UNIVERSIDADE FEDERAL DO RIO GRANDE
Instituto de Ciências Biológicas - ICB
Programa de Pós-Graduação em Ciências Fisiológicas
Dissertação de Mestrado

Respostas toxicológicas induzidas pela coexposição a diferentes formas cristalinas do nanomaterial dióxido de titânio (rutila e anatase) e cobre no mexilhão dourado *Limnoperna fortunei*.

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Março, 2017.

Agradecimentos

Gostaria de agradecer o apoio, incentivo e ensinamentos que recebi de minha orientadora Juliane Ventura Lima e coorientador José Maria Monserrat. Os ensinamentos do professor Marcos Alexandre Gelesky e suporte de Caroline Pires Ruas. A ajuda do Marcelo Estrella Josende em todas as etapas para a realização deste trabalho. E o auxílio do meu marido Juliano da Silva Barreto nos experimentos e análises.

A todos, muito obrigada!

Sumário

Lista de abreviações:

CAT: enzima catalase.

CE: enzima carboxilesterase.

Cytox: enzima citocromo c oxidase.

ERO: espécies reativas de oxigênio.

GSH: glutationa reduzida.

GST: enzima glutationa-S-transferase.

LPO: peroxidação lipídica.

MT: metalotioneína.

NM: nanomaterial.

$nTiO_2$: nano dióxido de titânio.

$nTiO_2A$: nano dióxido de titânio, forma cristalina anatase.

$nTiO_2R$: nano dióxido de titânio, forma cristalina rutila.

Se-GPX: enzima glutationa peroxidase dependente de selênio.

SOD: enzima superóxido dismutase.

1. Resumo Geral

A ampla produção e consequente utilização de nanomateriais (NM) em diversas áreas e aplicações conduzem para a potencial liberação dos mesmos nos ambientes, principalmente aquático, colocando em risco a biota. O NM de dióxido de titânio ($nTiO_2$) é um composto inorgânico usado na indústria para a fabricação de produtos variados e também tem sido utilizado com a finalidade de descontaminação ambiental, devido a sua capacidade de adsorver metais. Este NM pode existir como diferentes formas cristalinas (principalmente como anatase e rutila) que influenciam na sua aplicabilidade, toxicidade e provavelmente na sua capacidade de adsorção de metais. O cobre (Cu) é um metal de ocorrência natural, porém atividades antropogênicas podem aumentar a sua concentração no ambiente. Embora este metal seja essencial para muitas enzimas e proteinas, concentrações elevadas podem causar efeitos tóxicos aos organismos e consequentemente aos ecossistemas. Dificilmente um contaminante ocorre em forma isolada no ambiente, surgindo assim, a necessidade de se estudar os possíveis efeitos combinados dos mesmos. Como os NM são compostos emergentes, pouco se sabe sobre o efeito da coexposição com outros contaminantes mais conhecidos, como é o caso do Cu. Por esta razão, este trabalho teve como objetivo avaliar se a coexposição a diferentes formas cristalinas do $nTiO_2$ (rutila e anatase) poderiam influenciar na toxicidade e bioacumulação do Cu no mexilhão dourado *Limnoperna fortunei*. Para alcançar estes objetivos os seguintes parâmetros foram analisados nas brânquias, glândula digestiva e músculo adutor: **(1)** quantificação de Cu, **(2)** determinação da atividade das enzimas glutationa-S-transferase (GST), superóxido dismutase (SOD) e catalase (CAT) e **(3)** avaliação dos níveis de peroxidação lipídica (LPO). Os resultados mostraram que as coexposições aumentaram a acumulação de Cu nos três tecidos. Nas brânquias a GST foi aumentada pela rutila, Cu e coexposição rutila + Cu; a SOD foi aumentada pelo Cu e diminuída pela rutila e coexposições; todos os tratamentos inibiram a CAT, a rutila induziu dano lipídico e as coexposições reduziram os níveis de dano lipídico. Na glândula digestiva a GST foi aumentada pela anatase e diminuída pela coexposição rutila + Cu; a

SOD foi inibida pela anatase; todos os tratamentos inibiram a CAT e a anatase induziu dano lipídico. No músculo adutor a GST foi inibida pela rutila, Cu e coexposições; a SOD foi inibida pela rutila, Cu e coexposição rutila + Cu; a CAT foi diminuída pela rutila, Cu e coexposição anatase + Cu e todos os tratamentos reduziram o dano lipídico. De maneira geral, a rutila se mostrou mais tóxica nas brânquias e músculo adutor, enquanto que a anatase se mostrou mais tóxica na glândula digestiva. Além disso, a coexposição de diferentes formas do $nTiO_2$ com o Cu alterou alguns efeitos causados pelo Cu. Por exemplo, as coexposições aumentaram a acumulação de Cu nas brânquias e músculo adutor e causaram modulação da atividade das enzimas GST, SOD e CAT, uma vez que, as coexposições foram capazes de aumentar, diminuir ou bloquear os efeitos induzidos pelo Cu nos diferentes tecidos, induzindo o efeito sinérgico conhecido como Cavalo de Troia. Estes resultados sugerem que a coexposição de diferentes formas do $nTiO_2$ com o Cu foi tóxica aos organismos e o uso do $nTiO_2$ em remediação ambiental precisa ser melhor estudado.

Palavras-chave: nanomaterial de dióxido de titânio, nanotoxicologia, cobre, bivalve, estresse oxidativo.

2. Introdução Geral

2.1 Nanomateriais

Nanomateriais (NM) são compostos orgânicos ou inorgânicos que apresentam tamanho até 100 nm em pelo menos uma das dimensões e que estão sendo utilizados em diversas áreas, devido as suas propriedades físico-químicas peculiares (Aikten *et al.,* 2006). São utilizados em diferentes produtos e aplicações tais como: baterias, catalisadores, cerâmica, cosméticos, eletrônicos, plásticos, produtos farmacêuticos, protetores solares, tintas e vidros, entre outros (Bystrzejewska-Piotrowska *et al.,* 2009).

Devido à ampla utilização dos NM, os mesmos estão presentes em diferentes compartimentos ambientais (Gottschalk *et al.,* 2009). Uma vez no ambiente, os NM podem representar um risco aos organismos e ecossistemas. De fato, muitos estudos têm mostrado a toxicidade dos NM em diferentes organismos. Al-Subiai e colaboradores (2012) mostraram que a exposição ao fulereno (C_{60}) causou dano de DNA e anormalidades histológicas nas brânquias, glândula digestiva e músculo adutor do mexilhão marinho *Mytilus sp.* Também nas brânquias e gandula digestiva do molusco marinho *Ruditapes philippinarum* a exposição ao NM de óxido de zinco (nZnO) alterou o sistema de defesa antioxidante (**Figura 1**), uma vez que, aumentou a atividade das enzimas superóxido dismutase (SOD), catalase (CAT) e glutationa-S-transferase (GST), além de causar dano de DNA (Marisa *et al.,* 2016). Na carpa *Cyprinus carpio* houve aumento e inibição da atividade de enzimas antioxidantes e indução de dano lipídico após exposição ao NM de dióxido de titânio ($nTiO_2$) (Linhua *et al.,* 2009).

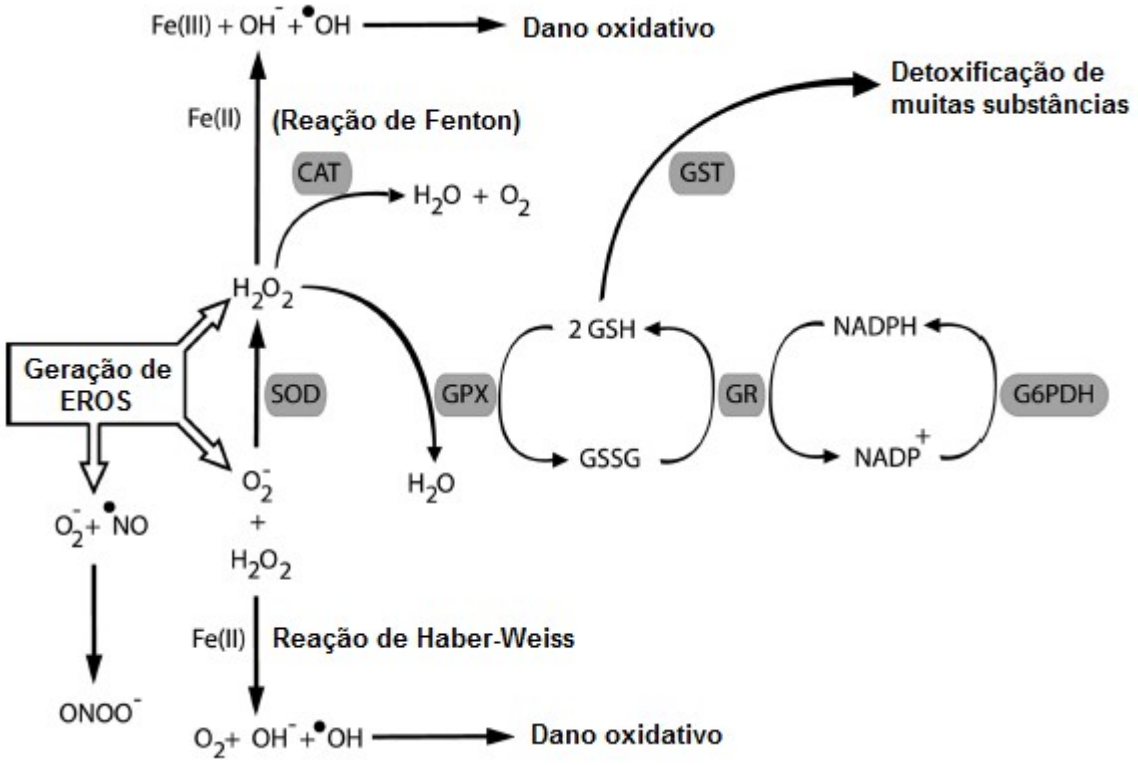

Figura 1: Sistema de defesa antioxidante. As abreviações SOD, CAT, GST, GR, GPx e G6PDH representam as enzimas superóxido dismutase, catalase, glutationa *S*-transferase, glutationa peroxidase e glicose-6-fosfato desidrogenase, respectivamente. As abreviações GSH e GSSG, representam a glutationa reduzida e glutationa oxidadada, respectivamente.

