UNIVERSIDADE FEDERAL DO RIO GRANDE – FURG
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS FISIOLÓGICAS

INFLUÊNCIAS DAS NANOPARTÍCULAS DE DIÓXIDO DE TITÂNIO NA METABOLIZAÇÃO, PADRÃO DE ACUMULAÇÃO E TOXICOLOGIA DO ARSÊNIO NO CAMARÃO BRANCO *Litopenaeus vannamei* (Penaeidae)

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Tese defendida no âmbito do Programa de Pós-Graduação em Ciências Fisiológicas como parte dos requisitos para obtenção do título de DOUTOR em Fisiologia Animal Comparada.

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SUMÁRIO

1. RESUMO GERAL

A toxicidade aguda de nanodióxido de titânio ($nTiO_2$) conjuntamente a estressores ambientais, como o arsênio (As), é pouco conhecida. Além disso, sabe-se muito pouco sobre como $nTiO_2$ pode afetar fatores como acumulação e metabolização de As, em experimentos com nanopartículas, expecialmente em água salgada. O objetivo do presente trabalho foi avaliar se a co-exposição de 48h ao $nTiO_2$ e As poderia potencializar os efeitos induzidos pelo As, bem como a capacidade de acumulação e metabolização do semi-metal, em hepatopâncreas e brânquias de *Litopenaeus vannamei*, em exposições em água salgada. Os experimentos consistiram de exposições ao As, ao $nTiO_2$ e exposições a ambos os compostos simultâneamente. Foram feitas exposições utilizando concetrações de 1 mg/L para ambos os compostos e outras com exposições de 0,01 mg/L também para ambos os compostos. Nas exposições a 0,01 mg/L para ambos os compostos, não foram observadas alterações significativas no padrão de acumulação e na toxicidade de As por influência de $nTiO_2$. O metabolismo do As, entretanto, foi afetado pela presença da nanopartícula, havendo um acúmulo importante de DMA apenas na co-exposição e não nas exposições individuais (apenas As ou $nTiO_2$). As brânquias também foram afetadas de forma mais significativa que hepatopâncreas, devido provavelmente pelo fato do órgão permenecer em contato direto com o ambiente e possuir uma capacidade antioxidante menor que hepatopêncreas. Nas exposições a 1 mg/L para ambos os compostos, houveram alterações na toxicidade de As importantes por ação de $nTiO_2$. A nanopartícula também afetou o padrão de acumulação de As, reduzindo a entrada de As em ambos os tecidos testados. Além disso, $nTiO_2$ afetou a metabolização de As de forma significativa, resultando em um aumento expresivo de espécies especialmente tóxicas em brânquias, como As inorgânicos e MMA. Independentemente da concentração, $nTiO_2$ parece reduzir a capacidade de metabolização ao As nos tecidos, o que pode resultar em um aumento na toxicidade do semi-metal.

Palavras-chave: arsênio, nanopartículas de dióxido de titânio, crustáceo, estresse oxidativo, bioacumulação, glutationa S-transferase ômega.

2. INTRODUÇÃO

2.1. Arsênio

O Arsênio (As) é um elemento químico comum encontrado no ar, solo, água e interiorizado em diferentes organismos, podendo ser encontrado como As elementar, arsenítos, sulfetos, óxidos e arsenato, salém de ser constituinte predominante em mais de 200 minerais (Chiban *et al*., 2012). No meio aquático, As costuma se apresentar na sua forma inorgânica pentavalente arsenato [As(V)] (Sakurai *et al*., 2005). Grande parte dos problemas ambientais decorrentes da presença de As na natureza ocorrem devido à mobilização de As por meio de condições e eventos naturais (Smedley & Kinniburgh, 2002). No entanto, atividades humanas como mineração, combustão de combustíveis fósseis, utilização de pesticidas, herbicidas e dissecantes empregados em colheitas são fontes de liberação importantes de As na natureza.

Existe uma grande quantidade de trabalhos que demonstram a toxicidade do As em vários organismos diferentes, dentre os quais os As inorgânicos demonstram-se mais tóxicos que os As orgânicos (Gbakuro, 2008). Tem-se conhecimento de que o As seja capaz de inibir mais de 200 enzimas (Abernathy *et al*., 1999). O As pode interagir com o receptor glucocorticóide, inibindo assim a transcrição mediada pelo mesmo (Kaltreider *et al*., 2001), possui a propriedade de causar apoptose nas células de barbatanas de peixes (Wang *et al*., 2004), além de poder causar inflamações em fígado, hiperplasia e necrose em peixes (Pedlar *et al*., 2002). Ele também é conhecido por causar a formação de micronúcleos (Huang *et al*., 2004) einibir o reparo em DNA (Brochmoller *et al*., 2000). Em termos de estresse oxidativo, o As tem se mostrado capaz de induzir uma série de efeitos que incluem danos oxidativos e modulação do sistema de defesa antioxidante em organismos aquáticos (Ventura-Lima *et al*., 2007, Ventura-Lima *et al*., 2009a, b).

O Arsênio inorgânico (iAs) é fortemente relacionado ao desenvolvimento de câncer de pele, fígado, pulmões e próstata em humanos (Wang *et al*., 2004). Ao ser internalizado em organismos,

entretanto, As passa por um processo de biometilação que o transformam em diversas outras formas de As (**Figura 1**), incluindo arsenato As(V), arsenito [As(III)] e compostos organometilados como o monometilarsênio (MMA) e o dimetilarsênio (DMA), além de formas consideradas não tóxicas como tetrametilarsênio (TETRA), arsenobetaína (AsB), arsenocolina (AsC) e óxido trimetilarsenio (TMAO).

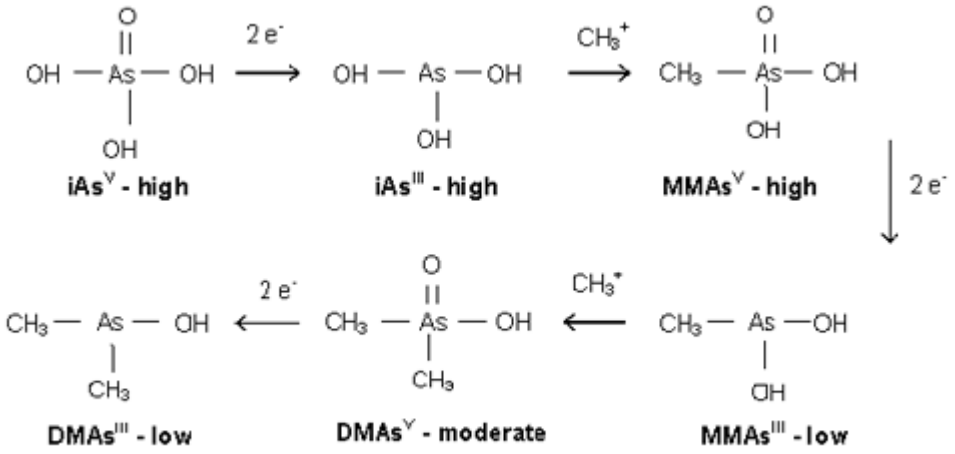

Figura 1: Parte inicial do processo de biometilação do arsênio. As palavras *high*, *moderate* e *low* correspondem a alta, média e baixa toxicidade, respectivamente. Fonte: http://arsenic.tamu.edu/

Crustáceos possuem a capacidade de reter grandes concentrações de As internalizado. Olmedo e colaboradores (2013), em um trabalho que analisou a capacidade de bioacumulação de metais pesados em animais marinhos, demonstraram que um crustáceo decápode, o camarão *Parapenaeus longirostris*, foi a espécie que apresentou os níveis mais altos de As incorporado entre 25 espécies analisadas, incluindo peixes e mexilhões. Outro crustáceo, *Crango crangon*, em um outro trabalho (Kubota *et al*., 2001), também mostrou ter uma grande capacidade de retenção de As. É importante notar, entretanto, que as espécies de As encontradas em diferentes espécies de animais é altamente variável devido a diferenças na capacidade de metabolização dentre eles (Fattorini *et al*., 2005; Ventura-Lima *et al*., 2009b; Ventura-Lima *et al*., 2011).

2.2. Nanodióxido de Titânio

Nanopartículas (NPs) são substâncias de tamanho muito reduzido, medindo, por definição, de 1 a 100 nm de diâmetro em pelo menos uma de suas dimensões (Oberdörster, 2004). Esta característica confere propriedades únicas as NPs quando comparados às mesmas substâncias, mas em maiores tamanhos. Isto ocorre pela grande relação superfície/volume característica desta classe de substâncias (Oberdörster *et al*., 2005).NPs são utilizados em diversas áreas da indústria mundial, sendo encontrados em roupas, protetores solares, tintas, pneus, pastas de dentes, aparelhos eletrônicos, células de combustível, agentes desinfetantes de água e estão cada vez mais cogitados para aplicações médicas, como o tratamento de cânceres (Colvin, 2003; Kim *et al*., 2010; Nel *et al*., 2006; Wijnhoven *et al*., 2009).

Na natureza o titânio (Ti) ocorre apenas na forma de óxido ou óxidos misturados com outros elementos (Clemente *et al*., 2012). Seus depósitos minerais são comumente de origem vulcânica, mas também são encontrados em lugares tão comuns quanto em areia de praias. O dióxido de titânio (TiO_2) é encontrado em três formas cristalinas distintas, sendo elas anatase (tetragonal), rutilo (tetragonal) e bruquita (ortorrômbico), cujas reservas principais encontram-se no Canadá, Estados Unidos da América, Escandinávia, África do Sul, Mar Mediterrâneo e Austrália (Titaniumart, 2010). O dióxido de titânio, também conhecido como óxido de titânio (IV) (NRC, 1999), tem sido usado comercialmente desde 1900, sendo empregado em tintas, pastas de dente, materiais plásticos, entre outros, devido a sua propriedade de clarear e dar opacidade a tais produtos (Clemente *et al*., 2012; Trouiller *et al*., 2009).

O dióxido de titânio em escala nanométrica, ou nano dióxido de titânio ($nTiO_2$), por sua vez, é um dos nanomateriais mais utilizados pela indústria, sendo empregado principalmente em cosméticos e protetores solares, além de possuir a capacidade de adsorver contaminantes de ambientes aquáticos, resultando em um possível agente de descontaminação de corpos d'água, incluindo contaminação por arsênio (Aitken *et al*., 2006; Trouiller *et al*., 2009). Em 2005, a

produção de TiO_2 nanoparticulado ($nTiO_2$) foi de cerca de 2000 toneladas, com 65% desta produção empregada apenas em cosméticos e loções bloqueadoras de luz solar (Clemente *et al*., 2012), resultados estes que indicam claramente a ampla utilização desta NP e demonstram a inevitável liberação da mesma no ambiente.

É fundamental notar, no entanto, que o TiO_2 (incluindo sua forma nanoparticulada) é hoje considerado substância cancerígena de classe 2B pela *Internatinal Agency for Research on Cancer* (IARC) (Baan, 2007). De fato, tanto o TiO_2 micro e nanoparticulado mostram induzir dano genético em ratos quando inalado (Borm *et al*., 2004; Dankovic *et al*, 2007). Ainda, estudos *in vitro* com $nTiO_2$ têm demonstrado que a NP induz a geração de espécies reativas de oxigênio (ERO) que podem provocar dano oxidativo, inflamação, danos celulares e genéticos em diversos organismos vivos (French *et al*., 2009; Linhua *et al*., 2009; Reeves *et al*., 2008). O $nTiO_2$ também pode se acumular em diversos órgão em camundongo (Wang *et al*., 2007), podendo causar efeitos diversos, como apoptose, necrose e fibrose nos órgãos onde foi observado maior acumulação do $nTiO_2$, sugerindo que quanto maior a capacidade de acumulação de um órgão, mais susceptível este está aos efeitos daNP (Chen *et al*., 2009). Em organismos aquáticos, há estudos que demonstram a toxicidade de $nTiO_2$ em *Daphnia magna* e peixes, embora estes estudos ainda sejam escassos (Federici *et al*., 2007; Lovern & Klaper, 2006; Zhang *et al*., 2007). Entre estes trabalhos, em especial, Zhang e colaboradores (2007), demonstraram que $nTiO_2$ pode aumentar a acumulação de outros compostos, como o cádmio em peixes, podendo causar uma acumulação 146% maior em comparação a exposição com cádmio apenas. Entretanto, ainda não se conhece o possível padrão de acumulação deste nanomaterial, nem sua influência no padrão de bioacumulação e na metabolização de As em crustáceos.

Com a crescente produção e o consumo das NPs aumenta também o descarte dos mesmos nos diferentes compartimentos ambientais (Canesi *et al*., 2011). A tendência é que tais substâncias cheguem aos ambientes costeiros, pois estes ambientes são considerados o destino final para diversos contaminantes, incluindo NPs. Uma vez nestes ambientes, o $nTiO_2$ pode acabar

interagindo com substâncias diversas, como o As podendo potencializar os efeitos induzidos pelo metalóide.

2.3. Modelo biológico: *Litopenaeus vannamei*, o camarão branco

O camarão branco *Litopenaeus vannamei* (Boone, 1931) (**Figura 2**), é uma espécie encontrada naturalmente desde o golfo da Califórnia até o norte Peru, no Oceano Pacífico, sendo, além disso, uma das espécies de penaídeos mais comuns de todo o hemisfério leste do planeta (Farfante & Kensley, 1997) e a espécie de camarão mais comumente cultivada do hemisfério oeste (Saoud *et al*., 2003). Paradoxalmente, existem poucos dados toxicológicos considerando parâmetros de estresse oxidativo sobre camarão exposto a nanopartículas, sendo que este tipo de dado pode ser utilizado para se identificar biomarcadores não apenas da qualidade da produção em cativeiro, mas de populações específicas no ambiente natural.

Figura 2: Camarão *Litopenaeus vannamei* em aquário doméstico. Fonte: www.aquakulturtechnik.de

2.4. Interações entre As e $nTiO_2$

Trabalhos que lidam com a interação de $nTiO_2$ e outros óxidos metálicos com As tendem a focar na capacidade de adsorção entre eles, e vários dentre esses objetivam analisar a capacidade de retirar o As do meio em que se encontra (Nabi *et al*., 2009; Pena *et al*., 2006; Hristovski *et al*., 2007; Jegadeesan *et al*., 2010). Entretanto, as interações toxicológicas e outros efeitos adversos relacionados à interação dos nanomateriais com o As deve ser levada em consideração. Sun e colaboradores (2007), por exemplo, encontraram que $nTiO_2$ pode aumentar a incorporação de As em vários órgão e tecidos de carpas (*Cyprinus carpio*), o que pode elevar a toxicidade do semi-metal ao organismo, efeito este denominado "cavalo-de-Tróia" (Limbach *et al*., 2007). Os autores desse trabalho relataram um aumento expressivo de 132% na incorporação de As neste mesmo organismo, quando na presença de $nTiO_2$. No entanto, as potenciais interações toxicológicas entre As e $nTiO_2$ são desconhecidas em crustáceos decápodes.

