 1 and 10 mg L^{-1}; 14 days) (Kaya et al., 2016), *D. rerio* exposed to oxide NMs (4.0 and 10.0 mg L^{-1}; 28 days) (Zhang et al., 2015) and Ag NPs (3.10 nm; 5 – 50 g L^{-1}; 2 day) (Griffitt et al., 2013). Results observed in the present study indicate that the MN and ENA frequency in fish are important biomarker of mutagenicity of IONPs used in nanoremediation process.

3.4. Principal component analysis (PCA)

The PCA on the data indicate differential genotoxic and mutagenic effects of the IONPs in the *P. reticulata* according to exposure time (Fig. 16 A-B). The two principal components represent 93.81 % (PC1 = 85.54 %, PC2 = 8.27 %) of total variance, showing a clear separation between unexposed fish and those exposed to IONPs for 14 and 21 days, while similar responses were observed between unexposed fish and those exposed to IONPs for 3 and 7 days (Fig. 7A), confirming that the genotoxicity and mutagenicity of IONPs is exposure time dependent. After 7 days of exposure to IONPs, fish response was associated to KN, MN and DNA damage (% DNA tail, OTM and tail length), while the response to IONP exposure for 21 days was associated to exposure time, BN, SN, LN and

DNA damage. Results agree with previous studies that showed that the IONPs induce genotoxic and mutagenic effects to brain, liver and erythrocyte mammals cells after *in vitro* exposure to IONPs (Liu et al., 2014; Petters et al., 2014) and to fish species, such as *A. Anguilla and D. rerio* and mice (Anjum et al., 2014; Gaharwar and Paulraj, 2015; Cáceres-Vélez et al., 2016).

4. Conclusions

Although the IONPs have been used in the environmental remediation, the environmental risk concerning the use of IONPs, their mode of action and

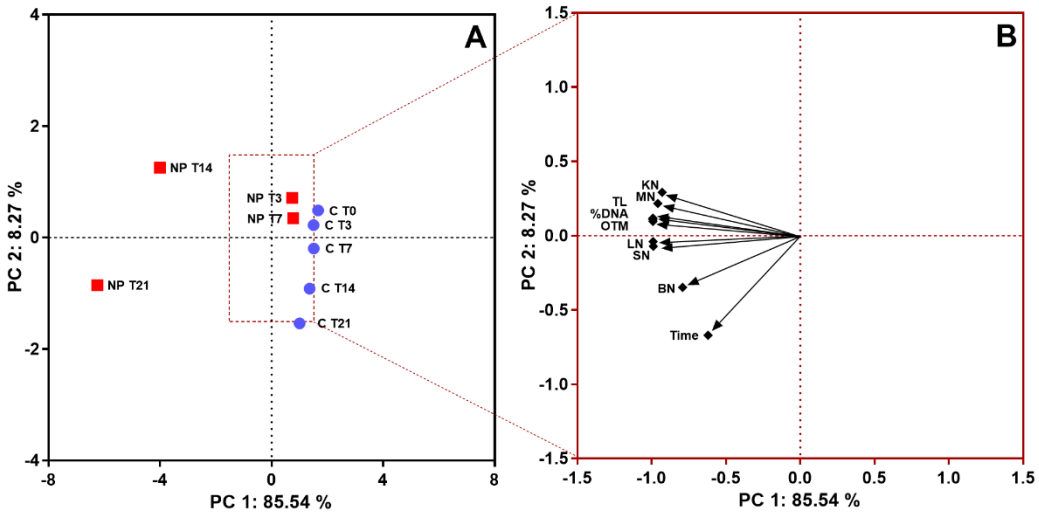

Figure 14 - Principal component analysis (PCA) of DNA damage (% DNA tail, OTM and TL) and nuclear alteration frequencies (LN, BN, SN, KN and MN) in *P. reticulata* from control and exposed to iron oxide (maghemite) nanoparticle (IONPs) for 21 days.

ecotoxicity in aquatic organisms remain insufficient. The present study indicates that the citrate-coated maghemite (Fe_2O_3) NPs at environmental relevant concentration (0.3 mg L^{-1}) are genotoxic and mutagenic in the female *P. reticulata* in an exposure time dependent pattern. Early genotoxic effects analyzed by comet assay were identified in fish exposed to 3 and 7 days, while higher genotoxic and mutagenic effects were observed after long-term exposure (14 and 21 days). Results indicate that the comet assay is a sensitive technique to

detected early DNA damage induced by IONP exposure in the peripheral erythrocyte of the *P. reticulata.* The association between ENA and MN test is a better mutagenic assessment approach than MN test alone to detected changes in nuclear erythrocyte morphology in fish exposed to NPs used in nanoremediation processes. The results indicated the *P. reticulata* as target species to carry out genotoxic and mutagenic tests of IONPs.

Acknowledgments

This work was funded by the by Fundação de Amparo à Pesquisa do Estado de Goiás (FAPEG; edital nº 003/2016) and by Postdoctoral National Program (PNPD) from the Coordination for the Improvement of Higher Education Personnel (CAPES). The authors also acknowledge CRTI, LabMic-UFG and Central analítica IQ-UFG for their collaboration in the characterization of IONPs.

O artigo pode ser acessado na integra pelo seguinte link: https://doi.org/10.1016/j.chemosphere.2017.05.061

Chemosphere 183 (2017) 305–314

Contents lists available at ScienceDirect

Chemosphere

journal homepage: www.elsevier.com/locate/chemosphere

Genotoxic and mutagenic assessment of iron oxide (maghemite-γ-Fe_2O_3) nanoparticle in the guppy *Poecilia reticulata*

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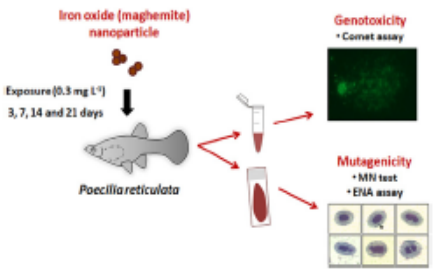
HIGHLIGHTS

- Genotoxicity and mutagenicity of IONPs in guppies are exposure time dependent.
- Early genotoxic effect in guppies after acute and long-term exposure to IONPs.
- Higher mutagenic effects in *P. reticulata* after long-term exposure to IONPs.
- Erythrocytes of *P. reticulata* are important target to IONP ecotoxicity.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 3 March 2017
Received in revised form
4 May 2017
Accepted 10 May 2017
Available online 13 May 2017

Handling Editor: Tamara S. Galloway

Keywords:
Nanoparticles
Nanoremediation
Bioaccumulation
DNA damage
MN test
Guppy

ABSTRACT

The environmental risk of nanomaterials (NMs) designed and used in nanoremediation process is of emerging concern, but their ecotoxic effects to aquatic organism remains unclear. In this study, the citrate-coated (maghemite) nanoparticles (IONPs) were synthesized and its genotoxic and mutagenic effects were investigated in the female guppy *Poecilia reticulata*. Fish were exposed to IONPs at environmentally relevant iron concentration (0.3 mg L^{-1}) during 21 days and the animals were collected at the beginning of the experiment and after 3, 7, 14 and 21 days of exposure. The genotoxicity and mutagenicity were evaluated in terms of DNA damage (comet assay), micronucleus (MN) test and erythrocyte nuclear abnormalities (ENA) frequency. Results showed differential genotoxic and mutagenic effects of IONPs in the *P. reticulata* according to exposure time. The IONP induced DNA damage in *P. reticulata* after acute (3 and 7 days) and long-term exposure (14 and 21 days), while the mutagenic effects were observed only after long-term exposure. The DNA damage and the total ENA frequency increase linearly over the exposure time, indicating a higher induction rate of clastogenic and aneugenic

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http://dx.doi.org/10.1016/j.chemosphere.2017.05.061

Capítulo 3

Resposta de melanomacrófagos e biomarcadores histopatológicos hepáticos no Guppy Poecilia reticulata expostos a nanopartículas de óxido de ferro (NOFs).

Este capitulo descreve resultados já finalizados e submetidos a revista “Aquatic Toxicilogy” (Fator: 4.129; A1) E já foi revisada e corrigida pelos revisores e autores e está aguardo a decisão final.

Melanomacrophage response and hepatic histopathological biomarkers in the guppy *Poecilia reticulata* exposed to iron oxide (maghemite) nanoparticles

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ABSTRACT

Although iron oxide nanoparticles (IONPs) have been widely used in nanomedicine and nanoremediation, their ecotoxic effects on aquatic organisms remain unclear. In this study, the melanomacrophage center (MMC) response and hepatic histopathologic biomarkers were investigated in female guppies, Poecilia reticulata, exposed to citrate-functionalized IONPs (γ-Fe2O3) at an environmentally relevant iron concentration (0.3 mg L-1) over 21 days. The animals were collected at the beginning of the experiment and after 3, 7, 14, and 21 days of exposure. Guppies exposed to IONPs showed increases in the number, area, and perimeter of MMC when compared with the unexposed ones, especially after 7 days of exposure. The results showed an increase in the frequency of histopathologic changes in fish after 7 days of exposure to IONPs, such micro- and macro-vesicular steatosis, melanomacrophage aggregates, exudate, and hemorrhagic foci. The acute (3 and 7 days) and long-term (14 and 21 days) exposure of P. reticulata to IONPs induced high histopathologic indexes associated with circulatory disorders and inflammatory responses. Results showed that the MMC response and histopathologic index are important biomarkers to indicate the environmental impact of IONPs, confirming that the guppy P. reticulata is a target of ecotoxicity of IONPs.