A toxicidade dos NM é determinada por características como tamanho, área de superfície, composição química, carga superficial, cristalinidade, forma, solubilidade e estado de aglomeração/agregação (Braakhuis *et al.,* 2014). O tamanho reduzido favorece a entrada dos NM nas células e organelas, comprometendo o seu funcionamento. Barmo e colaboradores (2013), observaram a presença do nTiO_2 nas microvilosidades e lisossomos de células da glândula digestiva de *Mytilus galloprovincialis.* Além do tamanho reduzido que auxilia na entrada em diferentes compartimentos biológicos, os NM apresentam uma área de superfície elevada para as reações químicas, o que aumenta a sua reatividade (Buzea *et al.,* 2007). Um estudo comparativo da toxicidade de óxidos de alumínio, silício, titânio e zinco em micro e nanoescala em diferentes espécies de bactérias, mostrou que todas as nanopartículas apresentaram maior toxicidade do que os

óxidos em microescala (Jiang *et al.,* 2009). Além disso, os NM podem aglomerar e formar partículas de maior diâmetro, o que pode afetar a captação celular e toxicidade dos NM. De fato, em diferentes linhagens celulares humanas, o $nTiO_2$ e nanopartículas de prata (nAg) foram capazes de induzir citotoxicidade, sendo as partículas com menor aglomeração as mais tóxicas (Lankoff *et al.,* 2012).

Outro aspecto importante para a toxicidade dos NM é o complemento genético de cada organismo, que determina como cada organismo irá responder a exposição a diferentes substâncias/contaminantes (Buzea *et al.,* 2007). De fato, o estudo realizado por Wong e colaboradores (2010) mostrou que o nZnO foi mais tóxico do que o ZnO nas algas marinhas *Skeletonema costatum* e *Thalassiosia pseudonana*, porém foi relativamente menos tóxico para os crustáceos *Tigriopus japonicus*, *Elasmopus rapax* e *Oryzias melastigma*. A exposição à nanopartículas metálicas em diferentes organismos mostrou que as nanopartículas de prata (nAg) e de cobre (nCu) foram tóxicas para todos os organismos estudados, mas o $nTiO_2$ não foi tóxico para nenhuma espécie (Griffitt *et al.,* 2008). Deste modo, são necessários estudos que considerem as propriedades dos NM em espécies distintas na tentativa de definir os mecanismos de ação de cada NM em cada espécie ou grupo.

Além disso, os NM podem interagir com outros contaminantes presentes na água devido a sua capacidade de adsorção, podendo provocar impactos indiretos aos organismos por causarem uma maior acumulação destes contaminantes e alterarem a toxicidade dos mesmos, efeito conhecido como *Cavalo de Troia* (Limbach *et al.,* 2007). Canesi e colaboradores (2014), por exemplo, observaram o efeito *Cavalo de Troia* no bivalve marinho *Mytilus galloprovincialis* causado pela coexposição do $nTiO_2$ com o poluente orgânico 2,3,7,8-TCDD (2,3,7,8-tetraclorodibenzo-p-dioxina).

2.2 Nanomaterial de dióxido de titânio

O mineral dióxido de titânio (TiO_2) ocorre naturalmente em três formas cristalinas denominadas rutila, anatase e brookita, além de uma forma amorfa (Reyes-Coronado *et al.,* 2008). Essas formas alotrópicas do TiO_2 possuem a mesma composição química, porém se diferenciam pela sua estrutura cristalina, ou seja, pelas diferentes posipções entre os átomos (**Figura 2**) e, consequentemente, apresentam propriedades químicas e físicas distintas (Buzea *et al.,* 2007).

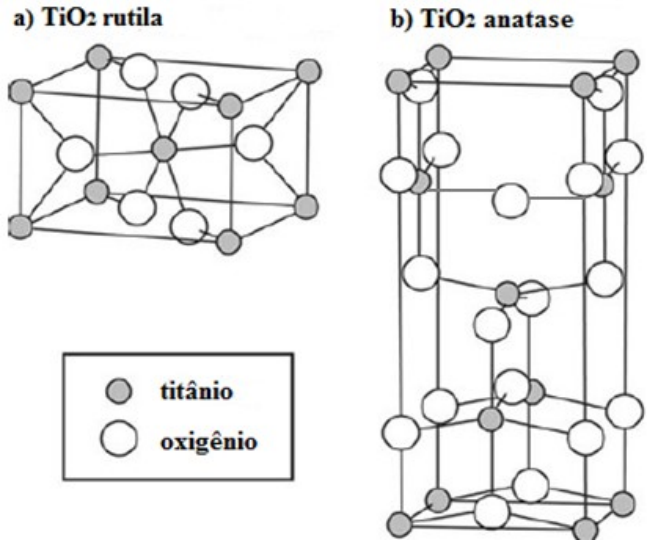

Figura 2: Formas cristalinas do dióxido de titânio: **(a)** rutila e **(b)** anatase. Extraído de Buzea *et al.* (2007).

Devido a isso, são utilizadas em diferentes aplicações e podem causar impactos ambientais distintos. A rutila é conhecida como pigmento branco e proporciona opacidade a tintas, papéis, plásticos, pasta de dentes, cosméticos e outras aplicações (Mueller e Nowack, 2008). Já a anatase é utilizada como eletrodo (Jiang *et al.,* 2002) e a atividade fotocatalítica é mais característica desta forma do que da rutila (Skokaj *et al.,* 2011).

Além disso, ambas formas cristalinas são produzidas em nanoescala e o tamanho das partículas pode alterar suas propriedades. A rutila é a forma mais comum encontrada na natureza (Menard *et al.,* 2011). Em diâmetros menores do que 11 nm a anatase é termodinâmicamente mais estável e a rutila é mais estável em tamanhos maiores que 35 nm (Kandiel *et al.,* 2010).

O nanomaterial de dióxido de titânio ($nTiO_2$) tem sido bastante produzido e utilizado em diversas aplicações como alimentos, catalisadores, cerâmica, cosméticos, papéis, plásticos, protetores solares e tintas, por conferir "brancura" e opacidade, apresentar atividade fotocatalítica, bloquear radiação ultravioleta (UV) e impedir o crescimento de microorganismos (Ge *et al.,* 2012; Kim *et al.,* 2012; Lin e Lin, 2011; Wang *et al.,* 2007). Além disso, o $nTiO_2$ tem sido aplicado para o tratamento de água contaminada por possuir uma área de superfície elevada e afinidade específica para a adsorção de metais a partir de sistemas aquosos (Hua *et al.,* 2012).

Devido a ampla aplicação do $nTiO_2$, existem predições de sua presença em diferentes compartimentos ambientais (Gottschalk *et al.,* 2009). No ambiente, o $nTiO_2$ pode causar efeitos tóxicos nos organismos e o estresse oxidativo parece ser o mecanismo chave responsável pelos efeitos adversos causados por este NM (Skokaj *et al.,* 2011). Em *L. fortunei* a exposição a diferentes concentrações do $nTiO_2$ (rutila + anatase) (1, 5, 10 e 50 µg/ml) inibiu a atividade da CAT e da SOD e reduziu o conteúdo de proteína sulfidrilada após 2 h (Girardello *et al.,* 2016b). Com base nestes resultados, os autores propuseram os possíveis mecanismos bioquímicos que podem ocorrer em *L. fortunei* após exposição ao $nTiO_2$ (**Figura 3**). O $nTiO_2$ pode ser fagocitado pelas células, liberado no citosol, e induzir a formação de espécies reativas de oxigênio (ERO), modular o sistema de defesa antioxidante enzimático e não enzimático (incluindo grupos sulfidrila de proteínas), induzir danos oxidativos (lipídios e DNA, por exemplo) causando uma situação de estresse oxidativo. De fato, a exposição a 1 mg/L de $nTiO_2$ causou diminuição da atividade da enzima antioxidante superóxido dismutase (SOD), redução dos níveis de glutationa reduzida (GSH) e peroxidação lipídica no bivalve marinho *Haliotis diversicolor supertexta* (Zhu *et al.,* 2011).

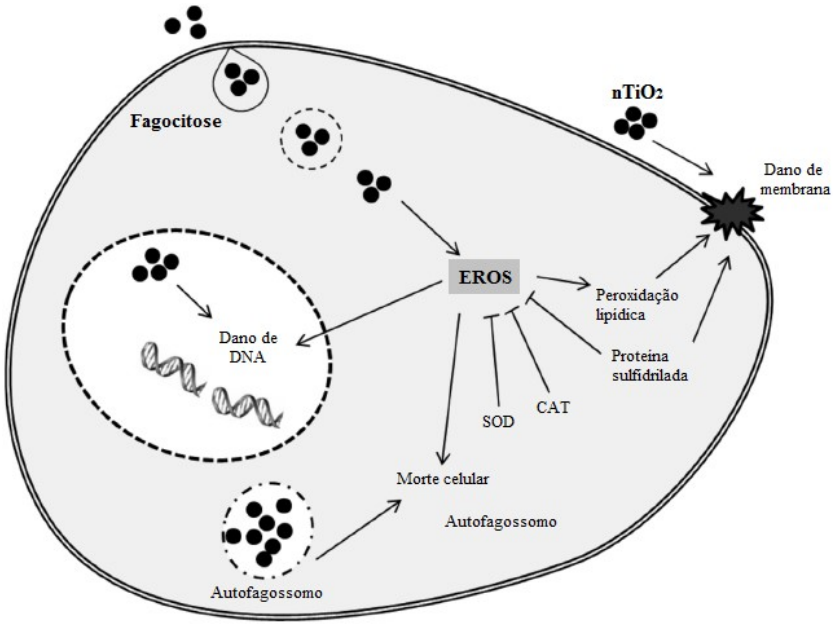

Figura 3: Indução de estresse oxidativo pela exposiçao ao $nTiO_2$ no mexilhão dourado *Limnoperna fortunei* (Girardello *et al.,* 2016b).

