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Efeitos do grafeno através de diferentes rotas de exposição considerando dois modelos biológicos (*Litopenaeus vannamei* e *Danio rerio*).

Bióloga Amanda Lucena Fernandes

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Sumário

1. Resumo

. O grafeno (GR) é um NM de carbono que tem ganhado grande destaque devido à suas características físico-químicas, que o permitem ser utilizado nas indústrias tecnológicas, farmacológicas e biomédicas. Ele possui formato de folha e é formado por uma camada bidimensional de carbono (2D), sua utilização se deve a grande estabilidade térmica, elétrica e química. Com o aumento do uso deste NM, é fundamental analisar se o provável descarte em ambientes aquáticos pode induzir efeitos tóxicos nos organismos vivos. Uma vez no ambiente, os animais podem ser expostos através de diferentes rotas de exposição como o ar, água e alimentação e exercer efeitos tóxicos nestes organismos. Diante destes fatos, o objetivo do presente estudo foi investigar os efeitos tóxicos do grafeno através de diferentes rotas de exposição (injeção intraperitoneal e alimentação) em animais aquáticos de diferentes ambientes (água doce e marinho) e se sua exposição é capaz de induzir estresse oxidativo nestes animais.

A presente dissertação é composta por dois estudos distintos, no primeiro estudo, camarões *Litopenaeus vannamei* foram expostos durante quatro semanas à concentração de grafeno de 500 mg/kg de ração e foram avaliados efeitos em brânquias, hepatopâncreas e músculo. Os resultados bioquímicos mostraram alteração do sistema de defesa antioxidante, com um aumento da concentração de glutationa reduzida (GSH) e da capacidade antioxidante total contra radicais peroxil, além de um aumento da concentração das espécies reativas de oxigênio (ROS), em brânquias e hepatopâncreas; aumento da atividade da enzima glutamato cisteína ligase (GCL) no hepatopâncreas e diminuição nas brânquias; enquanto que o hepatopâncreas mostrou um decréscimo na atividade da glutationa-S-transferase (GST) e um acréscimo em brânquias. Nos dois tecidos citados acima também foram encontrados danos oxidativo lipídicos, e alterações

de DNA sendo observada a capacidade de genotoxicidade do grafeno. Também, os resultados histopatológicos mostraram mudanças na morfologia do hepatopâncreas após a exposição ao grafeno, como hiperplasia das células basais, infiltração de hemócitos e decréscimo de células secretoras.

O segundo estudo avaliou as respostas toxicológicas da exposição intraperitoneal de 5 e 50 mg/L de grafeno no peixe *Danio rerio* durante 48 h em brânquias, intestino, músculo e cérebro. Os resultados analisando biomarcadores moleculares de estresse oxidativo, mostrou que os genes *nrf2* e *gclc* (subunidade catalítica da enzima GCL) não sofreram alteração em sua expressão após a exposição, enquanto que o sistema antioxidante enzimático do animal foi alterado em alguns tecidos após a exposição. Após a exposição de 5mg de GR/L, a concentração de GSH em brânquias diminuiu, enquanto que mostrou um aumento no intestino e em cérebro, sendo este resultado também observado após exposição a 50 mg/L no cérebro. A atividade da enzima GCL foi induzida após a exposição a ambos os grupos de GR em intestino e cérebro, já na enzima GST foi observado um acréscimo de atividade após a exposição de 5 mg/L de GR nestes mesmos tecidos. Danos a macromoléculas como lipídios foram observados em brânquias após a exposição à maior concentração; como também alterações morfológicas em brânquias, cérebro e músculo com histopatologias de grau moderado a severo, apresentando hiperplasia, inflamações e edemas nos tecidos.

Com estes estudos evidencia-se a capacidade do grafeno em produzir efeitos tóxicos para estes animais aquáticos, seja em uma exposição direta (*i.p*) ou através da alimentação por curta ou longa exposição. Como não existe hoje uma legislação que controle a liberação de nanomateriais de carbono no ambiente, e com o crescente uso dos mesmos, estudos sobre esses efeitos se torna importante para contribuir níveis de segurança para os seres vivos presentes nestes ambientes.

2. Abstract

Graphene (GR) is a carbon NM that has been having higher prominence due to their physical-chemical characteristics, which are used in the technological, pharmacological and biomedical industries. It has a sheet shape and consists of a one layer of carbon (2D), possessing high thermal, electrical and chemical stability. With increased use of this NM, it becomes necessary to analyze if their disposal in aquatic environments are not causing toxic effects for living organisms. Once into environment, the animals can be exposed through different routes of exposition such as air, water and food and cause toxic effects in these organisms. Considering these facts, the objective of this study was to investigate the toxic effects of graphene through different exposure routes (intraperitoneal injection and food) in aquatic animals from different environments (freshwater and marine) and their possibility of induce oxidative stress in these animals.

This dissertation is composed by two different studies, in the first study, the animals were exposed for four weeks to graphene concentration of 500 mg/kg of ration and changes in gills, hepatopancreas and muscle of *Litopenaeus vannamei* were evaluated. Biochemical results showed changes of the antioxidant defense system, with an increase in the concentration of reduced glutathione (GSH) and the total antioxidant capacity against peroxyl radicals, besides an increase of reactive oxygen species (ROS) concentration in gills and hepatopancreas; increase glutamate cysteine ligase (GCL) activity in hepatopancreas and decrease in gills; while hepatopancreas showed a decrease in glutathione-S-transferase (GST) activity and an increase in gills. In the tissues mentioned above were also observed lipid oxidative damages, and alterations in DNA, being observed genotoxicity of graphene. Histopathological results showed

changes in the morphology of hepatopancreas after exposure to graphene, such as basal cell hyperplasia, hemocyte infiltration and decrease of secretory cell.

The second study evaluated the toxicological responses of intraperitoneal exposure at 5 and 50mg GR/L in *Danio rerio* for 48 h in gills, intestine, muscle and brain. The results of molecular biomarkers of oxidative stress showed that *nrf2* and *gclc* genes (catalytic subunit of the GCL enzyme) did not change their expression after exposure, while the enzymatic antioxidant system of the animal was altered in some tissues. After exposure to 5 mg GR/L, the concentration of GSH in gills decreased, while in intestine and brain increased, the same result also was observed in brain after exposure to 50 mg GR/L. The activity of the GCL enzyme was induced after exposure to both GR groups in the intestine and brain, whereas an increase in the GST activity was observed after exposure of 5 mg GR/L in these same tissues. Damage to macromolecules such as lipids were observed in gills after exposure at the highest concentration, also were observed morphological changes in gills, brain and muscle with moderate to severe histopathology, presenting hyperplasia, inflammations and edema in tissues.

It those studies the ability of graphene to produce toxic effects for aquatic animals were demonstrated, either being by a direct (i.p) or through feed exposure during a short or long exposure time. Currently, there is no legislation that controls the release of carbon nanomaterials into the environment, and these materials are increasingly being used, the study of these effects becomes important to contribute with levels of safety for present living beings in this environment.

3. Introdução

3.1 Características físico-químicas do grafeno

Os nanomateriais (NM) são partículas com tamanho que varia de 1 a 100 nm em pelo menos uma dimensão. Estes NM estão sendo amplamente utilizados para novas tecnologias em diversas áreas como as biológicas, farmacológicas, da saúde e na informática (Aitken et al., 2006; Oberdörster, 2004). Eles podem ser incorporados, metabolizados pelos organismos através da água, dieta e/ou por inalação, se distribuindo em diferentes órgãos, entrando em contato com tecidos e células e podendo assim, se acumular e causar efeitos tóxicos nos mesmos (Fischer & Chan, 2007; Park et al., 2011; Zhang et al., 2011). Deste modo, os NM podem interagir com moléculas como o DNA além de induzir a produção de espécies reativas de oxigênio (ROS) que possuem a capacidade de reagir com estrutura de proteínas e lipídios de membrana, induzir danos ao DNA e modular respostas antioxidantes (Lee et al., 2008; Matés et al.,1999). Esta geração de ROS pode ser atribuída a fatores como concentração, tempo de exposição, tipo de dispersante utilizado junto ao NM, além do tipo de funcionalização dos mesmos (Aitken et al., 2006). Além disso, os NM tendem a se agregar em soluções aquosas devido à carga eletrostática do NM, além da força iônica presente no meio. Em contrapartida, muitas vezes a funcionalização aumenta sua biocompatibilidade e solubilidade podendo deste modo, reduzir sua citotoxicidade e genotoxicidade, assim polímeros e moléculas são utilizados para a modificação destes NM (Firme & Bandaru 2010; Liu et al, 2011 (a); Pan et al, 2012)

O grafeno (GR) é um nanomaterial formado por uma única camada de átomos de carbono possuindo duas dimensões com uma estrutura planar semelhante a uma folha (*nanosheets*) (Mafra, 2008). Por possuir propriedades físico-quimicas de grande interesse para o campo tecnológico, como sua grande área de superficie, alta

condutividade elétrica e estabilidade térmica, óptica, quimíca e eletroqúimica; seu uso tem crescido nos ultimos anos (Chen et. al, 2010; Huang e Shi, 2012). A produção de GR pode ser realizada por vários métodos, entretanto a forma mais utilizada é através da exfoliação do grafite, devido a seu custo baixo e a facilidade de produção (Latin e Henrard, 2006). Na estrutura do GR, cada átomo de carbono está ligado a outro átomo de carbono (carbono-carbono) no mesmo plano, fazendo desta uma forte ligação, o que lhe da à característica de um dos materiais mais resistente descoberto até o presente momento (Syama e Mohanan, 2016).

