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Efeitos toxicológicos do dimetilarsênio e influência da coexposição ao nanodióxido de titânio no poliqueto estuarino *Laeonereis acuta* (Annelida, Polychaeta)

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Lista de abreviações

As - Arsênio

As^{III} - Arsenito

As^{V} - Arsenato

AsB - Arsenobetaína

AsC - Arsenocolina

AsS – Arsenoaçúcar

AdoMet - Adenosilmetionina

CAT - Catalase

DMA - Dimetilarsênio

ERO – Espécie reativa de oxigênio

GCL - Glutamato cisteína ligase

GSH – Glutationa reduzida

GR – Glutationa redutase

GSSG – Glutationa oxidada

GST – Glutationa-S-transferase

GSTΩ - Glutationa-S-transferase isoforma ômega

iAs – Arsênio inorgânico

LPO – Lipoperoxidação

MMA - Monometilarsênio

$nTiO_2$ – Nanodióxido de titânio

NM - Nanomateriais

PAHs – Hidrocarbonetos policíclicos aromáticos

TBT - Tributilestanho

TETRA - Tetrametilarsênio

TMAO – Trimetilarsênio

1. Resumo geral

Quando os organismos marinhos são expostos ao arsênio (As), eles retém, acumulam e biotransformam este metaloide para formas menos tóxicas. Este processo de metabolização já se mostrou ser afetado pela coexposição à nanomateriais como o nanodióxido de titânio ($nTiO_2$), favorecendo o acúmulo de formas moderadamente tóxicas de As como o dimetilarsênio (DMA). O $nTiO_2$ vem sendo utilizado para biorremediação de corpos de água contaminados por As, mas os efeitos da sua interação com este em organismos aquáticos não foram ainda devidamente estudados. Embora alguns organismos aquáticos tenham a tendência de acumular altos níveis de DMA, pouco se sabe sobre a toxicidade deste composto em organismos aquáticos. Sendo assim, o objetivo deste trabalho foi avaliar os efeitos tóxicos do DMA bem como sua acumulação e metabolização no poliqueto estuarino *Laeonereis acuta* e se a interação com o $nTiO_2$ pode afetar sua toxicidade. A metodologia desenvolvida avaliou a acumulação e especiação de compostos arsênicos, bem como atividade da glutationa–S–transferase, níves de glutationa reduzida, concentração de espécies reativas de oxigênio (ERO) e medidas de danos a macromoléculas, como peroxidação lipídica e dano de DNA. Os organismos foram expostos durante 48 h ao DMA (50 ou 500 µg/L) sozinho ou em combinação com o $nTiO_2$ (1 mg/L). Os resultados mostraram que a exposição ao DMA (50 µg/L) aumentou os níveis de ERO e de As (500 µg/L) enquanto que a co-exposição ao $nTiO_2$ nas mesmas concentrações de DMA mostrou reverter este aumento em ambos grupos. Por sua vez, o $nTiO_2$ mostrou induzir peroxidação lipídica (sozinho ou em combinação com o DMA). Entretanto, DMA sozinho ou em co-exposição ao $nTiO_2$ mostrou efeito genotóxico em *Laeonereis acuta*, mostrando que a seus efeitos tóxicos precisam ser estudados com mais atenção.

Palavras-chave: *Laeoenereis acuta*, dimetilarsênio, nanotoxicologia, genotoxicidade.

Abstract

When marine organisms are exposed to arsenic (As), they retain, accumulate and biotransform this metalloid to less toxic forms. This metabolization process has already been shown to be affected by coexposure to nanomaterials such as titanium nanodioxide ($nTiO_2$), favoring the accumulation of moderately toxic forms of As such as dimethylarsium (DMA). The $nTiO_2$ has been used for bioremediation of water bodies contaminated by As, but the effects of its interaction with As on aquatic organisms have not yet been properly studied. Although some aquatic organisms tend to accumulate high levels of DMA, little is known about the toxicity of this compound in aquatic organisms. Thus, the objective of this work was to evaluate the toxic effects of DMA as well as its accumulation and metabolization in the estuarine polychaete *Laeonereis acuta* and whether the interaction with $nTiO_2$ can modulate As toxicity. The developed methodology evaluated the accumulation and speciation of arsenic compounds, as well as glutathione-S-transferase activity, reduced glutathione levels, concentration of reactive oxygen species (ROS) and measures of macromolecule damage such as lipid peroxidation and DNA damage. The organisms were exposed for 48 h to DMA (50 or 500 μg / L) alone or in combination with nTiO2 (1 mg / L). The results showed that exposure to DMA (50 μg / L) increased ERO and As (500 μg / L) levels while co-exposure to $nTiO_2$ at the same concentrations of DMA showed a reversal of this increase in both groups. In turn, $nTiO_2$ has been shown to induce lipid peroxidation (alone or in combination with DMA). In this study, co-exposure to $nTiO_2$ was not shown to affect the accumulation capacity or arsenic metabolism. However, DMA alone or in co-exposure to $nTiO_2$ showed a genotoxic effect in *Laeonereis acuta.*

Key words: *Laeonereis acuta*, dimethylarsinic acid, nanotoxicology, genotoxicity.

2. Introdução geral

O arsênio (As) é um metalóide distribuído nos mais variados ambientes, principalmente aquáticos, devido principalmente a atividades antropogênicas como as industriais e agrícolas (Zhang et al., 2012). A concentração nos ambientes aquáticos pode variar de 0,5 µg/L para mais de 5000 µg/L na água (Huang et al., 2003). Este metalóide pode ser encontrado tanto na forma inorgânica como arsenito (As^{III}) e arsenato (As^{V}), quanto formas orgânicas, tais como monometilarsênio (MMA), dimetilarsênio (DMA), óxido trimetilarsênio (TMAO), tetrametilarsênio (TETRA), arsenobetaína (AsB), e arsenocolina (AsC) (Fattorini et al., 2013), sendo que as formas inorgânicas são consideradas mais tóxicas do que as formas orgânicas (Ventura-Lima et al., 2011).

A toxicidade do arsênio varia de acordo com seu estado de oxidação, concentração e forma química (Akter et al., 2005; Jomova et al., 2011; Sharma and Sohn, 2009). Compostos de As inorgânicos (iAs) como As^{V} e As^{III} são as principais formas que predominam na água do mar e sedimentos. No entanto, e devido a atividade de microorganismos, formas dimetiladas são também encontradas no sedimento (Zeng et al., 2018). Outros compostos como arsenobetaína (AsB), arsenocolina (AsC) e arsenoaçúcares (AsS) são geralmente predominantes em organismos marinhos e considerados não tóxicos (Akter et al., 2005; Fattorini et al., 2004).

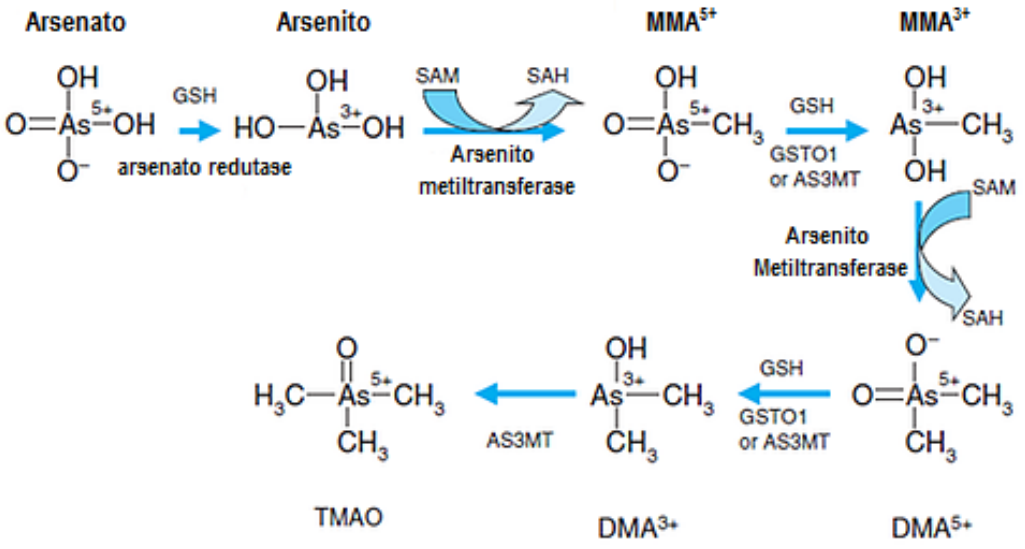

Figura 1: Processo de biotransformação do arsênio a partir da forma inorgânica pentavalente (As^{V}). Figura adaptada de Klaassen, C., (2013).

Uma vez incorporado pelos organismos, o As pode ser biotransformado em compostos menos tóxicos e ser acumulado ou excretado (**Fig. 1**). Independentemente da forma química, quando os organismos aquáticos são expostos, eles tendem a incorporar, acumular, e metabolizar o As (Fattorini et al., 2013). Este processo de metabolização envolve reações de oxidação/redução, utilizando glutationa reduzida (GSH) como doador de elétrons e adenosilmetionina (AdoMet) como um doador de grupos metilo, sendo a isoforma ômega da enzima glutationa-S-transferase (GSTΩ), a enzima chave na metabolização do As (Aposhian, 1997).