Além disso, estudos demonstram que a toxicidade do $nTiO_2$ é dependente da estrutura cristalina. Em hemócitos e células de brânquias de *Mytilus galloprovincialis* a mistura anatase + rutila foi mais tóxica do que a rutila (Katsumiti *et al.,* 2014). Em *Chlorella sp.* foi observada maior geração de ERO em algas tratadas com rutila (Iswarya *et al.,* 2015). Já em cérebro de camundongos a anatase causou peroxidação lipídica e diminuiu a capacidade antioxidante e a atividade de enzimas antioxidantes (Ma *et al.,* 2010).

Embora alguns estudos já tenham apontado diferenças em termos de toxicidade entre as duas formas cristalinas, pouco se sabe sobre a influência destas diferenças na toxicidade de outros contaminantes, como por exemplo, metais. Deste modo, são necessários estudos que avaliem o efeito da coexposição de diferentes formas do $nTiO_2$ com outros contaminantes ambientais.

2.3 Cobre

O cobre (Cu) é um metal de transição que ocorre naturalmente no ambiente, porém atividades antropogênicas como aplicação de pesticidas e fertilizantes, descargas industriais e mineração podem aumentar seus níveis ambientais (Nor, 1987).

Este metal atua como cofator para muitas enzimas e proteínas importantes em processos biológicos necessários para o crescimento, desenvolvimento e manutenção dos organismos. Dentre estas enzimas estão: álcool desidrogenase, citocromo c oxidase, cobre-zinco superóxido dismutase, dopamina beta hidroxilase, lisil oxidase, p-hidroxifenil piruvato hidrolase e tirosinase, entre outras (Ryu *et al.,* 2003; Serafim e Bebianno *et al.,* 2009).

A deficiência de Cu nos organismos pode diminuir a atividade de algumas enzimas que não contém este metal como grupo prostético, incluindo a CAT e a glutationa peroxidase selênio-dependente (Se-GPx), além de alterar os níveis de metalotioneínas (MT) e GSH (Adams e Keen, 2005). No entanto, quando presente em níveis elevados, o Cu pode ser tóxico e induzir diversos efeitos nos organismos. Essa toxicidade pode ser causada pelo radical hidroxila ($HO^{\cdot}$) formado na oxidação do íon Cu^{+} a Cu^{2+}, que pode interagir com proteínas, lipídeos e DNA e causar danos nestes componentes celulares (Halliwell e Gutteridge, 1985). Além disso, o Cu pode se ligar a grupos tióis de cisteínas podendo resultar na oxidação de proteínas, prejudicando a função das mesmas (Cecconi *et al.,* 2002).

Estudos têm demonstrado a toxicidade do Cu em organismos aquáticos (Maria e Bebianno, 2011; Trevisan *et al.,* 2011). Machado e colaboradores (2013) observaram que a exposição ao Cu induziu estresse oxidativo em diferentes tecidos do peixe *Poecilia vivipara*. No bivalve *Diplodon chilensis,* o Cu causou diminuição da atividade das enzimas SOD e glutationa-S-transferase (GST), aumento da atividade da CAT, aumento dos níveis de GSH e dano lipídico (Sabatini *et al.,* 2011). Em *Mytilus edulis* o Cu induziu dano de DNA, alterou os níveis de GSH e provocou anormalidades histológicas (Al-Subiai *et al.,* 2011).

2.4 *Limnoperna fortunei*

O mexilhão dourado *Limnoperna fortunei* (Mytilidae, Bivalvia) é uma espécie invasora de origem asiática, que atualmente está presente em diversos ecossistemas aquáticos de países sul-americanos como: Argentina, Uruguai, Paraguai, Bolívia e Brasil (Darrigran e Mansur 2006, 2009; Ricciardi, 1998). O sucesso invasivo desta espécie está relacionado à sua grande capacidade de adaptação a diversas condições ambientais, grande plasticidade fenotípica, alta fecundidade e capacidade de adesão a diferentes substratos, características estas que permitem que o *L. fortunei* colonize novos ambientes (Darrigran e Ezcurra, 2000; Iummato *et al.,* 2013).

Os bivalves têm sido reconhecidos como bons monitores de poluição ambiental por serem capazes de filtrar grandes volumes de água e processar microorganismos, sedimentos e partículas, acumulando diferentes substâncias presentes no ambiente em seus tecidos (Dagnino *et al.,* 2007). Devido a alta taxa de filtração do mexilhão dourado (125 a 350 ml/h por indivíduo) (Sylvester *et al.,* 2005), esta espécie pode ser utilizada como bioindicador dos níveis e distribuição de contaminantes em ecosistemas aquáticos. De fato, Villar e colaboradores (1999) observaram que esta espécie pode ser utilizada como biomonitor de poluição por metais. O estudo de Fiori e coautores (2012) mostrou que a concentração do herbicida glifosato na água foi dependente da presença do *L. fortunei* e variava com o tamanho dos organismos. Em outro estudo, a exposição ao glifosato causou aumento da peroxidação lipídica e diminuição da atividade das enzimas SOD e carboxilesterase (CE) nesta espécie (Iummato *et al.,* 2013). Além disso, bivalves tem sido considerados bons modelos para o estudo da toxicidade NM (Canesi *et al.,* 2012).

Embora alguns estudos já tenham avaliado o efeito da coexposição do $nTiO_2$ com o Cu em organismos aquáticos (Fan *et al.,* 2011), assim como o efeito da coexposição de diferentes formas do $nTiO_2$ com o Cu (Rosenfeldt *et al.,* 2015a) e também o efeito do $nTiO_2$ em *L. fortunei* (Girardello *et al.,* 2016a, b), ainda não existem estudos que tenham avaliado o efeito da coexposição de diferentes formas cristalinas do $nTiO_2$ com o Cu no *L. fortunei*.

3. Objetivos

3.1 Objetivo geral

Avaliar se a coexposição a diferentes formas cristalinas do nanomaterial dióxido de titânio (anatase e rutila) pode influenciar na toxicidade e bioacumulação do cobre no mexilhão dourado *Limnoperna fortunei*.

3.2 Objetivos específicos

Após a exposição ao Cu e/ou $nTiO_2R$ e ao Cu e/ou $nTiO_2A$, os seguintes objetivos foram levantados nos diferentes órgãos (brânquias, glândula digestiva e músculo adutor) analisados no bivalve *L. fortunei*:

a) Avaliar se a coexposição aos diferentes contaminantes (Cu, $nTiO_2R$ e $nTiO_2A$) pode induzir a dano lipídico.

b) Avaliar se a coexposição as diferentes moléculas acima mencionadas pode alterar o sistema de defesa antioxidante.

c) Evidenciar se a coexposição pode induzir mudanças no padrão de acumulação do Cu nos diferentes tecidos de *L. fortunei*.

Toxicological responses induced by coexposure of different cristalline forms of titanium dioxide nanomaterial (rutile and anatase) and copper in golden mussel *Limnoperna fortunei.*

(manuscrito a ser submetido à revista Aquatic Toxicology)

(Impact factor: 3.557)

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Abstract

The titanium dioxide nanomaterial ($nTiO_2$) has been produced and incorporated into various consumer products and commercial applications, due their catalytic, photocatalytic and ultraviolet (UV) light absorption properties. The wide applications of $nTiO_2$ can lead to the release of this nanomaterial into the environment endangering the organisms. In addition, $nTiO_2$ has been used in environmental remediation because of its ability to degrade or adsorb metals from water, and can coexist with metals such as Cu, for example. The $nTiO_2$ naturally exists in the crystalline forms rutile and anatase, which have different properties and can cause different effects in the organisms. This way, both $nTiO_2$ and Cu can occur together in the environment, and there is a need to study the potential combined effects of them. For this reason, this study aimed to evaluate if the coexposure to different crystalline forms of $nTiO_2$ (rutile and anatase) (1 mg/L) can influence in the toxicity and bioaccumulation of Cu (56 µg/L) in the golden mussel *Limnoperna fortunei* after 120 h of exposure. The following parameters were analyzed in the gills, digestive gland and adductor muscle: (1) quantification of Cu, (2) determination of the activities of glutathione-S-transferase (GST), superoxide dismutase (SOD), catalase (CAT) enzymes, and (3) assessment of the levels of lipid peroxidation (LPO). The results showed that coexpositions increased Cu accumulation in the three tissues independently of crystalline form. In the gills, rutile and coexposure rutile + Cu increased GST activity and decreased SOD activity. In the digestive gland, anatase increased GST activity and decreased SOD activity. In the adductor muscle, rutile and coexposure rutile + Cu decreased the activity of GST and SOD. All treatments inhibited CAT activity in the gills and digestive gland, while in the adductor muscle the CAT activity was inhibited by rutile and coexposure rutile + Cu. Only rutile caused lipid damage in the gills. These results suggest that both crystalline forms exhibited toxicity and that coexposure of $nTiO_2$ with Cu may be harmful to *L. fortunei*, thus more attention in use and release of $nTiO_2$ into environment are needs to avoid effects in aquatic biota.

Key words: titanium dioxide nanomaterial, nanotoxicology, copper, bivalves, oxidative stress.

1. Introduction

Nanomaterials (NM) are organic or inorganic compounds that have at least one of the dimensions up to 100 nm and have wide applicability in the most diverse areas (Aikten *et al.,* 2006). With increasing production and use of NM, the significant release of these into the environment seems to be inevitable (Galloway *et al.,* 2010). Gottschalk *et al.,* (2009) predicted the concentrations of some NMs in different environmental compartments (air, sediment, soil, surface water, and others) and stimated high concentrations of them in all compartments, for example, in sewage treatment plant sludge the concentrations of the NM titanium dioxide ($nTiO_2$) were 100 to 802 mg/kg in Europe, United States and Switzerland.

Among NM, titanium dioxide ($nTiO_2$) is one of the most produced and used in various products such as paints, pigments, varnishes, plastics, papers, catalysts, cosmetics, ceramics, and glasses (Kim *et al.,* 2012). In addition, $nTiO_2$ has been applied for the treatment of contaminated water because of their high surface area and specific affinity for the adsorption of heavy metals from aqueous systems (Hua *et al.,* 2012).