Key words: Nanomaterials; nanoecotoxicity; tissue-level biomarkers; immune response; guppy..

1. Introduction

The melanomacrophage centers (MMC), also known as macrophage aggregates, play an important role in the immune response and immunologic protection in fish species and have been used as biomarkers to assess fish health and aquatic environmental pollution (Agius and Agbede, 1984; Haaparanta et al., 1996; Capkin et al., 2017; Fu et al., 2017).

The melanomacrophages (MMs) are multifunctional cells that perform phagocytosis of red blood cells, hematopoietic cells, and cell debris; detoxification of exogenous and endogenous molecules; iron recycling/metabolism; and accumulation of different pigments such as melanin, lipofuscin and hemosiderin (Agius, 1984; Wolke et al., 1985). The pigment melanin neutralizes the free radicals released during cellular membrane breakdown, whereas the lipofuscin and hemosiderin are sub-product of non-saturated fatty acid peroxidation and breakdown of hemoglobin, respectively (Agius and Agbede, 1984; Wolke et al., 1985). Furthermore, clusters of adjacent B-cells, CD4, and T-cell receptor beta chain (TCRβ) were observed in the MMCs (Saunders et al., 2010; Diaz-Satizabal and Magor, 2015), indicating that the MMCs are analogs, or “primitive” evolutionary precursors, of the germinal center (GC) in mammalians, and represent a primitive site of adaptive immune system activation in organism poikilotherms (Steinel and Bolnick, 2017). The increase in the number and size of MMCs was reported in fish species exposed to metals (Sayed and Younes, 2016), herbicides (Rocha et al., 2015a; Santos et al., 2017), antimicrobial agents (Capkin et al., 2017) and in fish infected by parasites (Ventura et al., 2016). However, the knowledge about the effects of emerging pollutants such as nanoparticles (NPs) on the MMC role in fish pathology remains

unknown. The NPs are structures with at least one dimension between 1 and 100 nm and are considered to be an emerging concern for environmental and human health due to their nano-specific properties, dissolution capacity and release of toxic materials, as well as nano-specific mode of action (MoA) (Moore, 2006; Rocha et al., 2015b, 2017).

Among the engineered NPs, the iron oxide nanoparticles (IONPs) are used in nanomedicine, pharmacy, and electronics, as well as in nanoremediation for absorbing and removing contaminants in groundwater and wastewater treatment (Kolosnjaj-Tabi et al., 2015; Adeleye et al., 2016; Marcus et al., 2016). The major forms of manufactured IONPs with extensive applications include magnetite (Fe_3O_4), hematite (α-Fe_2O_3), and maghemite (γ-Fe_2O_3) (Su et al., 2017). However, despite their increasing production and use, the environmental impact and MoA of IONPs on aquatic organisms remain unclear. The genotoxic and mutagenic effects of citrate-functionalized (γ-Fe_2O_3) NPs (3.97 nm; 0.3 mg L^{-1}) were observed in the guppy *Poecilia reticulata* during 21 days of exposure (Qualhato et al., 2017), whereas the exposure to α-Fe_2O_3 NPs (100 × 200 nm) at 500 mg L^{-1} for 25 days induced changes in the hematologic parameters and ion regulatory process in *Labeo rohita* (Remya et al., 2015). Furthermore, the iron accumulation induced by exposure to Fe_2O_3 (80 – 90 nm) and Fe_3O_4 (140 – 160 nm) NPs at 4 and 10 mg L^{-1} for 28 days was reported in the zebrafish *Danio rerio* (Zhang et al., 2015), whereas transcriptional changes related to cell growth and proteins synthesis and mild lipid peroxidation were observed in the zebrafish exposed to γ-Fe_2O_3 coated with meso-2,3-di-mercaptosuccinic acid (DMSA) (5.7 nm; 4.7 – 74.4 mg L^{-1}) for 96 h (Villacis et al., 2017).

Although the effects of IONPs on immune response and their association with histopathologic biomarkers in fish species are unknown, the MMCs were recently indicated as immunohistologic biomarkers in the African catfish, *Clarias gariepinus*, exposed to Ag NPs (100 nm; 25 – 75 mg L^{-1}) for 14 days (Sayed and Younes, 2016) and in *Oreochromis niloticus* exposed to ZnO NPs in two different sizes (10 – 30 nm and 100 nm; 1 and 10 mg L^{-1}) for 7 and 14 days and the results showed that both sizes of particles have similar toxic effects (Kaya et al., 2016). In this sense, the aims of this study were (*i*) to investigate the MMC response in the guppy exposed to citrate-functionalized (γ-Fe_2O_3) IONPs by qualitative and histomorphometric analyses; (*ii*) analyse the histopathologic alterations induced by IONPs through the qualitative, histomorphometric and semi-quantitative approach based on hepatic histopathologic indices; and (*iii*) to describe the relationship between MMC response and the histopathologic condition induced in the guppy *P. reticulata* after acute (3 and 7 days) and long-term (14 and 21 days) exposure to citrate-coated IONPs.

P. reticulata was chosen as bioindicator in this study because this species is recommended for acute and long-term toxicity tests according to the OECD (OECD, 1992) due to its high sensitivity and ease and low cost of maintaining in the laboratory (Pelli and Connaughton, 2015; Rocha et al., 2015a; Santos et al., 2017). The IONP concentration used in this study (0.3 µg Fe L^{-1}) is under the recommendation of iron concentration detected in the aquatic environment, as reported by the OECD (2011). In addition, similar IONP concentrations were used in previous studies (Chen et al., 2013; Griffitt et al., 2013; Juganson et al., 2015; Zhang et al., 2015; Ates et al., 2016; Qualhato et al., 2017).

2. Materials and Methods

2.1. Nanoparticles

The citrate-functionalized maghemite (γ-Fe_2O_3) NPs used in this study were previously synthesized by Qualhato et al. (2017). The IONP stock solution at 6.8 mg Fe mL^{-1} was made in ultrapure water (18 MΩ cm^{-1}). After sonication for 30 min (Q500 Sonicator, Qsonica), the IONP solution (0.3 mg Fe L^{-1}) was characterized by transmission electron microscopy (TEM), dynamic light scattering (DLS), electrophoretic light scattering (ELS), x-ray diffraction (XDR), infrared spectroscopy in KBr pellet (IR-KBR) and ultraviolet–visible spectroscopy (UV-Vis) as described by Qualhato et al. (2017).

For zeta potential and hydrodynamic diameter (*dh*) analysis, the IONPs (0.3 mg Fe L^{-1}) were suspended in two aqueous media, ultrapure water (18 MΩ cm^{-1}) and reconstituted water, at pH 7.0 ± 0.01 (ISO, 1986). The d*h* of IONPs was determined by DLS using a Malvern ZetaSizer, model Nano-ZS90, and 12 mm square disposable polystyrene cells (DTS0012, Malvern, Inc.). The zeta potential was determined using the same equipment in a disposable polycarbonate capillary cell (DTS1061, Malvern Inc.) at 25 ºC. Each measurement was performed over a time period of 24 h.

2.2. Exposure

Female *P. reticulata* fish (total weight of 0.21 ± 0.09 g; total length of 2.63 ± 0.3 cm; standard length of 1.95 ± 0.3 cm) were collected in the Water Treatment Station (Saneamento de Goiás – SANEAGO – 16° 37’ 59” S and 49° 15' 44" W, Goiânia, Goiás, Brazil). After acclimation in the laboratory for 30 days in reconstituted water (ISO, 1986) at 27 ± 2 ºC, pH (7.1 ± 2) and photoperiod (14:10h

light/ dark), 360 fish were placed in 50 L tanks filled with 30 L of reconstituted water (ISO, 1986) (2 fish L^{-1}) and exposed to 0.3 µg Fe L^{-1} of citrate-functionalized γ-Fe_2O_3 NPs, jointly with a control group kept in clean reconstituted water in a triplicate design (3 tanks *per* treatment) for 21 days. Bioassays were conducted in a static-renewal condition under 14:10h light/dark cycles, and abiotic parameters were analyzed daily by measuring temperature 27 ± 2 °C, pH (7.1 ± 2) and oxygen saturation (8.3 ± 0.5 mg L^{-1}), such as described by Qualhato et al. (2017). The water was changed daily (15 L/day) with redosing of the IONP concentration. The fish were fed commercial fish food (Cardume® 36 %; VB alimentos). Fish (n = 10) from each experimental condition (control and IONP exposure) were collected at the beginning of the experiment and after acute (3 and 7 days) and long-term (14 and 21 days) exposure for histopathological assessment and the remaining fish were stored at -80 ° C for further analysis.

2.3. Qualitative and histomorphometric analyses of MMC response

After the exposure period, the animals were euthanized; the liver was dissected and immediately fixed by immersion in Karnovsky solution [paraformaldehyde 4 %; glutaraldehyde 2.5 % in phosphate-buffered saline (PBS) buffer 0.1 M], dehydrated through increasing ethanol gradient (70 % and 90 % for 20 min in each solution, followed by twice in 100 % for 30 min), immersed twice in xylene 100 % for 30 min and embedded in paraffin (Paraplast/McCormick) according to Gray (1954).