Algumas espécies de poliquetos mostram acumular DMA (**Fig. 2**) em quantidades consideráveis (Fattorini et al., 2005; Nunes et al., 2017; Ventura-Lima et al., 2007). Esta forma química por sua vez, é considerada moderadamente tóxica (Ventura-Lima et al., 2011). Embora poucos estudos tenham avaliado a potencial toxicidade desta forma de arsênio, estudos com mamíferos mostraram o efeito genotóxico e carcinogênico do DMA (Yamanaka et al., 2000, 1997, 1990; Yamanaka and Okada, 1994).

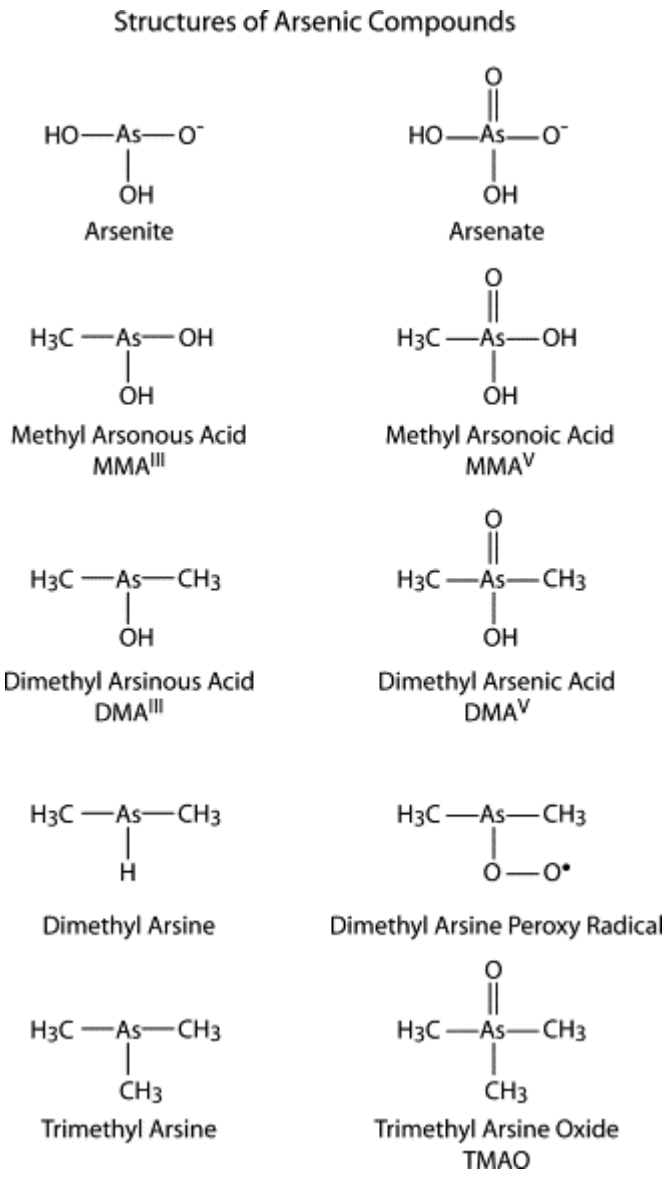

***Figura 2*:** estrutura química do dimetilarsênio e outros compostos de As (Rossman et al., 2003).

Muitos estudos têm mostrado que a exposição ao As pode induzir alterações no sistema de defesa antioxidante como aumento na atividade de enzimas antioxidantes e diminuição nos níveis de glutationa reduzida, bem como estresse oxidativo em organismo aquáticos (Kim and Kang, 2015; Sarkar et al., 2014; Zhao et al., 2017). Halliwell e Gutteridge (1999) descreveram o estresse oxidativo como "um desequilibro entre a concentração de pró-oxidantes e antioxidantes em favor dos primeiros, com possibilidade de induzir efeitos deletérios". Em (2006), Jones propôs outro conceito,

onde trata do estresse oxidativo como uma alteração nas reações de transferência de elétrons resultando em um desequilíbrio entre oxidantes/antioxidantes e danos oxidativos a macromoléculas. Para lidar com as situações pró-oxidantes, os organismos possuem o sistema de defesa antioxidante, que envolve defesas enzimáticas e não enzimáticas. Dentre as enzimáticas pode-se citar a GST (glutationa S-transferase), que desempenha um importante papel no processo de detoxificação celular, e a GR (glutationa redutase) uma enzima que catalisa a regeneração de da glutationa reduzida (GSH) a partir da glutationa oxidada (GSSG) e, desta forma, manter os níveis de GSSG sob controle. A GSH participa do sistema de defesa antioxidante não enzimático, sendo um tripeptídeo composto por glutamato, cisteína e glicina. A enzima glutamato cisteína ligase (GCL) é o passo limitante para a síntese da GSH (White et al., 2003). Os organismos vivos tem uma taxa basal de produção de ERO as quais, em condições normais, funcionam como importantes reguladores fisiológicos das vias de sinalização intracelular (Finkel, 2011). No entanto, um aumento elevado de ERO causa danos às células pela oxidação de biomoléculas celulares, incluindo ácidos nucléicos, proteínas e lipídios (Lobo et al., 2010).

Como mencionado anteriormente, a exposição ao As pode afetar a capacidade antioxidante bem como induzir estresse oxidativo em organismos aquáticos (Ventura-Lima et al., 2009, 2007). Ventura-Lima et al. (2007) mostraram que a exposição ao iAs^{III} em 48 h modulou a atividade de enzimas antioxidantes como a superóxido oxidase (SOD), catalase (CAT) e glutationa-S-transferase (GST), além de aumentar a lipoperoxidação (LPO) em *L. acuta*.

Ainda que poucos estudos tenham sido realizados avaliando os efeitos do DMA em organismos aquáticos, Yamanaka e colaboradores (2000) observaram efeitos tóxicos, como dano de DNA além da redução dos níveis de GSH em roedores. Também, no

crustáceo *Daphnia pulex*, esta forma dimetilada de As se mostrou mais tóxica do que o iAs (Shaw et al., 2007). Yamanaka e colaboradores (2001) mostraram que ratos expostos a DMA e As^{III} apresentaram altos níveis de 8-oxo-2'-deoxiguanosina (8-oxodG), um importante biomarcador de oxidação de DNA, sendo que os ratos expostos ao DMA apresentaram níveis ainda mais altos deste biomarcador. Ahmad e colaboradores (2000), relataram que entre o DMA e o iAs nas mesmas concentaçõees, o DMA foi o mais forte liberador de ferro da ferritina e a liberação de ferro sinérgico por DMA e ácido ascórbico (um conhecido liberador de ferro) da ferritina levou à formação de ERO. Além disso, alguns estudos tem demonstrado que formas orgânicas metiladas de arsênio como MMA e DMA, são mais tóxicas do que o iAs em exposição aguda, além de serem mais genotóxicas (Mass et al., 2001; Petrick et al., 2000).

No ambiente, bactérias presentes no sedimento participam do processo de biometilação do As, tornando o DMA disponível no ambiente (Zeng et al., 2018). Embora a forma predominante de As acumulada nos organismos marinhos seja AsB, alguns organismos como o poliqueto *L. acuta* acumulam DMA como forma predominante de As (Nunes et al., 2017; Ventura Lima et al., 2007). Além disso, a interação do As com nanomateriais pode alterar seu padrão de metabolização, acumulando predominantemente DMA ao invés de AsB no camarão branco *Litopenaeus vannamei* (Cordeiro et al., 2016).

Materiais em escala nanométrica (1-100 nm em pelo menos uma dimensão) são cada vez mais usados em indústrias e a nanotecnologia é uma importante área de crescimento econômico e científico (Aitken et al., 2006; Galloway et al., 2010). O nanodióxido de titânio ($nTiO_2$) é um dos nanomateriais inorgânicos mais utilizados na indústria, e suas aplicações incluem corantes alimentícios, cosméticos, protetores solares, e como descontaminantes de ambientes aquáticos (Aitken et al., 2006; Trouiller et al., 2009).

Sabe-se que alguns nanomateriais favorecem a entrada de substâncias através de membranas fosfolipídica e da barreira hematoencefálica (Disdier et al., 2017; Shrivastava et al., 2014) e, devido a isso, tem sido utilizado para distribuição de medicamentos no tecido nervoso (Masserini, 2013; Trouiller et al., 2009). Efeitos adversos, como disfunção celular, dano oxidativo, respostas inflamatórias, indução de trombose, prejuízo na memória de reconhecimento espacial e lesões no fígado foram demonstrados *in vitro* e *in vivo* após exposição a $nTiO_2$ em mamíferos (Hu et al., 2010; Li et al., 2008; Sha et al., 2011)

Zhu e colaboradores (2011) observaram em *Hediste diversicolor* um aumento nos níveis de peroxidação lipídica, diminuição nos níveis de GSH e aumento na atividade da superóxido dismutase quando expostos à 1 mg/L de $nTiO_2$. Além disso, o $nTiO_2$ em uma concentração inferior a 1mg/L pode causar disfunções respiratórias e perturbações no metabolismo de alguns metais traço, como zinco e cobre, na truta arco-íris (*Oncorhynchus mykiss*) após exposição crônica (14 dias) (Federici et al., 2007).

Estudos *in vitro* têm demonstrado que o $nTiO_2$ pode gerar ERO e induzir em danos oxidativos em organismos (Hattori et al., 2017; Nichols et al., 2018; Sonane et al., 2017). Essa geração de ERO pode ser promovida pela grande área de superfície, que é uma das principais características dos nanomateriais (NM). Em meio aquoso, o $nTiO_2$ facilita a transformação de moléculas de água adsorvidas na superfície da partícula, produzindo radicais hidroxila capazes de causar danos oxidativos aos componentes celulares e ao DNA (Wang et al., 2007).