O GR hoje compõe uma família de NM (**Figura 1**), que foram funcionalizados e modificados para atender diferentes demandas. A natureza do GR é hidrofóbica, portanto, quando entra em contato com soluções aquosas a tendência é que o material sofra agregação; para superar esse problema o óxido de grafeno (GO) foi desenvolvido, através da adição de grupos químicos reativos como epóxi (–O–), hidroxila (–OH) e ácido carboxílico (–COOH), gerando pontas hidrofílicas no NMs, aumentando desta forma a superfície além de possuir poucas folhas de grafite permitindo certa maleabilidade ao material, pois a resistência acaba sendo diminuída (Kiew et al., 2016; Park et al., 2009; Texter, 2014). Contudo, a presença destes grupos proporciona uma redução em suas propriedades químicas, térmicas e física, o que faz do GR um melhor material para se utilizar no âmbito tecnológico (Karlický. 2013).

Outra forma de funcionalização do GR é a adição de estruturas que modifiquem a superfície deste NM, incluindo polímeros hidrofílicos como o polietileno glicol (PEG) que é o mais utilizado; esta funcionalização melhora a dispersão e estabilidade do NM em soluções aquosas (Kiew et al., 2016). Estudos biológicos e farmacológicos têm utilizado este tipo de NM funcionalizado principalmente como carreador de fármacos,

devido a melhor circulação destes no sangue, além de melhor biodistribuição em diferentes órgãos do animal, quando comparado com a forma pristina do GR (Yang et al., 2013). Por outro lado, também devido à diferença em estruturas e grupamentos químicos presente nestes NM, ocorre uma alteração na capacidade de penetração e ligação destes NM nos diferentes compartimentos celulares, podendo exercer efeitos tóxicos (Jortner e Rao, 2002; Liu et al., 2011 (b)).

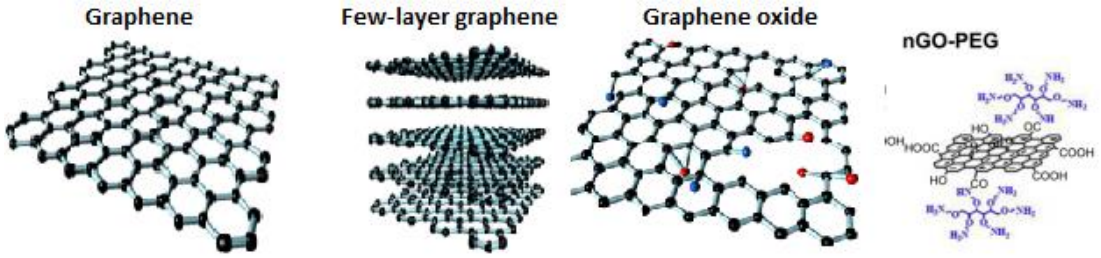

Figura 1. Diferentes formas químicas de grafeno (Adaptado de Kiew et al. (2016) e Yang et al. (2013)).

3.2 Diferentes rotas de exposição e efeitos do grafeno

O ambiente aquático engloba uma grande fauna, onde vertebrados e invertebrados estão constantemente expostos a qualquer tóxico nele presente, sendo suscetível à contaminação, pois a maioria dos contaminantes têm como destino final as grandes massas de água (Mieiro et al., 2011). Uma das formas de avaliar os efeitos de contaminantes ambientais nos organismos vivos é analisar parâmetros bioquímicos de estresse oxidativo, toda vez que uma grande variedade de compostos tóxicos possa direta ou indiretamente alterar defesas antioxidantes e exacerbar o dano oxidativo (Monserrat et al., 2007; Ventura-Lima et al., 2009). Além de parâmetros bioquímicos enzimáticos, a expressão de genes que codificam para enzimas e moléculas antioxidantes relacionadas ao estresse oxidativo é outro método muito utilizado para

analisar os efeitos gerados por exposição a diferentes tóxicos, considerando-se que os organismos podem estar respondendo a um nível molecular que pode anteceder algumas respostas bioquímicas, apesar do oposto ocorrer mais comumente (Storey, 2005; White et al., 2003).

Com a crescente utilização do GR pode também surgir risco de exposição dos organismos, principalmente aquáticos, através das mais variadas rotas de exposição, as frequentemente escolhidas para estudos toxicológicos, englobam exposições em água, via alimentação (**Figura 2**) e injeções intraperitoneais (*i.p.*) do material diretamente no animal (**Figura 3**) (Zhang et al., 2016). O tipo escolhido de exposição pode influenciar em muito nos resultados de um estudo, sendo que as exposições via água e sedimento geralmente estão associadas a recriar situações ambientais reais de contaminação. Efeitos tóxicos do óxido de grafeno quando exposto na água *Danio rerio* foram avaliados por Chen et al. (2016), onde os autores observaram alterações em enzimas do sistema oxidante como superóxido dismutase (SOD) e catalase (CAT), além de alterar os níveis de glutationa reduzida (GSH) e induzir a expressão de genes de citocinas inflamatórias, caracterizando uma situação geral de estresse oxidativo.

Figura 2: Exposição via ração de grafeno

Figura 3: Exposição intraperitoneal de grafeno

Assim como a rota de exposição aquática, as exposições orais tentam recriar situações que poderiam ocorrer no meio ambiente. Porém, poucos são os estudos com GR que utilizaram esta rota de exposição, por exemplo, Mao et al. (2016) observaram

que após exposição oral em ratos, o GR causou edema nos pulmões, porém estes efeitos foram transitórios, e nenhum grande dano foi observado. Exposições *i.p.* de GR foram realizadas em alguns estudos (Dziewiecka et al., 2016; Kurantowicz et al., 2015; Strojny et al., 2015; Yang et al., 2013), e foram observados efeitos tóxicos na maioria dos experimentos, com alterações morfológicas em tecidos e modificação na atividade de enzimas ou moléculas do animal. Esse tipo de exposição geralmente é escolhido quando se quer testar uma dose exata do contaminante, pois, sabe-se que NM podem ser comportar diferentemente quando em contato com soluções aquosas (da Rocha et al., 2013).

Estudos sobre o comportamento do grafeno em contato com células, mostram que este NM age em nível de membrana, sendo de difícil penetração nas células, provavelmente alterando as propriedades de membrana devido a morfologia plana em 2D, levando a uma alteração na solubilidade e gerando produção de espécies reativas de oxigênio (Lee et al., 2008; Nguyen e Berry, 2012). A toxicidade do óxido de grafeno em células foi exemplificada em estudo, onde a morte de bactérias foi causada pelo aumento de ROS dentro das células bacterianas (Liu et al., 2011 (c)).

3.3 Modelos biológicos

A escolha do modelo biológico em um experimento é de extrema importância, principalmente para experimentos toxicológicos, onde vários fatores precisam ser considerados: a espécie não pode ser frágil, precisa ser de fácil manutenção e pequena para haver um transporte facilitado, se tratando de espécies aquáticas que precisam ser realocadas de aquários; além de precisar se adequar a análise de diferentes tipos de estressores químicos (He et al., 2014).

O crustáceo *Litopenaeus vannamei*, conhecido como camarão branco do Pacífico, é uma espécie costeira de clima tropical, com ocorrência na costa do Pacífico, América Central e Sul, das Antilhas ao Rio Grande do Sul- RG. Possui ciclo de vida migratório, onde os adultos são encontrados em regiões marinhas de até 30 metros de profundidade e os juvenis em estuários, baías e enseadas (Iawi, 1973; Perez-Farfante, 1969; Silva, 1977). A espécie é de grande importância econômica, sendo amplamente cultivada para o comercio em diferentes regiões do globo, como América, China e Tailândia, assim utilizada para o consumo humano, devido a isso, a espécie tem sido escolhida cada vez mais para estudos toxicológicos, visto que pode ser uma fonte de contaminação para o consumidor final (Yang et al., 2010).