TiO_2 is a naturally occurring mineral that exists in three crystalline forms: rutile, anatase and brookite, beyond an amorphous form. Both crystalline forms are also found in the nanoparticulate form of TiO_2 (Reyes-Coronado *et al.,* 2008). Each form has different properties, and, consequently, different applications and potential environmental impacts. Rutile is the most form found in nature and has a larger surface area than anatase, which in turn is more stable than rutile in particle diameters up to 14 nm (Menard *et al.,* 2011). Although some studies have already pointed out the difference in toxicity of these two crystalline forms, little is known if these differences can influence the toxicity of other contaminants. In white shrimp *Litopenaeus vannamei*, the crystalline form rutile increased the activity of the enzyme glutamate-cysteine ligase (GCL), increased the antioxidant capacity and caused lipid damage in the gills after 48 h of exposure (Cordeiro *et al.,* 2015). In the marine bivalve *Haliotis diversicolor supertexta*, the crystalline form anatase increased

the activity of the enzyme superoxide dismutase (SOD), reduced levels of reduced glutathione (GSH) and increased levels of nitric oxide (NO) after 96 h of exposure (Zhu *et al.,* 2011). However, the difference in toxicity of both crystalline forms is still little studied for bivalves. For this reason, studies comparing toxicity between rutile and anatase are needed, to know which crystalline form of $nTiO_2$ would be most suitable for use in the remediation of contaminated environments.

On the other hand, the copper (Cu) is a naturally occurring metal in the environment, but anthropogenic activities such as application of pesticides and fertilizers, industrial discharges and mining can increase its environmental levels (Nor, 1987). Although it is an essential metal for acting as a cofactor of many enzymes and proteins (Gaetke and Chow, 2003), when present at high levels this metal can be toxic and induce diverse effects, including oxidative stress (Sabatini *et al.,* 2011). Besides, some studies have shown that Cu can induce toxic effects also in bivalves (Maria and Bebianno, 2011; Trevisan *et al.,* 2011). In fact, these organisms have been recognized as good indicators of environmental pollution by being able to filter large volumes of water and process microorganisms, sediments, and particles, accumulating different substances present in the environment in their tissues (Dagnino *et al.,* 2007).

The golden mussel *Limnoperna fortunei* (Mytilidae, Bivalvia) is an invasive species of Asian origin, which is present in several aquatic ecosystems of South American countries such as Argentina, Uruguay, Paraguay, Bolivia and Brazil (Darrigran and Mansur 2006, 2009; Ricciardi, 1998). The adaptability to distinct environmental conditions due to its high phenotypic plasticity, high fecundity index, and adhesion ability to different substrates, allow this species to colonize new environments (Darrigran and Ezcurra, 2000; Iummato *et al.,* 2013). Because it has a high filtration rate (Sylvester *et al.,* 2005), *L. fortunei* has been used as a bioindicator of environmental pollution (Fiori *et al.,* 2012, Iummato *et al.,* 2013, Villar *et al.,* 1999). In addition, bivalves have been considered good models for the study of NM toxicity (Canesi *et al.,* 2012) and the golden mussel began to be used to evaluate NM toxicity (Girardelo *et al.,* 2016 a, b).

For the present study gills, digestive gland and adductor muscle were used. The gills are considered the main route of entry of contaminants because the large surface of contact with the aquatic environment, favoring the diffusion of substances from the medium for this organ (Heath, 1995). The digestive gland has been repeatidly used in toxicological studies with mussels because it is the main metabolizing organ of some compounds and with biotransformation activity (Linvingstone, 1998). Finally, the adductor muscle is involved with the opening and closing of the shell (Hickman *et al.,* 2001), a mechanism of protection of bivalves against environmental variations, such as exposure to metals (Kádár *et al.,* 2001).

Several studies showed that NM can facilitate the uptake of other contaminants by the organisms and/or increase the toxicity of these contaminants, an effect known as a Trojan Horse (Limbach *et al.,* 2007). In the estuarine polychaete *Laeonereis acuta* the exposure of $nTiO_2$ with arsenic (As) affected the metabolization capacity of As and proved to be more harmful than the exposure to the isolated contaminants (Nunes *et al.,* 2017). In *Litopenaeus vannamei* the coexposure of $nTiO_2$ and As also affected the metabolism of this metalloid (Cordeiro *et al.,* 2015). For this reason, the purpose of this study was to evaluate if the coexposure to different crystalline forms of $nTiO_2$ (rutile and anatase) can influence in the toxicity and bioaccumulation of Cu in the golden mussel *Limnoperna fortunei* after 120 h of exposure.

2. Materials and Methods

2.1. Obtainment and maintenance of bivalves

L. fortunei organisms were obtained using "picard" type dredgers to facilitate the collection of submerged animals incrustated in the walls of the first rise of Rio Grande Sanitation Company (Corsan) channel (32° 3'14.39"S and 52° 22'18.28"W). After collection, the animals were transferred to the Biological Sciences Institute (ICB) of the Federal University of Rio Grande (FURG), where they were placed in monoblocks containing 20 L dechlorinated water, constant

aeration at 25 °C, feed with 500 mg of Supervit (beta-1.3/1.6-glucan) twice a week. Partial water exchanges were performed daily to prevent excreta accumulation. The collection of animals was authorized by Brazilian agency SisBio (process number 52321-1) to Juliane Ventura-Lima, PhD.

2.2. Experimental design

The animals were acclimatized for 2 weeks prior of experiment in glass recipients (6 animals per recipient), containing 200 ml of dechlorinated water and maintained as described in Section 2.1. The organisms were divided into six experimental groups: control group (CTL); rutile group ($nTiO_2R$); anatase group ($nTiO_2A$); copper group (Cu); copper + rutile group ($nTiO_2R$ + Cu) and copper + anatase group ($nTiO_2A$ + Cu). As the animals have a small size (3 cm of shell length for adult animals), to carry out the different biochemical analyzes it was necessary to perform a pool of samples (1 sample corresponds to 6 animals). During the experimental time (120 h) the animals were not fed.

Mussels were exposed to 1 mg/L (both crystalline forms) during 120 h. This concentration of $nTiO_2$ is based on the work of Nunes *et al.,* (2017), which observed the effects of rutile in the estuarine polychaete *Laeonereis acuta*. For the treatment with copper a concentration of 56 µg/L was choosen (Cu as $CuCl_2.2H_2O$, Sigma-Aldrich), this concentration was based on the study of Al-Subiai *et al.,* (2011), which used the marine bivalve *Mytilus edulis* as a model. The concentrations of $nTiO_2R$, $nTiO_2A$ and Cu from the coexposure treatments were the same as those used in the groups exposed separately to each contaminant. After exposure time, the different tissues were removed, weighed and stored at -80 °C.

2.3. Biochemical dosages

2.3.1. Preparation of homogenized samples

The organisms were euthanized at cold and the differents tissues of *L. fortunei* were dissecated and homogenized (1:4, w:v) in KCl buffer (0.154 M), dithiothreitol (DTT; 1 mM), pH 6.5. Then the homogenates were centrifuged at 10,000 *x g* for 20 min at 4 °C. After centrifugation, the supernatants were removed, aliquoted and stored in ultrafreezer (-80 °C) until biochemical analysis. The biochemical analyzes were relativized by the total amount of proteins present in the samples. Quantification of total proteins (550 nm) was performed from a commercial kit based on the Biureto method using a microplate reader (Biotel ELx 800).

2.3.2. Activity of glutathione *S*-transferase (GST)

Glutathione *S*-transferase activity was measured using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate as described by the method of Habig *et al.,* (1974). This method is based in the conjugation of reduced glutathione (GSH) with CDNB at 25 ºC. The absorbance of conjugate was measured at 340 nm.

2.3.3. Activity of superoxide dismutase (SOD)

Superoxide dismutase activity was performed based on the method proposed by McCord and Fridovich (1969). The radical superoxide anion ($O_2^{\cdot-}$) is generated by the xanthine oxidase system and the reduction of the cytochrome c was detected at 550 nm. The enzymatic activity was expressed in units of SOD where a unit is defined as the amount of enzyme required to inhibit 50% reduction of cytochrome c per minute and per mg of protein at 25 °C and pH 7.8.

2.3.4. Activity of catalase (CAT)

The catalase activity was performed by quantifying the H_2O_2 decomposition (50 mM) at 240 nm, according to the method described by Beutler (1975). The results were expressed as the unit of CAT, where a unit is the amount that the enzyme hydrolyses 1 μmol H_2O_2 per minute and per mg protein at 25 °C and pH 8.0.

2.3.5. Determination of lipid peroxidation level

The lipid peroxidation was performed using the FOX (ferrous oxidation in xylenol orange) method, following the methodology described by Hermes-Lima *et al.,* (1995) and Jiang *et al.,* (1991, 1992), with modification of incubation time without cumene hydroperoxide (CHP) for 2 hours, that was previously tested in this work for *L. fortunei.* The absorbance was measured at 580 nm and the results were expressed as CHP equivalents/mg wet weight.

2.4. Chemical analisys

2.4.1. Caracterization of $nTiO_2$ nanoparticles

Nanoparticles of $nTiO_2$ rutile (99.9% purity, rurile crystal structure) and anatase (99.7% purity, anatase crystal structure) were obtained from Sigma-Aldrich. The crystalline form of $nTiO_2$ nanoparticles was identified by comparison of X-ray diffraction patterns using Shimadzu XRD-6000 diffractometer which uses CuK radiation (1.5418A) in the range of 20 to 90 at a rate of 2°/min.

The surface morphology, particle size and shape of $nTiO_2$ nanoparticles were analyzed by scanning electron microscopy (SEM), using the microscope (JEOL JSM 6610) in the secondary ion mode (SEI) with a voltage of 20 kV and the samples were coated with carbon. The morphology was also analyzed by transmission electron microscopy (TEM), where the microscope (JEOL JEM 1400) was operated at an acceleration voltage of 100 kV. The stock suspensions of 1 g/L of $nTiO_2$

were prepared in milli-Q water and sonicated for 16 min with a 10% amplitude, then diluted (100x) and redispersed in water milli-Q, placed in an ultrasonic bath for 10 minutes and deposited three times on the carbon-coated copper grid. The energy dispersive spectrometry (EDS) was performed to analyze the presence of impurities of the different forms of $nTiO_2$ and also to analyze the presence of TiO_2 in the mucus filaments. Finally, the size distribution histogram was obtained by counting 300 measurements of diameter, using the program SigmaScan Pro 5.