The qualitative and histomorphometric analyses of MMCs were performed in images of liver sections (5-µm thick; 3 sections *per* animal, 30 sections *per* experimental group) obtained in the microtome (Spencer 820) and stained with hematoxylin and eosin (H&E). The images were randomly captured at 400 ×

magnification and analyzed using an imaging program (Motic Images Plus 2.0 software). For this purpose, several histomorphometric parameters were estimated: (*i*) area and perimeter of MMC (μm^2); (*ii*) ratio between MMC area and total liver area; (*iii*) number of MM nucleus *per* MMC area; (*iv*) number of MMC *per* total liver area; (*v*) and frequency of MMC in the hepatic parenchyma or associated with blood vessels and bile ducts. The quantitative approach was developed for each individual (mean of the 3 sections). Furthermore, for a better description of the MMC morphology, three liver sections (5-μm thick) *per* animal (3 sections *per* animal; n = 30 *per* experimental group) were stained with Mallory trichrome (Gray, 1954), and the images of MMC were obtained using Motic Images Plus 2.0 software.

2.4 Hepatic histopathological assessment

Sections of liver (5-μm thick) stained with H&E (*n* = 3 sections *per* animal; *n* = 30 *per* experimental group) were analyzed using the image capture system Moticam 2300 (Motic Inc., Hong Kong) coupled with an Olympus CH30 microscope, as recommended by Santos et al. (2017).

The semi-quantitative hepatic histopathologic condition indices (*Ih*) were estimated *per* individual according to the weighted indices approach previously described by Bernet et al. (1999) and modified by Costa et al. (2009) and Santos et al. (2017). Initially, histopathologic alterations in the liver of *P. reticulata* were identified and classified in five reaction patterns (circulatory disturbances, inflammatory response, regressive alterations, progressive alterations, and neoplasm), and an importance factor (*w*) ranging from 1 (minimally severe) to 3 (severe) was attributed to each tissue damage (Table 1). The *Ih* for each reaction pattern (*Ij1:* circulatory disturbance index*; Ij2:* inflammatory response index*; Ij3:*

regressive alteration index*; Ij4:* progressive alteration index) was determined using the following formula described by Bernet et al. (1999) and adapted by Costa et al. (2009):

$$Ih = n\sum_{J=1} WjOj$$

Where: *Ih* = histopathologic condition indices; *W*j = relative weight of the condition *j-th*; *Oj* = Boolean variable (1: presence; 0: absence); *n* = total number of histopathologic alterations analyzed in each reaction pattern. After this, the total hepatic histopathologic condition index (Ih_{liver}) was estimated by the sum of the five reaction indices of the liver *per* individual according to Bernet et al. (1999).

2.5. Statistical analysis

The results were compared using parametric tests [two-way analysis of variance (ANOVA), followed by the Tukey test] and/or non-parametric test (Kruskal-Wallis), depending on the distribution of the data and homogeneity of variance (Shapiro-Wilk and Levene tests) using the Statistica 7.0 software (Statsoft Inc., 2005, Tulsa, OK, USA). Linear regression analyses were also applied to verify existing relationships between parameters. All analyses were performed at the 0.05 significance level.

3. Results and discussion

3.1. NP characterization

Data on IONP characterization are summarized in Table 2. The TEM results showed that the IONPs have a rounded shape with an individual diameter of 3.97 ± 0.85 nm (Fig. 17 A-B) and interplanar distance of 0.13 ± 0.02 nm (Fig. 1C-D). The ionized citrate groups confer negative surface charge of IONPs in ultrapure water (-51.1 ± 7 mV) and in reconstituted water (-19.5 ± 6.5 mV), inducing the formation of the IONP aggregates in both media (14.11 ± 0.2 nm and 21.4 ± 0.39 nm, respectively) (Fig. 18 A-B), such as previously reported by Qualhato et al. (2017). The IONP stock solution was oxidized due to the synthesis technique used. According to Kang et al. (1996), the colloidal suspensions of the magnetite (Fe_3O_4) and maghemite (γ-Fe_2O_3) can then be directly oxidized to γ-Fe_2O_3 (maghemite) under constant oxygen aeration. This oxidation was confirmed by UV-Vis-NIR spectrum of the IONP suspension, which presents the characteristic absorption near the UV wavelength region ascribed as just the presence of Fe (III) (Sherman and Waite, 1985; Qualhato et al., 2017).

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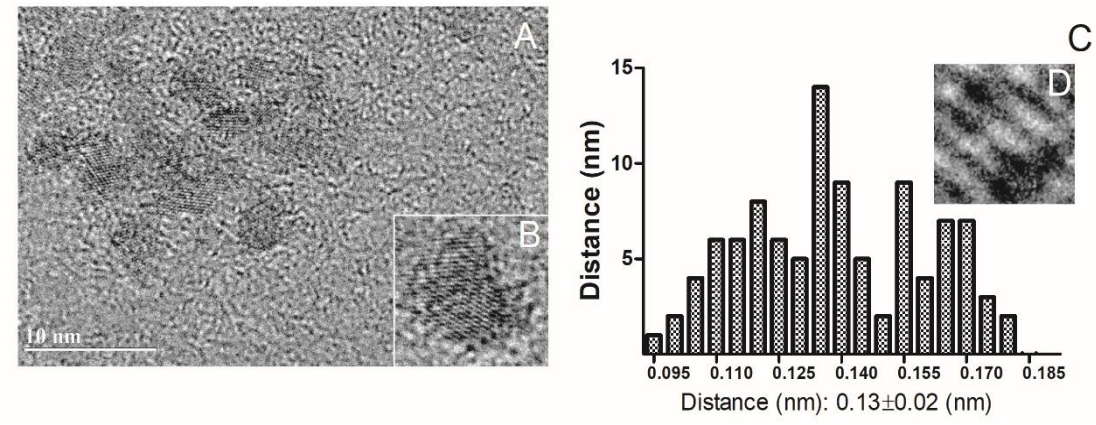

Figure 15- Transmission Electron Microscopic (TEM) image of citrate-coated iron oxide (maghemite) nanoparticle (IONPs) (A and B) and the individual diameter histogram of interplanar distance of the IONPs obtained from TEM figures (C and D).

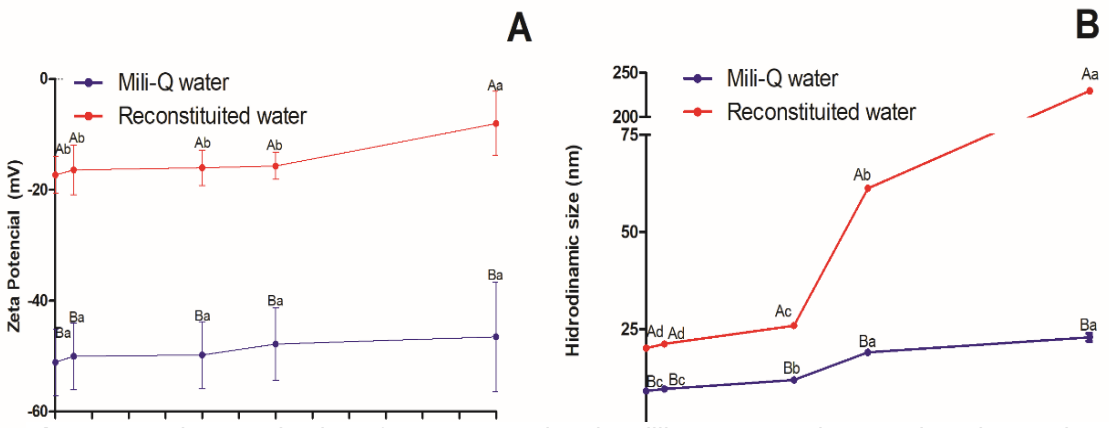

Figure 16- Characterization of INOPs over time in Milli-Q water and reconstituted water by DLS. Zeta potential (A) and hydrodynamic diameter (B) over 24 hours. Results are presented as means ± standard deviations.

3.2. MMC response

The MMC response of *P. reticulata* induced by IONP was exposure time dependent and confirmed by qualitative and histomorphometric assessment. The MMC parameters in the liver of the unexposed fish remained unchanged during the experimental period, confirming that the holding conditions did not induce a response or change in the control animals (Fig. 19 A-F). These results are according to Santos et al. (2017), who previously described the presence of isolated MM or MMCs in the hepatic parenchyma of the unexposed *P. reticulata*.

The fish exposed to IONPs showed a higher MMC response compared with the unexposed fish during the whole exposure period (Figs. 19-22). The unexposed and IONP-exposed fish showed MMC with absence of the encapsulation by matrix protein fibers (Fig. 19 B-D; Fig. 20-A), indicating its direct contact with the hepatocytes, such as reported in goldfish (Diaz-Satizabal and Magor, 2015). The IONP exposure induced a significant linear increase in the MMC area ($y = 4069.7\times + 27745$; $r^2 = 0.83$; $p<0.05$) and in the ratio between

MMC area and the total liver area ($y = 0.1100\times + 0.4274$; $r^2 = 0.93$; $p<0.05$) during the exposure period (Fig. 22 A, C), whereas the IONP-exposed fish showed a high perimeter of MMC compared with the unexposed ones ($p<0.05$; Fig 22 B). Furthermore, the long-term exposure (14 and 21 days) to IONPs induced a higher MMC area compared with acute exposure (3 and 7 days) ($p<0.05$; Fig. 22 A, C), indicating differential hepatic MMC response over the exposure period.