Além disso, a espécie possui características que a tornam um bom modelo para estudos toxicológicos, como grandes taxas de sobrevivência e adaptação a diferentes condições físico-químicas, como diferenças de salinidade e temperatura (Lotz, 2003). Por isso *L. vannamei* tem se mostrando um bom modelo biológico para estudos que avaliam os efeitos de diferentes contaminantes ambientais (Ren et al., 2015 (a,b); Lobato et al., 2013), entre eles contaminantes emergentes, como os NM (Juarez-Moreno et al., 2017).

O peixe *Danio rerio*, conhecido como zebrafish, é uma espécie comumente utilizada em experimentos de pesquisa científica devido a seu baixo custo e rápido desenvolvimento, genoma muito semelhante ao de mamíferos, assim como órgãos e tecidos, considerando aspectos anatômicos, fisiológicos, moleculares e celulares (He et al., 2014; Lawrence, 2007). A espécie também é um bom modelo para estudo de toxicidade, sendo usada em estudos com contaminantes ambientais e NM, pois mostra respostas de toxicidade para um amplo espectro de materiais como, por exemplo, NM de carbono; exibem respostas fisiológicas a xenobióticos semelhantes ao que ocorre em

mamíferos, como produção de enzimas antioxidantes para combater o estresse oxidativo; e devido ao genoma ser semelhante ao de mamíferos, possuem respostas fisiológicas e imunológicas equivalentes (Fako e Furgeson, 2009; Froehlicher et al., 2009; Pyati et al., 2007).

Devido a isso, a espécie se tornou a principal alternativa para testes de saúde humana e avaliação de riscos ecológicos por contaminantes potenciais (Esch et al., 2012). De fato, estudos demonstraram a toxicidade de nanomateriais de carbono em *D. rerio*, quando expostos a nanotubos de carbono e fulereno $C_{60,}$ induzindo um cenário de estresse oxidativo, com alterações do sistema antioxidante (da Rocha et. al, 2013; Usenko et al., 2008). Além disso, devido ao seu tamanho pequeno, avaliações histopatológicas de todos os órgãos do animal podem ser feitas facilmente e com baixo custo, o que é uma vantagem em relações a outras espécies de peixes utilizadas em experimentos toxicológicos, como *Cyprinus carpio* (Esch et al., 2012).

Entretanto, estudos analisando a toxicidade do GR tanto em zebrafish, como em outros seres vivos, atualmente é limitado, com poucos dados presentes na literatura, onde os já publicados analisam outras formas de grafeno, como por exemplo, o óxido de grafeno (Yang et al., 2013). Desta forma torna-se necessário o conhecimento da toxicidade apresentada por este NM considerando-se o constante aumento do uso do mesmo nos últimos anos.

4. Objetivos

4.1 Objetivo geral

Avaliar os efeitos toxicológicos do grafeno considerando diferentes rotas de exposição em organismos aquáticos de ambientes marinho e dulciaquícola.

4.2 Objetivos específicos

Associado a exposição do grafeno incorporado à ração no crustáceo *Litopenaeus vannamei* (Crustacea, decápoda):

- Avaliar a concentração de ROS e níveis de peroxidação lipídica nas brânquias, hepatopâncreas e músculo dos animais.
- Analisar os efeitos em parâmetros bioquímicos associados às defesas antioxidantes como: níveis de glutationa reduzida (GSH), atividade das enzimas relacionadas com a GSH (glutationa-S-transferase, glutamato cisteína ligase) e capacidade antioxidante total no músculo, hepatopâncreas e brânquias.
- Avaliar a indução de genotoxicidade em brânquias, hepatopâncreas e músculo.
- Analisar os efeitos histopatológicos em brânquias, hepatopâncreas e musculo.

Associado a exposição intraperitoneal de grafeno em *Danio rerio* (Cyprinidae)

- Analisar a expressão de genes associados à resposta antioxidante como o fator *nrf2* (fator nuclear eritróide 2) e *gclc* (subunidade catalítica da enzima GCL) em brânquias, intestino, e músculo de *D. rerio.*
- Avaliar se a exposição altera parâmetros de atividade antioxidante e estresse oxidativo incluíndo níveis de glutationa reduzida (GSH), atividade das enzimas: glutamato cisteína ligase (GCL) e glutationa-S-transferase (GST) e dano oxidativo lipídico em brânquias, intestino, músculo e cérebro.

- Analisar os efeitos histopatológicos do grafeno em brânquias, intestino, músculo e cérebro.

5. Capítulo 1

Exposure to few-layers graphene through diet induces oxidative stress and histological changes in the marine shrimp *Litopenaeus vannamei*.

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Amanda Lucena Fernandes[1,2], Marcelo Estrella Josende[1,2], Jefferson Patrício Nascimento[4], Adelina Pinheiro Santos[4], Sangram Keshai Sahoo[4], Flávio Manoel Rodrigues da Silva Júnior[1,3], Luis Alberto Romano[5], Clascídia Aparecida Furtado[4], Wilson Wasielesky[5], José Marìa Monserrat[1,2,5], Juliane Ventura-Lima[1,2*].

[1]Instituto de Ciências Biológicas (ICB), Universidade Federal do Rio Grande - FURG, Rio Grande, RS, Brasil.

[2]Programa de Pós-Graduação em Ciências Fisiológicas - FURG.

[3]Programa de Pós-Graduação em Ciências da Saúde- FURG

[4]Centro de Desenvolvimento da Tecnologia Nuclear – CDTN/CNEN, Belo Horizonte, MG, Brazil.

[5]Programa de Pós-Graduação em Aquacultura-FURG.

* Corresponding author: Juliane Ventura-Lima

Phone/Fax: +55 5332935249

E-mail: juliane_ventura@pesquisador.cnpq.br

Abstract

The production and use of graphene-based nanomaterials is rapidly increasing. However, little data are available regarding the toxicity of these nanomaterials in aquatic organisms. In the present study, the toxicity of few-layer graphene (FLG) (obtained by chemical exfoliation) was evaluated in different tissues of the shrimp *Litopenaeus vannamei* following exposure through diet for four weeks. Transmission electron microscopy and dynamic light scattering measurements showed a distribution of lateral sheet sizes between 100 and 2000 nm with average length and width of 800 and 400 nm, respectively. Oxidative stress parameters were analyzed, indicating that FLG exposure led to an increase in the concentration of reactive oxygen species, modulated the activity of antioxidant enzymes such as glutamate cysteine ligase and glutathione-S-transferase, and reduced glutathione levels and total antioxidant capacity. However, observed modulations were not sufficient to avoid lipid and DNA damage in both gill and hepatopancreas tissues. Further, graphene exposure resulted in morphological changes in hepatopancreas tissues. These results demonstrate that exposure to FLG through diet induces alterations in the redox state of cells, leading to a subsequent oxidative stress situation. It is therefore clear that this nanomaterial presenting these physico-chemical characteristics may be harmful to aquatic biota.

Key-words: few-layer graphene, nanotoxicology, oxidative stress, shrimp, antioxidant responses.

1. Introduction

Nanotechnology is expanding rapidly, and various nanomaterials (NM) are already being applied in technological and biomedical areas (Powell et al., 2010). Considering the growing production and use of these NM, their release into aquatic environments and interaction with biological systems appears inevitable (Handy et al., 2011). It is important to understand the effects NM may have on aquatic organisms, and to infer the potential ecotoxicological risks for ecosystems (Matranga and Corsi, 2012).

Graphene is an allotrope of carbon composed of a single two-dimensional layer (2D), which possesses unique electrical, mechanical, thermal and optical properties (Zhang et al., 2011). Different production processes have been proposed to explore each of these properties, using different experimental routes and yielding graphene-based materials with different physicochemical characteristics. Characteristics such as the number of graphene layers, average lateral size, and carbon-to-oxygen (C/O) atomic ratio may be modified. Graphene-based materials comprise not only single-layer graphenes but also few-layer graphenes (i.e., 2–10 layers), graphene oxide, reduced graphene oxide, graphite nanoplatelets (i.e., more than 10 graphene sheets but below 100 nm in thickness), graphene ribbons, graphene dots and other derivatives. The physicochemical characteristics of a graphene-based material can determine its integration into biological media and the environment, and consequently its toxicity to organisms and ecosystems. For example, graphene oxide and reduced graphene oxide were found to be readily accumulated by cells and exert toxicity on biological models. Specifically, these materials induced the generation of reactive oxygen species (ROS), modulation of antioxidant system and oxidative damage in lipids and DNA (Chatterjee et al., 2014). Further, acute exposure (24 h) to two different graphenes in the crustacean *Artemia salina* induced oxidative stress, evidenced by alterations in the antioxidant

system and lipid peroxidation. This toxicity increased as nanoparticle sizes decreased (Pretti et. al., 2014).