É importante salientar que as NPs tendem a se acumular nos bentos, ficando em menor quantidade na coluna d'água, devido principalmente a agregação promovida pela salinidade e substâncias diversas presente tanto em águas doces e salgadas, assim como existe a possibilidade de interagirem entre si, modificando suas toxicidades (Fortner *et al*., 2005; Tong *et al.*, 2007). Seguindo esta linha de raciocínio, animais bentônicos, como camarões, deveriam possuir maior risco de entrar em contato com tais substâncias, seja por contato corporal, pelas brânquias ou ingestão de matéria orgânica contaminada.

Ambientes estuarinos e costeiros representam o destino final para as NPs (Cannesi *et al*., 2011). No entanto, há uma importante falta de informação a respeito de seus efeitos na biota presente nestes ambientes (Reeves *et al*., 2008). Também é fundamental destacar que as indústrias que trabalham com NPs os produzem ou planejam produzi-los em larga escala, tornando inevitável que estes produtos e subprodutos acabem sendo liberados e consequentemente se acumulem no ambiente aquático, podendo assim induzir diversas alterações na biota (Reeves *et al*., 2008). Além disso, grande parte das criações de camarão localizam-se próximas à costa utilizando-se da própria

água do mar para o cultivo, sem nenhum tratamento adicional, o que configura risco aos organismos cultivados assim como à saúde humana, já que tais águas podem estar contaminadas com NPs e outros poluentes, como As, um elemento quase que onipresente, podendo este possuir interações de caráter nocivo com NPs como o $nTiO_2$ (Chiban *et al*., 2012; Smedley & Kinniburgh, 2002).

3. OBJETIVO

3.1. Objetivo geral

Avaliar se a co-exposição ao $nTiO_2$ pode influenciar na toxicidade do As bem como na capacidade de metabolização e acumulação deste metalóide em brânquias e hepatopâncreas do camarão *L. vannamei*.

3.2. Objetivos específicos

(a) Determinar a influência do $nTiO_2$ na metabolização e no padrão de acumulação de As em brânquias e hepatopâncreas de *L. vannamei*

(b) Avaliar a toxicidade de ambos os compostos e a influência de $nTiO_2$ sobre os efeitos do As, considerando parâmetros bioquímicos de respostas antioxidantes e de estresse oxidativo.

4. MANUSCRITO I

Evaluation of co-exposure to inorganic arsenic and titanium dioxide nanoparticles in the marine shrimp *Litopenaeus vannamei*

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RESEARCH ARTICLE

Evaluation of coexposure to inorganic arsenic and titanium dioxide nanoparticles in the marine shrimp *Litopenaeus vannamei*

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Abstract The acute toxicity of titanium dioxide nanoparticles ($nTiO_2$) that occur concomitantly in the aquatic environment with other contaminants such as arsenic (As) is little known in crustaceans. The objective of the present study is to evaluate whether coexposure to $nTiO_2$ can influence the accumulation, metabolism, and oxidative stress parameters induced by arsenic exposure in the gills and hepatopancreas of the shrimp *Litopenaeus vannamei*. Organisms were exposed by dissolving chemicals in seawater (salinity=30) at nominal concentrations of 10 μg/L $nTiO_2$ or As^{III}, dosed alone and in combination. Results showed that there was not a significant accumulation of As in either tissue type, but the coexposure altered the pattern of the metabolism. In the hepatopancreas, no changes were observed in the biochemical response, while in the gills, an increase in the glutamate-cysteine-ligase (GCL) activity was observed upon exposure to As or $nTiO_2$ alone, an increase in the reduced glutathione (GSH) levels was observed upon exposure to As alone, and an increase in the total antioxidant capacity was observed upon exposure to $nTiO_2$ or $nTiO_2$+As. However, these modulations were not sufficient enough to prevent the lipid damage induced by $nTiO_2$ exposure. Our results suggest that coexposure to $nTiO_2$ and As does not alter the toxicity of this metalloid in the gills and hepatopancreas of *L. vannamei* but does alter its metabolism, favoring its accumulation of organic As species considered moderately toxic.

Keywords Arsenic · Titanium dioxide nanoparticles · Oxidative stress · Antioxidant responses · Crustacean

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Introduction

Nanotechnology is an area of science that has presented extremely rapid development in recent years. Several nanomaterials (NM) have been produced and used in many commercial products without a concomitant evaluation of their potential effects on human and environmental health (Klaine et al. 2008).

Nano-titanium dioxide ($nTiO_2$) is one of the most used inorganic NM in industry, with applications including food dyes, cosmetics, sunscreens, and as a decontamination agent of aquatic environments (Aitken et al. 2006; Trouiller et al. 2009). Considering its long use in industry, the liberation and accumulation of $nTiO_2$ in the aquatic environment are considered extremely likely (Clemente et al. 2011).

$nTiO_2$ is considered a class 2B carcinogenic substance by the International Agency of Research on Cancer (Baan 2007), and it has been reported to induce genetic damage when inhaled by rats (Borm et al. 2004; Dankovic et al. 2007). In vitro studies have demonstrated that $nTiO_2$ may generate reactive oxygen species (ROS), which may in turn result in oxidative damage to organisms (French et al. 2009; Hao et al. 2009; Reeves et al. 2008).

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$nTiO_2$ is used for the decontamination of arsenic (As) and trace metals, substances that are potentially harmful to humans and aquatic ecosystems due to their possibility of accumulation in living organisms (Benhima et al. 2008; Chiban et al. 2012). Among trace elements, arsenic is commonly found in the atmosphere, soil, sediments, and water and in the tissues of living organisms. In addition to being a constituent element in over 200 different minerals, including arsenites, arsenates, sulfites, and oxides (Chiban et al. 2012), arsenic is also released into the environment by anthropogenic activities such as the smelting of nonferrous metals, burning of fossil fuels, and application of pesticides and wood preservatives (Järup 2003; Larsen et al. 1992). This metalloid may be found in both inorganic forms (as arsenite and arsenate, As^{III} and As^{V}, respectively) and organics (as methylarsonate (MMA), dimethylarsinate (DMA), oxide trimethylarsine (TMAO), and tetramethylarsonium (TETRA)) (Fattorini and Regoli 2004), with inorganic arsenic considered more toxic than the organic forms (Ventura-Lima et al. 2011). The environmental presence of arsenic compounds can be conspicuous, as documented in the region of Patos Lagoon (Southern Brazil), where arsenic contamination in water and sediments has been attributed to regional industry (Mirlean and Roseiberg 2006).

Some studies have shown that As can be found in the aquatic environment in a wide range of concentrations, from 0.5 μg/L to more than 5000 μg/L (Huang et al. 2003). In Brazil, current legislation has established 10 μg of As/L as the maximum safe concentration for aquatic life (Conselho Nacional do Meio Ambiente-CONAMA, resolution 357, 03/17/2005), but a recent study showed that this concentration modulate antioxidant responses in the gills of zebrafish *Danio rerio* (Ventura-Lima et al. 2009a). Crop vegetables, meat, and seafood may all present high arsenic levels (Abedin et al. 2002; Dabeka et al. 1993; Larsen et al. 1992; Meharg and Rahman 2003; Nasreddine and Parent-Massin 2002). Seafood can present notably high levels of this metalloid (Dabeka et al. 1993; Llobet et al. 2003), and it is considered the main source of dietary arsenic entry into humans (Wu et al. 2014).

In a study on trace metals in several fish and shellfish species, the shrimp *Parapenaeus longirostris* presented the highest levels of arsenic (median value of 0.739 mg/kg *w/w*) of the 25 species analyzed (Olmedo et al. 2013). Another crustacean, *Crangon crangon*, also showed a significant capacity for arsenic retention (Kubota et al. 2001), but the typology of arsenic compounds in the animal tissues is strongly influenced by the species and tissue due to marked differences in their As metabolization capability (Fattorini et al. 2005; Ventura-Lima et al. 2009b; Ventura-Lima et al. 2011).

At present, it is acknowledged that some nanomaterials favor substance entry through cellular phospholipid membranes or the blood-brain barrier (Stenehjem et al. 2009). Numerous medicinal and pharmacological applications arise from this property and are being studied and applied in the treatment of cancer (Kanwar et al. 2012; Olivo et al. 2011), brain delivery of drugs (Masserini 2013; Stenehjem et al. 2009), and diagnostic imaging (Boado et al. 2013; Kanwar et al. 2012; Olivo et al. 2011), among others. However, these benefits can also present a problem, as NM can facilitate the uptake of other molecules such as environmental contaminants, increasing their accumulation and/or effects. The term "Trojan horse" effect has been coined to describe this phenomenon (Limbach et al. 2007).

In *Daphnia magna*, $nTiO_2$ favors the accumulation and toxicity of copper (Fan et al. 2011). Other studies have tested the interactions between $nTiO_2$ (or other metallic oxides) and As as a strategy to remove this metalloid from the aquatic environment through its adsorption on NM (Hristovski et al. 2007; Jegadeesan et al. 2010; Nabi et al. 2009; Pena et al. 2006), but the toxicological interactions between the two contaminants should not be neglected.

Litopenaeus vannamei (Crustacea; Decapoda) originates in the Pacific Ocean and is found from the coast of Peru to the coast of Mexico. This is the most commonly cultivated shrimp species in the Western hemisphere, having being introduced to the Americas for this purpose during the late 1970s (Briggs et al. 2004). Some studies have shown that this species is a good biological model for toxicological studies (Tu et al. 2010).

Aquatic environments like estuaries are potential sinks for industrial products, including NM (Canesi et al. 2012). Animals inhabiting estuarine environments have not been extensively evaluated in terms of their toxicological responses to NM (Reeves et al. 2008). Most shrimp production is found along the coast, and their culture occurs in seawater without any special treatment, which may represent some risk to human health due to the presence of trace metals and NM that may interact with each other in unknown ways (Chiban et al. 2012; Smedley and Kinniburgh 2002).

This study tested the effects of arsenic and $nTiO_2$ on the gills and hepatopancreas of the white Pacific shrimp *L. vannamei*, focusing on oxidative stress responses, trying to elucidate the effects of the individual contaminants as well as their toxicological interactions. The tested arsenic concentration was the maximum allowed in water by Brazilian legislation. Additionally, the bioaccumulation and chemical speciation of arsenic were analyzed to test whether $nTiO_2$ influences arsenic bioaccumulation and metabolization in *L. vannamei*.

Materials and methods

Shrimp maintenance and exposure design

Adult specimens of *L. vannamei* from the Estação Marinha de Aquacultura–EMA (Federal University of Rio Grande-

FURG) were acclimatized for at least 1 week before exposure, being fed with commercial rations during that period. Experiments were conducted in four plastic boxes, each representing a distinct treatment group with different nominal concentrations: the control group, 10 μg/L of As^{III} (as $NaAsO_2$), 10 μg/L of $nTiO_2$, and both 10 μg/L of As^{III} and 10 μg/L of $nTiO_2$. Each box housed 6 animals in 12 L of artificial sea water, adjusted to a salinity of 30. The experiment lasted 48 h, the photoperiod was fixed at 12 h L/12 h D, and the temperature was adjusted to 20 °C. Animals were not fed during the period of exposure, aeration was kept constant, and light was partially blocked at all times to a degree of approximately 50 % by a crop net. Water was changed after 24 h, and exposures were renewed. Animals were sacrificed after 48 h by cooling the water, and the gills and hepatopancreas were removed and stored at −80 °C until analysis.

$nTiO_2$ characterization

Titanium dioxide nanoparticles (99.9 % purity, rutile crystal structure) were purchased from Sigma-Aldrich. The phase structure of the TiO_2 nanoparticles was characterized by X-ray diffraction (XRD). The phases present in the sample were identified by comparison of the diffraction patterns with the reference cards of the JCPDS powder diffraction file. The crystalline phases of the particles were identified by X-ray powder diffraction using a Shimadzu model XRD-6000 powder X-ray diffractometer using CuK radiation (1.5418A) over the range of 20 to 90 at a speed of 2°/min.

The morphology of the TiO_2 nanoparticles was analyzed by scanning electron microscopy (SEM) using a JEOL model JSM 6610 with 20 kV and 12000 magnification. The sample was gold coated for analysis. The morphology of the TiO_2 nanoparticles was also analyzed by transmission electron microscopy (TEM) (JEOL JEM 1400) operating at accelerating voltage of 100 kV. The samples were prepared by dispersion of the solutions at room temperature on a carbon-coated copper grid.

Total arsenic and arsenic speciation

The total arsenic concentration and the distribution of its different chemical forms were analyzed in lyophilized samples of the hepatopancreas and gills following the procedures described by Fattorini and Regoli (2004). For the total arsenic concentrations, the samples were digested in a microwave digestion system (CEM Mars5, CEM Corporation) and then analyzed through atomic absorption spectrophotometry (AAS) with a graphite furnace and the Zeeman effect (Varian AA240 Zeeman, Varian). Matrix effects and spectral interferences were resolved by adding a palladium solution (1 g/L, 10 % citric acid, 10 % nitric acid; Sigma-Aldrich products for AAS) as a matrix modifier and applying a standard addition technique. For the verification of the total arsenic determination, quality assurance and quality control were checked by processing with the same analytical procedures both blank samples (reagents only) and Standard Reference Materials (NIST2977, National Institute of Standards and Technology; DORM- 2, DOLT-1, DOLT-2, National Research Council, CNRC, Canada; BCR627, Institute for Reference Materials and Measurements, European Commission). Recoveries of SRM were always within 95 % of the confidence intervals of the certified values (n=5, means+standard error). Analyses were performed on 5 replicates for each tissue and experimental condition, and results are given as μg/g of dry weight, d.w. (means+standard error, n=5).

For the chemical speciation of arsenic, tissues were homogenized (1:10 *w/v*) in methanol (purity>99 %, HPLC grade, Fluka), and arsenic compounds were extracted using a microwave (Mars CEM, CEM Corporation) at 150 W and 55 °C for 15 min. Samples were centrifuged (2000*g* for 15 min), and the supernatants were then concentrated by a SpeedVac (RC1009; Jouan, Nantes, France) and recovered in 1 mL of a methanol/water (70:30) solution. Separation of arsenic compounds was performed by high-performance liquid chromatography (HPLC). 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. Procedures of extraction and separation were verified and checked using selected Standard Reference Materials, including DORM-2, DOLT-1, DOLT-2, and BCR627, and pure standards of As^{V}, dimethylarsinate (DMA), tetramethylarsonium (TMA), and arsenobetaine (AsB) to control for the accuracy, precision, and recovery of various arsenic species. Pellets obtained after centrifugation were recovered and washed 3 times with a saline solution (0.5 % NaCl in ultrapure water) before being digested and tested for insoluble and nonextractable arsenic, using the previously described procedure. Analyses of arsenic chemical speciation were applied on one representative sample for each tissue and experimental condition, obtained by pooling at least 3 replicates per group; arsenic compounds are expressed as μg/g of dry weight, d.w.