In IONP-exposed fish, the number of MM nucleus *per* MMC area increased linearly until 7 days of exposure ($y = 0.0035\times + 0.0063$; $r^2 = 0.99$; $p<0.05$) and remained unchanged until the end of the exposure period ($p<0.05$; Fig. 6 D), indicating the migration and recruitment of new cells into the MMCs during the acute exposure period (3 and 7 days). Previous studies indicated that the macrophages can be attracted by nitric oxide (NO), which is a response to lesions and reactive oxygen species (ROS) production (Flora Filho and Zilberstein, 2000), indicating a similar response in *P. reticulata.*

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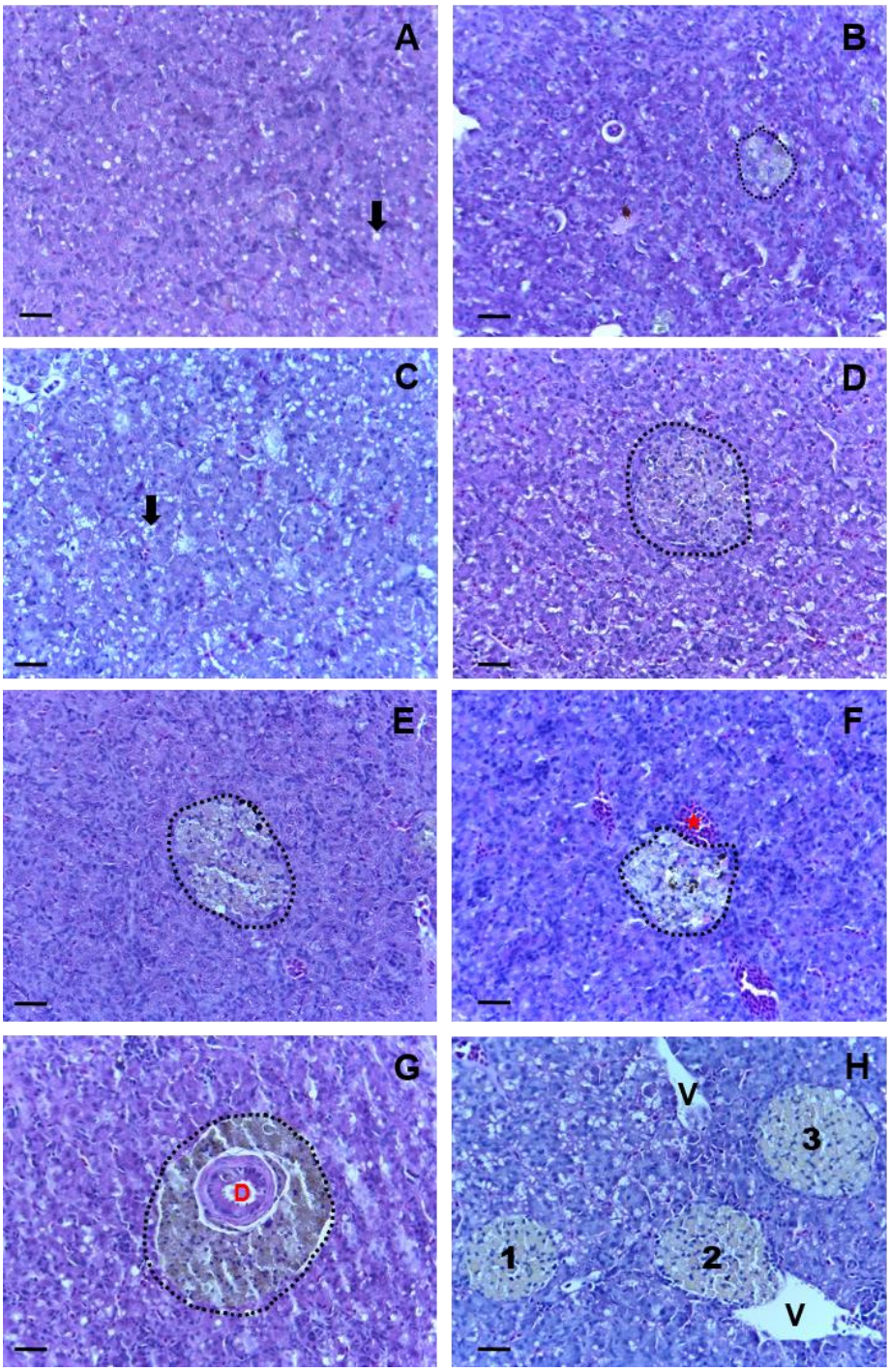

Figure 17- Hepatic histopathological profile of *P. reticulata* of the unexposed group and those exposed to INOPs. (A) Control group: the hepatocytes with a normal structural arrangement. Exposed group for 3 days (B), 7 days (C) and 14 days (D): Smalls MMC (traced circle) and isolated macrophage with hemosiderin (Black arrow) and micro-vesicular steatosis (Larger black arrow). After 21 days of exposure observe a MMC associated to parenchyma (E), to vessels (red star) (F), biliary ducts (D) (G) and 2 or 3 MMC (numbers 1, 2 and 3) associated to vessels (letter V) (H). Hematoxylin and eosin staining; 400x of magnificence and scale bar of 20 µm.

Similar to MMC area and perimeter, linear increase of the number of MMC

per total liver area was observed during the exposure to IONPs ($y = 1.561e^{007X} + 8.101e^{007}$; $r^2 = 0.82$; $p<0.05$; Fig. 22 E). Results showed higher MMC number in the IONP-exposed fish after 3 (5.73-fold; $p<0.05$), 7 (9.58-fold; $p<0.05$), 14 (5.92-fold $p<0.05$), and 21 (24.86-fold) days when compared with unexposed fish ($p<0.05$; Fig. 22 E), indicating that the IONP increased the area and perimeter of pre-existing MMCs, as well as promoted the formation of new hepatic MMCs.

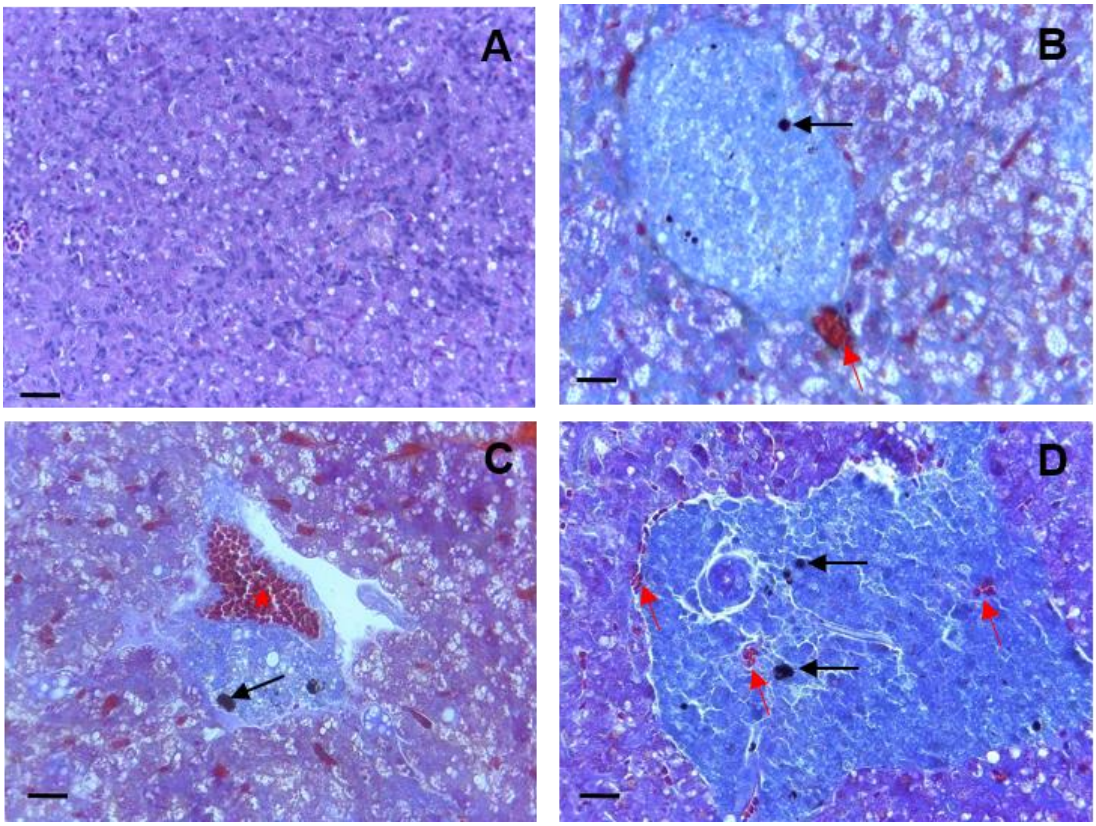

Figure 18- Hepatic histopathological profile staining by Mallory's Trichrome. Control Group (A). The MMC (in blue) with absence of the encapsulation by extracellular matrix with an isolated MM (black arrow) full of hemosiderin (B), association of MMC with blood vessels in red (C), and a bile duct surrounded by a MMC (D) in 21 days of exposure to INOPs. Note in the interior and perimeter of the MMC erythrocytes (red arrow) and isolated macrophage with hemosiderin (black arrow).

As the frequency of MMCs into hepatic parenchyma of IONP-exposed fish remain similar during the acute and long-term exposure (Fig. 22 F), results

indicated that the new MMCs of *P. reticulata* induced by IONP exposure were formed primarily associated with blood vessels or with bile ducts (Fig. 19 G-H; Fig. 22 G-H). Some fish (40 %) exposed to IONPs for 14 and 21 days showed 2 to 3 MMCs near the same hepatic vein (Fig. 19 H). In the same way, previous studies indicated that the piscine macrophages move to pre-existing aggregates and increasing the MMC area, as well as form new aggregates (Ziegenfuss and Wolke, 1991; Wolker, 1992), such as reported in *Carassius auratus* after intraperitoneal injection of polystyrene microsphere (1 micron; 5×10^6 particles/fish) (Ziegenfuss and Wolke, 1991).