Mudanças na atividade de enzimas antioxidantes também foram observadas em *Daphnia pulex* (Klaper et al., 2009), indicando que este NM afeta o estado redox das células. Também na truta *O. mykiss,* o $nTiO_2$ induziu peroxidação lipídica no organismo (Federici et al., 2007). Finalmente, Xiong e colaboradores (2011) encontraram altos

conteúdos de malondealdeído e proteínas carboniladas em fígado e brânquia de peixes (*Danio rerio*) expostos a $nTiO_2$.

Devido as suas características físico-químicas, os NM podem adsorver contaminantes, como hidrocarbonetos policíclicos aromáticos (PAHs) e alguns metais e metalóides, como o arsênio, diminuindo a concentração destes no ambiente (Tungittiplakorn et al., 2004). Porém, existe a possibilidade de que o tratamento com os NM ligados aos contaminantes, possam ser incorporados pelos organismos aquáticos podendo exercer efeitos tóxicos até mesmo superiores ao contaminante isolado, dado que alguns nanomateriais favorecem a entrada de substâncias através de membranas fosfolipídica e da barreira hematoencefálica (Disdier et al., 2017; Shrivastava et al., 2014). Zhu e colaboradores (2011) mostraram que a coexposição do tributilestanho (TBT) com o $nTiO_2$ aumentou a toxicidade do TBT em até 20 vezes em comparação com o TBT sozinho. Wang e colaboradores (2017) observaram que o $nTiO_2$ sozinho não exerceu citotoxicidade em cultura de células de mamíferos, mas quando coexposto ao As^{III} potencializou seus efeitos genotóxicos. Cordeiro e colaboradores (2016), observaram que o $nTiO_2$ coexposto com o As afetou a capacidade de metabolização deste metalóide, uma vez que houve um aumento na porcentagem de DMA em brânquias do camarão branco *Litopenaeus vannamei*, mostrando um predomínio da acumulação desta espécie de As quando comparada com o grupo controle, que acumulou principalmente na forma não tóxica (AsB). Um resultado semelhante também foi observado no poliqueto *L. acuta* quando exposto ao iAs e ao $nTiO_2$ (Nunes et al., 2017).

Estes resultados, sugerem que a co-exposição ao $nTiO_2$ afeta uma fase precoce da metabolização do As, diminuindo a capacidade de metabolização, evidenciada pelo acumulo de DMA. No entanto, não se sabe se este NM pode afetar a capacidade de

metilação a partir deste composto. Além disso, pouco se conhece sobre os efeitos da exposição a formas dimetiladas de arsênio em organismos estuarinos, e se a co-exposição à nanomateriais como o $nTiO_2$ pode influenciar nos possíveis efeitos induzidos pelo DMA.

Para avaliar se a co-exposição ao $nTiO_2$ pode influenciar nos efeitos induzidos pelo DMA, foi escolhido como modelo biológico o poliqueto estuarino *L. acuta,* tendo em vista que esta espécie mostrou ser um bom modelo para avaliar os efeitos do As, uma vez que possui respostas claras para exposição deste metalóide (Nunes et al., 2017; Ventura-Lima et al., 2011, 2007). Além disso, *L. acuta* é um elo importante na cadeia alimentar marinha, sendo predada por peixes, aves e invertebrados aquáticos (Pamplim et al., 2007), e por ser um animal que possui pouca mobilidade reflete as condições do local onde vive (Geracitano et al., 2004).

2. Objetivos

2.1. Objetivo Geral

Avaliar a influência da co-exposição do arsênio dimetilado (DMA) ao nanomaterial dióxido de titânio (nTiO2) na acumulação, metabolização e toxicidade em *Laeonereis acuta* frente à exposição aguda ao DMA.

2.2. Objetivos específicos

a) Avaliar se a exposição ao DMA pode induzir uma situação pró-oxidativa através do aumento da concentração de ERO.

b) Verificar se o $nTiO_2$ pode influenciar na capacidade de acumulação e metabolização do arsênio na forma dimetilada.

c) Avaliar se a exposição ao DMA (sozinho ou em combinação com o $nTiO_2$) pode modular defesas antioxidantes como atividade da GST e níveis de GSH.

d) Avaliar se a exposição ao DMA pode induzir danos a lipídios e ao DNA e se a co-exposição ao $nTiO_2$ pode influenciar nos potenciais efeitos do DMA.

MANUSCRITO

Genotoxic effect of dimethylarsinic acid and influence of coexposure to titanium nanodioxide ($nTiO_2$) in *Laeonereis acuta* (Annelida, Polychaeta).

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Chamada especial: "New research on Arsenic in Latin America, a still unrevealed continent: Occurrence, health effects and mitigation"

Genotoxic effect of dimethylarsinic acid and influence of coexposure to titanium nanodioxide ($nTiO_2$) in *Laeonereis acuta* (Annelida, Polychaeta).

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Abstract:

Arsenic (As) is a metalloid widely distributed into the aquatic environment, and although the inorganic forms are predominant in these environments, dimethylated forms also can be found. However, few data are available about the effect of dimethylated forms of arsenic (DMA) in living organisms and, particularly, in aquatic ones. As As contamination is a global problem, several strategies are being used as alternative to decontamination of aquatic environment. Titanium dioxide nanoparticles ($nTiO_2$) are being tested as decontaminant of arsenic due to its high adsorption capacity. So, this study evaluated the toxicological effects of DMA in acute exposure condition and if $nTiO_2$ can influence in the effects induced by DMA in polychaete *Laeonereis acuta*. The animals were exposed during 48 h to DMA (50 and 500 μg/L) alone or in combination with $nTiO_2$ (1 mg/L). Biochemical parameters such as generation of reactive oxygen species (ROS) concentration, glutathione-S-transferase (GST) activity, levels of reduced glutathione levels (GSH), and macromolecules (lipid and DNA) damage were evaluated. In addition, it was determined accumulation of total As and chemical speciation of the metalloid in organisms. Only the group exposed to 500 μg of DMA/L accumulated As and when co-exposed to $nTiO_2$ this accumulation was not observed. The levels of ROS increased in the group exposed to 50 μg/L of DMA alone and when this group was co-exposed to $nTiO_2$, the effect was reversed. None of the treatments showed altered GST activity or GSH levels. However, all groups that received $nTiO_2$ (alone or in combination with DMA) showed lipid damage. Also, the exposure to DMA (both concentrations) alone or in combination with $nTiO_2$ induced DNA damage in *L. acuta*. These results showed that DMA exhibit genotoxic effect and that co-exposure with $nTiO_2$ has little influence on its toxicity.

Key-words: Dimethylated arsenic, estuarine polychaete, arsenic genotoxicity

1. Introduction

Arsenic (As) is an important metalloid distributed in aquatic environments due to natural events and anthropogenic activities (Zhang et al., 2012). In Brazil, Mirlean et al. (2003) reported high As levels in the sediments of the Patos Lagoon estuary near the city of Rio Grande in southern Brazil (RS). In 2006, other study showed that sediments were partially contaminated by As and this contamination probably was related to fertilizer factories located in this region (7.5 mg kg^{-1} to 27.5 mg kg^{-1}) (Mirlean and Roisenberg, 2006). Actually there was an increase in the number of fertilizer factories in the industrial zone that surround the estuary of Rio Grande city. As the arsenic coexists with phosphate group, the intensification in the production of fertilizer rich in phosphate also increases the release of As into aquatic environment.

The toxicity of arsenic varies according to its chemical form (Jomova et al., 2011). Inorganic forms of arsenic (iAs) such as arsenate (As^{V}) and arsenite (As^{III}) are considered to be more toxic (Ventura-Lima et al., 2011). Among the organic forms, methylated species such as monomethylarsenic and dimethylarsenic acid are considered moderately toxic (Yamanaka et al., 1990), while arsenobetaine (AsB), arsenocholine (AsC), trimethylarsinic oxide (TMAO) and tetramethylarsonic (TETRA) are considered non-toxic (Fattorini et al., 2013).

In aquatic environments, the arsenic occur mostly as inorganic forms (Akter et al., 2005), however, organic forms as MMA and DMA also are detected in sediments and aquatic organisms (Zeng et al., 2018). Once released in the water, the organisms can incorporate, accumulate and biotransform As in less toxic compounds (Hasegawa et al., 2001). Although marine organisms tend to accumulate forms of non-toxic As, such as AsB, studies have shown that some species of polychaetes accumulate high concentrations of methylated compounds such as MMA and DMA

(Fattorini et al., 2005). Although many studies have evaluated the effects of iAs on aquatic organisms, there is very little information about the potential toxic effects of DMA. The few data available showed that DMA promotes reactive oxygen species (ROS) generation and DNA damage in mammalian cells (Kitchin, 2001; Sakurai et al., 2005; Yamanaka and Okada, 1994). Regardless of the chemical form, the presence of As in the environment is a fact, mainly in regions of fertilizer factories (Mirlean and Roisenberg, 2006). For this reason, several strategies are being developed to remove this metalloid from the aquatic environments (Pena et al., 2005).