Enzymatic analysis

The gills and hepatopancreas tissues were homogenized (1:4 *w/v*) in a buffer of Tris-HCl 100 mM, EDTA 2 mM, and $MgCl_2 \cdot 6\,H_2O$ 5 mM, pH 7.75 (Gallagher 1992), and a cocktail

of protease inhibitors (Sigma-Aldrich) (dilution, 1:1000). Homogenized samples were centrifuged at 10,000×*g* for 20 min at 4 °C. Supernatants were used for the biochemical analysis, and total protein concentrations were determined through a commercial kit (Doles Ltda, Brazil) based on the Biuret method and following the specifications of the developer.

Determination of reduced glutathione (GSH) level and glutamate cysteine ligase (GCL) activity

The reduced glutathione (GSH) level and glutamate-cysteine ligase (GCL) activity were determined according to White and coworkers (2003). This method makes use of the reaction of naphthalene dicarboxialdehyde (NDA) with GSH or γ-glutamylcysteine (γ-GC), forming fluorescent cyclic products that can be detected on a fluorescence microplate reader (Victor 2, Perkin Elmer) at wavelengths of 485 and 530 nm for excitation and emission, respectively.

Determination of antioxidant capacity against peroxyl radicals

The antioxidant capacity was analyzed according to Amado et al. (2009). The method employs a fluorescence microplate reader (Victor 2, Perkin Elmer), in which peroxyl radicals are produced at 35 °C by the thermal decomposition of 2,2′azobis (2-methylpropionamidine) dyhidrochloride) (ABAP) (Winston et al. 1998). Immediately before reading, 10 μL of the fluorescent probe 2′,7′ dichlorofluorescein diacetate (H_2DCF-DA) was added at a final concentration of 40 mM (Ferreira-Cravo et al. 2007). After the H_2DCF-DA cleavage by esterases naturally present in the samples, the nonfluorescent H_2DCF is oxidized by peroxyl radicals into the fluorescent compound DCF, which is then detected at wavelengths of 485 nm for excitation and 530 nm for emission. The total fluorescence was calculated by the integration of the fluorescence generated with and without ABAP over 30 min after adjusting the fluorescence units to a second-order polynomial function. The difference between the areas indicates the total antioxidant capacity of the sample, where a high relative area means a low antioxidant capacity against peroxyl radicals.

Lipid peroxidation measurement

Lipid peroxides were determined as thiobarbituric acid reactive substances (TBARS), following Oakes and Van Der Kraak (2003), measured by fluorescence at wavelengths of 515-nm excitation and 550-nm emission. The concentration of lipid peroxides was expressed as nmol TBARS/mg of protein, and tetramethoxypropane (TMP) was used as a standard.

Statistical analysis

Significant differences between the selected parameters were tested through a one-way analysis of variances (ANOVA). Assumptions of normality and homogeneity of variances were previously tested, and mathematical transformations were applied when necessary (Zar 1984). A pairwise mean comparisons were performed using Tukey's test. In all cases, a significance level of 5 % was employed.

Results

The analyses of total arsenic bioaccumulation reveal no significant differences ($p>0.05$) between the various treatments in either the gills or the hepatopancreas (Figs. 1 and 2a, b, respectively).

In the gills, the predominant form of arsenic was arsenobetaine (AsB) (average of 60.7 %) in all treatments. In the groups of organisms exposed to As, $nTiO_2$, and As+$nTiO_2$, it was possible to detect inorganic arsenic (iAs, average of 5 %), while in the control group, this chemical form was below the limit of detection. Additionally, in the group exposed to only $nTiO_2$, higher values of iAs and monomethylarsenic (MMA) were identified than in the As or $nTiO_2$ groups (Table 1). In the hepatopancreas, in the group exposed to only As, the predominant forms detected were DMA (44.7 %) and AsB (41.4 %). In the group exposed to only $nTiO_2$, AsB was the predominant form of As (82.1 %), while DMA represented only 9 %. In the group exposed to As+$nTiO_2$, the predominance of DMA (54.5 %) and AsB (39.2 %) was observed.

In the gills, the glutathione-cysteine ligase (GCL) activity was raised significantly in the arsenic and $nTiO_2$ treatments ($p>0.05$) compared with the control and the As+$nTiO_2$ groups (Fig. 3a). In the hepatopancreas, no significant change (pμ 0.05) in the GCL activity was detected in any of the experimental groups (Fig. 3b).

Arsenic significantly raised the reduced glutathione (GSH) level in the gills compared to the control group ($p<0.05$) (Fig. 4a). In the hepatopancreas, none of the treatments showed significant difference in the GSH levels when compared with the control group ($p<0.05$), although the $nTiO_2$ group showed a significantly ($p<0.05$) lower GSH level than the As and As+$nTiO_2$ groups (Fig. 4b).

For the total antioxidant capacity against peroxyl radicals, an increase (lower relative area) was observed in the $nTiO_2$ and As+$nTiO_2$ groups ($p<0.05$) when compared with the control and As groups (Fig. 5a), while in the hepatopancreas, no changes were observed in all treatments ($p<0.05$) (Fig. 5b).

In gills, significantly higher lipid peroxidation levels were observed in the $nTiO_2$ group when compared with the other treatments ($p<0.05$) (Fig. 6a). No differences between treatments ($p>0.05$) were observed in the hepatopancreas (Fig. 6b).

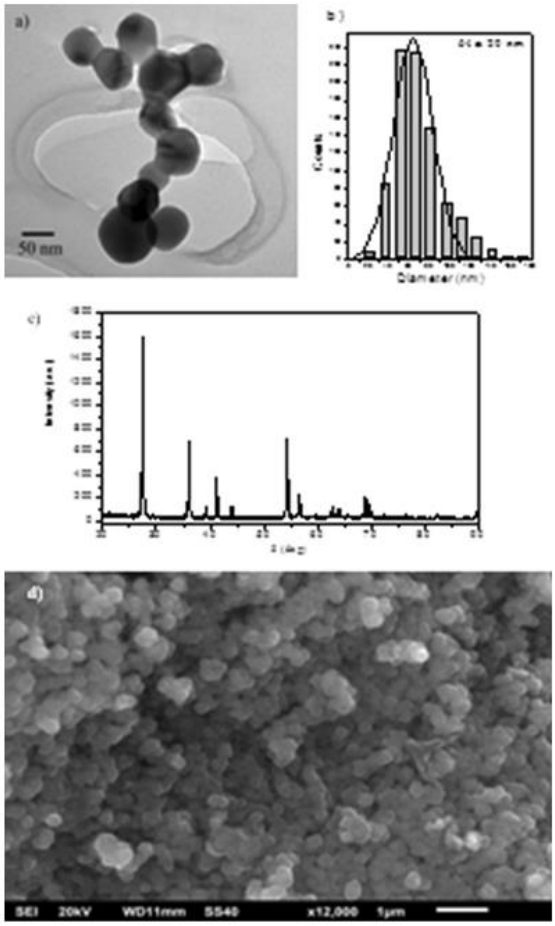

Fig. 1 **a** Transmission electron microscopy (TEM) image of $nTiO_2$. **b** Histogram showing the size distribution of $nTiO_2$. **c** X-ray diffraction (XRD) pattern of $nTiO_2$. **d** Scanning electron microscopy (SEM) image of $nTiO_2$

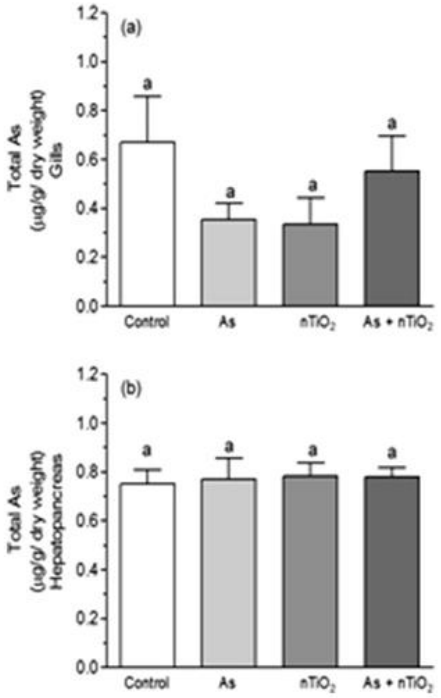

Fig. 2 Total arsenic concentrations (expressed as μg/g/dry weight) in gills (**a**) and hepatopancreas (**b**). Concentrations are expressed as the mean+1 standard error (n=5). *Equal letters* indicate absence of significant difference (p>0.05) between the means of different treatments

Discussion

Arsenic is an environmentally ubiquitous contaminant, being found in a broad range of concentrations. Considering the contamination of groundwater in several countries, some techniques are being developed aimed at the remediation of these water bodies. An alternative proposed by Ma and Tu (2011) is the use of TiO_2 nanoparticles to remove As from the water through the adsorption of the metalloid onto $nTiO_2$ (Pena et al. 2005).

However, some studies have suggested that nanomaterials can facilitate the incorporation of other toxic compounds, increasing their accumulation or adverse effects (Costa et al. 2012; Ferreira et al. 2014). As noted previously, the property of NM to facilitate the entry and/or toxic effect of contaminants is known as "the Trojan horse" effect (Limbach et al. 2007; Park et al. 2010). The objective of our study was to analyze if the low concentrations of $nTiO_2$ that are typically found in several environmental compartments (Mueller and Nowack 2008) can influence the accumulation pattern, metabolization, and toxicity of As in the gills and hepatopancreas of *Litopenaeus vannamei*.

Shrimps were exposed to a low concentration of As (10 μg of As/L, the maximum concentration allowed by Brazilian legislation) (Conselho Nacional do Meio Ambiente 2005), and after 2 days, no significant differences were observed in terms of As accumulation between treatments in either the gills or hepatopancreas (Fig. 2a, b, respectively). A similar result was found by Ventura-Lima et al. (2011) in estuarine polychaeta *Laeonereis acuta* after 2 days of exposure to inorganic As. Additionally, in different tissues of the rockfish *Sebastes schlegeli*, it was only possible to observe a significant As accumulation at higher concentrations (up to 100 μg of As/L) after 10 and 20 days of exposure (Kim and Kang 2015). These results indicate that the time of exposure to a low concentration is crucial to inducing a conspicuous accumulation. Coexposure to $nTiO_2$ and As did not alter the accumulation capacity in either the gills or hepatopancreas. Sun et al. (2007) showed that $nTiO_2$ enhanced the accumulation of As in different tissues of *Cyprinus carpio*, but they used a very

Table 1 Distribution (in percentage) of arsenic compounds in gills and hepatopancreas of *Litopenaeus vannamei* after exposure for 48 h to arsenic and/or $nTiO_2$

Tissue	Treatment	As-ins	iAs	MMA	DMA	TMAO	TETRA	AsB	AsC
Gills	Control	nd	nd	11.8 %	13.5 %	11.0 %	nd	63.8 %	nd
Gills	As	8.3 %	3.2 %	3.7 %	14.1 %	5.6 %	nd	60.7 %	4.0 %
Gills	As + $nTiO_2$	2.2 %	2.0 %	2.0 %	27.1 %	nd	nd	66.8 %	nd
Hepatopancreas	Control	nd	nd	3.1 %	16.5 %	7.9 %	7.4 %	55.1 %	10.0 %
Hepatopancreas	As	2.9 %	nd	2.2 %	49.7 %	1.3 %	0.7 %	41.4 %	1.7 %
Hepatopancreas	As + $nTiO_2$	2.1 %	nd	nd	54.5 %	1.9 %	nd	39.2 %	2.3 %

Arsenic compounds analyzed: insoluble arsenic (As-ins), inorganic arsenic (iAs), monomethylarsenic acid (MMA), dimethylarsinic acid (DMA), trimethylarsine oxide (TMAO), tetramethylarsonium (TETRA), arsenobetaine (AsB), and arsenocholine (AsC). Insoluble arsenic was determined in pellets after extraction and centrifugation of samples to arsenic speciation analysis. As-ins may be associated with cellular compounds such as proteins and lipids that settle to the bottom of tube after centrifugation

nd not detectable

much higher As concentration (200 μg/L) than used in the present study. Fan et al. (2011) observed in *Daphnia magna* that $nTiO_2$ exposure increased copper bioaccumulation and its toxicity, although the assayed $nTiO_2$ concentration was 200 times higher than that used in this study.

Although no variations could be observed in the total content of arsenic, some effects were evident in the chemical speciation and distribution of different arsenic compounds in the tissues of exposed shrimps.