2.4.2. Copper accumulation

The accumulation of Cu in the different tissues analyzed was determined according to the method of Fattorini and Regoli (2004). The samples are dried to a constant weight at 60 °C for 8 hours and digested in concentrated nitric acid using microwaves. The analytical determination was carried out using an atomic absorption technique using graphite atomizing furnace and Zeeman effect (Varian Spectra 300 Zeeman). For this analysis, quality control and quality assurance were checked using a blank and reference standard materials (Mussel Tissue Standard Reference Materials [SRM] 2977, National Institute of Standards and Technology). The concentrations obtained from these SRM were always within the 95% confidence intervals of the certified values. Cu tissue concentrations were expressed as μg/g dw.

2.5 Statistical analysis

The data obtained in the different parameters were tested through analysis of variance (ANOVA). To verify the significant differences, the Newman-Keuls post-hoc test ($\alpha = 0.05$) was used. The normality and homogeneity of the variance were previously analyzed and mathematical transformations were applied when necessary (Zar, 1984).

3. Results

The **Figure 1a** show X-ray diffraction diagram, where all diffraction peaks corresponding to $nTiO_2$ in the rutile crystalline form (peaks at 2Θ = 27.4; 36.1; 39.1; 41.27; 44.02; 54.4; 56.7; 62.7; 64.15; 69.02 and 69.8). While the **Figure 1e** show the X-ray diffraction diagram with peaks corresponding to $nTiO_2$ in the anatase crystalline form(peaks of 2Θ = 25.36; 36.86; 37.86; 38.84; 48.08; 53.93; 55.22; 62.69; 68.86; 70.30 and 75.2).

Microscopic analyzes (SEM and TEM) showed that the $nTiO_2R$ particles have a spherical shape, a well defined surface and size of 67 ± 20 nm (**Figure 1b, c and d**). The particles of $nTiO_2A$ also have a spherical shape, a well defined surface and size of 21 ± 5 nm (**Figure 1f, g and h**). In addition, the analysis by EDS showed that the rutile and anatase nanoparticles presented just titanium (Ti) and oxygen (O), thus both $nTiO_2$ nanoparticles used in this study are pure.

During the exposition to contaminants, mucus filaments from mussels were observed in the treatments with $nTiO_2R$, $nTiO_2A$, $nTiO_2R$ + Cu and $nTiO_2A$ + Cu, being more conspicuous in the treatment with $nTiO_2R$ (**Figure 2a, b**). EDS analysis showed that the mucous filaments of the treatment with $nTiO_2R$ and $nTiO_2A$ presented Ti and O. In the control and Cu groups no mucus formation was observed.

In terms of Cu accumulation, in the gills and digestive gland, there was a significant increase in Cu content in the organisms exposed only to this metal when compared with the control group ($p < 0.05$) (**Figure 3a, b**). In gills, the coexposure to both crystalline forms of $nTiO_2$ showed to increase the Cu accumulation when compared with group exposed only metal (**Figure 3a**). While in the digestive gland, the coexposure mantained the levels of cooper accumulation similar to group exposed only metal (**Figure 3b**). In the case of muscle, the group exposed only Cu not showed to accumulate this metal, but when coexposed with $nTiO_2R$ or $nTiO_2A$ was possible to observe a significantly increase in terms of copper accumulation ($p < 0.05$) (**Figure 3c**).

In the gills, there was a significant induction of GST activity in the organisms exposed only to $nTiO_2R$, Cu and in the coexposure $nTiO_2R$ + Cu ($p \leq 0.05$) (**Figure 3d**). In the digestive gland, there was a significant induction of GST activity in the organisms exposed only to $nTiO_2A$ and there was also a significant reduction of GST activity in the coexposure $nTiO_2R$ + Cu when compared to the treatment only with Cu ($p \leq 0.05$) (**Figure 3e**). In the adductor muscle, there was a significant reduction in GST activity in the organisms exposed only to $nTiO_2R$, to Cu and in both treatment of coexposure ($p \leq 0.05$) (**Figure 3f**).

In the gills, there was a significant increase in SOD activity in organisms exposed only to Cu and a significant decrease in SOD activity after exposure to $nTiO_2R$, in the coexposure $nTiO_2R$ + Cu and $nTiO_2A$ + Cu ($p \leq 0.05$) (**Figure 4a**). In the digestive gland, was observed a significant decrease in SOD activity in organisms exposed only to $nTiO_2A$ ($p \leq 0.05$) (**Figure 4b**). In the adductor muscle, there was a significant decrease in SOD activity in organisms exposed only to $nTiO_2R$, Cu and in the coexposure $nTiO_2R$ + Cu ($p \leq 0.05$) (**Figure 4c**).

In the gills and digestive gland, all treatments significantly reduced CAT activity when compared with control group ($p \leq 0.05$) (**Figure 4d, e**). However, in the adductor muscle there was a significant reduction of CAT activity only in the organisms exposed to $nTiO_2R$, Cu and in the coexposure $nTiO_2A$ + Cu ($p \leq 0.05$) (**Figure 4f**).

In the gills, there was a significant induction of the levels of lipid peroxidation in organisms exposed only to $nTiO_2R$ ($p < 0.05$). Also it was observed a significant reduction ($p < 0.05$) in the levels of lipid peroxidation in organisms coexposed to $nTiO_2R$ + Cu and to $nTiO_2A$ + Cu (**Figure 5a**). However, this result was not observed in the organisms exposed only to $nTiO_2A$ ($p < 0.05$) (**Figure 5b**). Interestingly, in adductor muscle all treatments significantly decreased levels of lipid peroxidation when compared with control group ($p < 0.05$) (**Figure 5c**).

4. Discussion

The use of $nTiO_2$ in various consumer products and their application in environmental remediation make possible its presence in the environment (Kim *et al.,* 2012; Liang *et al.,* 2004), generating concerns about the potential effects of this compound on living organisms, for this reason, many studies have been conducted to evaluate the effects of this NM on different species (Girardello *et al.,* 2016a, b; Iswarya *et al.,* 2015). These studies have showed that the main mechanism for $nTiO_2$ toxicity is oxidative stress, since it induces an increase in ROS levels, modulates the antioxidant defense system and may induces damage to biomolecules (Linhua et *al.,* 2009, Nunes *et al.,* 2017). On the other hand, the Cu also occurs in the environment due to natural and anthropogenic sources (Nor, 1987). Thus, studies have been carried out on the toxicity of this metal in different organisms, which also show to induce oxidative stress (El-Gendy *et al.,* 2009; Maria and Bebianno, 2011).

Bivalves have been widely used in toxicological studies because of their ability to filter and accumulate contaminants at levels close to that in the environment (Fukunaga and Anderson, 2011; Gregory *et al.,* 2002). In the present study, significant accumulation of Cu was observed in the gills and digestive gland of animals exposed only Cu and both coexposure conditions (1 mg/L of $nTiO_2$ (rutile or anatase) and 56 μg/L of Cu (**Figure 3a, b, c**). A similar result was also observed in a study that evaluated the effects of exposure to Cu in bivalves (Serafim and Bebianno, 2009; Trevisan *et al.,* 2011). In the groups coexposed, in the digestive gland, the Cu levels was maintened similar to group exposed only copper, while in gills and muscle the coexposure showed increase the cooper levels when compared with the group exposed to only cooper. In fact, some studies showed that NM can cause an increase in the concentration of other contaminants, for example, $nTiO_2$ increased the bioaccumulation of Cd in *Cyprinus carpio*, *Daphnia magna* and *Lumbriculus variegatus* (Zhang *et al.,* 2007), corroborating with the results observed in this study to gills and muscle.

GST is family of detoxification enzymes, which catalyzes the conjugation of reduced GSH with contaminants to facilitate the elimination of them (Strange *et al.,* 2001). Cu stimulated GST activity in the gills of *L. fortunei*, and inhibited this enzyme activity in the adductor muscle (**Figure 3d** and **3f**). In the gills of *Mytilus edulis* exposed the concentration of 56 µg/L Cu during 72h, also was not observed changes this parameter (Trevisan *et al.,* 2011). While in muscle of *P. vivipara* the exposure to Cu showed modulate the GST activity. These results, suggests that the effect of Cu on this biomarker is dependent on several factors such as: concentration, exposure time, tissue and species analysed. Considering the effects os NM, the rutile form increased the activity of GST in the gills and decreased in the adductor muscle, whereas the anatase caused alteration only in the digestive gland. In the freshwater bivalve *Corbicula fluminea*, the exposure to anatase (1 mg / L) for 72 h and 240 h did not alter GST activity (Vale *et al.,* 2014). In the digestive gland of the *Mytilus galloprovincialis* bivalve the exposure to different concentrations (0.2, 1 and 5 mg / L) of $nTiO_2$ (80% anatase + 20% rutile) during 24 h increased GST activity (Canesi *et al.,* 2010). With respect to the effect of the coexposure of $nTiO_2$ with Cu, the coexposure rutile + Cu decrease this enzyme activity in gills and digestive gland when compared with group exposed only to metal. A different result was observed in *Corbicula fluminea*, where the coexposure (0.1 e 1 mg/L of rutile + 112 µg/L of cadmium) did not modify the effects of Cd on GST activity (Vale *et al.,* 2014).

SOD is the enzyme responsible for catalyze the dismutation of the superoxide anion radical ($O_2^{\cdot-}$) in hydrogen peroxide (H_2O_2) and oxygen (O_2), transforming $O_2^{\cdot-}$ into a less reactive molecules (Hermes-Lima, 2004). In this study, SOD activity was stimulated in the gills, inhibited in the adductor muscle and did not suffer alteration in the digestive gland after exposure to Cu (**Figure 4a, b** and **c,** respectively). Modulation of the activity of this enzyme seem be one of the mechanisms of Cu toxicity (Machado *et al.,* 2013). In the digestive gland of bivalve *Diplodon chilensis*, the same result was observed by Sabatini *et al.,* (2011) after 1 week of exposure to this metal. In the bivalve *Pinctada fucata* the Cu exposure induced increased SOD activity in the gills and digestive gland at

different concentrations and exposure times (Jing *et al.,* 2006). These studies suggest that this enzyme is sensitive to Cu exposure being a good biomarker in aquatic organisms. Rutile was more toxic for SOD activity in the gills and adductor muscle (decreasing enzyme activity), whereas anatase caused inhibition only in the digestive gland. This provides evidence that the toxicity of $nTiO_2$ is dependent on the crystalline form, as has been seen in previous studies (Iswarya *et al.,* 2015; Sekar *et al.,* 2011), showing differences in terms of their impairment of the antioxidant system. Regarding the effect of $nTiO_2$ + Cu treatment, only in the gills the coexposure caused a distinct response to Cu treatment.