Nanoparticles such as titanium dioxide ($nTiO_2$) have been employed for bioremediation of water bodies contaminated with As (Hua et al., 2012). Due to their small size, the nanomaterials shown large relative surface area, allowing the adsorption of metals and metalloids as As (Jegadeesan et al., 2010). However, the same characteristic that allow the decontamination of water also can facilities the uptake both of nanomaterial and As by organisms (Luo et al., 2018; Pincus et al., 2018; Yang and Yan, 2018). In fact, Sun et al. (2007) showed that the $nTiO_2$ increased the incorporation and effect of As in fish *Cyprinus carpio.* Also, this nanomaterial showed to affect the arsenic metabolism in shrimp gills (*L. vannamei*) and in polychaete *Laeonereis acuta* when exposed to iAs (Cordeiro et al., 2016; Nunes et al., 2017). *L. acuta* has been previousyly used as biological model because this species possesses classic responses when exposed to environmental contaminants (Nunes et al., 2017; Ventura-Lima et al., 2007). This species is of environmental relevance, having little mobility reflects the conditions of the environment where it lives, in addition to being predated by fish, sea birds and other aquatic invertebrates (Geracitano et al., 2004, Pamplim et al., 2007).

Based on the informations cited above, two main objectives were proposed in this study: (1) to evaluate the toxicological effects of a dimethylated form of As in acute exposure condition; and (2) to evaluate if $nTiO_2$ can influence in the effects induced by DMA in *Laeonereis acuta.*

2. Material and methods

2.1. Sampling and maintainence of animals

Specimens of *L. acuta* (n=120) were collected at reference site known as "Saco do Justino", a shallow cove in the Patos |Lagoon estuary (southern Brazil, 32°03' S, 52°05' W) This site is known to present low metal content in the sediment (Costa et al., 2016). After collection, the animals were transferred to the laboratory in saline water (10 ppm), placed in a glass container (6.0 cm diameter, one animal per container) in artificial saline water (10 ppm), pH 7.8, during 6 days, following the procedure described by Geracitano et al. (2004). During acclimation period the animals were feed with fish food (TETRA) every 48 h. After the acclimation period, the animals were divided into six experimental groups (10 animals per treatment, individually) as follows: **Control group** (animals received the same conditions of acclimation period); **DMA50** group (50 µg/L of DMA); **DMA500** group (500 µg/L of DMA); **$nTiO_2$** group (1mg/L of $nTiO_2$); **DMA50 + $nTiO_2$** group (50 µg/L + 1 mg/L of $nTiO_2$) and **DMA 500** + **$nTiO_2$** group (500 µg/L + 1 mg/L of $nTiO_2$). The animals were not fed during the experiment period (48 h) and the water was renewed each 24 h and a new adding of DMA and $nTiO_2$ was made at each change of water. After this, the animals were killed by cooling, and stored at -80 °C. The authorization for animal sampling was approved by Brasilizian Agency SISBio (process number 58385) conceded to Juliane Ventura Lima, PhD.

2.2. Chemical analysis

2.2.1. $nTiO_2$ characterization

Titanium dioxide nanoparticles (99.9% purity, rutile crystal structure from Sigma-Aldrich. weres identified through 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 2º/min.

The transmission electron microscopy (TEM) was used to analyze the morphology, particle size and shape of $nTiO_2$, using the microscope (JEOL JEM 1400) at an acceleration voltage of 100 kV. The morphology and shape of $nTiO_2$ was also verified by scanning electron microscopy (SEM), using the microscope (JEOL JSM 6610) at secondary ion mode (SEI) with a voltage of 20 kV and the samples were coated with carbon. The size distribution histogram was obtained by the count of 300 measures of diameter, using the SigmaScan Pro 5 program.

2.2.2. Total As content and chemical speciation of arsenic

The total content of As was determined according to Fattorini et al. (2013). The samples lyophilized (SPeedVAc) were dried to a constant weight at 60° C during 8 h and digested under pressure with nitric acid and hydrogen peroxide (5:1 v/v) using microwaves for sample digestion (CEM Xpress, CEM Mars6, CEM Holding Corporation, Matthews, NC, USA). The total As content determination was performed by atomic absorption technique using an electro-furnace atomization with Zeeman effect (Agilent SpectrAA 240Z, Agilent Technologies, Santa Clara, CA, USA). A blank and standard reference material (SRM) specific to arsenic was used (mussels tissue standard reference materials [SRM] 2977, National Institute of Standart and Technology (NIST), USA) were treated with the same procedures cited above as control for accuracy and precision. The total arsenic was expressed as µg of As/g of tissue (d.w). The chemical speciation of As was analyzed after methanolic extraction using a

microwave (Mars CEM, CEM Corporation) and separation by high performance liquid chromatography (HPLC) as previously detailed by Fattorini et al. (2013) Anionic forms were obtained using a Supelcosil liquid chromatography-SAX1 column (25 cm, 4.6 mm ID, 5 mm, Supelco, Bellefonte, PA, USA) with 15 mM KH_2PO_4 (pH 6.1) as the mobile phase at a flow rate of 1 mL/min. The cationic exchange was realized through a Supelcosil liquid chromatography-SCX column (25 cm, 4.6 mm ID, 5 mm, Supelco, Bellefonte, PA, USA) with 2.5 mM pyridine (pH 2.65) as the mobile phase at a flow rate of 1 mL/min. Forty fractions were collected every 30 s from injection with 0.5 mL of nitric acid (purity>65%, Fluka), and the arsenic content was determined as previously described. Accuracy, precision and efficiency of recovery were tested by processing standard reference materials DORM-2 and BCR-625 containing certified levels of As compounds (DMA, TETRA and AsB), and selected standards of arsenate, MMA, DMA, TMAO and AsB. The results are expressed relative to the percentage of total As in *L. acuta* samples.

2.3. Biochemical analysis

For enzymatic activity determinations, the animals were homogenized (1:4 w/v) with 0.5 M of sacarose, Tris-Base 20 mM, EDTA 1 mM, dithiothreitol (DTT) 1 mM and KCl 0.15 M with pH adjusted in 7.60. Homogenates were then centrifuged at 10,000 × *g* for 20 min at 4°C and the supernatants kept. Total protein content was analyzed using a commercial kit (Doles Ltda, Brazil) based on the Biuret method. After, the samples were stored (-80 ºC) for biochemical measurements except to ROS measurement that was performed immediately after homogenization (see below).

2.3.1. **Determination of reactive oxygen species concentration (ROS)**

After 48 h of exposure, the animals were killed by cooling and immediately homogenized and processed as described in Section 2.3,. The supernatants were used for determination of ROS using 2',7'-dichlrofluorescein-diacetate (H_2DCF-DA, InvitroGen), which generates a fluorochrome detected at 485 e 530 nm for excitation and emission wavelengths, respectively (Ferreira-Cravo et al., 2007). Readings were performed with a fluorescence microplate reader (Victor 2, Perkin Elmer) at 5 min intervals during 60 min.

2.3.2. Glutathione-S-transferase (GST) activity

GST activity was determined through the monitoring of 1chloro-2,4-dinitrobenzene (CDNB, Sigma-Aldrich) conjugation with GSH, measuring the absorbance at 340 nm (Habig and Jakoby, 1981).

2.3.3. Reduced glutathione (GSH) levels determination

GSH levels determination was analyzed from the reaction of naphthalene dicarboxialdehyde (NDA) with GSH to form highly fluorescent cyclized products detected on a fluorescence microplate reader at 485 and 530 nm for excitation and emission wavelengths, respectively (White et al., 2003).

2.3.4.Lipid peroxidation (LPO) level determination

Lipid peroxidation (LPO) was measured by means of the ferric/xylenol orange reaction, as described by Rosa et al. (2005). The animals were homogenized in methanol (10% w/v) and centrifuged at 1000 ×g, for 10 min. Lipid hydroperoxides were detected using $FeSO_4$ (0.25 mM) The sample absorbance (580 nM) was measured on a fluorescence microplate reader after 1 h of incubation at room temperature. After that, LPO values were expressed in terms of cumene hydroperoxide (CHP) equivalents, used as standard (5 nmol/ml).

2.4. Comet assay

Previously, the samples were macerated and pass through a mesh (< 100 mm), then were mixed with low melting point agarose (0.7%) and pipetted onto pre-coated slides with normal melting agarose (1%). After gelatinized, the samples were incubated in a lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% Triton X-100, and 10% DMSO, pH 10) for at least 2 h. The slides were incubated in an electrophoresis alkaline buffer (300 mM NaOH and 1 mM EDTA, pH > 13) for 30 min. Electrophoresis was carried out at 1 V/cm for 20 min (300 mA and 25 V). Slides were fixed using methanol P.A. (Merck) and were stained with SYBR1 Safe DNA gel stain and visualized in fluorescence microscopy; around of 50 nucleoids were captured per slide. The parameters evaluated were: % of DNA in the tail, tail length and moment. The software used to analysis was ImageJ. This analysis was based in the methodology described by Singh et al. (1988).

2.5. Statistical analysis

The dates obtained in the different analysis were tested through variance analysis (ANOVA) followed by Newman-Keuls (a = 0.05). The normality and homoscedasticity

were previously analyzed and mathematical transformations were applied when necessary (Zar, 1984). The results were compared as follows: $nTiO_2$ and the two concentrations of isolated DMA (DMA 50µg/L and DMA 500µg/L) were compared with the control group. Co-exposure groups were compared to the corresponding concentration of the isolated DMA (DMA 50µg/L vs. DMA 50µg/L + $nTiO_2$ and DMA 500µg/L vs. DMA 500µg/L + $nTiO_2$).

3. Results

XRD analysis showed diffraction peaks characteristic of rutile phase (**Figure 1**). TEM image shown that TiO_2 nanoparticles exhibited spherical shapes (**Figure 2**) and the histogram showed the monomodal distribution with mean diameter of 64 ±20 nm (**Figure 3**).