Inorganic arsenic (iAs), including both the arsenate (As^V) and arsenite (As^{III}) species, is the predominant chemical form in water and sediments, while in marine organisms, As is typically found mostly in nontoxic organic forms (Akter et al. 2005). This is due to the capacity of some organisms to biotransform the inorganic arsenic through enzymatic

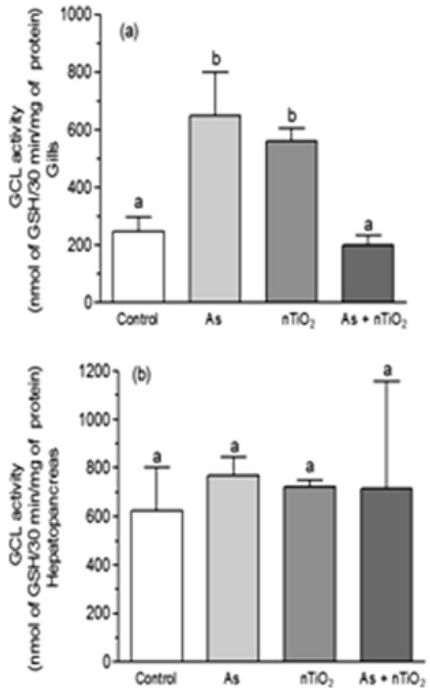

Fig. 3 Reactive oxygen species (ROS) concentrations (expressed as area) in gills (**a**) and hepatopancreas (**b**). Data are expressed as the mean+1 standard error (n=5). *Equal letters* indicate absence of significant difference (p>0.05) between the means of different treatments

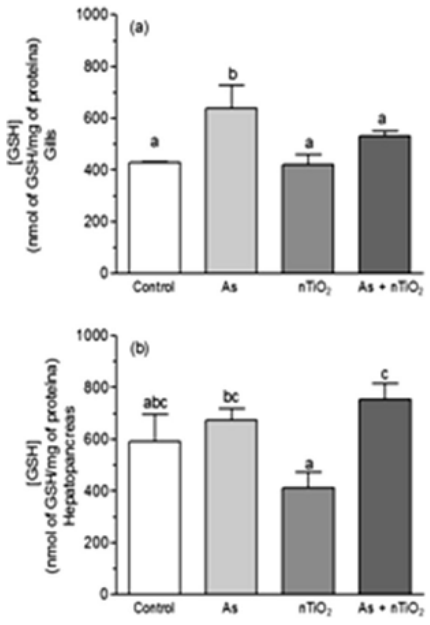

Fig. 4 Glutamate cysteine ligase (GCL) activity (expressed as nmol of GSH/30 min/mg of protein) in gills (**a**) and hepatopancreas (**b**). Data are expressed as the mean±1 standard error (n=5). *Equal letters* indicate absence of significant difference (p>0.05) between means of different treatments. *GSH* reduced glutathione

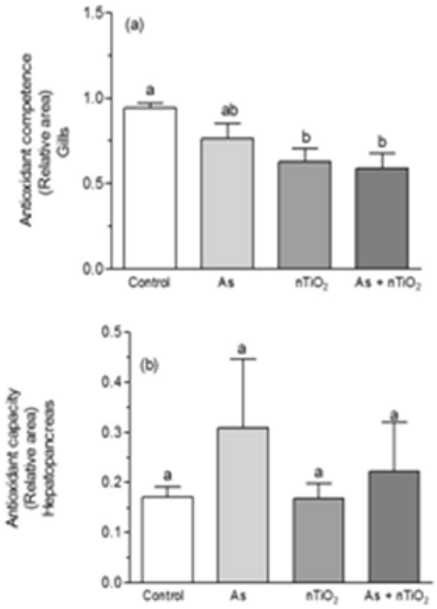

Fig. 5 Reduced glutathione (GSH) levels (expressed as nmol of GSH/mg of protein) in gills (**a**) and hepatopancreas (**b**). Data are expressed as the mean±1 standard error (n=5). *Equal letters* indicate absence of significant difference (p>0.05) between means of different treatments

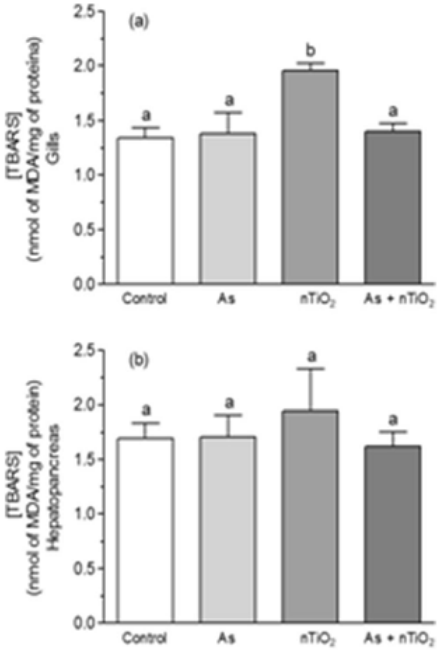

Fig. 6 Total antioxidant capacity against peroxyl radicals (expressed as relative area) in gills (**a**) and hepatopancreas (**b**). Data are expressed as the mean±1 standard error (n=5). *Equal letters* indicate absence of significant difference (p>0.05) between means of different treatments

processes using reduced glutathione (GSH) as a substrate and adenosylmethionine (AdoMet) as a donor of methyl groups (Aposhian 1997). This biotransformation is considered by many authors to be a detoxification pathway of the inorganic forms, which are more toxic than the organic forms (for a revision, Ventura-Lima et al. 2011). The predominant As forms accumulated in aquatic organisms are arsenobetaine (AsB) and arsenocholine (AsC), both considered nontoxic (Fattorini et al. 2006; Zhang et al. 2010).

In the shrimp gills, the predominance of AsB was observed in all treatments (Table 1), but in the group exposed to nTiO2+As, there was an increase in the percentage of dimethylarsinic acid (DMA). In the hepatopancreas, exposure to As altered this pattern, favoring the accumulation of DMA, which is a moderately toxic form (Akter et al. 2005). The same was observed in the group coexposed to $nTiO_2$ and As (Table 1). These results suggest that the coexposure interferes with the metabolization capacity, diminishing the percentage of nontoxic compound as AsB and increasing the accumulation of a more toxic form of As (DMA), mostly in the gills.

Some arsenic compounds increased in both tissues of the exposed shrimps, and this can be useful in explaining some effects even in the absence of a significant accumulation. Although DMA is considered a moderately toxic form of As, some studies showed that long-term exposure to this As species can induce deleterious effects such as reduction of GSH levels, modulation of the antioxidant system, and DNA damage (Yamanaka et al. 1991) and induce progression of skin tumors in mice (Yamanaka et al. 2000). In the crustacean *Daphnia pulex*, this dimethylated As form was shown to be more toxic than iAs (Shaw et al. 2007).

Both As and $nTiO_2$, once released into aquatic environments, can be taken up by organisms and exert several toxic effects, including the modulation of the antioxidant system and oxidative damage (Menard et al. 2011; Bagnyuokova et al. 2007; Schuliga et al. 2002). However, in crustaceans, the effect of these two compounds administered together is unknown.

To cope with oxidative situations, organisms possess efficient antioxidant systems formed of enzymatic and nonenzymatic defenses (Halliwell and Gutteridge 2007). Glutamate-cysteine ligase (GCL) is a key enzyme for GSH synthesis and is part of the defense antioxidant system of living organisms (White et al. 2003). Although few studies have been performed considering GCL activity in response to As and/or $nTiO_2$ exposure, this study revealed that both As or $nTiO_2$ administered alone increased GCL activity in the gills of *L. vannamei* when compared to the control group (Fig. 3a). Ren et al. (2015) showed that a low concentration of As stimulates GCL activity in the liver of mammals, while high concentrations cause enzyme inhibition. In the gills of *Danio rerio*, exposure to a low concentration of As also stimulated GCL activity (Ventura-Lima et al. 2009a).

Although no study has analyzed the effect of $nTiO_2$ exposure on GCL activity in aquatic organisms, Petković et al. (2011) showed that $nTiO_2$ exposure induced the increase of GCL gene expression in mammalian cells (HepG2). The increase of the GCL activity observed in gills can be related to the alteration in the cell redox state by As or $nTiO_2$ that can stimulate the nuclear factor erythroid 2 (Nrf2) transcription factor that regulates the expression of several antioxidant genes including GCL. Some studies demonstrated that both As and $nTiO_2$ activate Nrf2 transcription factor in mammals, thus resulting in increased GCL gene expression (Li et al. 2011; Sun et al. 2012). However, when administered together, As and $nTiO_2$ did not induce changes in the GCL activity, suggesting that the interaction between these compounds could prevent the modulation of Nrf2.

Reduced glutathione (GSH) protects organisms against oxidative insults generated by stressful agents such as metals and other contaminants. Several studies showed that As exposure increases the GSH levels (Schuliga et al. 2002; Costa et al. 2012; Lobato et al. 2013). The same effect was observed in the gills of As-exposed shrimps (Fig. 4a), a result previously reported in gills of *Danio rerio* after exposure to the same As concentration (10 μg of As/L) for 48 h (Ventura-Lima et al. 2009a). In both cases, the gills seem to modulate GSH levels in response to As exposure, concomitant to the increase in GCL activity. The increase in GSH levels can be related to As metabolism because the formation of nontoxic species of As require that this tripeptide be recruited as a substrate for the activity of GSTΩ, a key enzyme involved in the metabolism of this metalloid (Ventura-Lima et al. 2009b).

In the hepatopancreas, no changes in GSH levels were observed for all treatments (Fig. 4b). In the liver of *Carassius auratus*, the exposure to low As concentrations for 6 to 96 h also did not induce any changes in GSH levels. An increase in GSH was observed at higher As concentrations and exposure times (Bagnyuokova et al. 2007). In the hepatopancreas of *L. vannamei*, exposure to a high As concentration (1 mg of As/L) for 48 h stimulated GSH levels (Lobato et al. 2013). These results suggest that in organs with a high antioxidant capacity such as the liver and hepatopancreas, higher As concentrations are needed to induce GSH changes, while in the gills, which possess a lower antioxidant capacity than these organs, lower As concentrations can stimulate the synthesis of this tripeptide.

The nearly constant GSH levels observed in the hepatopancreas for all treatments should contribute to maintain the total antioxidant capacity (Fig. 6b) once that variable is affected by nonenzymatic antioxidants as GSH (Amado et al., 2009). This condition should also explain the absence of oxidative damage observed in this organ. The gills presented quite different biochemical responses: the antioxidant capacity was augmented in the $nTiO_2$ and As+$nTiO_2$ groups, although this response seems to be insufficient to prevent lipid peroxidation in the $nTiO_2$ group. A similar result was observed by Linhua et al. (2009) in the gills, liver, and brain of the carp *C. carpio* after exposure to high concentrations of $nTiO_2$ (100–200 mg/L), suggesting that *L. vannamei* should be sensitive to $nTiO_2$, even when the gills antioxidant response was induced.

As a general conclusion, it can be stated that coexposure with $nTiO_2$ did not alter the effects of As in terms of the accumulation and biochemical response. In the gills, however, coexposure seemed to affect As metabolization capacity once there was an increase in the percentage of DMA. In *L. vannamei*, the gills were more responsive to a low concentration of As and $nTiO_2$ over 48 h than the hepatopancreas, likely due to their lower antioxidant capacity.

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Conflict of interest The authors declare that there are no conflicts of interest.

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5. MANUSCRITO II

Toxicological interactions between arsenic and titanium dioxide nanoparticles in the gills and hepatopancreas of shrimp *Litopenaeus vannamei.*

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Toxicological interactions between arsenic and titanium dioxide nanoparticles in the gills and hepatopancreas of shrimp *Litopenaeus vannamei.*

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Abstract

The present study had the objective to evaluate the effects of the 48h co-exposure of high nominal concentrations of As and $nTiO_2$ (1 mg of As or/and $nTiO_2$) in gills and hepatopancreas of *Litopenaus vannamei*. The results showed: **(1)** both in gills and hepatopancreas the group exposed only to As presented a higher accumulation of this metalloid. **(2)** Glutamate cysteine ligase (GCL) activity was decreased in both tissues in the groups exposed to $nTiO_2$ alone or in combination with As. **(3)** The reduced glutathione (GSH) levels and **(4)** glutathione reductase (GR) activity **(5)** were diminished in response to co-exposure to both contaminants in gills and hepatopancreas. Also, GSH levels were raised by individually applied As and $nTiO_2$, in hepatopancreas and GR activity was also raised by As in gills. **(6)** In gills, glutathione S-transferase (GST) activity was negatively modulated in all treated groups. **(7)** Total antioxidant capacity against peroxyl radicals was not modulated in the group co-exposed to As and $nTiO_2$ in any tissue, but the antioxidant capacity was raised by As in hepatopancreas **(8)** Even if the exposure to each contaminants alone induced lipid damage in hepatopancreas, in the group co-exposed to As and $nTiO_2$ it was not observed any damage. **(9)** In terms of As metabolization, the co-exposure induced a decrease in the percentage of non-toxic or moderately toxic compound and increased the percentage of more toxic ones. The results showed that the co-exposure to As and $nTiO_2$may decrease antioxidant and metabolization capacity in both tissues, although no oxidative damage was observed in this time length of exposure.

Key words: arsenic, titanium dioxide nanoparticles, crustacean, oxidative stress, biotransformation, glutathione S-transferase omega isoform.

1. Introduction

Titanium dioxide nanoparticles ($nTiO_2$) are among the first produced nanoparticles (NPs) in industrial scale and it is actually one of the most utilized in the world (Schaumann et al. 2015). This NP is utilized in a wide range of areas such as medicine, consumer products, paints and surface coatings, and environmental decontamination, in the food industry, dietary supplements, crop production and in vitamin manufacturing (Gui et al. 2013; Skocaj et al. 2011; Wang et al. 2013). Additionally, $nTiO_2$ may also be used as decontaminant agents against heavy metals, including As (Trouiller et al. 2009). However, $nTiO_2$ has also important toxicological characteristics, like inducing reactive oxygen species (ROS) generation (French et al. 2009; Hao et al. 2009; Reeves et al. 2008), affecting the modulation of the antioxidant system and inducing oxidative damage to macromolecules (Trouiller et al. 2009; Saquib et al. 2012). Although it is difficult to quantify the concentration of this NP into the aquatic environment, Gottschalk and co-workers (2009), in an environmental concentrations modeling of different NPs, suggested that $nTiO_2$ presented generally the highest predicted concentrations, mainly due to the highest levels of production of $nTiO_2$ compared to the other NPs analyzed. In fact, the prediction in surface waters is of μg/L, but there is no data regarding $nTiO_2$ concentrations in marine environments (Gottschalk et al. 2013), but its presence in the aquatic environment is expected (Gottschalk et al. 2009; Ward and Kach 2009).

NPs are known as being able to go through the cellular phospholipid membrane and even the blood-brain barrier (Stenehjem et al. 2009). The ability to carry other substances together with them, is called “Trojan horse” effect, a characteristic which may result in an increasing uptake/accumulation of harmful substance and/or their toxic effects (Limbach et al. 2007). The knowledge about not only the effect of this NPs but its interactions with other environmental contaminants is fundamental to predict the potential ecotoxicological impact of NPs on aquatic biota.

Arsenic (As) is widely distributed in the environment, whose natural sources include weathering of rocks, wind-blown dirt and volcanic activity, while its anthropogenic sources include

activities like mining, industries and use of fertilizers and pesticides (Chen et al. 2009; Mardirosianet al. 2015).

Arsenic is found in a wide range of concentrations in aquatic environments around the world, varying as much as 0.5 μg/L to more than 5000 μg/L (Huang et al. 2003). In Brazil, the maximum permitted concentration in national aquatic environments is defined as 10 μg As/L (Conselho Nacional do Meio Ambiente-CONAMA, resolution 357, 03/17/2005), however, this concentration was enough to modulate the antioxidant system in gills of *Danio rerio* (Ventura-Lima 2009b; Cordeiro et al. 2015). In aquatic compartments, As exists predominantly as its inorganic forms As^{+3} and As^{+5}; arsenite and arsenate, respectively (Jain and Ali 2000). Once into the aquatic environment, As may be uptaken and metabolized by living organisms generating organic forms that are considered moderately toxic or even non-toxic (Vahter, 2002). The metabolization process involves the reduced glutathione (GSH) as electrons donor and the activity of the limiting-step enzyme glutathioneS-transferase omega isoform (GSTΩ) (Thomas, 2004). Besides, the exposure of aquatic organisms to different forms of As is known to induce changes in biochemical and molecular parameters of oxidative stress (Sarkar et al. 2014; Altikat et al. 2015).