The conversion of H_2O_2 into H_2O and O_2 is performed by the CAT enzyme (Hermes-Lima, 2004). After exposure to Cu, there was a decrease in CAT activity in all tissues analyzed from *L. fortunei* (**Figure 4d, 4e** and **4f,** respectively). While in another species of bivalve (*Bathymodiolus azoricus*), an increase in the activity of this enzyme was observed (Company *et al.,* 2008) after Cu exposure. $nTiO_2R$ also reduced CAT activity in the three tissues, but anatase decrease this parameter only in the gills and digestive gland. In fact, seem that CAT activity is modulated by $nTiO_2$ independently of crystalline form. Canesi *et al.,* (2010) showed that exposure to 1 and 5 mg/L of $nTiO_2$ (80% anatase + 20% rutile) during 24 h increased the activity of this enzyme in *Mytilus galloprovincialis*. In this study, the coexposure showed to have an effect different from that induced by Cu in all tissues.This result was also observed in freshwater bivalve *Corbicula fluminea* after coexposure of different forms of $nTiO_2$ with cadmium (Cd) during 3 and 10 days (Vale *et al.,* 2014).

Lipid peroxidation has been used as a biomarker of oxidative stress in many studies (Radwan *et al.,* 2010). Cu and $nTiO_2$ exposure cause ROS generation, which in turn can cause oxidation to biomolecules (Drazkiewicz *et al.,* 2004; Park *et al.,* 2008; Zhu *et al.,* 2011). In this study, Cu did not cause lipid damage in the evaluated tissues (**Figure 5a, 5b** and **5c,** respectively). However, Geret and co-authors (2002) observed in other bivalve specie (*Ruditapes decussates)*, an

increase in peroxidized lipid levels in gills after 72h of exposure to 25 µg/L of Cu. On the other hand, our results showed that the $nTiO_2R$ caused lipid peroxidation in the gills, a similar result was observed in white shrimp *Litopenaeus vannamei* exposed to 10 µg/L of $nTiO_2$ (rutile) for 48 h (Cordeiro *et al.,* 2015). In the gills of *Cyprinus carpio* exposed to 100 and 200 mg/L of $nTiO_2$ (rutile) there was also a significant increase in levels of peroxidized lipids (Linhua *et al.,* 2009). These results suggested that, the gills appear to be a target tissue for the toxicity of $nTiO_2$ in the rutile crystalline form.

After 24 h of exposure to different forms of $nTiO_2$, rutile or anatase (alone or in coexposure with cooper) caused the formation of mucus filaments. In this mucus, was detected $nTiO_2$ and the degree of mucus formation was dependent on the crystalline form, once that rutile induced higher mucus formation when compared to anatase (**Figure 2a, b**). Studies have suggested that the production of mucus is responsible for the elimination of pollutants (David and Fontanetti, 2009). In *Mytilus edulis* was observed mucus formation after 24 h of exposure to 5 mg/L of $nTiO_2$ and carbon nanomaterial (Canesi *et al.,* 2010). In two other species of bivalves, *Perna viridis* and *Septifer virgatus*, the exposure to Cu increased the production of mucus in both species, showing that the mucus seems to be an effective barrier against Cu entry in these organisms (Sze and Lee, 1995). However, the formation of mucus observed in this study was not enough to avoid or decrease the copper levels mostly in all tissues analysed.

The results observed in this study showed that the different crystalline forms of $nTiO_2$ exert their toxicity in different tissues. Where the rutile appeared to have greater toxicity in the gills and adductor muscle, and anatase had greater toxicity in the digestive gland. The coexposure to Cu and $nTiO_2$ (rutile and/or anatase) showed to modulate the antioxidant responses and increase the Cu accumulation in all tissues analysed in this study, suggesting that condition of coexposure to long time can culminate in oxidative stress situation endangering the aquatic biota.

Acknowledgments

The authors wish to thank CNPq for financial support (MCTI/CNPq process nº 17/2011) and the CEME-SUL (Centro de Microscopia Eletrônica - FURG) for characterization of $nTiO_2$ nanoparticles. Silvana Manske Nunes and Marcelo Estrella Josende are graduate fellows at Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES). José Maria Monserrat is research fellows at CNPq. Juliane Ventura-Lima and José Maria Monserrat are members of the nanotoxicology network "Nanotoxicologia ocupacional e ambiental: subsídios científicos para estabelecer marcos regulatórios e avaliação de riscos" (CNPq, Proc. 552131/2011-3).

Conflict of interest

The authors declare that there are no conflicts of interest.

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6. Legends of figures

Figure 1: Characterization of $nTiO_2$ nanoparticles. **(a,e)** X-ray diffraction (XRD) pattern of rutile and anatase, respectively. **(b,f)** Scanning electron microscopy (SEM) image of rutile and anatase, respectively. **(c,g)** Transmission electron microscopy (TEM) image of rutile and anatase, respectively. Histogram showing the size distribution of rutile **(d)** and anatase **(h)**.

Figure 2: Images of mussels after 24 h exposure to $nTiO_2$ (1 mg/L), showing the mucous filaments of rutile **(a)** and anatase **(b)**.

Figure 3: Total copper concentration (expressed as µg/g/dry weight) in gills **(a),** in digestive gland **(b)** and in adductor muscle **(c)**. Glutathione-S-transferase activity (expressed as nmol of conjugated CDNB/min/mg of proteins) in gills **(d),** in digestive gland **(e)** and in adductor muscle **(f).** Different letter indicates significantly differences ($p<0.05$), while equal letters indicates absence of significantly differences ($p>0.05$) between means of treatments. All data are expressed as the mean ± 1 standard error (n=5).

Figure 4: Superoxide dismutase activity (expressed as units of SOD/mg of protein) in gills **(a)**, digestive gland **(b)** and adductor muscle **(c)**. Catalase activity (expressed as µmol of H_2O_2/min/mg of protein) in gills **(d)**, digestive gland **(e)** and adductor muscle **(f)**. Different letter indicates significantly differences ($p<0.05$), while equal letters indicates absence of significantly differences ($p>0.05$) between means of treatments. All data are expressed as the mean ± 1 standard error (n=5).

Figure 5: Levels of lipid peroxidation (expressed as nmol of CHP/g of wet tissue) in gills **(a)**, digestive gland **(b)** and adductor muscle **(c)**. Different letter indicates significantly differences

($p<0.05$), while equal letters indicates absence of significantly differences ($p>0.05$) between means of treatments. All data are expressed as the mean ± 1 standard error (n=5).