Only the group exposed to 500 µg/L of DMA accumulated As significantly ($p<0.05$) and nTiO2 showed to decrease the accumulation of As in this group, resembling the values of the control group (**Figure 4a).**

Considering As metabolization, it was observed the predominance of DMA follow by MMA, AsB, TMAO and TETRA, in any treatment showed induced accumulation of iAs. Co-exposure with $nTiO_2$ did not interfere in As metabolization (**Table 1**).

In the group exposed to 50 µg/L of DMA it was observed an increase in ROS concentration ($p<0.05$), while the co-exposure showed to decrease the ROS (Figure 3b).

None of the treatments showed a significant difference in the GST activity or GSH levels ($p>0.05$) (**Figure 5a** and **5b**, respectively**)**

The content of LPO showed an increase in the $nTiO_2$ group ($p<0.05$). Both co-exposures with DMA also showed an increase ($p<0.05$) in the LPO levels compared with groups exposed only DMA (both 50 and 500 µg of DMA/L) (**Figure 6)**

In all the parameters evaluated in the comet assay (tail length, tail moment and %DNA), the groups treated with DMA presented greater damage than the control ($p<0.05$) and the co-exposure to $nTiO_2$ did not reverse this effect. In the case of DMA500 + $nTiO_2$ it was observed an increase of DNA damage compared to group exposed only DMA 500 both in tail moment and %DNA (**Figure 7**).

4. Discussion

Due to the wide environmental distribution of As, it is important to differentiate the various chemical forms of this element, which in many circumstances can be accumulated at moderately high levels (Fattorini et al., 2004).

Previous studies have shown that 48 h of exposure is not sufficient to induce a significant As accumulation in *L. acuta* when exposed to inorganic As (50 µg of As/L) (Nunes et al., 2017; Ventura-Lima et al., 2011) and the same was observed in this study. However, at the higher concentration (500 µg of DMA/L) it was registered an increase in the accumulation of As.

A previous study from our group indicated that the exposure to the same concentration of iAs during 48 h did not induce a significant accumulation in *L. acuta* (Ventura-Lima et al. 2007). So, it seems that As organic forms can be accumulated more efficiently than inorganic forms, perhaps in virtue of iAs forms be more toxic that organic and the organism expend to more energy to metabolize and eliminate iAs in less time However, when co-exposed to $nTiO_2$, there was a reversion of As accumulation at

the concentration of 500 µg/L. When *L. acuta* was exposed to this nanomaterial it was observed a higher mucus production that should interfere in As entry. In fact, some studies suggest that $nTiO_2$ stimulate the production of mucus in bivalves and this may be linked to the elimination of metals (Canesi et al., 2010; David and Fontanetti, 2009).

Recent studies have shown that when incorporated together with $nTiO_2$, arsenic changes its accumulation pattern in some aquatic organisms such as *L. vannamei,* which tends to accumulate arsenic in the form of AsB. However, when co-exposed to iAs and $nTiO_2$, shrimps showed an increase in the accumulation of DMA, a compound moderately toxic (Cordeiro et al., 2016). In this study, we observed that *L. acuta* presented a predominance in the accumulation of moderately toxic forms (MMA and DMA) in all groups. These results corroborate with Ventura-Lima et al. (2007) and Nunes et al. (2017), since these authors reported in *L. acuta* a high content of moderately toxic forms such as MMA and DMA after exposure to iAs.

The observed increase in ROS concentration after exposure to 50 µg of DMA/L can be related to Kitchin (2001), who suggested that the generation of free radicals in exposure to As is formed mainly from DMA, because when DMA^{V} is reduced to DMA^{III}, this metabolite can indirectly generate superoxide and hydroxyl radicals. Also Naranmandura et al. (2012) found that DMA was a more potent ROS generator than iAs in rat liver cell culture.

Changes in the redox state of the cells can generate a stressful situation, and to cope with this situation the cell recruits the antioxidant defense system through activation of enzymes such as GST, expression of antioxidant genes, and non-enzymatic responses such as increase in GSH levels (Halliwell and Gutteridge, 2007). In fact, some studies have shown that GSH levels are positively modulated in aquatic organisms in response to exposure to iAs (Bagnyukova et al., 2007; Lobato et al., 2013). However, several

reports indicate absence of GSH induction in *L. acuta* after exposure to iAs during 48 h (Nunes et al., 2017; Ventura-Lima et al., 2011). The same was observed for GST activity and previous evidences indicate that short-term exposure (48 h) to As, regardless of chemical form, not alters GST activity in different aquatic organisms (Lobato et al., 2013; Nunes et al., 2017; Ventura-Lima et al., 2009).

Although the exposure to 50 μg of DMA/L induced the increase of ROS concentration and the group exposed to 500 μg/L increased the As accumulation, no lipid damage was observed in any group treated with DMA alone in both concentration. However, in the experimental groups that were exposed to $nTiO_2$ it was showed an increase in lipid damage compared with groups exposed only DMA. This effect probably is linked to $nTiO_2$, once that the group treated only with the nanomaterial also presented lipid damage. In liver of fish *Danio rerio,* the $nTiO_2$ also induced lipid peroxidation, indicating its pro-oxidant potency (Diniz et al., 2013).

Faced with exposure to metals, cell macromolecules damage occurs due to the formation of reactive oxygen species and/or the formation of reactive metabolites. As the pollutants are metabolized, reactive molecules are produced and can interact with DNA causing damage to genetic material such as single-strand breaks, chromosomal aberrations and replication errors (Ren et al., 2015; Yamanaka et al., 2001). Among the reactive oxygen species formed during the metabolism of metals, the hydroxyl radical is considered the reactive species that directly attacks the DNA. For the hydroxyl radical to be involved in the genotoxicity of arsenic, a free transition metal (such as iron) is commonly considered necessary for a Haber-Weiss-like process to damage DNA (Kitchin and Ahmad, 2003). Arsenic is generally a potent iron-releasing agent of ferritin, but DMA has been by far one of the most active arsenic species in this process (Ahmad et al., 2000). Although As is considered a genotoxic agent, the type of damage

it exerts on genetic material depends on the chemical form of As exposure. The genotoxicity of iAs is associated with inactivation of DNA repair system, leading to cell replication with defective DNA, which can lead to tumor formation (Kumar et al., 2016; Tong et al., 2015; Xie et al., 2014). Yamanaka et al. (1989) proposed that during DMA metabolism, DMA radicals are formed that interact with the DNA molecule causing single-strand breaks, chromosomal aberrations, and cell cycle arrest, but did not observe inhibition of repair enzyme activity of DNA damage. In the present study, we have seen that exposure to both concentrations of DMA induced DNA damage and in some parameters the co-exposure to $nTiO_2$ generated even more. Wang et al. (2017) found that $nTiO_2$ alone did not exert genotoxicity in mammalian hybrid cell culture, but when co-exposed to iAs it potentiated its genotoxic effects. In contrast, Ventura-Lima et al. (2007) and Nunes et al. (2017) observed no damage to the DNA in 48 hours of exposure to the same concentrations of iAs, indicating that iAs in *L. acuta* tis not a genotoxic agent, whereas DMA seem to be.

The results obtained in this study showed the potential toxic effect of DMA (both concentrations), mainly considering the DNA. Also, was observed that nTiO2 little influenced in the effect induced by DMA exposure. Considering the previous studies performed by our research group using iAs (same conditions of this study) was possible to observe that DMA can more toxic than iAs, showing that the discussion of DMA toxicity needs to be seen more closely.

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Conflict of interest statement

The authors declare that there are no conflicts of interest.

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6. Figure captions

Figure 1: Characterization of $nTiO_2$ nanoparticles. **(a)** X-ray diffr**act**ion (XRD) patter of $nTiO_2$.

Figure 2: (a) Transmission electron microscopy (TEM) image of $nTiO_2$. **(b)** Scanning electron microscopy (SEM)

Figure 3. Histogram illustrating the particle size distribution of the TiO_2 nanoparticles.

Figure 4: (a) Total arsenic concentration (expressed as µg/g/dry weight) in *L. acuta*. **(b)** Reactive oxygen species (ROS) concentration (expressed as area in fluorescen units.min). Different letter indicates significantly differences ($p < 0.05$). All were expressed as the mean + 1 standard error (n = 5).

Figure 5: (a) Glutathione-S-transferase (GST) activity (expressed as nmol of conjugated CDNB/ min/mg of proteins). **(b)** Reduced glutathione (GSH) levels (mmol of GSH/mg of protein).. All were expressed as the mean + 1 standard error (n = 5).

Figure 6: LPO content (expressed as nmol of CHP/g of wet tissue). Different letter indicates significantly differences ($p<0.05$). All were expressed as the mean ± standard error (n = 5).

Figure 7: DNA damage expressed as: **(a)** percentage of tail DNA, **(b)** tail length and **(c)** moment. Different letter indicates significantly differences ($p < 0.05$). Both date are expressed as the mean ± 1standard error (n = 5).

7. Figures

Figure 1.