However, the present study is the first report of the increase of the MMC frequency associated with blood vessels and bile ducts in fish exposed to metal-based NPs. This increase of number, size, and perimeter of the MMCs indicated the inflammatory response in guppies induced by IONP exposure. Equally, previous studies in bullfrog tadpoles (*Lithobates catesbeianus*) exposed to chitosan-alginate NPs (200 to 1000 nm; 0.5 mg L^{-1}) associated with clomazone herbicide (0.5 mg L^{-1}) showed an increase of MMCs, indicating that the MMC in amphibians also are good biomarkers to quantify and identify the ecotoxicologic effects of NPs (Oliveira et al., 2016).

The presence of erythrocytes in the MMC of the fish exposed to IONP was confirmed by the Mallory trichrome stain (Fig. 20 B-D), especially those associated with blood vessels, confirming the participation of the MMC in the metabolism of the erythrocyte, as well as its role in the renewal of circulating erythrocytes and in the iron recycling/metabolism during the exposure to IONPs. Similarly, the role of MMC in metal metabolism was previously reported in

Cyprinus carpio exposed to copper NPs (40 nm; 0.25 mg L^{-1}) (Hoseini et al., 2016). A higher induction rate of clastogenic and aneugenic effects was recently reported in *P. reticulata* erythrocytes at the same exposure condition to IONPs (Qualhato et al., 2017). In this sense, the present study indicated that the MMCs uptake and degrade erythrocytes with DNA damage or nuclear alterations induced by IONP exposure.

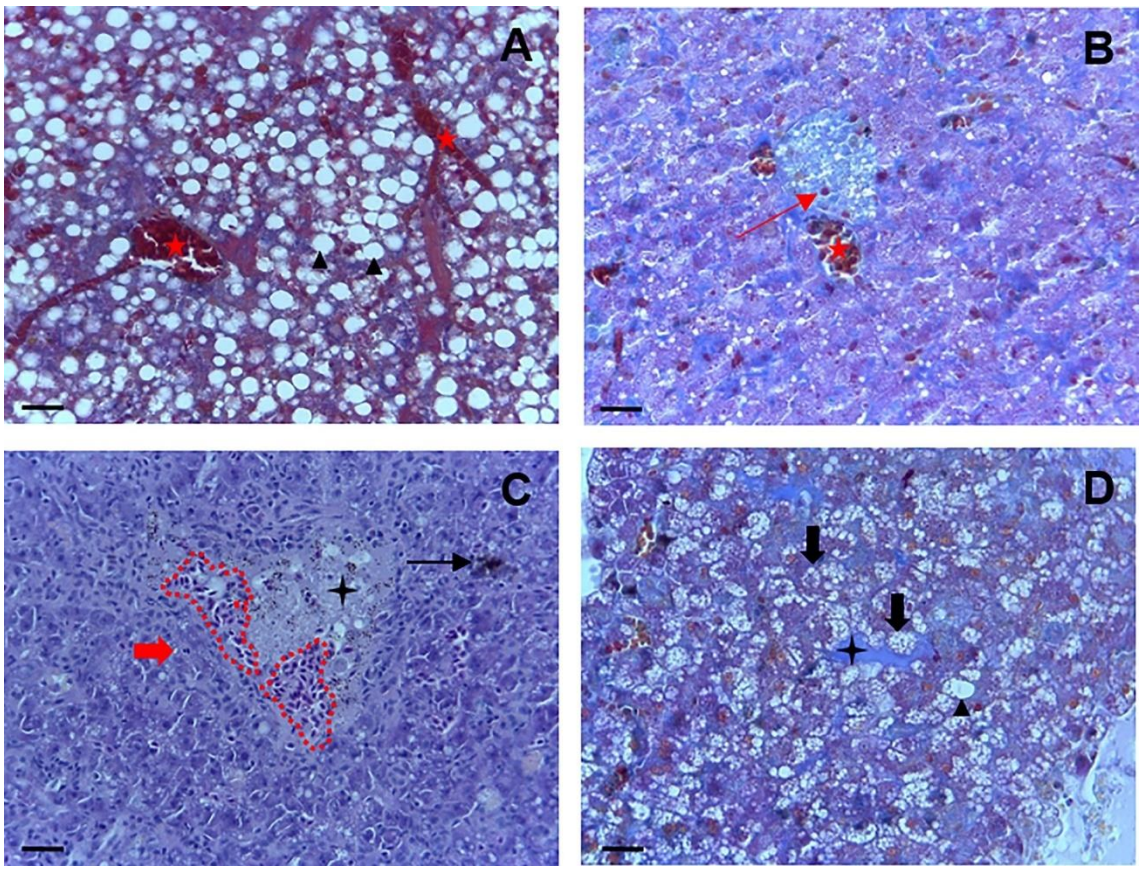

Figure 21- Histopathological lesion observed in the liver of *P. reticulata* from exposed to iron oxide (maghemite) nanoparticle (IONPs) for 21 days. (A and B) steatosis macrovesicular (head arrow) and vessels with hyperemia and dilatations (red star); (C and D) exudate (black star), pyknotic nuclei (bigger red arrow), isolated macrophages with hemosiderin (black arrow), steatosis microvesicular (bigger black arrow) and hemorrhagic focus (red traced circle).

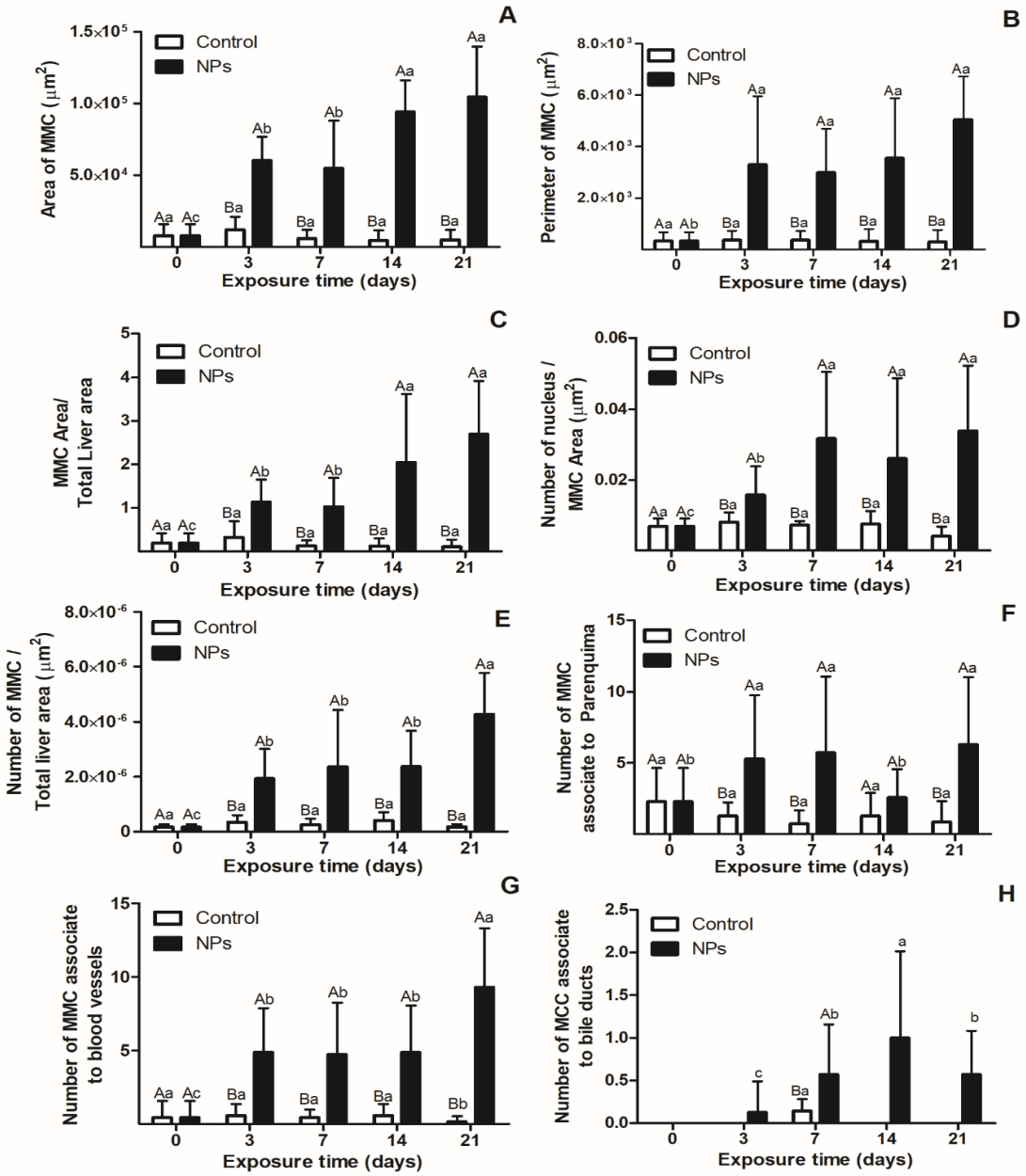

Figure 19- MMC response in the hepatic tissue of *P. reticulata* from control and exposed to iron oxide (maghemite) nanoparticle (IONPs) for 21 days. Area of MMC (A), perimeter of MMC (B), ratio of MMC area and total liver area (C), ratio of number of nucleus counted in MMC and MMC area (D), ratio of number of MMC and total liver area (E), and number of MMC associated to parenquima (F), vessels (G) and bile ducts (H). Results are presented as means ± standard deviations (n = 30 sections per experimental group). Different capital and lower-case letters indicate significant differences between treatments at each time of exposure and within treatment during the exposure period, respectively ($p<0.05$).