7. Figures

Figure 1

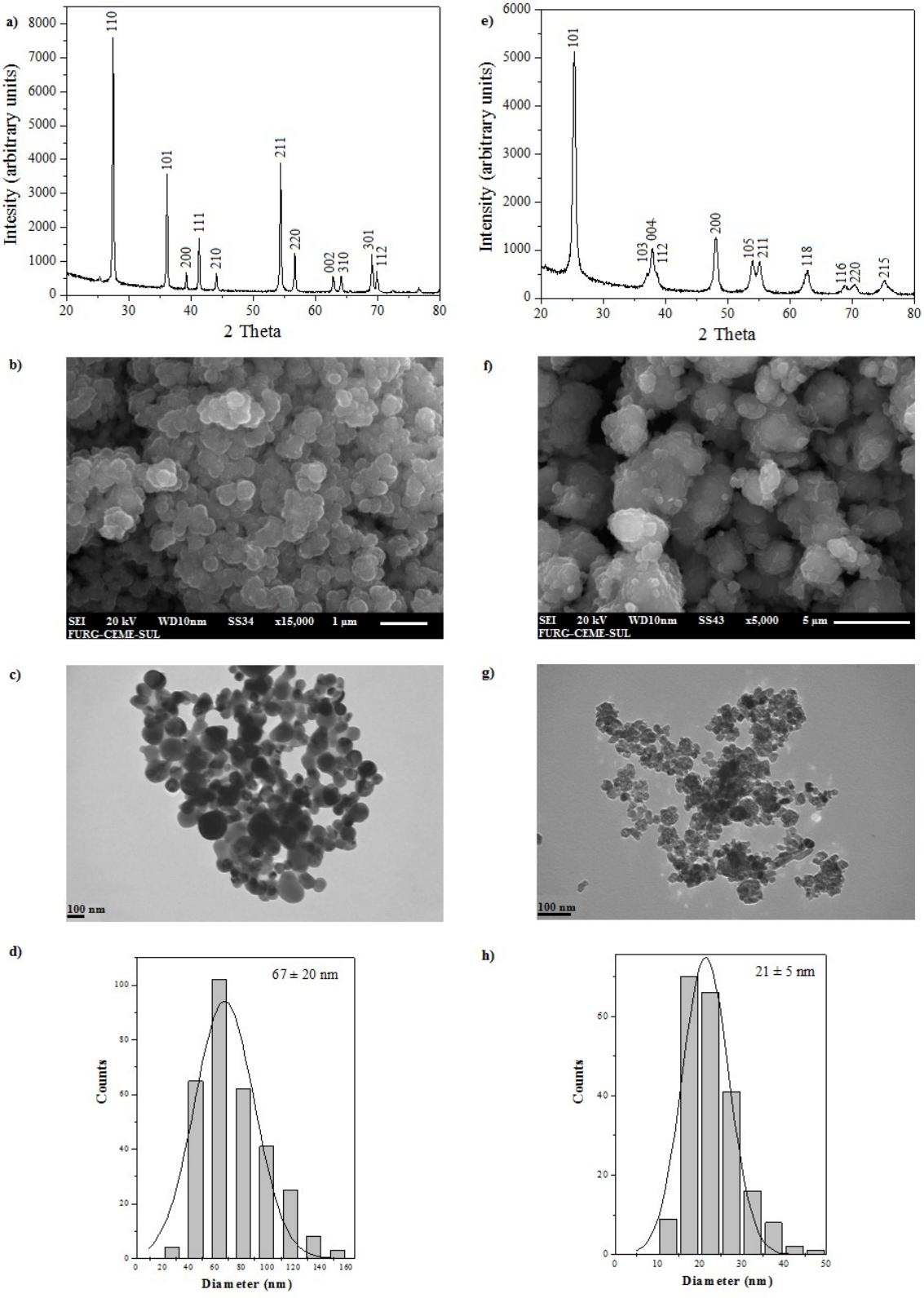


Figure 2

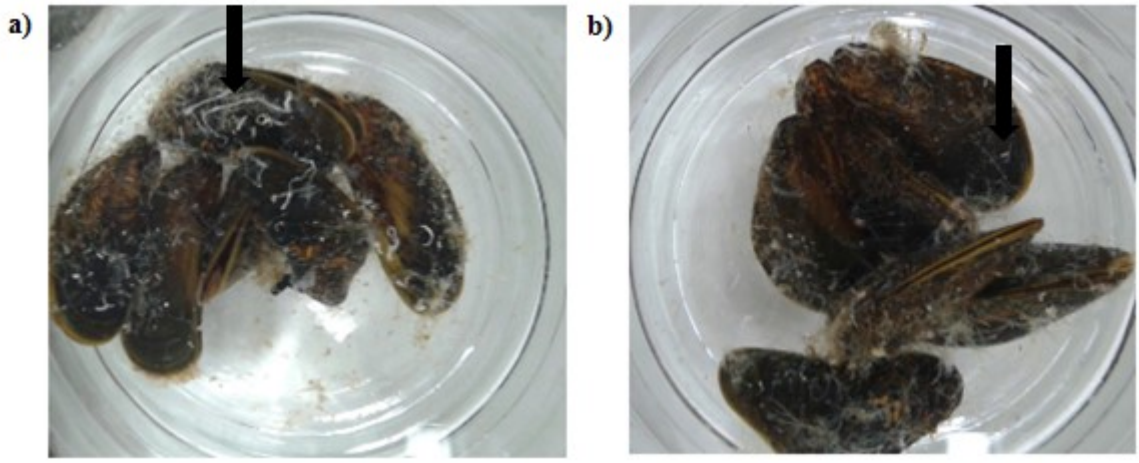


Figure 3

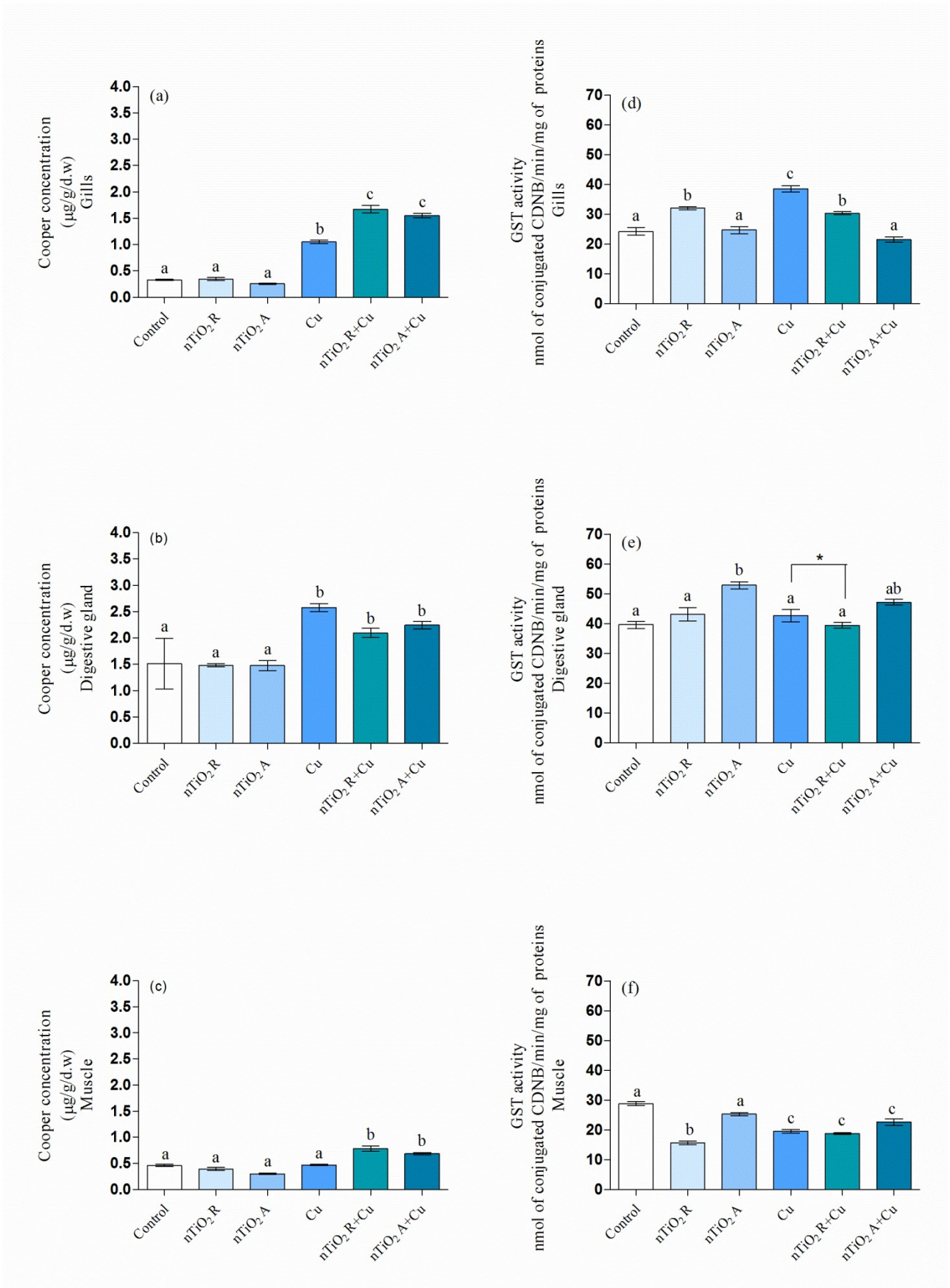

(a)
Cooper concentration (µg/g/d.w) Gills
(b)
Cooper concentration (µg/g/d.w) Digestive gland
(c)
Cooper concentration (µg/g/d.w) Muscle
(d)
GST activity nmol of conjugated CDNB/min/mg of proteins Gills
(e)
GST activity nmol of conjugated CDNB/min/mg of proteins Digestive gland
(f)
GST activity nmol of conjugated CDNB/min/mg of proteins Muscle
Control
nTiO2 R
nTiO2 A
Cu
nTiO2 R+Cu
nTiO2 A+Cu