Carbon nanomaterials (CNM) tend to deposit in sediment, as predicted by environmental modeling by Gottschalk et al. (2009). This deposition likely endangers organisms that live in close proximity to sediment. In fact, Waisse-Leinonen and co-authors (2012) showed that fullerene (C_{60}) exerts toxicity on the benthic organism *Chironomus riparius* after contact with contaminated sediment. Diet represents another environmentally relevant exposure route, as NM tends to adsorb to surfaces such as in food matter, and are subsequently ingested by organisms (Zhu et. al., 2010).

The Pacific white shrimp *Litopenaeus vannamei* (Crustacea, Decapoda) is a species widely distributed along the Pacific Coast and in South and Central America and possess high economic value as they are a main species employed in shrimp farming around the world (Wang et al., 2015). Beyond this, *L. vannamei* exhibit a high tolerance to stressful conditions and has been used extensively in toxicological studies (Lobato et al., 2013).

Until now, few studies have assessed the toxicity of graphene-based materials on aquatic organisms. The objective of the present study was to evaluate the toxicological effects of exposure to chemically exfoliated FLG via diet in *L. vannamei*. Specifically, oxidative stress parameters and histological alterations in different tissues of shrimps were analyzed. The authors present that this is the first study evaluating the toxicity of this NM through dietary exposure in a benthic species such as *L. vannamei*.

2. Materials and Methods

2.1. Obtainment and characterization of FLG

FLG was obtained according to the work of Khan and co-authors (2011). This procedure was performed with the following two major steps. In the first step, 3.3 g of natural graphite (Graflake 99580, supplied by Nacional de Grafite LTDA-Brazil) was added to 1000 mL of NMP (1-methyl-2-pyrrolidinone, Sigma-Aldrich). This mixture was then placed in an ultrasonic bath (Cole-Parmer 08895-50 –100 – 250 W a 42 kHz) and sonicated for 168 h. The mixture was then centrifuged (Heraeus Multifuge X1R) at 110 *g* for 45 min. The supernatant was collected (around 90% of flask volume) and filtered through a nylon membrane (0.2 μm diameter). A washing was performed using 400 mL deionized water, 200 mL of ethanol (95% P.A) and 100 mL of diethyl ether (Synth P.A) in order to remove waste NMP. FLG was dried for 24 h at 150°C under vacuum. In the second step, the dried FLG obtained in the first step was added to 16 mL of NMP (1-methyl-2-pirrolyde; 24 $mg.mL^{-1}$) and sonicated for 24 h. Posteriorly, the dispersion obtained was kept standing for 8 days and the supernatant was collected and filtered through a 0.2 μm membrane. Again, the material was washed as described above. Finally, the derived sample of FLG was dried for 24 h at 150 °C under vacuum.

The morphology, structure and size of exfoliated FLG were characterized by transmission electron microscopy (TEM), Raman spectroscopy and dynamic light scattering (DLS). Images of TEM were collected in a microscope (Tecnai G2–Spirit-FEI-2006) operated with tension of 80 kV. A drop of supernatant was collected in step 2 (described above) and deposited over a copper grid covered with carbon film (holey carbon 300 mesh) and dried for 24 h under vacuum at room temperature. Raman spectroscopy was performed in Raman Horiba Jobin Yvon iHR 550 using a 514 nm laser (2.41 eV), 4.6 mW power, three accumulations of 60 s and objective of 50 x. For

Raman measurements, a drop of supernatant collected in step 2 was deposited and diluted 100 x in NMP over substrate of Si/SiO_2, dried under vacuum for 24 h at 150 °C. DLS measurements were carried out using a Zetasizer Nano ZS equipped with a laser of 633 nm.

2.2. Diet supplemented with graphene nanosheets.

The FLG concentration used was 500 mg/kg of ration; the nanomaterial in power was mixed to the feed previously macerated and this mixture subsequently was sealed with 3.3% bovine gelatin (Sigma-Aldrich, Inc.) and MilliQ water the bovine gelatin was used to avoid that the FLG was released from ration. Following this procedure, the feed (FLG or control) was dried in an oven at 50 °C and then stored in glass vials at 4°C. A control diet was prepared in the same way but was not supplemented with graphene. The choice of FLG nanosheet concentration was based on work analyzing the effect of carbon nanomaterials in the diet of *Oncorynchus mykiss* by Fraser et al. (2011).

2.3. Detection of graphene in the ration

To identify if the incorporation of FLG in the feed had been efficient, was performed vibrational spectral measurements of control and FLG supplemented food samples were carried out using Raman spectroscopy in the near IR (NIR) region of the electromagnetic spectrum to detected FLG into the ration. The Raman experiments were carried out using a Bruker RFS 100/S Fourier Transform (FT) Raman spectrometer in macro sampling mode with a 1064 nm excitation source from a Nd:YAG laser attached to a liquid nitrogen cooled Ge detector. The choice of NIR excitation wavelength helps in maintaining low noise as well as an effective rejection of naturally occurring unwanted food autofluorescence. Laser power and spectrometer resolution were kept at 150 mW and 4 cm^{-1}, respectively, for the spectral acquisition for 256 scans. Control and

FLG subjected food samples were ground to a fine powder using a mortar and pestle, sandwiched between two glass cover slips and exposed to the laser focus in the sample chamber. The resultant spectrum was chosen on the basis of average of the spectra acquired for each sample.

2.4. Maintenance of *L. vannamei* shrimps

A total of 64 animals (~ 15 g each) were obtained from Marine Station of Aquaculture (EMA) of the Federal University of Rio Grande (FURG) and immediately transferred to laboratory. Animals were then acclimated in a tank of 100 L of seawater for at least two weeks prior to the beginning of the experiment. The shrimps were fed twice day with commercial ration (45% crude protein) under laboratory conditions (temperature of 20 ºC and photoperiod of 12 hours light/12 hours dark with a screen shading for stress reduction in the animals, pH 8.0, and constant aeration in order to maintain dissolved oxygen at 7.2 mg O_2/L). No mortality was observed during the acclimation period.

2.5. Experimental design

Two replicates were performed in this study, and in each replicate, the animals were divided into two experimental groups: **1) Treatment group (n=8** per aquarium in duplicate**):** received ration supplemented with FLG (as described previously), and **2) Control group (n=8** per aquarium in duplicate**):** received ration without FLG. All experimental groups were fed twice per day. Each group was maintained in aquarium with 10 L of seawater with constant aeration (7.2 mg O_2/L) and pH 8.0. The quantity of feed offered to animals was based on 2.8% of the average weight of animals per aquarium. Shrimps were weighed weekly during all experiment periods for adjustments

421 in the quantity of ration offered over four weeks (exposure period). There was no
422 mortality in both groups during exposure time.

423 **2.6. Biochemical analysis**

424 **2.6.1. Preparation of homogenates**

425 After each experiment the animals were sacrificed via freezing, while gill,
426 hepatopancreas and muscle tissues were immediately removed and stored at -80^{o}C.
427 Tissues were homogenized (1:4 w/v) in crustacean homogenization buffer (Tris-base
428 buffer (20 mM) with sucrose (0.5 M), EDTA (20 mM), DDT (1 mM), KCl (0.15 mM))
429 and protease inhibitor cocktail (Sigma-Aldrich), with pH adjusted to pH 7.75. After
430 homogenization, samples were centrifuged at 10,000 x *g* at 4 °C for 20 min.
431 Supernatants were then aliquoted and stored (-80 °C) for assays of enzyme activities,
432 concentration of reduced glutathione (GSH), total antioxidant capacity and lipid
433 peroxidation. However ROS determination was performed with fresh tissue
434 immediately after dissection.

435 Measurements of enzymatic activities were standardized by the total amount of
436 protein present in the extracts. Measurements of total protein concentration were
437 performed using the commercial kit based on the Biuret method with microplate reader
438 (ELX 800 Biotel) at 550 nm.

439 **2.6.2. Determining the concentration of reactive oxygen species (ROS)**

440 For ROS detection, 2',7' dichlorofluorescein diacetate (H_2DCF-DA) probe was
441 used, which generates a detectable fluorochrome at wavelengths of 485 and 530 nm for
442 excitation and emission, respectively (Viarengo et al., 1999) (after deacetylation and in

443 the presence of ROS). The readings were performed with microplate reader (Victor 2
444 fluorometer, Perkin Elmer).