Wolke (1992) reported that the hemosiderin contain iron in the ferric form (Fe^{+++}), which is accumulated in the MMCs after destruction of erythrocytes and breakdown of hemoglobin. The iron present in the MMCs can be recycled for use in the erythropoiesis or designated to metabolic routes for detoxification (Wolke,

1992; Sayed and Younes, 2017), confirming the MMC role in the iron metabolism. Moreover, future studies and new technologies are needed to differentiate if the iron present in the MMCs is in the dissolved form or remains as NP.

3.3. Histopathological biomarkers

The hepatic tissue of unexposed *P. reticulata* showed hepatocytes with polyhedral shape, homogeneous cytoplasm, and evident nucleus, as well as the presence of the lipid vacuoles (micro-vesicular steatosis) (Fig. 19 A;20 A), such as reported by Santos et al. (2017). On the other hand, the increase of the micro- and macro-vesicular steatosis was observed in the fish exposed to IONP for 3 (micro: 0.33-fold; macro: 1.5-fold), 7 (micro: 1.25-fold; macro: 5-fold), 14 (micro: 1.02-fold; macro: 4.01-fold), and 21 (micro: 2.07-fold; macro: 5.01-fold) days compared with the unexposed group ($p<0.05$; Fig. . 21 A, D, E). This alteration has been regarded as a general failure in lipid metabolism after exposure to toxic compounds, indicating the specific response in the liver (Bernet et al., 1999; Costa et al., 2009). Recent studies showed that the ingestion of metal-based NPs promotes the micro- and macro-vesicular steatosis in the liver of rats exposed to ZnO NPs (20 and 30 nm; 50 mg kg^{-1}; 14 days) and hepatocyte vacuolization in *C. carpio* exposed to Cu NPs (0.25 mg L^{-1};14 days) (Hoseini et al., 2016; Nazdar et al., 2016; Khorsandi et al., 2016).

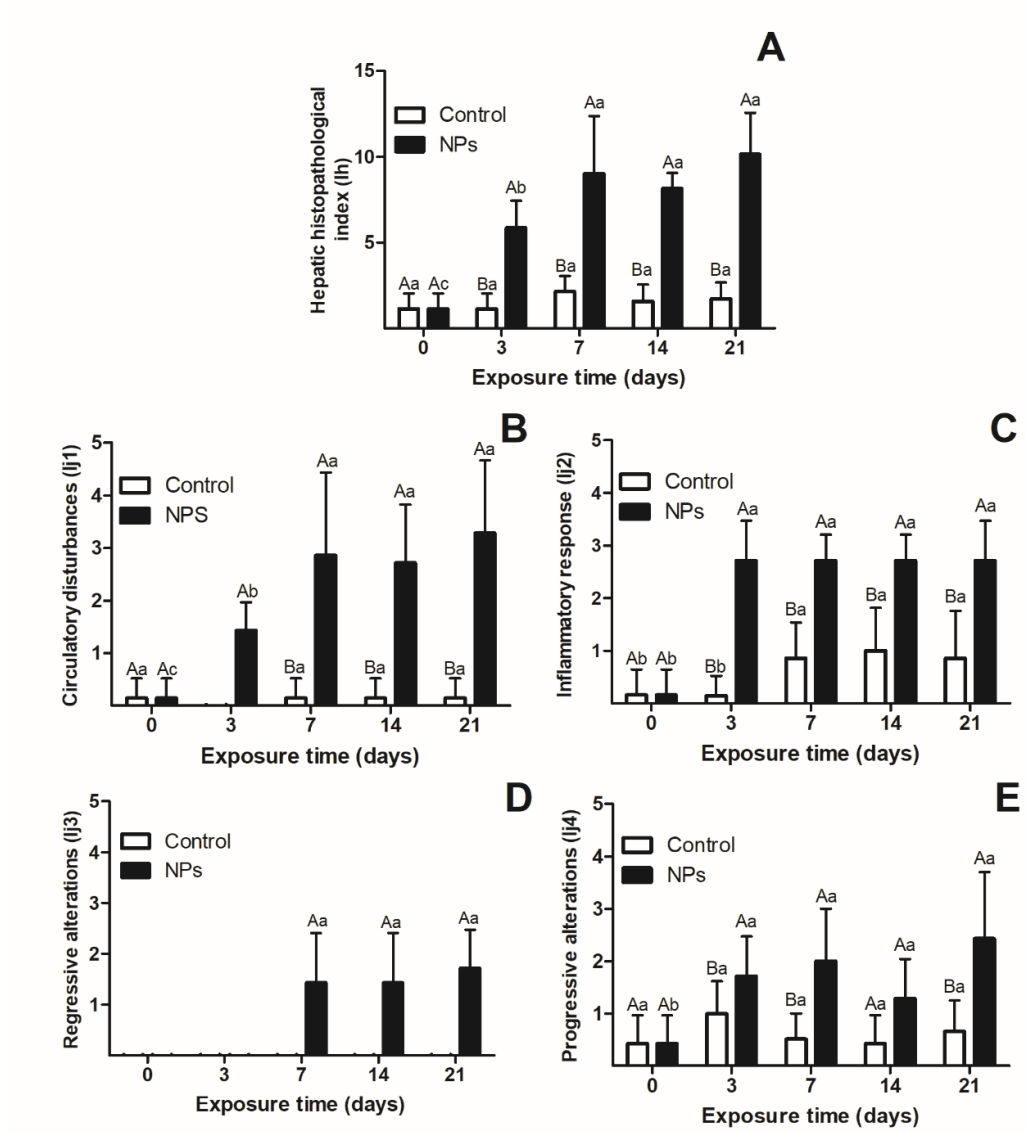

Figure 20- Histopathological index in *P. reticulata* from control and exposed to iron oxide (maghemite) nanoparticle (IONPs) for 21 days. Total hepatic histopathological index – Ih (A); circulatory disturbances – Ij1 (B); inflammatory response – Ij2 (C); regressive alterations – Ij3 (D); progressive alterations – Ij4 (E). Results are presented as means ± standard deviations (n = 30 sections per experimental group). Different capital and lower-case letters indicate significant differences between treatments at each time of exposure and within treatment during the exposure period, respectively ($p<0.05$).

The IONP exposure induced circulatory disturbances in the fish, primarily hyperemia and dilatation of sinusoid vessels after 14 and 21 days, whereas the hemorrhagic focus was observed only in the fish exposed to IONP for 21 days (Fig. 21 A-C). However, occasional focus of exudate was observed after 7 and 14 days of exposure to IONPs (Fig. 21 C-D). Circulatory alterations are a response of the blood cells to toxic agents. According to other studies, the NPs

can penetrate into the red blood cell membrane (Rothen-Rutishauser et al., 2006). Due to the characteristics of transport, storage, and mediation of blood (Bruslé et al., 1996; Santos et al., 2017), the liver showed circulatory and inflammatory responses to the acute and long-term exposure to IONP, such as observed in the present study.

No significant differences were observed for hepatic histopathologic index (*IhL*) in unexposed fish during the experimental period (p>0.05; Fig. 23). However, the *IhL* showed a significant difference between all the exposed groups when compared with the control groups (p<0.05; Fig. 23 A). After 3 and 7 days, a significant increase in the *IhL* was observed in the fish exposed to IONPs (5.1-fold and 6.2-fold, respectively; p<0.05) when compared with unexposed fish. This response remained high until the end of the exposure period (5.2-fold; p<0.05), with similar effects between 7, 14, and 21 days of exposure to IONPs (p>0.05; Fig. 23 A). These results indicated that the IONP changed the cellular and hepatic metabolism, reducing the hepatic health, metabolic efficiency and the animal health (Costa et al., 2011; Connolly et al., 2016).

The acute and long-term exposure to IONP induced high *IhL* associated with regressive alterations (*Ij3*), inflammatory responses (*Ij2*), circulatory disturbances (*Ij1*) and progressive alterations (*Ij4*), respectively (p<0.05; Fig. 23 B-D). The fish exposed to IONP for 3 and 7 days showed a significant increase in the circulatory disturbances index (*Ij1*) compared with unexposed fish (1.5-fold and 20-fold, respectively). The *Ij1* remained more pronounced until the end of the exposure period in the IONP-exposed fish compared with unexposed fish (p<0.05; Fig. 23 B). After 3 days of exposure, the IONP induced high *Ij2* (18-fold)

and *Ij4* (2-fold) compared with the control group (p<0.05; Fig. 23 A-E). The *Ij1* and *Ij2* reflect an immunologic response of guppies exposed to NPs, because IONPs can activate the complementary system, which is a normal response of the immune system to external materials. In this system, the absorption of plasma protein on the surface of NPs causes their detection, clearance and digestion by the reticuloendothelial system of the macrophages (Ai et al., 2011).

Regressive alterations (*Ij3*) and inflammatory response (*Ij2*) were associated with specific MoA of IONPs in the *P. reticulata*. Conversely, the guppies exposed to glyphosate-based herbicide showed a higher response associated with progressive alterations (*Ij4*) and circulatory disturbance (*Ij1*) (Santos et al., 2017; Antunes et al., 2017). Therefore, the *P. reticulata* showed differential hepatic responses when exposed to IONPs. Ates et al. (2016), using a similar γ-Fe_2O_3 NPs, showed a dissolution rate less than 1 % (for 0.1 mg L^{-1} suspensions, Fe concentrations were < 1 µg L^{-1}), confirming the specific responses observed in the present study due to nano-specific properties.