The white shrimp *Litopenaeus vannamei* (Crustacea; Decapoda) is a species native from the coastal region between Peru and Mexico in the Pacific Ocean. The species holds an important economical value, as it is the most commonly cultivates shrimp in the western hemisphere since its introduction during the late 1970s (Briggs et al. 2004). The specie hasbeen used as biological model in toxicological studies because exhibit good response in biomarkers front to different contaminants (Lobato et al. 2013; Cordeiro et al. 2015). In a previous study from our research group (Cordeiro et al. 2015), $nTiO_2$ was not able to affect the toxicity or bioaccumulation of As. Since this could have happened because of the relatively low concentrations selected for both toxicants (10 μg/L each), in the present study we decided for a higher concentration of $nTiO_2$ and As, as a way of analyzing, mechanistically, to whether $nTiO_2$ can possibly affect As toxicity, accumulation patterns and metabolization capacity in *L. vannamei*.

The objective of this study was evaluating the effects of As and $nTiO_2$ alone or in combination on gills and hepatopancreas tissues of the Pacific white shrimp *L. vannamei* through oxidative stress parameters, focusing on the effects of their toxicological interactions and isolated toxicities as well. Arsenic bioaccumulation and chemical speciation were also realized to show if $nTiO_2$ can influence uptake, metabolization and toxicological effects of As in *L. vannamei*.

2. Materials and methods

2.1. Shrimp maintenance and exposures design

Adult *L. acuta* specimens were obtained in collaboration with Estação Marinha de Aquacultura – EMA (Federal University of Rio Grande-FURG), and acclimatized for at least one week before exposure. The animals were fed with commercial rations during acclimatization period. Experiments were held in four distinct groups separated in four plastic boxes: the control group (only sea water); one group exposed to nominal concentration of 1 mg/L of As^{III} ($NaAsO_2$); another exposed to 1 mg/L of $nTiO_2$ and a group treated with both 1 mg/L of Arsenic and 1 mg/L of $nTiO_2$. Each box was filled with 12 L of artificial sea water (30 ppm of salinity, pH 8.0) and contained a *n* = 7 animals. The experiment had a duration of 48 h with an extra 24 h acclimatization period, temperature was adjusted to 20 ^{0}C and the photoperiod was 12 h L/D. Animals were maintained in a fastening condition during the exposure, with constant aeration and light was blocked by roughly 50% by a crop net. Water renewal was realized at every 24 h with consequential new exposure thereafter. The animals were killed after 48h of exposure by freezing. Tissues (gills and hepatopancreas) were separated after the procedure and stored at -80 ^{0}C until posterior analysis.

2.2. Titanium dioxide nanoparticles preparation and characterization

The solution of $nTiO_2$ was the same used in the previous study performed by Cordeiro and co-workers (2015), where was performed widely characterization that is clearly described in the mentioned study.

2.3 Total arsenic and arsenic speciation

Total arsenic levels and speciation in hepatopancreas and gills were carried on lyophilized samples. Procedure was based on Fattorini and Regoli (2004). For total arsenic concentrations, lyophilized samples were digested in a microwave digestion system (CEM Mars5, CEM Corporation), analyzed using Atomic Absorption Spectrophotometry (AAS) with graphite furnace and Zeeman Effect (Varian AA240 Zeeman, Varian). Matrix effects and spectral interferences were done adding a palladium solution (1 g/L, 10% citric acid, 10% nitric acid; Sigma–Aldrich products for AAS) as matrix modifier and realizing standard addiction technique. Control and quality assurance were carried on by processing standard reference materials (NIST 2977, National Institute of Standards and Technology; DORM- 2, DOLT-1, DOLT-2, National Research Council, CNRC, Canada; BCR627, Institute for Reference Materials and Measurements, European Commission). Recoveries were within 95% of confidence intervals of certified values. Arsenic chemical speciation was realized by first homogenizing tissues (1:10 w/v) in methanol (purity > 99%, HPLC grade, Fluka) and extraction of arsenic compounds were realized using a microwave (Mars CEM, CEM Corporation) at 150 W and 55 ^{0}C for 15 min. Samples were centrifuged at 2000 g for 15 min and supernatants were then concentrated by SpeedVac (RC1009; Jouan, Nantes, France). Later, supernatants were recovered in 1 mL of a methanol/water (70:30) solution. Separations of arsenic compounds were realized using liquid performance chromatography (HPLC). Anionic forms were acquired through Supelcosil liquid chromatography-SAX1 column (25 cm, 4.6mmID, 5 mm, Supelco, Bellefonte, PA, USA) with 15mM KH_2PO_4 (pH 6.1) as mobile phase at a flow rate of 1 mL/min. The cationic exchange was performed utilizing Supelcosil liquid chromatography-SCX column (25 cm, 4.6mmID, 5 mm, Supelco, Bellefonte, PA, USA) and 2.5 mM pyridine (pH 2.65) as mobile phase at a flow rate of 1 mL/min. The amount of 40 fractions were collected every 30 s from injection, 0.5 mL of nitric acid (purity > 65%, Fluka) was added and arsenic determined. Extraction procedures and separation were realized to Standard Reference Materials, including DORM-2, DOLT-1, DOLT-2 and BCR627 and to pure standards of As^{V},

DMA, TMA, AsB to control for accuracy, precision and recovery of various arsenic species. The pellets that were obtained after centrifugations were recovered and then washed 3 times with saline solution (0.5% NaCl in ultrapure water). Pellets were digested and tested for insoluble and not extractable arsenic. Total arsenic concentrations and concentrations of arsenic compounds were expressed as μg/g dry weight (μg/g.d.w).

2.4. Enzymatic analysis

Tissues (gills and hepatopancreas) were homogenized (1:4 w/v) in a buffer of Tris-HCl 100 mM, EDTA 2mM and $MgCl_2.6H_2O$ 5mM, pH 7.75 (Gallagher, 1992) and cocktail of proteases inhibition (Sigma-Aldrich). Homogenized samples were then centrifuged for 20 min at 10.000 x *g* and 4 ^{0}C. Supernatant phases were used for biochemical analyses. Total protein concentrations were analyzed using a commercial kit (Doles Ltda, Brazil) based on the Biuret method.

2.4.1. Reduced glutathione content and Glutamate-cysteine ligase activity

Concentrations of reduced glutathione (GSH) and glutamate-cysteine ligase (GCL) activity were performed following White and co-workers (2003), whose method makes use of naphthalene dicarboxialdehyde (NDA) reaction with γ-glutamylcysteine (γ-GC) or GSH which forms fluorescent cyclic products that can be detected on a fluorescence microplate reader (Victor 2, Perkin Elmer) at 485 and 530 wavelengths for excitation and emission, respectively.

2.4.2. Glutathione S-transferases activity

Glutathione S-transferases (GST) activity was performed according to the method described by Habig and Jakoby (1981) monitoring the conjugation of CDNB (1-Chloro-2,4-dinitrobenzene) with GSH, in absorbance at 340 nm. The GST activity was expressed as μmol of CDNB/min and per mg of protein, at 25 °C and pH 7.4.

2.4.3 Glutathione reductase activity

Gluthione reductase (GR) activity was measured based on the method described by Nagalakshimi and Prasad (2001) monitoring the absorbance at 340 nm due to consumption of NADPH. The GR activity was expressed as nmol of NADPH consumed by min and mg of protein at pH 7.4 and 25ºC.

2.5. Lipid peroxidation measurement

Levels of lipid peroxides were determined as thiobarbituric acid reactives substances (TBARS), following methodology by Oakes and Van Der Kraak (2003). These substances may then be measured by fluorescence at 515 nm excitations and 550 nm emission wavelengths. Concentration of lipid peroxides was expressed as nmol TBARS/g of wet tissue and tetramethoxypropane (TMP) was used as standard.

2.6. Total antioxidant capacity against peroxil radicals

The antioxidant capacity against peroxyl radicals was measured according to the method of Amado and co-workers (2009) using a fluorescence microplate reader (Victor 2; Perkin Elmer), at a programmed temperature of 35°C, at which peroxyl radicals were produced by thermal decomposition of ABAP (2,2′-azobis 2 methylpropionamidine dihydrochloride) (Winston et al. 1998). Immediately before the reading, 10 µl of the fluorescent probe 2′, 7′ dichlorofluorescein diacetate (H2DCF-DA) was added to each well (Ferreira-Cravo et al. 2007). H_2DCF-DA is cleaved by esterases present in the samples and the non-fluorescent compound H_2DCF is oxidized by peroxyl radicals to the fluorescent compound DCF, which is detected at wavelengths of 485 and 530 nm, for excitation and emission, respectively. The difference between fluorescence area with and without ABAP, relative to area without ABAP, was considered a measure of antioxidant capacity, with high area difference meaning low antioxidant capacity since high fluorescence levels were obtained after adding ABAP, meaning low competence to neutralize peroxyl radicals.

2.7. Glutathione S-transferase omega isoform gene expression

In the gene expression of Glutathione S-transferase omega isoform (GSTΩ)**,** total RNA of gills and hepatopancreas were isolated with Trizol® reagent (Invitrogen, Carlsbad, CA, USA) and the total RNA was quantified by spectrophotometry and after treated with Deoxyribonuclease I (Invitrogen) to eliminate genomic DNA contamination. The cDNA was synthesized with ImProm-II™ Reverse Transcription System (Promega) following the manufacturer´s instruction. Quantitative PCR was performed using SYBR® Green I (Invitrogen) to detect double-strand cDNA synthesis. Reactions were done in a volume of 25 µL using 12.5 µL of diluted cDNA, containing a final concentration of 0.2x SYBR® Green I (Invitrogen), 100 µM dNTP, 1x PCR Buffer, 3 mM $MgCl_2$ 0.25 U Platinum® Taq DNA Polymerase (Invitrogen) and 200 nM of each reverse and forward primers (**Table 1**). *18S*, *L8* and *APTsy* were used as reference genes for normalization. The accuracy per sample was calculated using LinRegPCR 2012.3 Software (http://LinRegPCR.nl) and the stability of the references genes, and the optimal number of reference genes according to the pairwise variation (*V*) were analyzed by GeNorm 3.5 Software (http://medgen.ugent.be/genorm/). Relative mRNA expression levels were determined using the $2^{-\Delta\Delta CT}$ method.

2.8. Statistical analysis

Statistical differences between selected parameters were performed through of one way analysis of variances (ANOVA). Assumptions of normality and homogeneity of variances were previously checked and mathematical transformations were applied when necessary (Zar, 1984).

3. Results

As mentioned in **Materials and Methods Section**, a characterization of $nTiO_2$ was previously performed and all results were described in previous study of Cordeiro and co-workers (2015).

Both in gills and hepatopancreas, the group exposed to only As has presented a significant ($p<0.05$) accumulation of this metalloid, while the group co-exposed to $nTiO_2$ did not show significant differences ($p>0.05$) when compared with the group exposed to As and the control group (**Figure 1a and 1b**, respectively). In gills, theAs compound that accumulated the most was dimethylarsine (DMA) in all treatments followed by arsenobetaine (AsB). However, in the group co-exposed to $nTiO_2$ and As at then sametime it was observed a higher percentage of more toxic compounds such as inorganic arsenic (iAs) and monomethylarsonate (MMA) **(Table 2)**. In hepatopancreas, the group exposed only to As alone or in combination with $nTiO_2$presented an accumulation of mostly DMA, while in the control group there was higher accumulation of AsB. Again, the group co-exposed with both contaminants showed higher percentage of inorganic As than the group exposed only to the metalloid (**Table 2**).

Both in gills and hepatopancreas, it was not observed changes in GSTΩ gene expression in any treatment (**Figure 1c and 1d, respectively**).

In gills and hepatopancreas, GCL activity decreased significantly ($p<0.05$) in the groups exposed to $nTiO_2$ administered alone or in combination with As (**Figure 2a and 2b, respectively**).

The GSH levels was decreased significantly ($p<0.05$) in gills in the co-exposure to $nTiO_2$ and As (**Figure 2c**) while in hepatopancreas, in the groups exposed to As or $nTiO_2$ alone, there was a significant increase ($p<0.05$) in GSH levels. In the group co-exposed with both contaminants there was a drop in GSH levels, however (**Figure 2d**).

The GST activity in gills, showed a negative modulation ($p<0.05$) in all treated groups when compared with the control group (**Figure 2e**) while in hepatopancreas, no statistically significant changes ($p>0.05$) in the activity of this enzyme was observed (**Figure 2f**).

In gills, only the group exposed to As alone showed an increase in the GR activity, while the treatments with $nTiO_2$ in combination with Asthere was an enzymatic activity reduction (**Figure 3a**). In hepatopancreas only the group exposed to $nTiO_2$ and As together showed a negative ($p<0.05$) GR activity modulation (**Figure 3b)**.

The total antioxidant capacity against peroxyl radicals was not induced in gills after any treatment (**Figure 3c**). In hepatopancreas, the exposure to As alone increased significantly ($p>0.05$) the total antioxidant competence compared to the control and As coupled with $nTiO_2$ treatments (**Figure 3d**).

Nolipid oxidative damage was observed in gills after the exposure to both contaminants (**Figure 3e**), while in hepatopancreas the treatment with As or $nTiO_2$ administered alone induced lipid peroxidation, damage that was not observed in the co-exposure experimental group. (**Figure 3f**).

4. Discussion

This study investigated the effect of the co-exposure of arsenic, a metalloid well known for its bioaccumulation properties and the capacity to induce oxidative stress in aquatic organisms (Ventura-Lima et al. 2007, 2011) and $nTiO_2$, a widely produced NP that have shown potential toxic effects in biological models (Linhua et al. 2009; Li et al. 2015).

Marine organisms have the property to accumulate significant levels of As in their tissues (Fattorini and Regoli 2004). In this study, both in gills and hepatopancreas, it was observed an important increase in the As content in the group exposed only to this metalloid (**Figure 1a** and **1b**, respectively). On the other hand, Cordeiro and co-workers (2015) did not observe a significant bioaccumulation of As neither in gills nor hepatopancreas of *Litopenaeus vannamei,* when exposed to low concentration of As (10 μg of As/L). It is probable that the difference between both results is in virtue of the concentration of As that the animals were exposed, sincein the present study, a concentration one thousand times higher than the concentration utilized in our previous work was

applied. Lobato and co-workers (2013) also observed a remarkable accumulation of As in hepatopancreas of *L. vannamei* in similar conditions used in the present study. These results suggest that, in *L. vannamei*, high concentrations of As are needed to induce a significant accumulation after a short time of exposure (48 h).