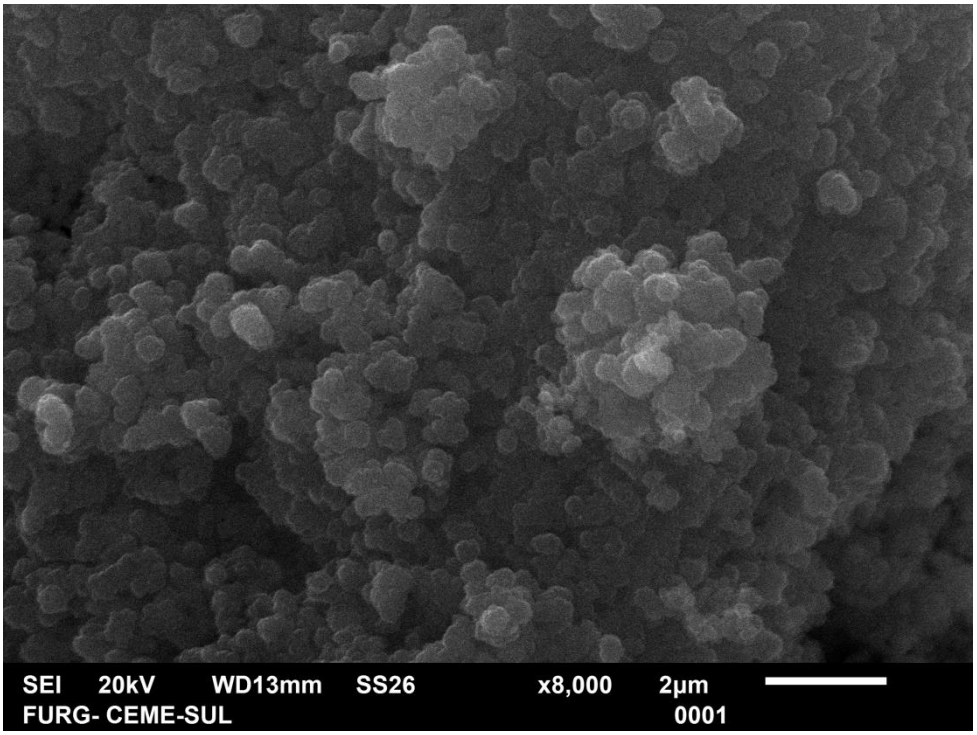
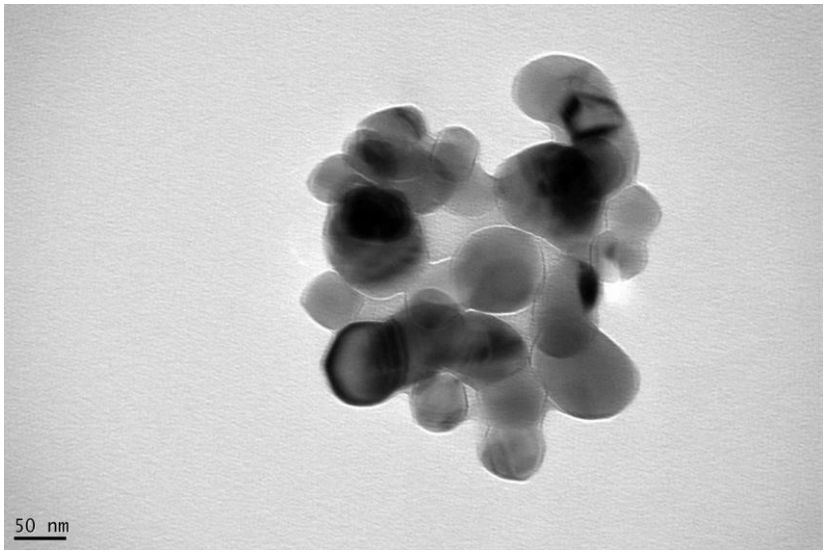


Figure 2.

(a)



(b)

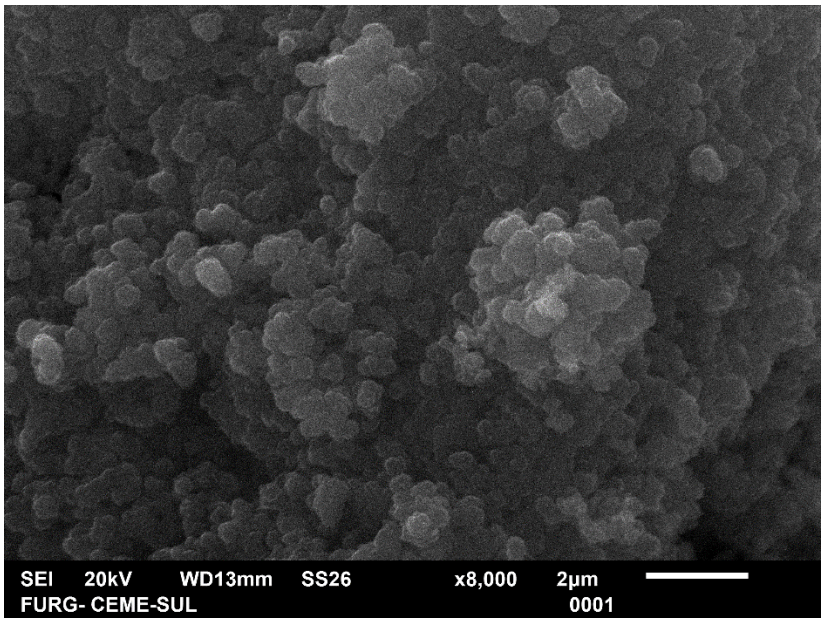


Figure 3.

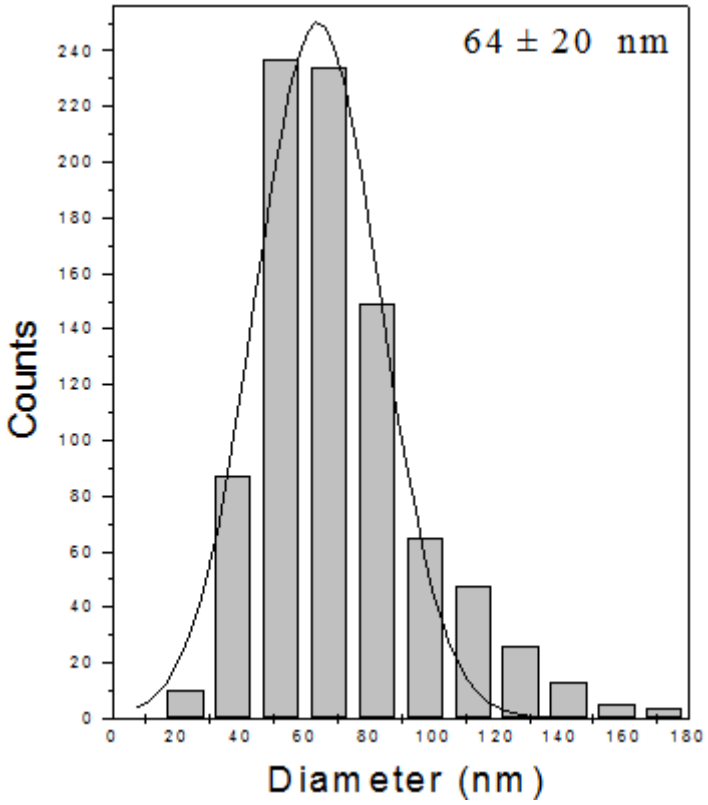

64 ± 20 nm
Counts
Diameter (nm)
0
20
40
60
80
100
120
140
160
180
200
220
240

Figure 4.