445 **2.6.3. Enzymatic assays and reduced glutathione (GSH) levels**

446 The methodology for glutamate cysteine ligase (GCL) activity and GSH levels
447 was based on White et al. (2003). This analysis is based on the ability of the compound
448 2,3-naphtalenedicarboxaldehyde (NDA) to react with γ-glutamylcysteine (γ-GC) or
449 glutathione (GSH), forming a fluorescent cyclic compound (GC-NDA and GS-NDA,
450 respectively). The fluorescence of the complex NDA- γ-GC and GS-NDA was
451 measured using a fluorometer (2 Victor, Perkin Elmer) with wavelengths of 485 and
452 530 nm for excitation and emission respectively.

453 Glutathione-S-transferase (GST) activity was determined according to Habig et al.
454 (1974). This analysis measures the conjugation of 1 mM GSH (Sigma) with 1 mM of
455 the reagent 1-chloro-2.4-dinitrobenzene (CDNB, Sigma), a reaction catalyzed by GST.
456 The complex formed has a maximum absorbance at 340 nm. Absorbance was measured
457 using a microplate reader (Victor2, Perkin Elmer).

458 **2.6.4. Determination of lipid peroxidation and total antioxidant capacity**

459 Lipid peroxidation was measured fluorometrically via the thiobarbituric acid
460 reactive substance (TBARS) method in accordance with Oakes and Kraak (2003). This
461 method involves the reaction of malondialdehyde (MDA), a degradation by-product of
462 peroxidized lipids, with thiobarbituric acid (TBA) under conditions of high temperature
463 and acidity, resulting in a detectable fluorescent chromogen (wavelength of 520 nm
464 (excitation) and 580 nm (emission)). Tretramethoxypropane (TMP, Sigma) was used as
465 a standard and MDA content was expressed as nmol of TMP equivalent/mg of protein.

The total antioxidant capacity against peroxyl radicals was assessed following the protocol of Amado et al. (2009). This analysis consists of the thermal decomposition of ABAP (2,2 '-azobis (2-methylpropionamidine dihydrochloride) at 37 °C, which generates peroxyl radicals. 2', 7' dichlorofluoreceine diacetate (H_2DCF-DA) was added before reading, as this compound reacts with esterases to produce a non-fluorescent compound (H_2DCF). This is subsequently oxidized by ROS, generating a fluorescent compound that is detectable at a wavelength of 485 and 530 for excitation and emission respectively. The calculation of total antioxidant capacity is based on a second order polynomial function that integrates ROS area with or without ABAP. In this way, smaller area corresponds with greater antioxidant capacity due to neutralization/interception of peroxyl radicals.

2.6.5. DNA damage

DNA damage was assessed by an alkaline version of the comet assay, according to Singh et al. (1998). Aliquots (15 uL) of muscle, hepatopancreas and gill tissue homogenates were mixed with low melting point agarose (37 °C) and placed on slides containing agarose. These were left in lysis buffer for at least 2 h, after which electrophoresis was conducted under the following conditions: 30V (1 V / cm), 300 mA, 20 min in an ice bath. Slides were stained with SYBR Safe and analyzed using a fluorescence microscope. The % DNA in tail, the tail length and tail moment of nucleoids (100 per animal) were analyzed using ImageJ software.

2.7. Histological analysis

Whole animal bodies were fixed by injection of Davidson solution. Different parts of the shrimps were kept immersed in the same solution for 24 h. Afterward, body sections corresponding with muscle, gill and hepatopancreas tissues were cut away and

were processed in an automatic tissue processor LUPE PT 05 (dehydration, rinsing, clearing and impregnation) and embedded in Paraplast (Sigma-Aldrich). Following embedment, the tissues were sectioned in microtome (LUPETEC MRPO3) with 4 μm thin sections. The sections were stained with hematoxylin and eosin (H&E). A light optic microscope (Primo star, Zeiss) was used to analyze the histological cuts, and the images were captured using a 400x magnification camera (ERc5s, AxioCam). 5 animals were used in both experimental groups (control and FLG), and two histological sections were measured for each shrimp. The analyses were performed by a single-board certified medical pathologist.

3. Statistical analysis

Statistical differences were tested using one-way analysis of variance (ANOVA) followed by Tukey *post hoc* comparison. Normality and variance were previously checked and mathematical transformations were made where necessary. The significance level was fixed at 5% for all statistical analyses (Zar, 1984).

4. Results

TEM images confirmed exfoliated graphene sheets as mostly with rectangular shape and well-defined borders (**Figure 1a, b and c**). The nanosheets were agglomerated in several regions due to the deposition process and drying of samples over the TEM grid (Figure 1a). **Figure 1d** shows the statistics of the lateral dimensions of 150 nanosheets measured from several TEM images. A large distribution of sheet sizes between 100 nm and 2000 nm with average length and width values of 800 and 400 nm, respectively, can be observed in the histogram. The distribution of the hydrodynamic diameter found by DSL ranged from 200 to 1000 nm (data adjusted

513 according to Lotya (2013), with average diameter of 500 nm (**Figure 1e)**. These
514 represent results of the same order of distribution magnitude found via TEM.

515 The efficiency of the exfoliation of the natural graphite to graphenes, as well as
516 the structural quality of the nanosheets obtained, was evaluated by Raman spectroscopy
517 with excitation in the visible range (**Figure 1f**). Raman spectra display the characteristic
518 bands of graphenic structures: G band (~1580 cm^{-1}) assigned to primarily in-plane C=C
519 vibrational mode, D band (~1350 cm^{-1}) activated by disorder and G' (~2700 cm^{-1})
520 assigned to the second order overtone of D band. The increase of the I_D/I_G ratio (0.39)
521 for the exfoliated graphene compared with the I_D/I_G ratio (0.09) for the natural graphite
522 (0.09) characterizes the increase of structural disorder and suggests the effectiveness of
523 the chemical exfoliation process. Due to the dependence on the number of layers of
524 graphene, G' band has been used to characterize the number of layers of graphene-based
525 nanomaterials. The inset in Figure 1f shows a 2D band for the exfoliated graphene
526 typical of few layer graphene.

527 **Figure 2a**. displays the Raman spectra of control food samples in the range of 600
528 to 3500 wavenumber region obtained using two different laser exposures of 256 and
529 512 scans, keeping the laser power constant at 150 mW. The reason for this procedure is
530 to show the consistency of the Raman data at different acquisition times. Both spectra
531 are normalized with respect to 1452 cm^{-1} band. Some of the most prominent bands
532 along with their vibrational assignment are explained as below. All the band
533 assignments were carried out using published data. The most intense Raman peak at
534 2933 cm^{-1} is assigned to the C-H stretch related to the proteins in food sample. The
535 1654 cm^{-1} is assigned to the well-known Amide I band that consists of primarily ν(C=O)
536 involving protein α-helix, lipids and other unsaturated fatty acids. The band around
537 1600 cm^{-1} corresponds to the Tyrosine (Tyr) signal. The Raman peak around 1450 cm^{-1}

538 is assigned to the CH_2/CH_3 deformation ($\delta(CH_2)$, $\delta_{as}(CH_3)$) involving proteins. The
539 band around 1300 cm^{-1} is assigned to C-H deformation involving protein and lipids. The
540 peak at 1271 cm^{-1} corresponds to Amide III band, primarily dominated by the
541 component from protein α-helix. The Raman signatures at 1122 and 1093 cm^{-1} were
542 assigned to ν(CC), ν(CN) from lipids and proteins whereas the bands at 1003 cm^{-1}
543 ($\rho(CH_3)$) and 849 cm^{-1} (ν(CC)) are assigned to the ring breathing mode involving
544 proteins (Gelder, 2007).

545 **Figure 2b** presents the Raman spectra of control and the food sample subjected to
546 FLG at 256 scans using 150 mW of laser power. The latter clearly shows all the
547 explained biological signatures along with the FLG marker peaks marked as asterisks:
548 G band at ~1590 cm^{-1}, D band at ~1280 cm^{-1} and 2D band at 2560 cm^{-1}. The D and 2D
549 bands are dispersive in nature, depending on the laser excitation wavelength. The
550 unchanged position of biological markers in food sample in the presence of FLG further
551 concludes that the introduction of FLG appears to not have any significant impact on
552 the integrity of structure and composition of the components at a molecular level, and
553 can be used as an effective nondestructive biological and biomedical Raman label.

554 The biochemical analysis shows that ROS concentration was significantly
555 ($p<0.05$) increased in gill and hepatopancreas tissues after exposure to FLG when
556 compared with the control group, while in muscle tissue this result was not observed
557 ($p>0.05$, **Figure 3a.**).