Some studies have showed that the co-exposure ofNPs can augment the accumulation of heavy metals in different organisms (Fan et al. 2011; Nigro et al. 2015; Sun et al. 2007). In this study, the co-exposure to $nTiO_2$ did not increase the accumulation of As neither in gills nor hepatopancreas when compared with the group exposed only to As (**Figure 1a** and **1b**; respectively). The same result was also observed by Cordeiro and co-workers (2015) in the tissues of *L. vannamei* exposed to the same experimental condition of this study, however using a lower concentration for both As and/or $nTiO_2$ (10 μg/L). Contrarily, in *Daphnia magna*, the co-exposure to copper and $nTiO_2$ induced higher bioaccumulation of this metal when compared with animals exposed only to copper (Fan et al. 2011). It is known that factors like salinity can interfere in properties of NPs as their aggregation states (Chesne and Kim 2014). In this study, the exposure was realized in seawater. Due to its high ionic strength, seawater probably favors the aggregation of NPs, which, in turn, decreases its superficial area limiting the adsorption capacity of NPs. In virtue of this characteristic, freshwater animals appear to be more sensible in terms of accumulating heavy metals by the action of NPs than marine organisms, as can be observed in data available in the scientific literature (Cordeiro et al. 2015; Fan et al. 2011; Nigroet al. 2015; Sun et al. 2007).

Once incorporated by organisms, As is metabolized thought an enzymatic process that involves oxidations/reductions and methylation reactions (Aposhian et al. 2004). The GSTΩ enzyme (also known as monomethylarsonate reductase) have a pivotal role in the As metabolization (Sampayo-Reyes and Zacharyan 2006). A study performed by Ventura-Lima and co-workers (2011) showed that the inhibition of this enzyme resulted in a decrease in methylated As compounds in the estuarine polychaeta *Laeonereis acuta.* In this study, the exposure to both chemicals did not induce changes in GSTΩ gene expression in neither gills nor hepatopancreas (**Figure 1c** and **1d**,

respectively). In our best knowledge, no previous study was performed considering the gene expression of this enzyme in aquatic organisms after As exposure. However, Saadat and Saadat (2013) observed, in mammalian cells line (HeLa), that the arsenite exposure during 24 h did not induce changes in the expression of GSTΩ. Although data about gene expression of this enzyme are limited, the few data available indicate that short exposure time is not enough to induce the activity or gene expression of this enzyme in aquatic organisms (Ventura-Lima et al. 2009, 2011).

As shown in the **Table 2,** the As compound that accumulated the most in both tissues was dimethylarsine (DMA) followed by arsenobetaine (AsB) in all treatments. However, in the group co-exposed to $nTiO_2$ it was possible to observe a decrease in AsB content and the increase of other compounds as inorganic arsenic (iAs) and monomethylarsonate (MMA) considered high and moderately toxic, respectively. Similar result was observed by Cordeiro and co-workers (2015) in gills and hepatopancreas of *L. vannamei*, indicating that the co-exposure to $nTiO_2$ do not increase As accumulation in this short time of exposure but affects its metabolization capacity, favoring the accumulation of compounds considered toxic with the potential capacity of endangering aquatic organisms.

Changes in redox state of cells can generate a stressful situation, and to cope this situation, the cell recruit several antioxidants such GCL, GST and GR enzymes activities, antioxidant genes expression, besides non enzymatic responses as modulation in GSH levels (Halliwell and Gutteridge, 2007).

The GCL enzyme is the key element in the GSH synthesis (White et al. 2003), and some studies showed that it can be modulated by As exposure in different biological systems (Schuliga et al. 2002; Ventura-Lima et al. 2009b). Previously, we showed that the exposure to a lower concentration of As or $nTiO_2$ induced an increase in GCL activity in gills of *L. vannamei* and no changes in hepatopancreas (Cordeiro et al., 2015). In this study, in both gills and hepatopancreas, $nTiO_2$ treatment group and $nTiO_2$ and As treatment grouppresenteda decrease in GCL activity (**Figure 2a** and **2b**, respectively). The difference between the responses observed in these studies

are probably related to concentrations utilized. In fact, a study performed by Ren and co-workers (2015a) suggests that As concentration is determinant to stimulate GCL activity and gene expression in mammals systems. In the present study, however, the co-exposure to $nTiO_2$ diminished the GCL activity in both tissues. The same result also was observed in the group exposed solely to $nTiO_2$, indicating that this enzyme is sensible to this NPs exposure. Although few data are available on the effect of $nTiO_2$ in GCL activity in aquatic organisms, Gui and co-workers (2013) showed, in mouse kidney, that gene expression of GCL is associated to the exposure concentration of $nTiO_2$, since a higher concentration (10μg/ml) induced a gene expression drop when compared with lower concentrations (1 and 5 μg/ml). Some studies have showed that $nTiO_2$ exposure induces the nuclear factor erythroid related factor 2 (Nrf2) that is responsible to activate antioxidant genes such as GCL and GST (Gui et al. 2013; Shi et al. 2015) resulting in the increase of antioxidant responses. Perhaps, a high concentration of the NPs may block Nrf2 translocation of cytoplasm to nucleus avoiding the activation of antioxidant genes and consequently enzymatic activity. This hypothesis can explain the result observed in this study considering the GCL activity in the groups exposed to $nTiO_2$ alone or in combination with As.

The tripeptide GSH is a powerful antioxidant present in high concentrations in cells (Halliwell and Gutteridge 2007). Heavy metals as As, are known to induce an increase in the GSH levels, which in turn raises cell defenses against oxidative stress situations (Bagnyukova et al. 2007; Ventura-Lima et al. 2009a, b). Lobato and co-workers (2013) observed in hepatopancreas of *L. vannamei* an increment in GSH levels after Asexposure (1mg of As/L). A similar result was also observed in the present study in hepatopancreas exposed only to this metalloid (**Figure 2d**), while in gills this result was not observed (**Figure 2c**). Though in hepatopancreas it was observed an elevation in the basal antioxidant capacity, a higher concentration of As seems necessary to induce an increase in GSH levels. Corroborating with our hypothesis, Cordeiro and co-workers (2015) showed that the gills of *L. vannamei*, exposed to a hundred times lower As concentration induced an increase in GSH levels. The same result also observed in gills of *Danio rerio* (Ventura-Lima et al.

2009b). Independently of the tissue, however, the groups that received $nTiO_2$ and As concomitantly, presented a drop in GSH levels (**Figure 2c and 2d).** In addition, in zebrafish larvae the co-exposure to $nTiO_2$ and pentachlorophenol induced a decrease in GSH content (Fang et al. 2015). This suggests that when administered concomitantly with other toxicant, $nTiO_2$ induces a decrease in the antioxidant capacity, leaving the organism more vulnerable to changes in the redox state of cells.

Besides, the GSH participates in the arsenic metabolization process, serving as substrate of GSTΩ activity (Sampayo-Reyes and Zacharyan 2006). The decrease in GSH levels can trigger the reduction in As compounds considered non-toxic as arsenobetaine and arsenocholine. In fact, in this study, it was observed that in the groups co-exposed to As and $nTiO_2$ there was a higher percentage of compounds that are toxic or moderately toxic than groups exposed only to As, and this result was more prominent in the gills (**Table 2**). A similar result was observed previously by Cordeiro and co-workers (2015) both in gills and hepatopancreas of *L. vannamei* co-exposed to As and $nTiO_2$. These results indicate that $nTiO_2$ exposure can interfere in As metabolization, favoring the accumulation of more toxic compounds that, given time, can exert toxicity to organisms and consumers.

GST is a family of phase II enzymes that act in detoxification processes (Cui et al. 2010). Exposure to metals such as cadmium and As showed their capacity to modulate GST activity in aquatic invertebrates (Ventura-Lima et al. 2007; Sandrini et al. 2008; Ventura-Lima et al. 2011). In this study, in hepatopancreas, it was not observed any changes in this enzyme activity independently of treatment (**Figure 2f**). Corroborating our results, Lobato and co-workers (2013) also did not observe changes in terms of GST activity in hepatopancreas of *L. vannamei*exposed to As (1mg of As/L) during 48 h. In gills, however, all treated groups exhibited a decrease in the GST activity (**Figure 2e).** The gills are organs in direct contact with the environment and are the animal first contact with environmental contamination. This factor may explain why GST activity is prominent in gills. Ren and co-workers (2015b) observed in gills of *L. vannamei* a positive modulation after exposure to benzo-(a)-pyrene, while in gills of *C. carpio* the exposure to $nTiO_2$ induced a drop in this enzyme activity (Lee et al. 2012). This last result, together with the observed

in the present study indicates that the exposure to $nTiO_2$ reduces the antioxidant competence decreasing the detoxification capacity, leaving the organisms more vulnerable to other environmental stressors.

The GR enzyme is responsible to maintain the normal ratio of GSH through the reduction of oxidized glutathione (GSSG) using NADPH as reducing power (Halliwell and Gutteridge 2007), it is therefore an index of redox cell state. In a work by Ventura-Lima and co-workers (2009a), in *Cyprinus carpio,* the exposure to high concentration of As induced a decrease in the GR activity in liver, while in gills this result was not observed. In the present study, in gills, the As exposure induced an increase in the GR activity (**Figure 3a**) indicating that this tissue suffered changes in redox state, perhaps in virtue of modulation in the GR activity, the GSH levels remained constant in the group exposed only As (**Figure 2c**). Some metallic NPs are known to modulate the GR activity in biological systems. In brain of *Oreochromis niloticus* and *Tilapia zillii,* the exposure to zinc NPs (2mg/ L) resulted in a reduction in this enzyme activity (Saddick et al. 2015) a similar result also was observed in gills of *Crossostrea gigas* exposed to zinc oxide NPs (Trevisan et al. 2014). Although in the animals exposed to only $nTiO_2$ no change in the GR activity, in respect to control group, was observed (both gills and hepatopancreas), the co-exposure to $nTiO_2$ and As showed a significant decrease in the GR activity (**Figure 3a** and **3b**, respectively). Concomitantly, it was observed a negative modulation in the GSH levels (**Figure 2c** and **2d,** respectively), which indicates that the co-exposure induces changes in the redox state, favoring a pro-oxidant scenario.

To evaluate the total antioxidant capacity of tissues it was used the methodology developed by Amado and co-workers (2009) to measure the capacity of tissues to cope peroxyl radicals formed through fluorometry readings. In this study, the gills did not show changes in this parameter, while in hepatopancreas, in the group exposed only to As, the total antioxidant capacity was increased (**Figure 3c** and **3d**, respectively). A very different result was observed by Cordeiro and co-workers (2015), where in gills of *L. vannamei* the treatment with $nTiO_2$ alone or in combination with As increased the total antioxidant capacity while in hepatopancreas no change

was observed. Again, these differences may be explained by the concentrations used. Some biomarkers are more sensible to lower concentration of toxicants, which may explain the result in gills, while in hepatopancreas; a higher concentration may be needed to induce antioxidant responses. Besides, the constant content of reduced glutathione observed in gills may have contributed to maintain the levels of total antioxidant capacity, which, in turn, is highly influenced by GSH levels (Amado et al. 2009). This hypothesis is strengthened by the results observed in hepatopancreas where the increase in GSH levels observed in the groups exposed to As or $nTiO_2$ was accompanied by the increase in total antioxidant capacity (**Figure 2d** and **3d**, respectively).

However, this pro-oxidative situation not was sufficient to induce lipid peroxidation in gills (**Figure 3e**), while in hepatopancreas, both exposure to As and $nTiO_2$ administered alone induced oxidative damage in lipids; but when both toxicants were administered together, however, this effect was not observed (**Figure 3f**). The effects observed in this study compared with our previous one (Cordeiro et. al. 2015) suggest that gills are more sensible to low concentration of NPs while in hepatopancreas higher concentration are need to induce antioxidant responses and/or oxidative damage.

5. Conclusions

The results obtained in this study, showed that GR and GST activity are good biomarkers in gills of *L. vannamei* after exposure to both contaminants. The GSH levels showed to decrease after co-exposure to $nTiO_2$ and arsenic indicating a drop in the antioxidant system and metabolization capacity of metalloid, both in gills and hepatopancreas. The co-exposure has not induced any raise of As accumulation, but a decrease in the metabolization capacity was observed, evidenced by a decrease in the percentage of nontoxic As compounds favoring the accumulation of compounds considered moderate or highly toxic. In fact, this result was also observed in our previous study when shrimps where exposed to low concentration of both contaminants. Independently of the concentration used, the co-exposure to $nTiO_2$ induced changes in the As metabolization. These

changes may decrease this detoxification process that, given enough time, may exert severer toxic effects than those observed in this study**.**

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7. Conflict of interest statement

The authors declare that there are no conflicts of interest.

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9. Figure Legends

Figure 1. Total arsenic concentrations are expressed as µg/g/dry weight: **(a)** gills and **(b)** hepatopancreas. Gene expression of GSTΩ is showed as relative mRNA expression: **(c)** gills and **(d)** hepatopancreas of *L. vannamei*. Concentrations are expressed as the mean ±1 standard error. Different letters indicate significant difference ($p<0.05$) between means of different treatments.

Figure 2. Glutamate cysteine ligase (GCL) activity is expressed as nmol of GSH/30 min/mg of protein: **(a)** gills and **(b)** hepatopancreas. Reduced glutathione (GSH) levels are expressed as nmol of GSH/mg of protein:**(c)** gills and **(d)** hepatopancreas. Activity of glutathione S-transferase (GST) is expressed as nmoles of CDNB conjugated/min/mg of proteins: **(e)** gills and **(f)**

hepatopancreas. The GCL and GST activity and GSH levels are expressed as the mean ±1 standard error. Different letters indicate significant difference ($p<0.05$), between means of different treatments.

Figure 3. Glutathione reductase (GR) activity is expressed as nmol NADPH/min/mg/protein: **(a)** gills and **(b)** hepatopancreas. Total antioxidant capacity against peroxyl radicals is expressed as relative area: **(c)** gills and **(d)** hepatopancreas. TBARS content is expressed as nmol of MDA/mg of protein: **(e)** gills and **(f)** hepatopancreas**.** The results are expressed as the mean ±1 standard error. Different letters indicate significant difference ($p<0.05$) between means of different treatments.

Figure 1.