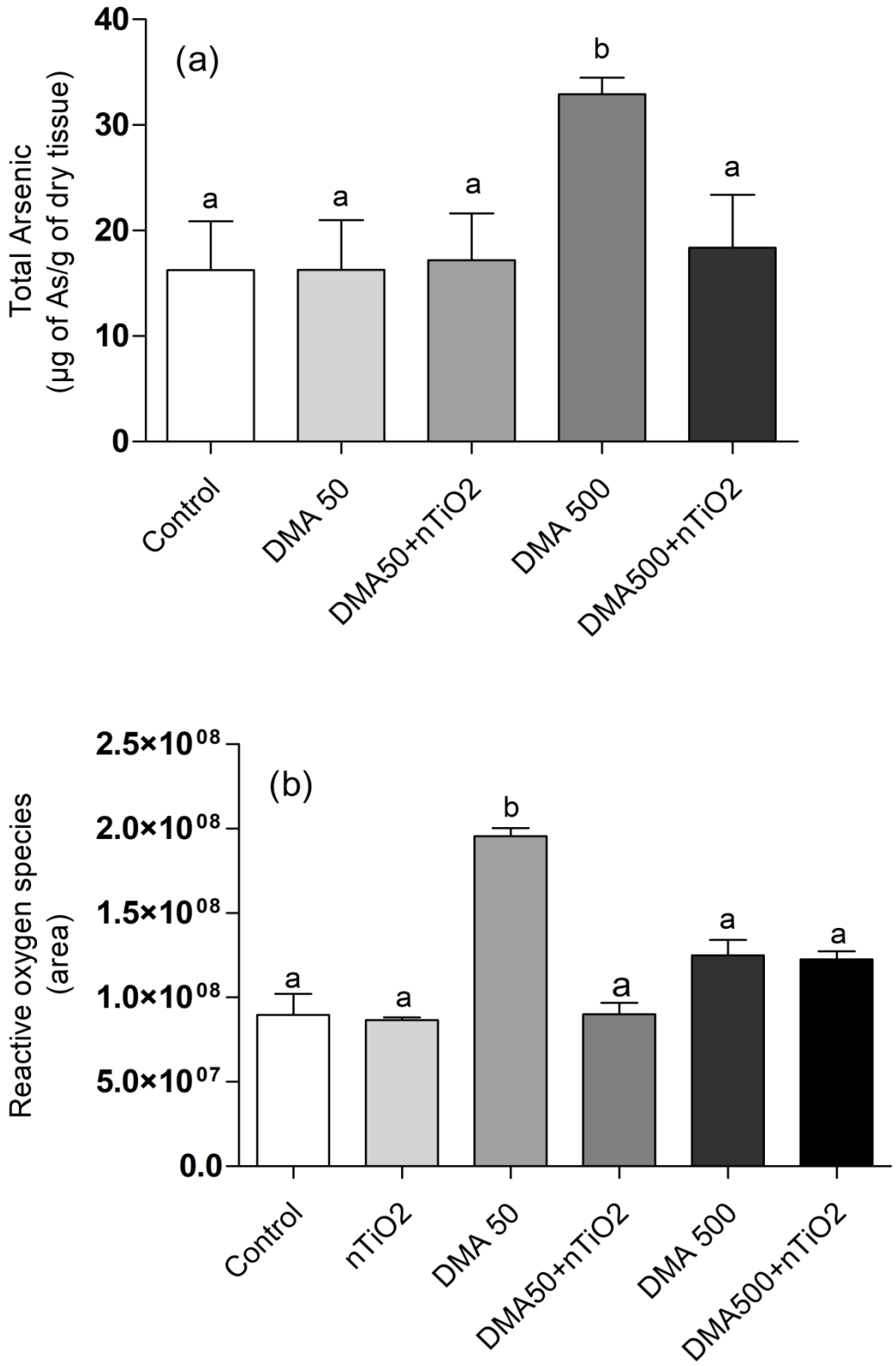

(a)
Total Arsenic
(µg of As/g of dry tissue)
40
30
20
10
0
a
a
a
b
a
Control
DMA 50
DMA50+nTiO2
DMA 500
DMA500+nTiO2
(b)
Reactive oxygen species
(area)
2.5×10 08
2.0×10 08
1.5×10 08
1.0×10 08
5.0×10 07
0.0
a
a
b
a
a
a
Control
nTiO2
DMA 50
DMA50+nTiO2
DMA 500
DMA500+nTiO2

Figure 5.

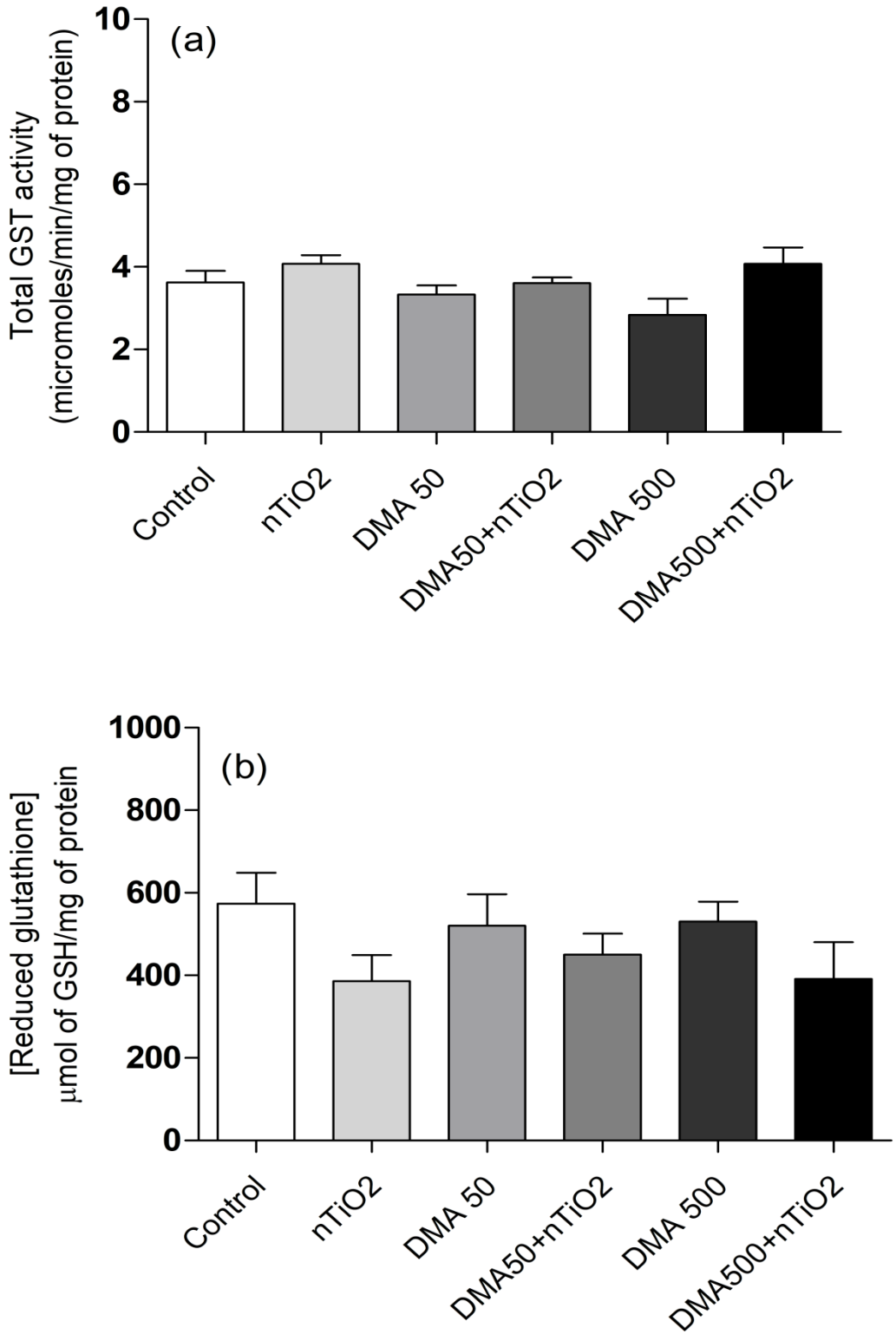

(a)
Total GST activity
(micromoles/min/mg of protein)
10
8
6
4
2
0
Control
nTiO2
DMA 50
DMA50+nTiO2
DMA 500
DMA500+nTiO2
(b)
[Reduced glutathione]
µmol of GSH/mg of protein
1000
800
600
400
200
0
Control
nTiO2
DMA 50
DMA50+nTiO2
DMA 500
DMA500+nTiO2

Figure 6.

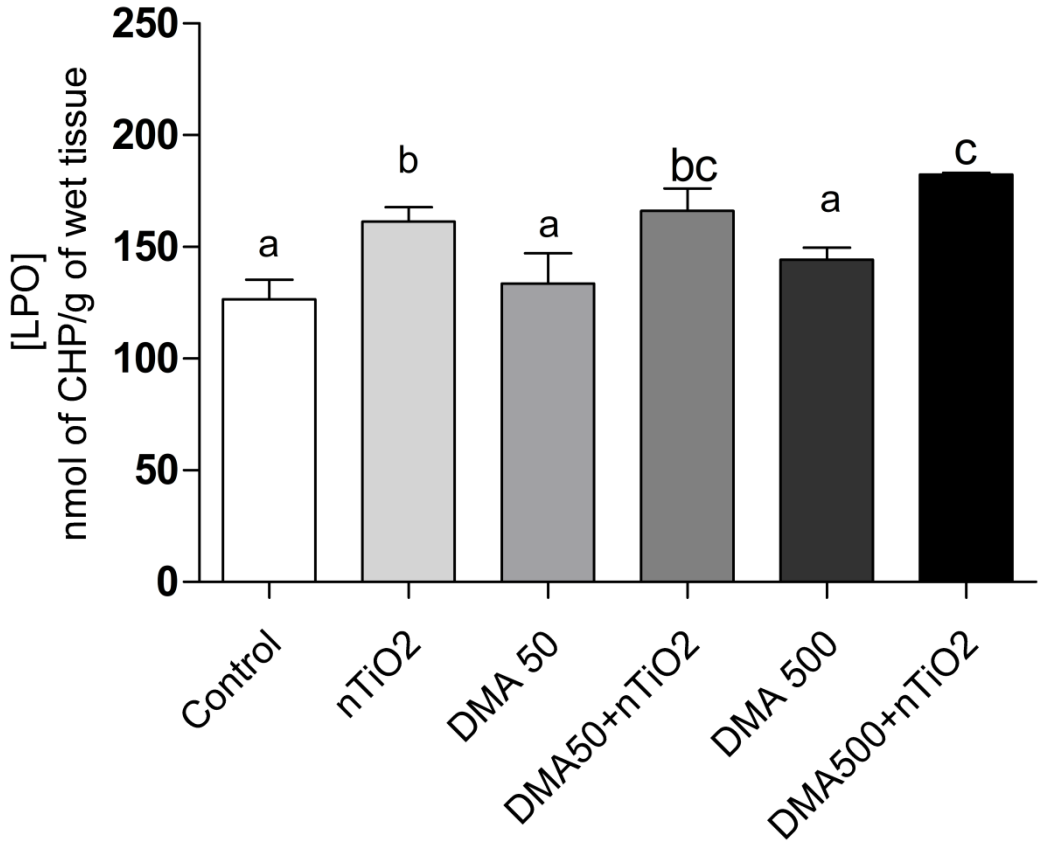
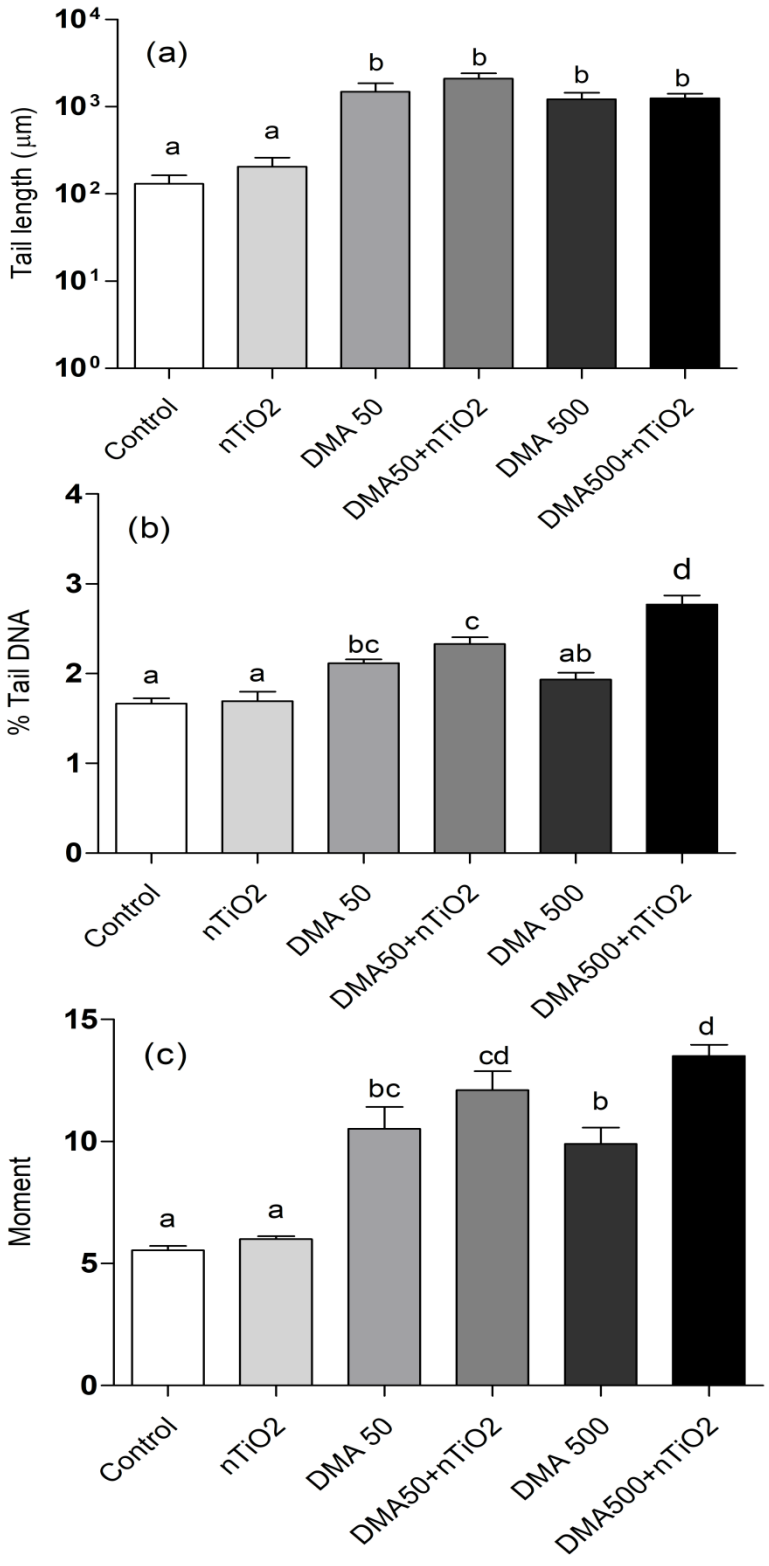