558 Glutamate cysteine ligase (GCL) activity demonstrated a different pattern of
559 activity, as a decrease ($p<0.05$) was observed in gill tissue while in hepatopancreas
560 tissue there was an increase in GCL activity after treatment with FLG (**Figure 3b**). In
561 muscle tissue GCL activity was unchanged in response to FLG exposure ($p>0.05$,
562 **Figure 3b**).

563 GSH levels were increased in gill and hepatopancreas tissues in the groups
564 exposed to FLG when compared with their respective control groups ($p<0.05$, **Figure**
565 **3c**). However, in muscle tissue this result was not observed ($p>0.05$, **Figure 3c**).

566 The activity of GST was positively modulated in gill tissue and negatively in
567 hepatopancreas tissue ($p<0.05$, **Figure 4a**) after FLG exposure. In muscle tissue GST
568 activity remained unchanged ($p>0.05$, **Figure 4a**).

569 Total antioxidant capacity against peroxyl radicals was increased (lower relative
570 area) in gill and hepatopancreas tissues ($p< 0.05$, **Figure 4b**) following FLG exposure,
571 while in muscle tissue this result was not observed ($p>0.05$, **Figure 4b**).

572 In both gill and hepatopancreas tissues the treatment with dietary FLG resulted in
573 increased lipid peroxidation compared with their respective control groups ($p< 0.05$,
574 **Figure 4c)**. However, no lipid peroxidation was observed in muscle tissue in response
575 to FLG exposure ($p>0.05$, **Figure 4c**). Treatment with graphene induced DNA damage
576 in gill and hepatopancreas tissues, but not in muscle tissue, when compared with their
577 respective control groups. This increase in DNA damage in gill and hepatopancreas
578 tissues was detected with respect to the three evaluation parameters of the comet assay
579 (tail length, tail moment and % of DNA in the tail (**Figure 5a**, **5b** and **5c**, respectively).
580 Histological analysis demonstrates that: normal architecture of the tissue was observed
581 in hepatopancreas tissue of the control group (**Figure 6a and 6b**), while tubule
582 deformities with light tubular alterations (LTA) were observed in the FLG group.
583 Hyperplasia of basal cells and a decrease in secretory cells (**Figure 6c**) were also
584 observed, along with hemocyte infiltrators (**Figure 6d**). No histological changes were
585 observed in both gill and muscle tissues (data not shown).

5. Discussion

NM possesses physical-chemical characteristics that enable their application in several areas. Among NM, the carbon allotropes, such as fullerenes, nanotubes and graphene are some of the most used in technology and biomedicine (Qu et al., 2013; Orecchioni et al., 2014). However, with rapid development and increasing production of nanotechnology, environmental contamination and specifically aquatic contamination by these products is highly likely (Kahru and Doubourguier, 2010). Once into the aquatic environment, NM may be dispersed in the water column and incorporated by organisms and/or sediments (Koelmans et. al., 2009). Further, previous work regarding NM has demonstrated the capacity of NM to be ingested by small organisms that serve as food species for higher trophic levels (Baun et al., 2008). In the present study, the shrimp *Litopenaeus vannamei* was used as a biological model, as it represents a benthonic species widely used in aquaculture and consumed in the diet of humans (Zhou et al., 2009). Although some studies have been performed evaluating the toxicity of fullerene (C_{60}) and nanotubes in aquatic organisms (Canesi et al., 2010; Britto et al., 2012; Da Rocha et al., 2013), few data are available regarding the effects of graphene in these kinds of organisms. Evaluating the potential adverse effects of graphene in an aquatic species with high commercial value such as *L. vannamei* is important to understand the ecotoxicological impacts of graphene exposure.

Some studies have indicated that oxidative stress is a primary mechanism of toxicity within CNM exposure (Britto et al., 2012; Da Rocha et al., 2013). In mammal cells, exposure to graphene induced ROS production in a time and dose-dependent manner with subsequent cellular apoptosis (Zhang et al., 2010). Here, an increase in ROS concentration in gill and hepatopancreas tissues of shrimps was observed after 4 weeks of exposure to FLG (**Figure 3a**). A similar result was found in *C. elegans* after

exposure to graphene oxide, where an increase in ROS levels was associated with physiological changes in the gut of this organism (Wu et al., 2013). The increase in ROS levels after exposure to graphene and their derivatives may be related to the structural form of this NM; graphenes possess a planar structure that tends to associate with mitochondrial membranes, causing disruptions in the electron transport chain (ETC) (Duch et. al., 2011). Zhou et al. (2014) observed alterations in electron transport and subsequent decreases in ATP production in mammal cell lines exposed to graphene. The ability of graphene to alter electron transport can explain the increase of ROS levels observed in the present study.

Previous literature indicates that increases in ROS levels can induce the expression of antioxidant genes, including those coding for GCL, GSH and GST. This occurs through the action of the transcription factor *Nrf2*, which migrates to the nucleus and interacts with antioxidant response elements (ARE) of antioxidant genes. In fact, Da Rocha et al. (2013) observed up-regulation of the *Nrf2* gene following i.p. exposure to single walled carbon nanotubes (SWCNT). GSH is the first line of cellular defense against exposure to contaminants and oxidative damages because this tripeptide acts as a scavenger of reactive species and serves as substrate for antioxidant enzymes such as glutathione-S-transferase and glutathione peroxidase (GPx) (Halliwell and Gutteridge, 2007). In the present study, an increase in GSH content after exposure to FLG (**Figure 3c**) was observed in both gill and hepatopancreas tissue**,** indicating that this NM induced a pro-oxidant condition that triggered an antioxidant response in these organs.

De novo GSH synthesis is related to GCL activity (White et al., 2003). Interestingly, an organ-dependent response in terms of GCL activity was observed in the present study. In gill tissue, GCL activity decreased, while an increase was observed in hepatopancreas tissue (**Figure 3b**). Previous studies indicate that other carbon

636 nanomaterials may modulate both expression and activity of this enzyme in other
637 organisms such as freshwater fishes (Britto et al., 2012, Da Rocha et. al., 2013). As
638 GSH synthesis is an energetically expensive process, GCL activity should be regulated
639 so that enzyme activity is decreased when the reduced glutahione levels are sufficient to
640 maintain homeostasis of tissue (White et al., 2003). The increase in GSH levels
641 (approximately 4.5 fold compared with control group) observed in gills tissue may be
642 responsible for the decrease observed in GCL activity. However, in hepatopancreas
643 tissues, the increase in GCL activity induced by FLG exposure was accompanied by
644 increases in GSH content (around twice compared with control group). This result is
645 likely due to the role in metabolization/detoxification of several endogenous and
646 exogenous compounds as performed by this organ (Vogt, 1994), wherein GSH is used
647 as substrate, thus requiring high levels of this antioxidant to maintain function (about 5
648 fold higher than gills, **Figure 3c).**

649 Glutathione-*S*-transferase (GST) is a superfamily of enzymes involved in
650 detoxification and oxidative stress response processes, and is useful as a biomarker in
651 ecotoxicological studies (Kim et al., 2010). In fact, some studies have demonstrated that
652 GST activity is modulated in response to toxic compounds both in the gills and
653 hepatopancreas of *Litopenaeus vannamei* (Ren et al., 2015a,b). Here, an increase in
654 GST activity in gill tissue and a decrease in hepatopancreas tissue was observed
655 following FLG exposure (**Figure 4a**). Contrarily, a decrease in GST activity in gill
656 tissue of *Mytilus galloprovincialis* exposed to carbon black nanoparticles and C_{60} was
657 observed by Canesi et al. (2010), while similar exposure induced an increase in the
658 activity of this enzyme in the digestive gland. These results suggest that each organ of
659 different species may modulate GST activity in response to different CNM. However,
660 either positive or negative modulation of GST activity represents an unfavorable

cellular situation, as an increase in GST activity expends GSH and leaves the tissue more vulnerable to pro-oxidant conditions, while a decrease in GST activity affects the detoxification capacity, allowing the accumulation of toxic compounds.

A similar methodology to Amado et al. (2009) was employed in the present study to understand how graphene exposure can interfere in antioxidant capacity. This methodology offers the advantage of evaluating total antioxidant capacity, including antioxidants such as GSH. In this study, an increase in the total antioxidant capacity in gill and hepatopancreas tissues was observed (**Figure 4b**). This result was expected as a response to the increase in ROS levels. Further, an increase in GSH levels was observed that likely significantly contributed to the observed increase in total antioxidant capacity.

Although both gill and hepatopancreas tissues were observed to positively modulate antioxidant responses in the present study, these were not sufficient to avoid lipid peroxidation (**Figure 4c**). A similar result was observed in gill tissue of *C. carpio* exposed to C_{60} (Britto et al., 2012) and in mammal cells after graphene oxide exposure (Chatterjee et al., 2014), suggesting that different CNM can induce oxidative damage in lipids of distinct biological systems. Also, lipid peroxidation in the present study fits with the observed increase in ROS levels in the same organs.