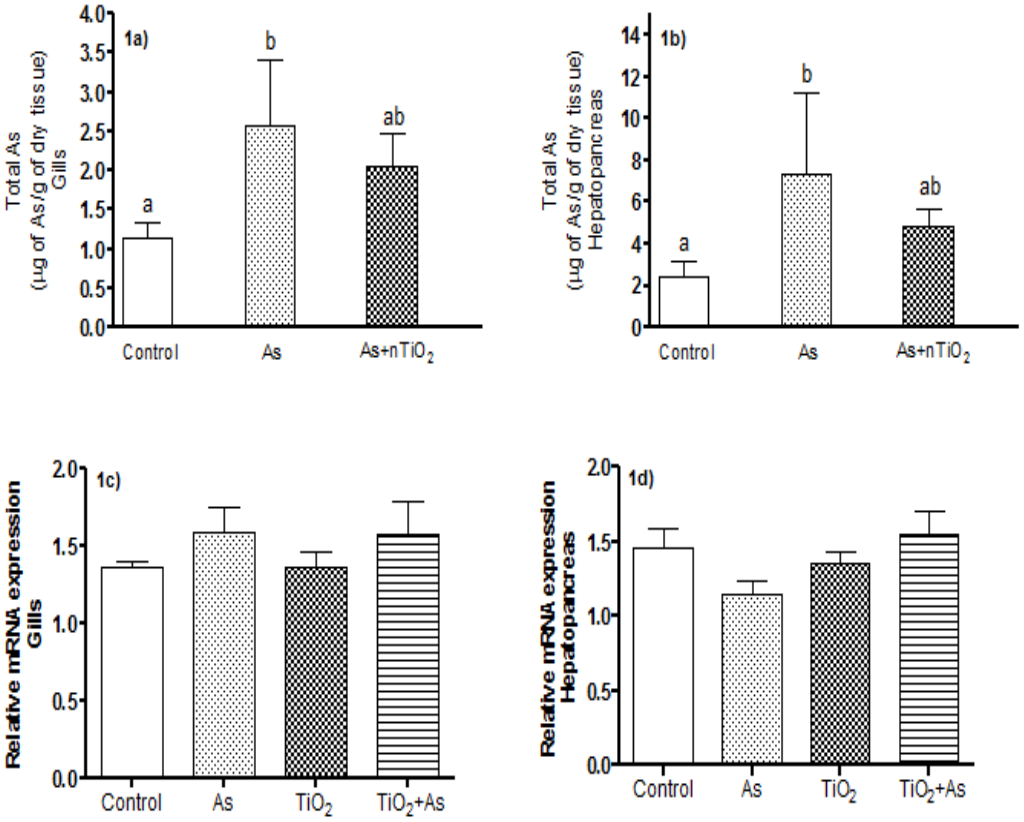
1a)
Total As (µg of As/g of dry tissue) Gills
a
b
ab
Control
As
As+nTiO2
1b)
Total As (µg of As/g of dry tissue) Hepatopancreas
a
b
ab
Control
As
As+nTiO2
1c)
Relative mRNA expression Gills
Control
As
TiO2
TiO2+As
1d)
Relative mRNA expression Hepatopancreas
Control
As
TiO2
TiO2+As

Figure 2.

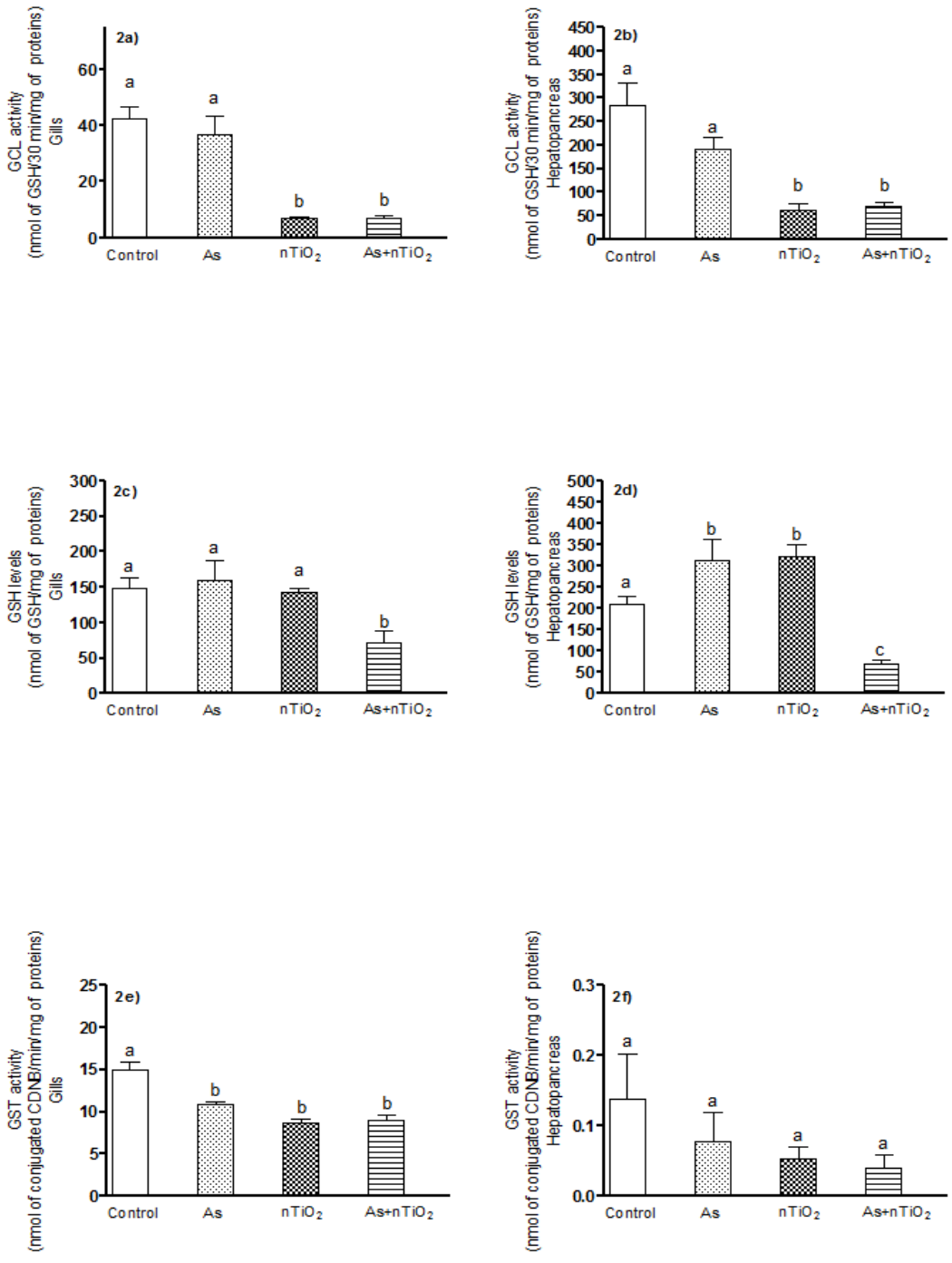
2a)
GCL activity (nmol of GSH/30 min/mg of proteins) Gills
2b)
GCL activity (nmol of GSH/30 min/mg of proteins) Hepatopancreas
2c)
GSH levels (nmol of GSH/mg of proteins) Gills
2d)
GSH levels (nmol of GSH/mg of proteins) Hepatopancreas
2e)
GST activity (nmol of conjugated CDNB/min/mg of proteins) Gills
2f)
GST activity (nmol of conjugated CDNB/min/mg of proteins) Hepatopancreas
Control
As
nTiO2
As+nTiO2

Figure 3.

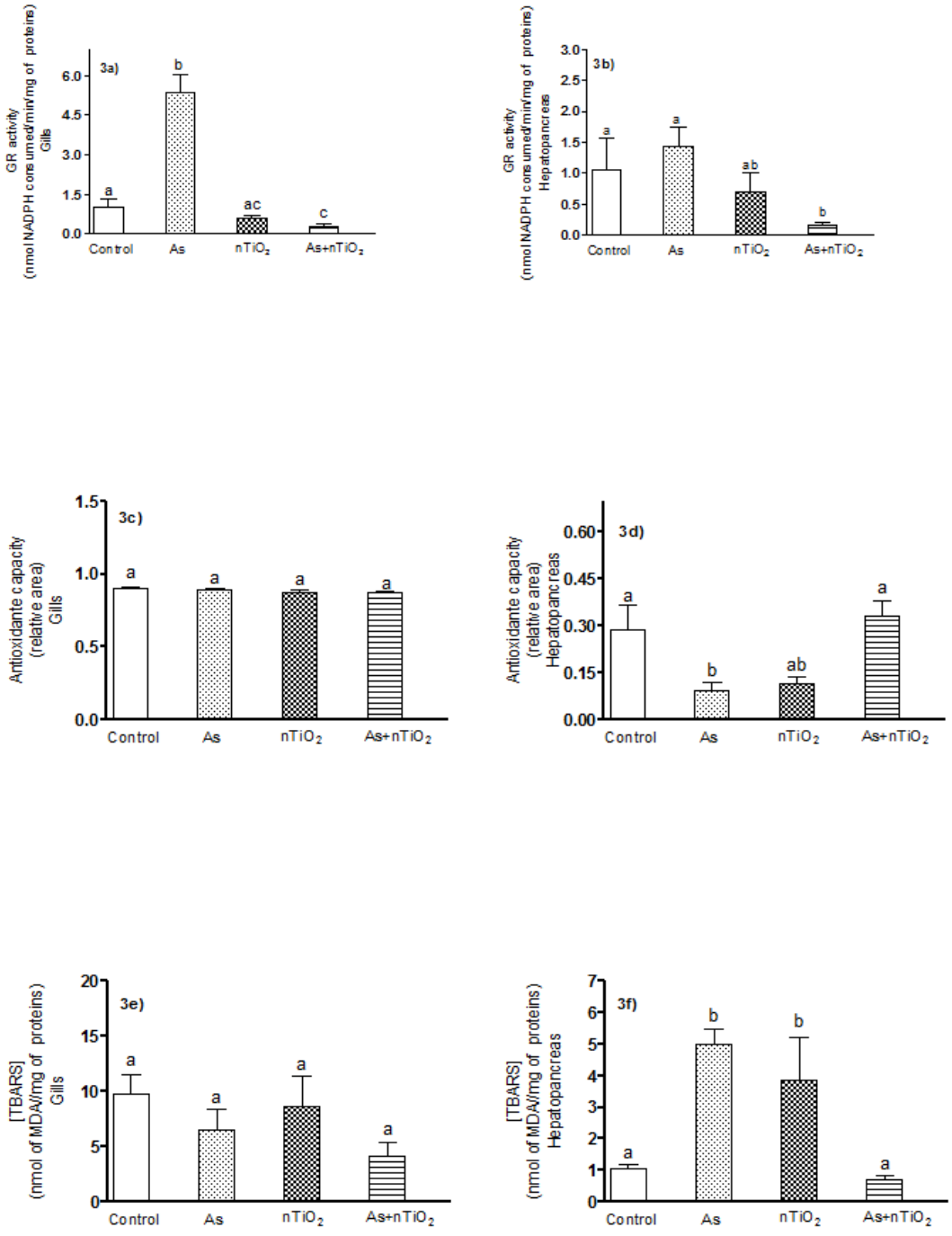
3a)
GR activity
(nmol NADPH consumed/min/mg of proteins)
Gills
Control
As
nTiO2
As+nTiO2
3b)
GR activity
(nmol NADPH consumed/min/mg of proteins)
Hepatopancreas
3c)
Antioxidante capacity
(relative area)
Gills
3d)
Antioxidante capacity
(relative area)
Hepatopancreas
3e)
[TBARS]
(nmol of MDA/mg of proteins)
Gills
3f)
[TBARS]
(nmol of MDA/mg of proteins)
Hepatopancreas

Table 1. Primer sequences for RT-qPCR experiments performed in the study

Gene	Primer sequences (5'-3')	Amplicon size (bp)
18S	F-CCGAATGGTCGTGCATGGAATGAT R-GAATTTCACCTCTAGCGTCGCAGT	127
L8	F-TAGGCAATGTCATCCCCATT R-TCCTGAAGGGAGCTTTACACG	166
ATPsy	F-TGTCTTATGATGGCCTTCCTGTTG R-GGAAGTGGTCCACAAACACCATGC	121
ΩGST	F-ACTTGGACGAGGCCTACCCTGAACC R-AGCGCCTCTTGATCACCCTTTGC	145

According to Gonçalves-Soares et al.2012.

Table 2. Percentage of As compounds in gills and hepatopancreas of *Litopenaeus vannamei*. **iAs:** inorganic arsenic as sum of As^{+3} and As^{+5}; **MMA:** monomethylarsonate; **DMA:** dimethylarsine; **TMAO:** trimethylarsine oxide; **TETRA:** tetramethylarsonium; **AsB:** arsenobetaine; **AsC**: arsenocholine; **n.e As**: not extractable arsenic: usually inorganic arsenic associated to insoluble concretions.

As compounds	Gills			Hepatopancreas		
	Ctl	As	As+nTiO_2	Ctl	As	As+nTiO_2
n.e As	2.9%	2.0%	9.7%	0.8%	1.8%	3.7%
iAs	n.d	n.d	7.4%	n.d	0.6%	1.6%
MMA	n.d	n.d	13.2%	n.d	1.0%	n.d
DMA	48,9%	63.8%	54.2%	38.0%	72%	66.2%
TMAO	n.d	n.d	n.d	n.d	4.6%	3.0%
TETRA	10.2%	n.d	n.d	n.d	n.d	n.d
AsB	38.0%	28.6%	15.6%	61.2%	18.6%	25.6%
AsC	n.d	5.6%	n.d	n.d	1.3%	n.d

6. DISCUSSÃO GERAL

Esta tese é composta de dois trabalhos experimentais. Ambos tiveram como objetivo analisar as interações toxicológicas de As e $nTiO_2$ e a influência de $nTiO_2$ sobre a metabolização e a capacidade de bioacumulação de As em diferentes tecidosdo camarão branco *L. vannamei*. Embora metodologicamente semelhantes, a concentrações tanto de As quanto de $nTiO_2$ utilizadas foram distintas,o primeiro trabalho foi realizado utilizando uma concentração de As e $nTiO_2$ de 0,01 mg/L, concentração esta baseada na resolução 357 do CONAMA, de março de 2005, que define que a concentração máxima permitida por lei de As em ambientes aquáticos é de 10 µg/L. O segundo trabalho foi realizado com uma concentração significativamente maior, sendo 1 mg/L, tanto de As quanto de $nTiO_2$, com o objetivo de ressaltar os efeitos de ambos os tóxicos para assim gerar um trabalho de cunho principalmente mecanístico.