[LPO]
nmol of CHP/g of wet tissue
250
200
150
100
50
0
a
b
a
bc
a
c
Control
nTiO2
DMA 50
DMA50+nTiO2
DMA 500
DMA500+nTiO2

Figure 7.


(a)
Tail length (µm)
10^4
10^3
10^2
10^1
10^0
a
a
b
b
b
b
Control
nTiO2
DMA 50
DMA50+nTiO2
DMA 500
DMA500+nTiO2
(b)
% Tail DNA
4
3
2
1
0
a
a
bc
c
ab
d
Control
nTiO2
DMA 50
DMA50+nTiO2
DMA 500
DMA500+nTiO2
(c)
Moment
15
10
5
0
a
a
bc
cd
b
d
Control
nTiO2
DMA 50
DMA50+nTiO2
DMA 500
DMA500+nTiO2

.

Table 1. Percentage of As compounds in *Laeonereis acuta*. **iAs:** inorganic arsenic as sum of As^{+3} and As^{+5}; **MMA:** monomethylarsonate; **DMA:** dimethylarsine; **TMAO:** trimethylarsine oxide; **TETRA:** tetramethylarsonium; **AsB:** arsenobetaine.

As compounds iAs	**Ctl** -	**DMA 50**	**DMA 500**	**DMA 50 + $nTiO_2$**	**DMA 500 + $nTiO_2$**
		-	-	-	-
MMA	24,4%	23,4%	10,9%	18,6%	16,9%
DMA	40,6%	34,8%	54,9%	49,5%	51,4%
TMAO	7,9%	16,2%	20,6%	8,2%	8,3%
TETRA	6,9%	6,6%	3,0%	5,9%	6,4%
AsB	20,3%	19,0%	10,5%	17,7%	16,9%

3. Discussão geral

A toxicidade do arsênio está intimamente relacionada com a sua forma química, sendo as formas inorgânicas consideradas mais tóxicas do que as orgânicas. Nos ambientes aquáticos e no sedimento, as formas de As que predominam são As^{V} e As^{III}, enquando nos organismos marinhos o As é encontrado principalmente na forma orgânica não tóxica (Asb) (Fattorini et al., 2004). Embora as formas encontradas no ambiente, sejam predominantemente inorgânicas, existem bactérias que metilam o arsênio inorgânico substituindo o grupo hidroxila pelo grupo metila, tornando as formas orgânicas metiladas (MMA e DMA) disponíveis nos compartimentos ambientais (Lemmo et al., 1983; Zeng et al., 2018). Além disso, ainda que a forma de arsênio predominante nos organismos aquáticos seja AsB, existem organismos que possuem um padrão de metabolização de As diferente, e acumulam de forma predominante intermediários moderadamente tóxicos como o DMA. O poliqueto *Sabella spallanzanii* possui um alto padrão de acumulação de DMA, perfazendo cerca de 85% do As total. Alguns autores trazem este acúmulo de DMA como uma estratégia anti-predatória. O camarão branco, *L. vannamei* acumula arsênio predominantemente na forma de AsB, mas quando co-exposto ao $nTiO_2$ aumenta o acúmulo de DMA que se torna a forma predominante de As nesta espécie (Cordeiro et al., 2016). No presente estudo vimos que o poliqueto *L. acuta* acumula As na forma de DMA predominantemente, mas o $nTiO_2$ não influencia no seu padrão de metabolização, mostrando não afetar formas orgânicas de As.

Embora muitos estudos tenham mostrado que o As^{III} é um potente um gerador de estresse oxidativo, através do aumento na concentração de ERO, modulação de enzimas do sistema de defesa antioxidante e dano lipídico além de um agente genotóxico em peixes e linhagens celulares de mamíferos (Kim and Kang 2015; Rossman 2003), em *L.*

acuta essas respostas não se mantêm. Ventura-Lima e colaboradores (2007) e Nunes e colaboradores (2017) ao expor o poliqueto *Laeonereis acuta* às mesmas concentrações de arsênio na forma inorgânica, não observaram um aumento significativo na concentração de espécies reativas de oxigênio, tampouco indução a danos no DNA. Neste trabalho, foi observado após 48h, que 50 µg de DMA induziu um aumento na concentração de espécies reativas de oxigênio, enquanto na concentração de 500 µg de DMA foi observado um aumento no acúmulo de As. Estes dois efeitos observado para ambas concentrações de DMA não mostraram ser influenciadas pela co-exposição ao $nTiO_2$.

Considerando os efeitos genotóxicos, o DMA (50 e 500 µg/L) mostrou induzir danos no DNA em todos os parâmetros avaliados (momento e comprimento da cauda, % de DNA) e somente na co-exposição a 500 µg/L que o $nTiO_2$ mostrou potencializar efeito. De fato, Yamanaka e colaboradores (1994, 2001) sugerem que iAs é um potente gerador de radicais peroxil, enquanto o DMA induz a geração de radicais hidroxila. Os radicais peroxil afetam de forma mais direta os lipídios, enquanto os radicais hidroxila interagem com o DNA levando à danos no material genético. Outros estudos considerando o *L. acuta* como modelo biológico utilizando o iAs mostram não induzir danos ao DNA mas mostram valores superiores de peroxidação lipídica dos que encontrados neste estudo, onde vimos níveis mais altos de lipoperoxidação no grupo tratado com $nTiO_2$, do que nos grupos tratados com DMA.

4. Conclusão geral

Embora o DMA seja considerado menos tóxico que o iAs, nossos resultados mostram que a forma dimetilada induz danos genotóxicos. Também foi possível observar neste estudo, que o $nTiO_2$ não teve grande influência nos efeitos induzidos pela exposição ao DMA tanto nos parâmetros de estresse oxidativo quanto em

parâmetros de acumulação ou metabolização, um resultado diferente do que foi observado em *Laeoenereis acuta* quando exposto ao iAs e co-exposto ao $nTiO_2$. Embora o DMA seja considerado moderadamente tóxico, existem poucos trabalhos que busquem resultados de sua exposição em organismos aquáticos para comparação com as outras formas químicas deste metaloide. Com base nos resultados obtidos neste estudo, vemos o potencial genotóxico do DMA em *L. acuta*, mostrando que sua toxicidade precisa ser discutida com mais atenção.

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