Although the methodology used in this study to evaluate DNA damage was not specific for identifying DNA fragmentation by action of ROS, the three parameters evaluated in the alkaline comet assay were in the same direction as those observed from production of ROS, oxidative damage and antioxidant systems. In the present study, the gill and hepatopancreas tissues of animals exposed to FLG showed more DNA damage when compared to cells from unexposed animals.

Even though genotoxicity studies have a powerful appeal because of their close relationship with mutagenesis and cancer, the number of studies is reduced when compared to studies of cytotoxicity (Seabra et al., 2014). Qiao et al. (2013) investigated the genotoxicity of a series of nanomaterials, observing that graphene was the most harmful nanomaterial to the DNA of all the tested types. Siddique et al. (2013, 2014) reported the genotoxicity of Graphene Oxide in the midgut of the fly *Drosophila melanogaster*, even after short exposure periods (24 and 48 h). Our study reinforces the genotoxicity of graphene but indicates that this action is organ-dependent.

Histological observations in hepatopancreas tissues showed changes such as hyperplasic basal cells, infiltration of hemocytes and lower amount of secretory cells in these tissues after treatment with FLG (**Figure 6b**, **c**) suggesting that this NM can alter the structure of tissue and compromise their function. In a study by Smith and co-workers (2007), several tissue disorders caused by single walled carbon nanotubes (SWCNT) in liver and other tissues of the rainbow trout *Oncorhynchus mykiss*, were observed, supporting evidence that CNM have the potential to induce histological changes in different tissues. It is important to note that the hepatopancreas is the organ responsible for metabolization and nutrition in shrimps (Sánchez-Paz et al., 2007), and changes to this organ can affect detoxification capacity. In fact, GST activity in this organ was severely diminished following FLG exposure (**Figure 4a**).

6. Conclusions

The results of the present study demonstrate that FLG exposure (below 2000nm lateral size) induced a pro-oxidant scenario, as evidenced by an increase in ROS concentration and modulations in the antioxidant system (GSH levels and GST and

709 GCL activity). Histological alterations and lipid peroxidation were observed in
710 hepatopancreas tissues of the shrimp *Litopenaeus vannamei*, characterizing an oxidative
711 stress situation. The results taken together indicate that exposure to FLG via diet may be
712 harmful to shrimps, endangering this aquatic biota. The present study offers important
713 information regarding the risks of this FLG to aquatic and environmental health,
714 especially in the context of the rapid development, production and potential
715 environmental release of these NM.

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723

7. Figure captions

Figure 1. (a), (b) and **(c)** Transmission electron microscopy **(**TEM) images of graphene nanosheets obtained from diluted dispersion 1800x in NMP. **(d)** Histogram of lateral dimensions obtained from TEM images. **(e)** Dynamic light scattering (DLS) of graphene nanosheets dispersed in NMP. **(f)** Raman spectroscopy in the regions 1000-4000 cm^{-1} obtained from natural graphite and graphene nanosheets. (λ_{exc} = 514 nm, 4.6 mW laser power).

Figure 2. (a) FT Raman spectra of control food sample at 256 and 512 scans. Laser power and spectrometer resolution were kept at 150 mW and 4 cm^{-1} respectively. (b) FT Raman spectra of control (C) and graphene subjected (Gr) food samples obtained using 150 mW laser power and 256 scans.

Figure 3. (a) Reactive oxygen species (ROS) concentration (expressed as area). **(b)** Glutamate cysteine ligase (GCL) activity (expressed as nmol of GSH/30min/mg of protein). **(c)** Reduced glutathione (GSH) content (expressed as μmol of GSH/mg of protein). Data are expressed as the mean ±1 standard error, n=4-5. Asterisks indicate the significant difference ($p<0.05$) to respect control group of same tissue.

Figure 4. (a) Glutathione-S-transferase (GST) activity (expressed as nmol of CDNB conjugated/min/mg of protein). **(b)** Total antioxidant capacity against peroxyl radical (expressed as relative area). **(c)** Substances reactives to thiobarbituric acid (TBARS) levels (expressed as nmol od MDA/mg of protein). Data are expressed as the mean ±1 standard error, n=4-5. Asterisks indicate the significant difference ($p<0.05$) to respect control group of same tissue. **CDNB:** 1-chloro-2.4-dinitrobenzene; **MDA:** malondialdehyde.

749

750 **Figure 5.** DNA damage: **(a)** Tail length (in μm). **(b)** Olive tail moment. **(c)**
751 Percentage of DNA tail. Data are expressed as the mean ±1 standard error, n= 5.
752 Asterisks indicate the significant difference ($p<0.05$) to respect control group of same
753 tissue.

754

755 **Figure 6.** Histological analyzes of hepatopancreas. **(a)** and **(b)** control group,
756 arrow indicating tubular cells (TC). **(c)** Graphene group, arrow indicating hyperplasia of
757 basal cells and **(d)** Graphene group, long arrow indicating hyperplasia of basal cells and
758 short arrow indicating decrease of secretory cells and hemocyte infiltration.

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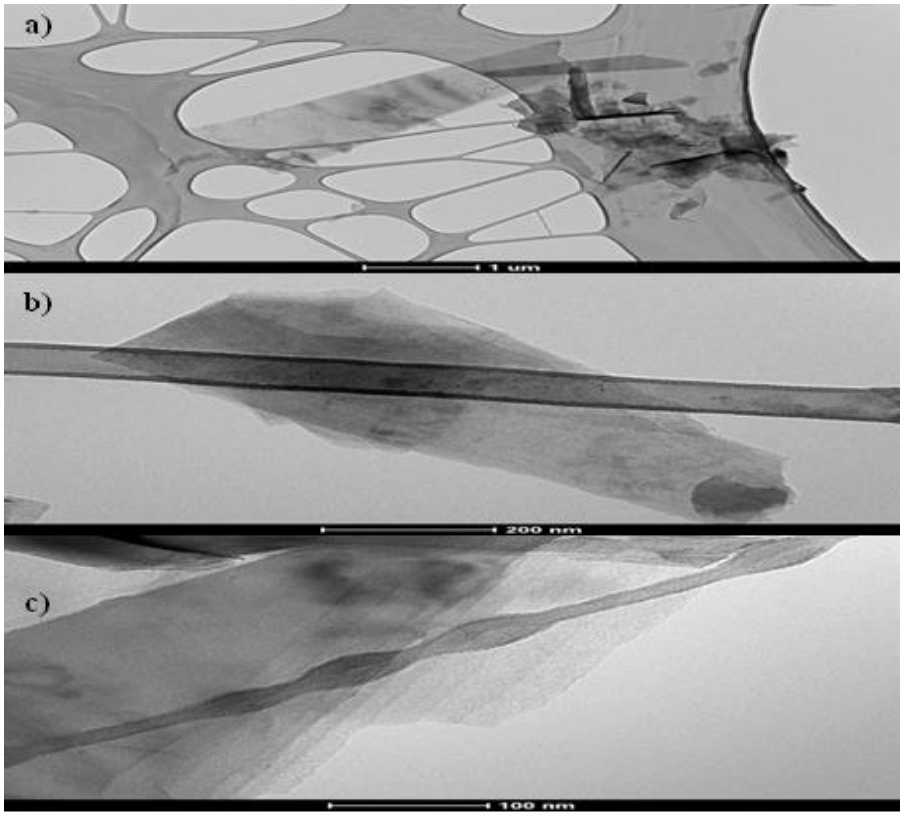
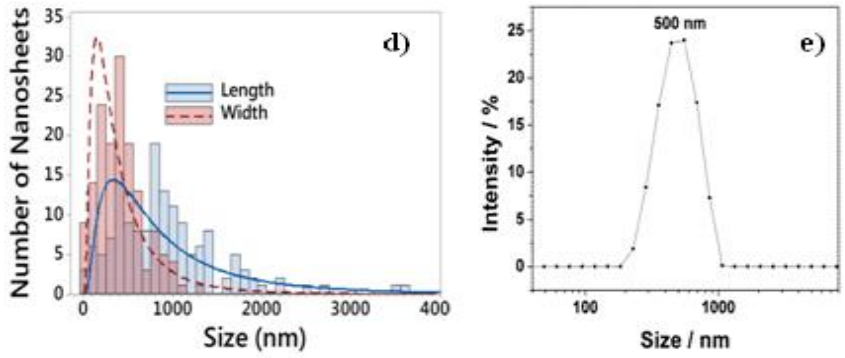
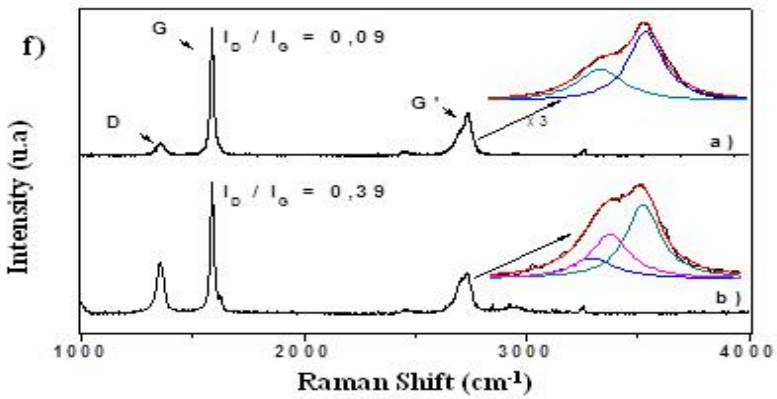
9. Figures