The IONP induced high *Ij2* and *Ij4* levels after 7 days of exposure. These responses remained higher until the end of exposure compared with the unexposed fish (Fig. 23 C-E). Conversely, the *Ij3* was observed only in the fish exposed to IONP after 7 (1.42 ± 0.8), 14 (1.42 ± 0.8), and 21 (1.7 ± 0.7) days (Fig. 23 D). The regressive alterations are a result of severe structural damage caused by previous damage indicated in *Ij1, Ij2*, and *Ij4*, which terminates in a functional reduction or loss of an organ (Bernet et al., 1999; Costa et al., 2009), confirming the hepatotoxic effects of IONPs in *P. reticulata.* These effects induced by IONP exposure are consequence of ROS production, oxidative stress

damage, alterations of cell structure, and immune response changes, such as the oxidative stress previously reported in *Oncorhynchus mykiss* exposed to TiO_2 NPs (21 nm; 0.1, 0.5, or 1.0 mg L^{-1}; 14 days) (Federici et al., 2007).

Reaction pattern	Alteration	W*
Circulatory disturbances	Hyperaemia	1[a]
	Dilatations of vessels	1[a,b]
	Hemorrhagic focci	1[a]
	Aneurysm	2[a]
	Exudate	2[c]
Inflammatory	Isolated melanomacrophages	1[b]
	Center of melanomacrophages	2[c]
Regressive	Pyknotic nuclei	2[a,b]
Progressive	Lipidosis Microvesicular	1[a]
	Lipidosis Macrovesicular	2[a]
	Leukocyte infiltration	1[a]

* Importance factor (w) according to Bernet et al. (1999) (a), Costa et al. (2009) (b) and described in this work(c).

Table 1- Histopathological response in the liver of *P. reticulata* exposed to IONPs and their respective importance factor (w), ranging from 1 to 3.

The present study confirms that the MMCs are good tissue-level biomarkers in fish exposed to environmental pollution (Agius and Agbede, 1984; Haaparanta et al., 1996; Wolke et al., 1985; Steinel and Bolnick, 2017). To the best of our knowledge, this is the first study about the immune response and use of the MMC as an immunological biomarker associated with hepatic histopathologic indices in fish species exposed to metal-based NPs.

Parameters [1]	Techniques [2]	Data
Individual particle size (nm)	TEM	3,97 ± 0,85 [a]
Shape [a]	TEM	Crystalline and rounded [a]
Hydrodynamic diameter (nm)	DLS	14.11 ±0,2[a]; 21.4± 0.39
Polydispersity index	DLS	0,206 ± 0,013 [b]
ζ-potential (mV)	ELS	-51,1 ±7 [a]; -19,5 ± 6,5 [b]
Concentration in stock solution (synthesis) [a]	ABS	6,8 mg.mL^{-1}

[1] Aqueous medium: Milli-Q water ([a]) and reconstituted water ([b]).
[2] TEM: Transmission Electron Microscopy; DLS: Dynamic Light Scattering, ELS: Electrophoretic Light Scattering; ABS: Atomic absorption spectroscopy.

Table 2- Summarized data of IONP characterization in Milli-Q water and reconstituted water.

3.5. Conclusions

The present study indicates that the citrate-funcionalizated maghemite (γ-Fe2O3) NPs at iron environmentally relevant concentration (0.3 mg L−1) increased the number, size and cellular density of MMC and histopathological damage in the female P. reticulata during the 21 days of exposure. The long-term exposure (14 and 21 days) to IONPs induced a higher number of MMC, indicating an inflammatory and immune cellular response to exposure of the IONPs. Theses responses are reflected in histopathological alterations associated to circulatory disturbances (Ij1) and inflammatory responses (Ij2) found in the liver of animals exposed to IONPs after 3 and 7 days. Results indicated that the further studies are needed to confirm the MMC role in the adaptive immune system activation in fish exposed to metal-based NPs. The results indicated that the study of histopathological biomarkers associated quantitative and semi-quantitative responses is important to understand the ecotoxicity of the IONPs. The MMCs are a suitable biomarker to understand the hepatotoxicity of INOPs in fish species.

Acknowledgments

This work was funded by the by Fundação de Amparo à Pesquisa do Estado de Goiás (FAPEG; edital nº 003/2016) and by Postdoctoral National Program (PNPD) from the Coordination for the Improvement of Higher Education Personnel (CAPES). The authors also acknowledge CRTI, LabMic-UFG and Central Analítica IQ-UFG for their collaboration in the characterization of IONPs.

O artigo pode ser acessado na integra pelo seguinte link: https://doi.org/10.1016/j.aquatox.2018.02.014

Aquatic Toxicology 198 (2018) 63–72

Contents lists available at ScienceDirect

Aquatic Toxicology

journal homepage: www.elsevier.com/locate/aqtox

Melanomacrophage response and hepatic histopathologic biomarkers in the guppy *Poecilia reticulata* exposed to iron oxide (maghemite) nanoparticles

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ARTICLE INFO

Keywords:
Nanomaterials
Nanoecotoxicity
Tissue-level biomarkers
Immune response
Guppy

ABSTRACT

Although iron oxide nanoparticles (IONPs) have been widely used in nanomedicine and nanoremediation, their ecotoxicological effects on aquatic organisms remain unclear. In this study, the melanomacrophage center (MMC) response and hepatic histopathologic biomarkers were investigated in female guppies, *Poecilia reticulata*, exposed to citrate-functionalized IONPs (γ-Fe_2O_3) at an environmentally relevant iron concentration (0.3 mg L^{-1}) over 21 days. The animals were collected at the beginning of the experiment and after 3, 7, 14, and 21 days of exposure. Guppies exposed to IONPs showed increases in the number, area, and perimeter of MMC when compared with the unexposed ones, especially after 7 days of exposure. The results showed an increase in the frequency of histopathologic changes in fish after 7 days of exposure to IONPs, such micro- and macro-vesicular steatosis, melanomacrophage aggregates, exudate, and hemorrhagic foci. The acute (3 and 7 days) and long-term (14 and 21 days) exposure of *P. reticulata* to IONPs induced high histopathologic indexes associated with circulatory disorders and inflammatory responses. Results showed that the MMC response and histopathologic index are important biomarkers to indicate the environmental impact of IONPs, confirming that the guppy *P. reticulata* is a target of ecotoxicity of IONPs.

1. Introduction

The melanomacrophage centers (MMC), also known as macrophage aggregates, play an important role in the immune response and immunological protection in fish species and have been used as biomarkers to assess fish health and aquatic environmental pollution (Agius and Agbede, 1984; Haaparanta et al., 1996; Capkin et al., 2017; Fu et al., 2017).

The melanomacrophages (MMs) are multifunctional cells that perform phagocytosis of red blood cells, hematopoietic cells, and cell debris; detoxification of exogenous and endogenous molecules; iron recycling/metabolism; and accumulation of different pigments such as melanin, lipofuscin and hemosiderin (Agius and Agbede, 1984; Wolke et al., 1985). The pigment melanin neutralizes the free radicals released during cellular membrane breakdown, whereas the lipofuscin and hemosiderin are sub-product of non-saturated fatty acid peroxidation and breakdown of hemoglobin, respectively (Agius and Agbede, 1984; Wolke et al., 1985). Furthermore, clusters of adjacent B-cells, CD4, and T-cell receptor beta chain (TCRβ) were observed in the MMCs (Saunders et al., 2010; Diaz-Satizabal and Magor, 2015), indicating that the MMCs are analogs, or "primitive" evolutionary precursors, of the germinal center (GC) in mammals, and represent a primitive site of adaptive immune system activation in poikilothermic organisms (Steinel and Bolnick, 2017). An increase in the number and size of MMCs was reported in fish species exposed to metals (Sayed and Younes, 2016), herbicides (Rocha et al., 2015a; Santos et al., 2017), antimicrobial agents (Capkin et al., 2017) and in fish infected by parasites (Ventura et al., 2016). However, the knowledge about the effects of emerging pollutants such as nanoparticles (NPs) on the MMC role in fish pathology remains unknown. The NPs are structures with at least one dimension between 1 and 100 nm and are considered to be an emerging concern for environmental and human health due to their nano-specific properties, dissolution capacity and release of toxic materials, as well as nano-specific mode of action (MoA) (Moore, 2006; Rocha et al., 2015b, 2017).

Among the engineered NPs, the iron oxide nanoparticles (IONPs) are used in nanomedicine, pharmacy, and electronics, as well as in nanoremediation for absorbing and removing contaminants in

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https://doi.org/10.1016/j.aquatox.2018.02.014
Received 23 October 2017; Received in revised form 8 February 2018; Accepted 16 February 2018
Available online 21 February 2018
0166-445X/

Capítulo 4

Considerações finais e Conclusão

Foi definido recentemente pela comissão europeia o que seria um nanomaterial:

> *"Um material natural, incidental ou fabricado contendo partículas em um estado não ligado ou como agregado ou aglomerado, e que para 50% ou mais do montante destas partículas esteja em uma ou mais dimensões externas medindo de 1 a 100 nm "(2011/696 / UE)*

Além do mais a Organização Internacional de Padronização (ISO) define o termo nanoescala como *“intervalo de comprimento aproximadamente de 1 nm a 100 nm",* enquanto que os nanomateriais foram definidos como *"materiais com qualquer dimensão externa em nanoescala ou com uma superfície interna estruturada nas dimensões (entre 1 e 100 nm) que são projetadas para uma finalidade ou função específica "(*ISO / TS 27687: 2008; ISO / TS 80004-4: 2011; ISO / TS 80004-2: 2015).