Figure 4

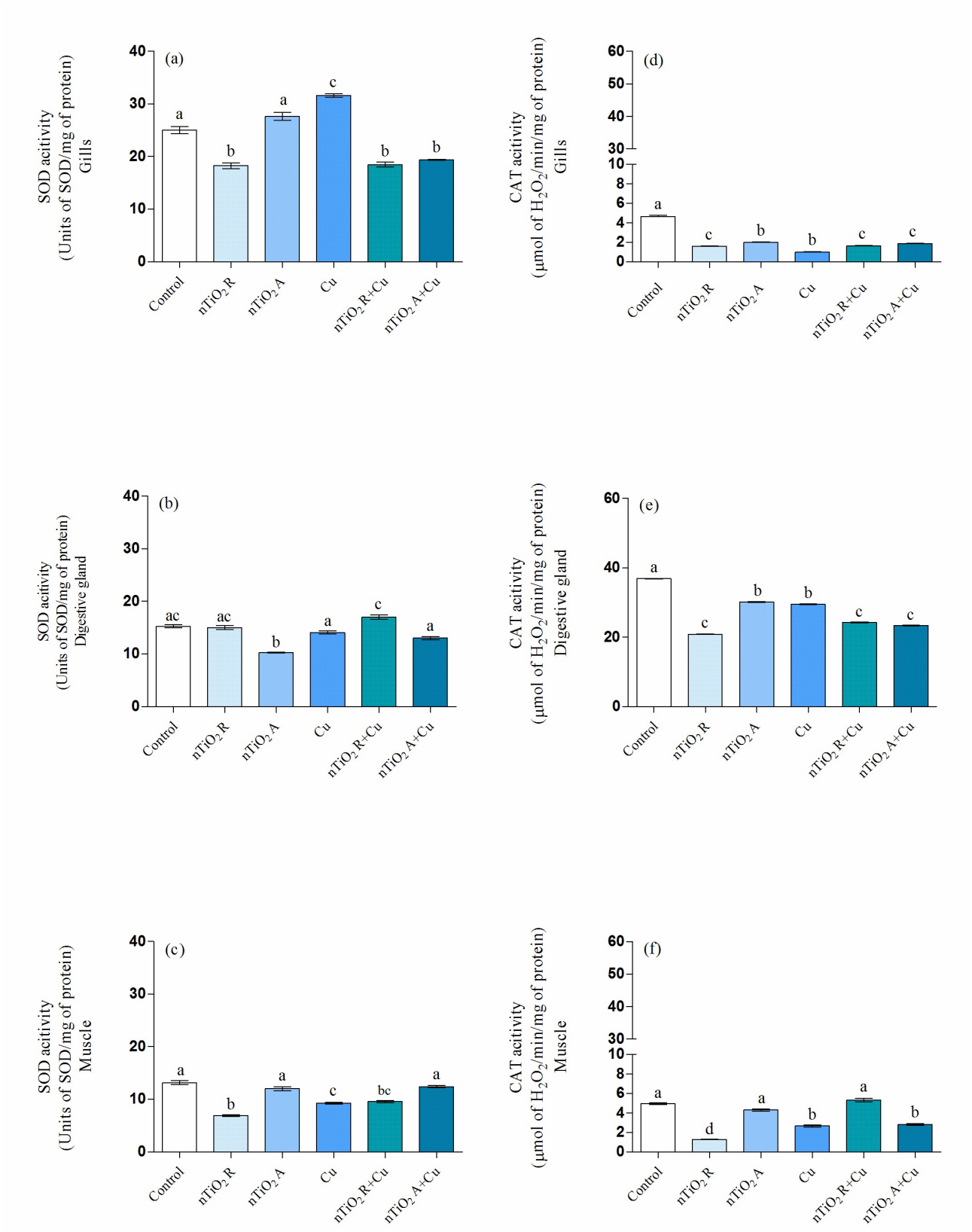
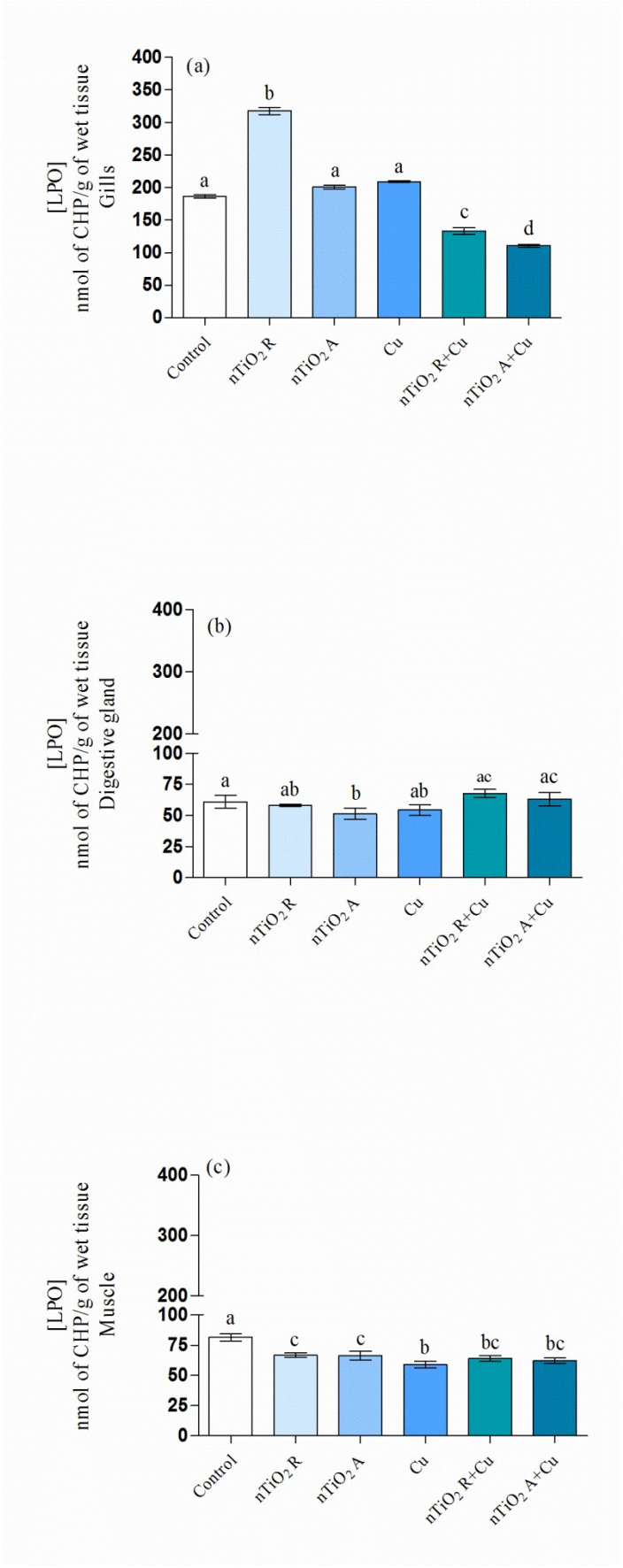

(a)
SOD acitivity
(Units of SOD/mg of protein)
Gills
(b)
SOD acitivity
(Units of SOD/mg of protein)
Digestive gland
(c)
SOD acitivity
(Units of SOD/mg of protein)
Muscle
(d)
CAT acitivity
(µmol of H2O2/min/mg of protein)
Gills
(e)
CAT acitivity
(µmol of H2O2/min/mg of protein)
Digestive gland
(f)
CAT acitivity
(µmol of H2O2/min/mg of protein)
Muscle
Control
nTiO2 R
nTiO2 A
Cu
nTiO2 R+Cu
nTiO2 A+Cu

Figure 5


(a)
[LPO]
nmol of CHP/g of wet tissue
Gills
400
350
300
250
200
150
100
50
0
a
b
a
a
c
d
Control
nTiO2 R
nTiO2 A
Cu
nTiO2 R+Cu
nTiO2 A+Cu
(b)
[LPO]
nmol of CHP/g of wet tissue
Digestive gland
400
300
200
100
75
50
25
0
a
ab
b
ab
ac
ac
Control
nTiO2 R
nTiO2 A
Cu
nTiO2 R+Cu
nTiO2 A+Cu
(c)
[LPO]
nmol of CHP/g of wet tissue
Muscle
400
300
200
100
75
50
25
0
a
c
c
b
bc
bc
Control
nTiO2 R
nTiO2 A
Cu
nTiO2 R+Cu
nTiO2 A+Cu

4. Discussão Geral

A utilização do $nTiO_2$ em diversos produtos de consumo e a sua aplicação na remediação ambiental torna provável a presença do mesmo no ambiente (Kim *et al.,* 2012; Liang *et al.,* 2004). De fato, Gottschalk *et al.,* (2009) observaram altas concentrações do $nTiO_2$ em diferentes compartimentos ambientais (água, ar, sedimento, solo, dentre outros). Devido a isso, surge a preocupação com os possíveis efeitos deste composto para os organismos. Por este motivo, muitos estudos têm sido realizados para avaliar os efeitos deste NM em diferentes espécies (Girardello *et al.,* 2016a, b; Iswarya *et al.,* 2015). Estes estudos têm demonstrado que o principal mecanismo para a toxicidade do $nTiO_2$ parece ser o estresse oxidativo (Lee *et al.,* 2012; Saquib *et al,* 2012; Xue *et al.,* 2010). Além disso, devido a utilização do $nTiO_2$ como adsorvente para a remoção de metais de sistemas aquosos (Hua *et al.,* 2012), o mesmo pode coexistir com outros contaminantes ambientais, como por exemplo o Cu, surgindo a necessidade de estudar os efeitos combinados destes compostos.

Embora alguns estudos já tenham avaliado os efeitos da coexposição do $nTiO_2$ com o Cu (Fan *et al.,* 2016; Rosenfeldt *et al.,* 2015b), pouco se sabe sobre o efeito de diferentes formas cristalinas do $nTiO_2$ na toxicidade e acumulação deste metal. No presente trabalho, foi observado que a rutila e a anatase exibiram diferentes respostas toxicológicas em diferentes tecidos, corroborando com estudos que têm demonstrado a influência da forma cristalina na potencial toxicidade do $nTiO_2$ em diferentes organismos (Clément *et al.,* 2013; Seitz *et al.,* 2014). Além disso, neste estudo as coexposições da rutila ou anatase com o Cu aumentaram a acumulação de Cu nos tecidos e foram capazes de alterar alguns efeitos induzidos pelo Cu (aumentando, diminuindo ou bloqueando a toxicidade do mesmo, dependendo do parâmetro e tecido analisado). Rosenfeldt *et al.,* 2015a observaram que diferentes formas do $nTiO_2$ (anatase, rutila e mistura de anatase com rutila) foram capazes de reduzir a toxicidade do Cu no crustáceo *Daphnia magna* após 48 h de exposição, além de diminuir as concentrações de Cu na coluna d'água. De fato, estudos têm demonstrado que o $nTiO_2$ pode aumentar a captação de outros contaminantes pelos organismos,

bem como, alterar a toxicidade destes contaminantes (Canesi *et al.,* 2014; Fan *et al.,* 2016; Li *et al.,* 2016).

7. Conclusão geral

Os efeitos do $nTiO_2$ estão relacionados com as características químicas e físicas da partícula de $nTiO_2$ como: área de superfície específica, estrutura cristalina, forma da partícula, tamanho, pureza, solubilidade e taxa de aglomeração. De fato, os resultados obtidos no presente trabalho corroboram com esta afirmação, uma vez que, a toxicidade do $nTiO_2$ foi dependente da forma cristalina. Além disso, as diferentes formas do $nTiO_2$ exercem sua toxicidade em diferentes tecidos. A rutila pareceu ter maior toxicidade nas brânquias e músculo adutor, uma vez que, modulou a atividade da GST (aumento nas brânquias e diminuição no músculo adutor), diminuiu a atividade da SOD e da CAT e, aumentou os níveis de lipídeos peroxidados nas brânquias. Enquanto que a anatase teve maior toxicidade na glândula digestiva, pois causou um aumento na atividade da GST e diminuiu a atividade da SOD e da CAT.

Com relação ao efeito das coexposições de diferentes formas do $nTiO_2$ com o Cu, este estudo mostrou diferenças causadas pelas coexposições nos efeitos já esperados para o Cu. Por exemplo, ambas coexposições causaram um aumento nos níveis de Cu nas brânquias e músculo adutor, quando comparado com o grupo exposto somente ao Cu. Além disso, a coexposição rutila + Cu diminuiu a atividade da GST nas brânquias e glândula digestiva, quando comparado ao grupo exposto somente ao Cu. Ambas coexposições diminuiram a atividade da SOD nas brânquias e a coexposição rutila +Cu aumentou a atividade da SOD na glândula digestiva, quando comparado ao grupo exposto somente ao Cu. Finalmente, ambas coexposições aumentaram a atividade da CAT nas brânquias e diminuiram na glândula digestiva quando comparado ao grupo exposto somente ao Cu e, a coexposição rutila + Cu bloqueou a toxicidade do Cu no músculo adutor. Essas diferenças demonstram que a coexposição de diferentes formas cristalinas do $nTiO_2$ com o Cu induziu o efeito

sinérgico conhecido como Cavalo de Troia. Portanto, os resultados observados neste estudo, juntamente com outros que estão sendo realizados na área, sugerem que a utilização deste nanomaterial (independentemente da forma cristalina) pode representar um perigo para a biota aquática, embora seja efetiva na remoção de contaminantes na água.

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