No primeiro trabalho não houve acumulação estatisticamente significativa em nenhum tratamento, em ambos os tecidos testados (brânquias e hepatopâncreas). Entretanto, no segundo trabalho, foi observada uma significativa acumulação de As no grupo que foi exposto somente ao metaloide, nos dois tecidos. Este é um resultado que, muito provavelmente, está relacionado à concentração cem vezes maior de As utilizadano último estudo. De fato, tal resultado é corroborado por Lobato e colaboradores (2013), que em condições semelhantes às utilizadas no segundo trabalho desta Tese, também observaram significativa acumulação de As em hepatopâncreas de *L. vannamei*. No entanto, a co-exposição ao $nTiO_2$ não mostrou aumentar a incorporação de As em nenhum dos dois estudos quando comparados com os outros grupos experimentais. NPs, quando em ambientes com alta salinidade, tendem a agregar e consequentemente reduzir sua área superficial e reatividade (Chesne & Kim, 2014). Como a salinidade utilizada nos experimentos foi baseada na salinidade marinha (salinidade experimental de 30 ppm), este parâmetro pode ter contribuído para a agregação das NPs, o quê, consequentemente, contribuiria para a diminuição da área superficial da NP, reduzido sua reatividade. Em virtude destes fatores, animais de água doce aparentam ser mais

sensíveis à acumulação de metais pesados pela ação de NP do que animais de água salgada (Fan *et al*., 2011; Nigro e*t al*., 2015; Sun *et al*., 2007).

No primeiro trabalho, em brânquias, foi observada uma predominância de AsB (arsenobetaína) em todos os tratamentos. Também foi observado um aumento de DMA (dimetilarsênio) no grupo exposto simultaneamente ao $nTiO_2$ e As. Em hepatopâncreas, houve uma predominância do moderadamente tóxico DMA na exposição ao As. O mesmo foi observado ao grupo que foi exposto a ambos os tóxicos. A co-exposição parece afetar a metabolização de As por *L. vannamei*, aumentando a concentrações de espécies de As considerada mais tóxicas (DMA), enquanto reduz os níveis de espécies menos tóxicas como AsB. Mesmo que DMA seja considerado como uma forma moderadamente tóxica de As, alguns estudos demonstram que exposições mais longas a esta espécies de As podem induzir efeitos deletérios como redução nos níveis de GSH, modulação do sistema antioxidante e dano no DNA (Yakanama *et al*., 1991) e induzir a progressão de câncer de pele em ratos (Yakanama *et al*., 2000). Além disso, em *Daphnia pulex*, DMA se demonstrou mais tóxico do que iAs (Shaw *et al*., 2007).

No segundo trabalho, o DMA e AsB foram os principais compostos acumulados em ambos os tecidos após exposição ao As. Entretanto, no grupo da co-exposição foi possível observar uma diminuição nos níveis de AsB e o aumento de outros compostos, inclusive de iAs e MMA, respectivamente espécies de arsênio altamente e moderadamente tóxicas. Este foi um resultado parecido com o encontrado no primeiro trabalho em ambos os tecidos, o que sugere que a exposição simultânea com $nTiO_2$ não aumenta a acumulação de As de forma significativa neste tempo de exposição, mas afeta a capacidade de metabolização de As, favorecendo assim a acumulação de compostos mais tóxicos.

A enzima glutationaS-tranferase (GST) é uma enzima de fase II que age no processo de detoxificação das células (Cui *et al*., 2010). A análise de GST foi realizada apenas no segundo trabalho presente nesta tese. Vários trabalhos demonstram que metais pesados como As e cádmio

podem modular a atividade da GST em invertebrados aquáticos (Sandrini *et al*., 2008; Ventura-Lima *et al*., 2007; Ventura-Lima *et al*., 2011). Neste trabalho, em hepatopâncreas, não foram observadas mudanças na atividade desta enzima independentemente do tratamento, um resultado que é corroborado porLobato e colaboradores (2013). Por outro lado, nas brânquias todos os grupos tratados exibiram diminuição na atividade de GST. De fato, as brânquias são o órgão de primeiro contato do animal com o ambiente e consequentemente com a contaminação ambiental. Este fator pode ser a explicação de porque a atividade da GST é especialmente alta em brânquias quando comparado com hepatopâncreas. Ren e colaboradores (2015b) observaram, em brânquias de *L. vannamei*, uma modulação positiva após exposição ao benzo-(a)-pireno, enquanto que, em brânquias de *C. carpio*, a exposição ao $nTiO_2$ induziu uma diminuição na atividade desta enzima (Lee *et al*., 2012). Estes resultados indicam que a exposição ao $nTiO_2$ é capaz de reduzir a competência antioxidante e de tetoxificação dos tecidos, o que deixa o organismo mais vulnerável a outros estressores ambientais.

A enzima GSTΩ (monometil arsenato redutase) é uma enzima chave no processo de metabolização de As (Sampayo-Reyes & Zacharyan, 2006). A análise da expressão da GSTΩ foi realizada apenas no segundo trabalho presente nesta Tese, e todos os tratamentos apresentaram resultados estatisticamente idênticos. Não há trabalhos anteriores a este que tenham demonstrado o efeito de As sobre GSTΩ em organismos aquáticos. Entretanto, Saadat & Saadat (2013) observaram que, em células de mamíferos (HeLa), a exposição a arsenito durante 24h não causou mudanças na expressão de GSTΩ. Apesar de também existirem poucos trabalhos que lidem com a expressão gênica desta enzima, acredita-se que exposições agudas, de curta duração, podem não ser suficientes para causar expressão gênica significativa desta enzima (Ventura-Lima *et al*., 2009; 2011).

A enzima glutationa redutase (GR) é responsável em manter a taxa normal de GSH na célula, através da redução de glutationa oxidada (GSSG) utilizando NADPH como poder redutor

(Halliwell & Gutteridge, 2007). Esta enzima também só foi estudada no segundo trabalho desta Tese. Em um trabalho de Ventura-Lima e colaboradores (2009a), em *C. carpio*, uma concentração mais alta de As foi capaz de induzir uma redução na atividade da GR em fígado, enquanto que em brânquias este resultado não foi observado. No presente trabalho, em brânquias, a exposição ao As causou um aumento na atividade de GR, o que sugere que este tecido sofreu mudanças no estado redox. Algumas NPs metálicas e de óxidos metálicos são conhecidas como sendo capazes de modular a atividade da GR em sistemas biológicos. Em cérebro de *Oreochromis niloticus* e *Tilapia zillii*, a exposição à NPs de zinco (2 mg/L) resultou em uma redução na atividade desta enzima (Saddick *et al*., 2015), um resultado também observado em brânquias de *Crossostrea gigas* expostos a NPs de óxido de zinco (Trevisan *et al*., 2014). Embora o grupo exposto ao $nTiO_2$ não tenha apresentado mudanças na atividade de GR em relação ao controle, no grupo exposto de forma conjunta a ambos os tóxicos foi notada uma redução na atividade da GR em ambos os tecidos. Como também foi observada uma redução nos níveis de GSH, é provável que a exposição conjunta tenha induzido mudanças no estado redox, favorecendo um estado pró-oxidante nas células.

Glutamato cisteína ligase (GCL) é uma enzima chave na síntese de glutationa reduzida (GSH) (White *et al*., 2003). No primeiro trabalho, foi possível observar nas brânquias, que os tratamentos de As e $nTiO_2$ administrados individualmente causaram aumento naatividade da GCL em comparação ao grupo controle. Um resultado semelhante foi mostrado por Ventura-Lima e colaboradores (2009) em que uma concentração semelhante de As também estimulou a atividade de GCL em brânquias de *D. rerio*. Também, Ren e colaboradores (2015) também conseguiramobservar que baixa concentração de As estimulam a atividade de GCL em fígado de mamíferos, enquanto que, com uma concentração mais alta, induz inibição da atividade desta enzima. No segundo trabalho desta Tese, entretanto, a exposição conjunta de As e $nTiO_2$ diminuiu a atividade da GCL em ambos os tecidos. O mesmo resultado foi encontrado no grupo exposto apenas ao $nTiO_2$, indicando que a enzima é sensível a alta concentração desta NP. Embora existam poucos dados disponíveis sobre o efeito de $nTiO_2$ na atividade da GCL em organismos aquáticos, Gui e

colaboradores (2013) observaram em mamíferos, que a expressão gênica de GCL é associada a concentração de $nTiO_2$, pois a concentração mais alta empregada no trabalho (10 μg/ml) induziu uma diminuição na expressão gênica quando comparada com concentrações mais baixas (1 e 5 μg/ml). Alguns estudos têm demonstrado que a exposição ao $nTiO_2$ induz o fator nuclear Nrf2, que por sua vez é responsável pela ativação de genes antioxidantes responsáveis pelos níveis de GCL e GST (Gui *et al*., 2013; Shi *et al*., 2015). Talvez uma maior concentração de NPs possam bloquear a translocação de Nrf2 para o núcleo, o que impediria a ativação de genes antioxidantes. Esta hipótese pode explicar o resultado observado no segundo trabalho desta Tese, considerando a atividade de GCL no grupo exposto ao $nTiO_2$ sozinho ou em combinação com o As.

O tripeptídeo GSH é um poderoso antioxidante presente em altas concetrações nas células (Halliwell & Gutteridge, 2007). O As é conhecido por ser capaz de elevar os níveis de GSH, o que, por sua vez, aumenta as defesas celulares contra o estresse oxidativo (Costa *et al*., 2012; Lobato *et al*., 2013; Schuliga *et al*., 2002). No primeiro trabalho, uma elevação nos níveis de GSH causado por As foi observada em brânquias. Resultado similar foi observado por Ventura-Lima e colaboradores (2009a), em *Danio rerio* (10 μg de As/L em 48 h de exposição). Em ambos os casos, os níveis de GSH parecem estar sendo elevados pelo tecido em resposta à presença de As. No segundo trabalho, em hepatopâncreas, foi observado um aumento nos níveis de GSH nos grupos experimentais expostos a As ou $nTiO_2$, administrados individualmente, embora isto não tenha sido observado em brânquias. Resultado similar foi encontrado por Lobato e colaboradores (2013), em *L. vannamei* exposto a 1 mg/L, com aumento nos níveis de GSH também em hepatopâncreas. Apesar das diferenças nos tecidos, no segundo trabalho, os grupos experimentais que foram tratados com As e $nTiO_2$ simultaneamente apresentaram redução nos níveis de GSH. De forma semelhante, em um trabalho realizado por Fang e colaboradores (2015), uma diminuição nos níveis de GSH foi observada em co-exposição de $nTiO_2$ e pentaclorofenol. Tais resultados sugerem que, quando administrados concomitantemente com outros agentes tóxicos, $nTiO_2$pode induzir uma redução na capacidade antioxidante, o que deixa o organismo mais vulnerável a mudanças no estado redox

celular. É importante ressaltar que GSH também participa no processo de metabolização de As como substrato para a atividade de GSTΩ (Sampayo-Reyes & Zacharyan, 2006). No advento de ocorrer diminuição nos níveis de GSH, a falta de substrato para a ação da GSTΩ pode ocasionar em uma diminuição do próprio metabolismo do As, dificultando que este seja transformado em espécies menos tóxicas. Estes resultados indicam que $nTiO_2$ pode interferir na metabolização de As, o que pode até mesmo favorecer a acumulação de compostos mais tóxicos de As em determinado tempo.

No primeiro trabalho (exposição de 0,01 mg/L), em brânquias, foi observado aumento na capacidade antioxidante total contra radicais peroxil no grupo exposto ao $nTiO_2$ e no grupo da co-exposição a ambos os agentes tóxicos, o que não foi suficiente para prevenir a peroxidação lipídica observada no grupo exposto ao $nTiO_2$. Resultado similar foi observado por Linhua e colaboradores (2009) em brânquias, fígado e cérebro de *C. carpio*, após exposição a altas concentrações de $nTiO_2$, (100 – 200 mg/L), sugerindo a sensibilidade do *L. vannamei* ao $nTiO_2$, mesmo quando as respostas antioxidantes em brânquias tenham sido induzidas. Já em hepatopâncreas, os níveis praticamente constantes de GSH, em todos os tratamentos, parece ter sido suficiente para prevenir dano lipídico.

No segundo trabalho (exposição de 1 mg/L), houve aumento da capacidade antioxidante total contra radicais peroxil no grupo exposto ao As. Nos outros grupos de exposição, assim como em todos os grupos em brânquias, não houveram mudanças estatisticamente significativas em comparação ao controle. Este é um resultado bastante distinto do observado no primeiro trabalho. Tal diferença pode, mais uma vez, ser explicada pela diferença de concentração empregada nos dois trabalhos. Alguns biomarcadores são mais sensíveis a concentrações mais baixas de certos agentes tóxicos, o que pode explicar este resultado encontrado em brânquias, enquanto que em hepatopâncreas, uma maior concentração pode ser necessária para induzir respostas antioxidantes. Além do mais, o conteúdo constante de GSH observado em brânquias pode ter contribuído para manter o nível da capacidade antioxidante nas células, já que esta molécula é extremamente

importante para manter este fator elevado (Amado *et al*., 2009). Esta hipótese é corroborada pelos resultados observados em hepatopâncreas, onde o aumento nos níveis de GSH observados nos grupos expostos ao As ou $nTiO_2$ foram acompanhadas por um aumento na capacidade antioxidante total. Também se observou, em hepatopâncreas, dano lipídico nas exposições de As e $nTiO_2$, quando administrados individualmente, pois quando adicionados simultaneamente este efeito foi anulado.

Os resultados de ambos os trabalhos, quando comparados, mostraram que as brânquias de *L. vannamei* foram mais afetadas por baixas concentrações de $nTiO_2$ enquanto que em hepatopâncreas, concentrações maiores foram necessárias para induzir respostas antioxidantes e dano oxidativo. A exposição de As conjuntamente com $nTiO_2$ não aumentou a acumulação de As em nenhum tecido, independentemente da concentração utilizada. O efeito "cavalo-de-tróia", portanto, não foi observado no presente trabalho, mas uma redução na capacidade de metabolização de As foi evidenciada através da diminuição de espécies de As menos tóxicas e o aumento de espécies mais tóxicas, em ambos os trabalhos. Este resultado pode ser explicado pelo efeito observado na co-exposição da maior concentração utilizada, tratamento este que causou redução nos níveis de GSH e atividade da GR, em ambos os tecidos, efeitos não observados nas exposições com os tóxicos utilizados separadamente. Ainda, efeitos induzidos por $nTiO_2$, (também obervados na co-exposição) como a redução na atividade da GCL em ambos os tecidos, redução na atividade da GST em brânquias também servem para explicar o motivo da diminuição na capacidade de metabolização de As no animal. Independentemente da concentração utilizada, a interação entre $nTiO_2$ e As parece ser capaz de causar mudanças importantes na capacidade de metabolização de As, o que pode ocasionar em um aumento na toxicidade do semimetal sobre animais marinhos.

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