Desta maneira verifica-se que a nanotecnologia é a área da ciência que estuda o desenvolvimento, criação e manipulação de materiais, compostos ou associados a nanopartículas. Indubitavelmente, a nanotecnologia é um dos ramos da ciência que mais se desenvolve atualmente, fruto dos altos investimentos em pesquisa, sobretudo por parte de países desenvolvidos como os Estados Unidos, cujos recursos financeiros são os mais vultuosos, seguidos da Alemanha e Japão, enquanto que o Brasil integra o grupo de países emergentes com avanços importantes, neste estão também a China e Índia.

Então, é crescente a demanda por investimento em pesquisa nesta área o que tem gerado expectativas pelo significativo aumento das verbas. Consequentemente o aumento de novos produtos e tecnologias, sendo

necessário estudos com objetivo de compreender além do uso industrial, o potencial perigo que as NOFs podem oferecer ao meio ambiente.

De maneira que aliado ao grande impulso tecnológico devem ser envolvidos esforços para se investigar as ações toxicológicas, avançando assim para a recente proposição de avaliações ambientais, a nanoecotoxicologia, especialidade que pode ser definida como a ciência que estuda os efeitos adversos de agentes de natureza física, química ou biológica sobre os sistemas biológicos, tendo como meta o tratamento, o diagnóstico e, principalmente, a prevenção da intoxicação.

De acordo com René Truhaut (1969), a ecotoxicologia é "um ramo investigativo da toxicologia que visa compreender efeitos tóxicos causados por poluentes naturais ou sintéticos, sobre quaisquer constituintes dos ecossistemas: animais, vegetais ou microorganismos, em um contexto integral". Em que pese o fato de ter sido definida há apenas 49 anos, ela é uma ciência relativamente nova, cujo ponto de análise se fundamenta sobre contaminantes e seus efeitos sobre os constituintes da biosfera. Dentre estes os seres humanos (Bols et al., 2001).

Assim, pode-se considerar a nanoecotoxicologia como uma divisão emergente e atual das ciências toxicológicas, especializada em estudar o potencial ecotoxicológico dos NMs. A preocupação com a nanotoxicidade surge na medida em que diversificados NMs são sintetizados, manipulados e descartados em diferentes ambientes, sejam naturais, urbanos ou industriais, sem o devido controle e regulamentação. (Martinez and Alves, 2013).

Dentre os mais diversos tipos de NMs, as nanopartículas de oxido de Ferro (NOFs) diferem das demais partículas metálicas por possuir características exclusivas, principalmente no que diz respeito às suas propriedades magnéticas e ao tamanho controlável, ressaltando que tal tamanho pode estar na ordem de poucos nanômetros (2 a 20 nm). Estas organizações estruturais as tornam adequadas às aplicações que envolvam entidades biológicas diminutas como partes de organelas e moléculas, como o DNA. Além disso, a grande superfície relativa das NPs pode ser apropriadamente modificada para receber agentes biológicos e serem manipuladas por um gradiente de campo magnético externo para transporte e imobilização no corpo humano de modo não-invasivo. Possibilitando assim uma grande aplicação de tais partículas à saúde e à nanoremediação.

Porém estas possibilidades e benefícios das NOFs para a melhorias ambientais e na saúde, não retiram seu potencial danoso ao meio ambiente. As mesmas características que as tornam interessantes do ponto de vista de aplicação tecnológica, podem ser indesejáveis quando essas são liberadas no meio ambiente. O pequeno tamanho das NPs facilita sua difusão e transporte na atmosfera, em águas e em solos, ao passo que dificulta sua remoção por técnicas usuais de filtração. Oferecendo assim um potencial acumulativo ambiental e trófico, sendo necessário estudos para elucidar os riscos que estes NMs podem oferecer ao ambiente.

Para se compreender a estrutura, dimensão, organização morfológica de uma NOF, bem como avaliar seu potencial toxicológico, são necessários exaustivos ensaios experimentais e uma minuciosa preparação destas para uso

em pesquisa. Portanto, o presente estudo se ocupou da caracterização da maghemita. Para tanto, foram utilizadas as seguintes técnicas: para a forma da partícula e o diametro individual foi utilizada a Microscopia Eletrônica de Transmissão (TEM), enquanto a carga superficial (potencial zeta) e o diametro hidrodinâmico foram analisados através de espalhamento de luz dinâmica (DLS) e espalhamento de luz eletroforética (ELS). Estas metolologias foram utilizadas por muitos autores que fizeram trabalhos similares (El-Temsah and Joner, 2013; Lim et al., 2013; Sun et al., 2016; Yan, 2011).

Embora, de maneira geral, as NOFs tenham sido utilizados na biorremediação, o risco ambiental relativo ao seu uso, seu modo de ação e impacto sobre organismos aquáticos, permanece insuficientemente esclarecidos. Como descrito anteriormente, sintetizamos e caracterizamos NPs de maghemita e indicamos que estas partículas funcionalizadas com citrato na concentração ambientalmente relevante (0,3 mg L^{-1}) apresentam potencial genotóxico e mutagênico para fêmeas *P. reticulata*. Este indicativo genotóxico foi dependente do tempo de exposição. Os efeitos genotóxicos precoces foram identificados pelo ensaio cometa em peixes expostos a 3 e 7 dias, enquanto que os efeitos genotóxicos e mutagênicos mais intensos foram observados após exposição prolongada (14 e 21 dias) (Capitulo 2 figuras 12 e 13).

Devido aos dados obtidos neste trabalho, indicamos as células sanguíneas de peixes como importantes biomarcadores. Uma vez que seu uso associado às análises realizadas como o ensaio cometa, uma técnica sensível para detectar danos precoces no DNA, foram suficientes para detectar os danos induzidos pela exposição ao NOFs em eritrócito de *P. reticulata*. No entanto, esta

técnica associada com as alterações nucleares (ANE) e a contagem de micronúcleos (MN) nos eritrócitos se mostraram importantes ferramentas de avaliação mutagênica e genotóxica. Portanto, a associação das análises permitiu resultados melhores do que o uso separado destas metodologias, visto que comumente apenas o MN e ANE são utilizados em peixes expostos tanto em laboratório quanto em condições ambientais para avaliar o potencial ecotoxicológico das NPs (De Souza Filho et al., 2013; Seriani et al., 2011). Em comparação às análises realizadas e aos dados apresentados no corpo do presente trabalho, o segundo capitulo ainda concluiu que fêmeas de *P. reticulata* são excelentes biomonitores para testes genotóxicos e mutagênicos de NOFs.

Contudo, para além das ações moleculares e celulares há importantes reflexos da ação das NOFs em tecidos e órgãos, sobretudo naqueles órgãos cujas células estão comprometidas na detoxificação, alterando seu comportamento em resposta a ação de agentes causais diversos. Por isso, a continuidade das avaliações do presente estudo se focou no terceiro capitulo, onde as concentrações de maghemita (γ-Fe_2O_3) funcionalizadas com citrato na concentração ambiental de ferro (0,3 mg L^{-1}) induziram um aumento e desenvolvimento dos MMC em número, tamanho e densidade celular juntamente com os danos histopatológicos na fêmea *P. reticulata* durante os 21 dias de exposição.

A exposição a longo prazo (14 e 21 dias) às NOFs aumentaram o número de MMC, por certo esta resposta celular é resultado da ação inflamatória e consequente ativação da resposta imune à exposição das NOFs (capitulo 3, figuras 19 a 20). Esta resposta imune resulta em alterações histopatológicas

associadas a distúrbios circulatórios (Ij1) e respostas inflamatórias (Ij2) encontradas no fígado de animais expostos a IONPs após 3 e 7 dias (capitulo 3, figuras 22 e 23).

Os resultados indicaram que o estudo de respostas quantitativas e qualitativas associadas as análises semi-quantitativas juntamente com o uso de técnicas histológicas especificas são parâmetros de extrema importância para entender a ecotoxicidade das NOFs (Sinhorin et al., 2014; Rocha et al., 2015).

As alterações no tecido hepático juntamente com a análise dos MMCs são biomarcadores adequados para entender a hepatotoxicidade das NOFs em espécies de peixes.

Conclusões gerais

- O presente estudo indica que os NPs de maghemita funcionalizadas com citrato na concentração relevante ambiental (0,3 mg L-1) apresentam potencial genotóxicos e mutagênicos em fêmeas P. reticulata em um padrão dependente do tempo de exposição;
- Os animais expostos às NOFs apresentam danos ao fígado, refletindo a saúde do animal. O efeito genotóxico antecede os efeitos mutagênicos, sendo que os maiores efeitos mutagênicos foram observados na exposição de longo período;
- As respostas histopatológicas e imune dos guppies expostos às NOFs foram tempo dependentes;
- Existe um aumento no número e área de CMM em animais expostos às NOFs;
- Os centros de melanomacrofagos (MMC) foram o melhor parâmetro quantitativo para avaliar a exposição às NOFs;
- A exposição prolongada às NOFs induziu uma resposta histopatológica elevada em população celular residente e transitória do fígado;
- Os resultados indicaram P. reticulata como espécies alvo para realizar testes genotóxicos e mutagênicos;
- CMM e índice histopatológico são biomarcadores para a toxicidade às NOFs.

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