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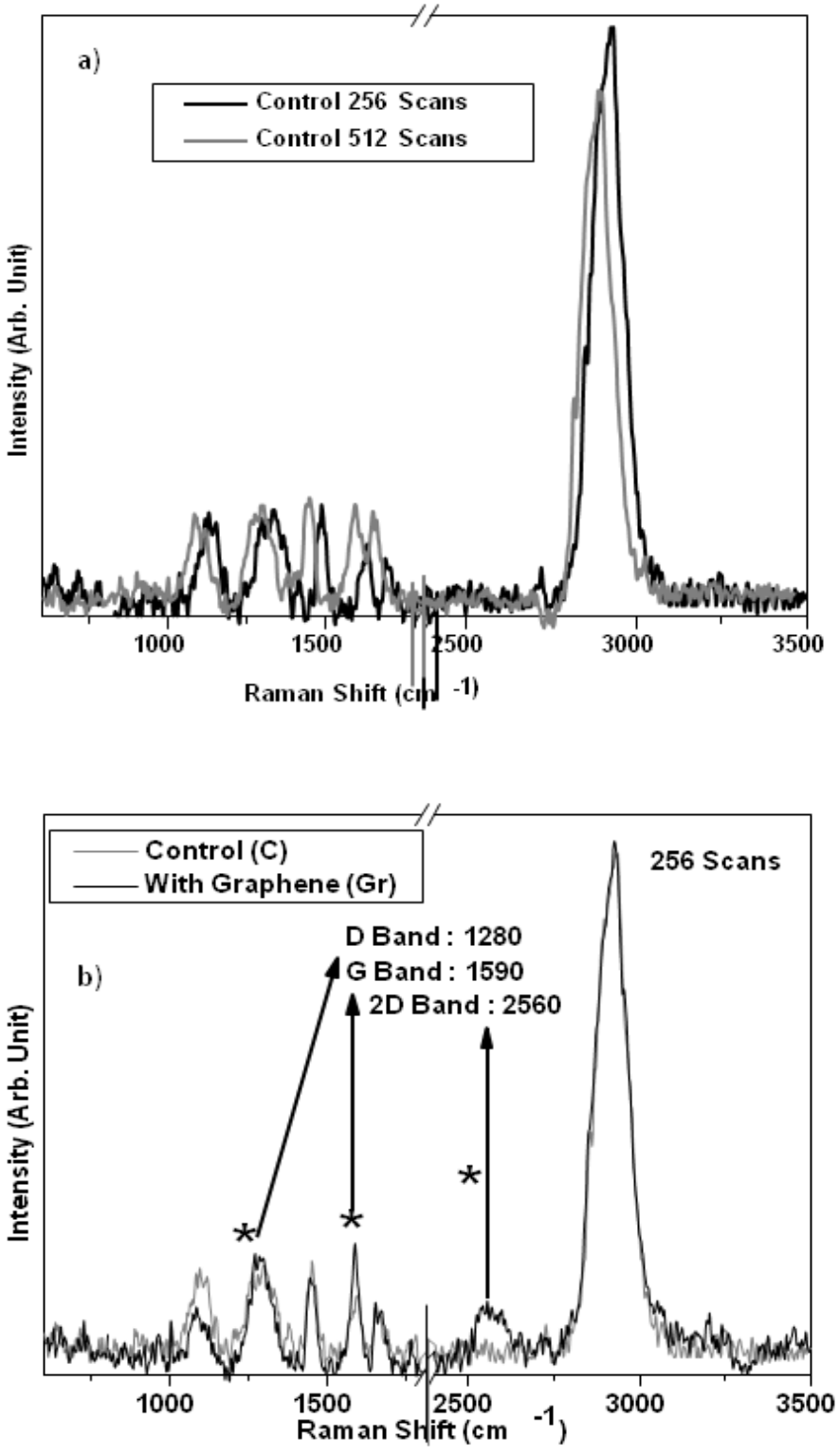

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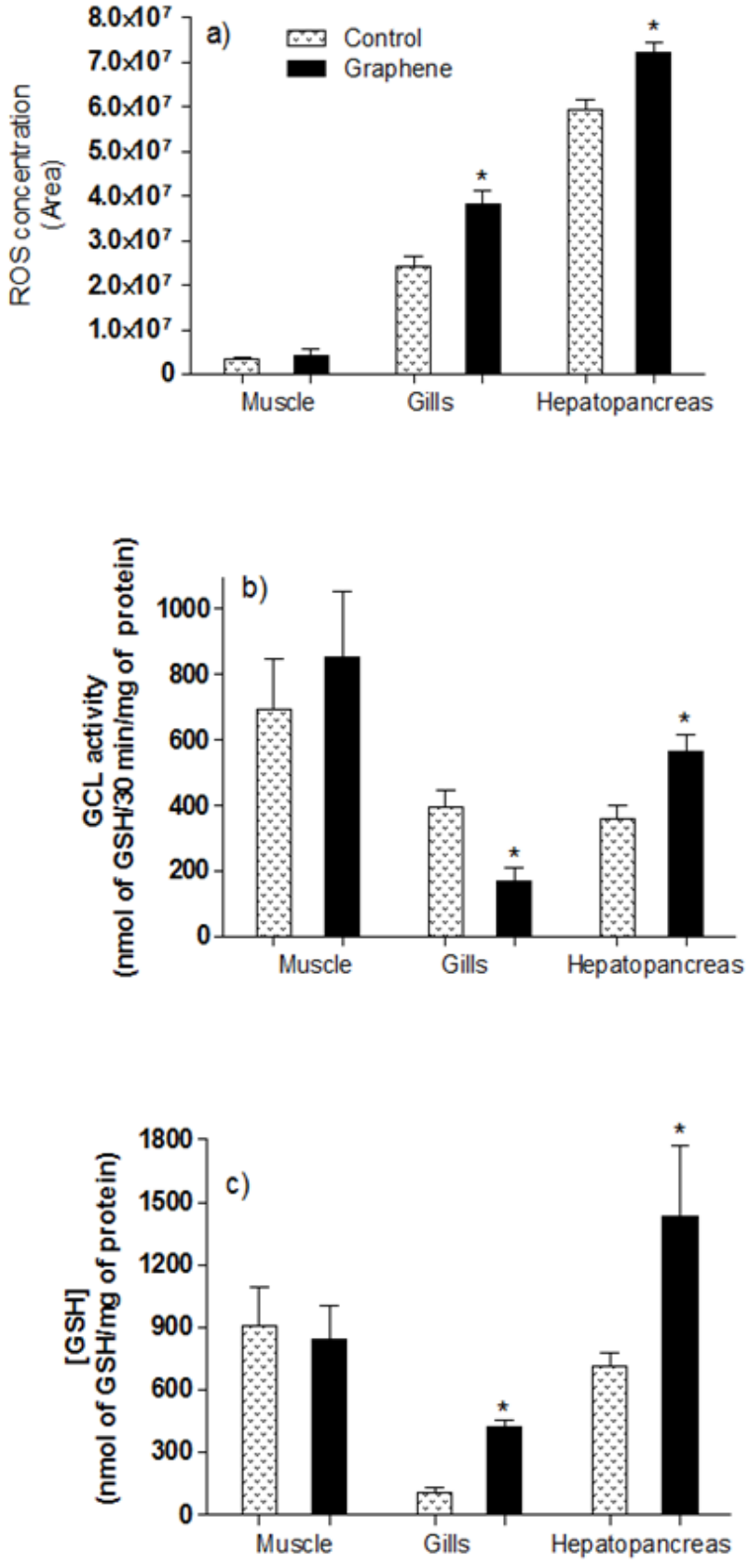

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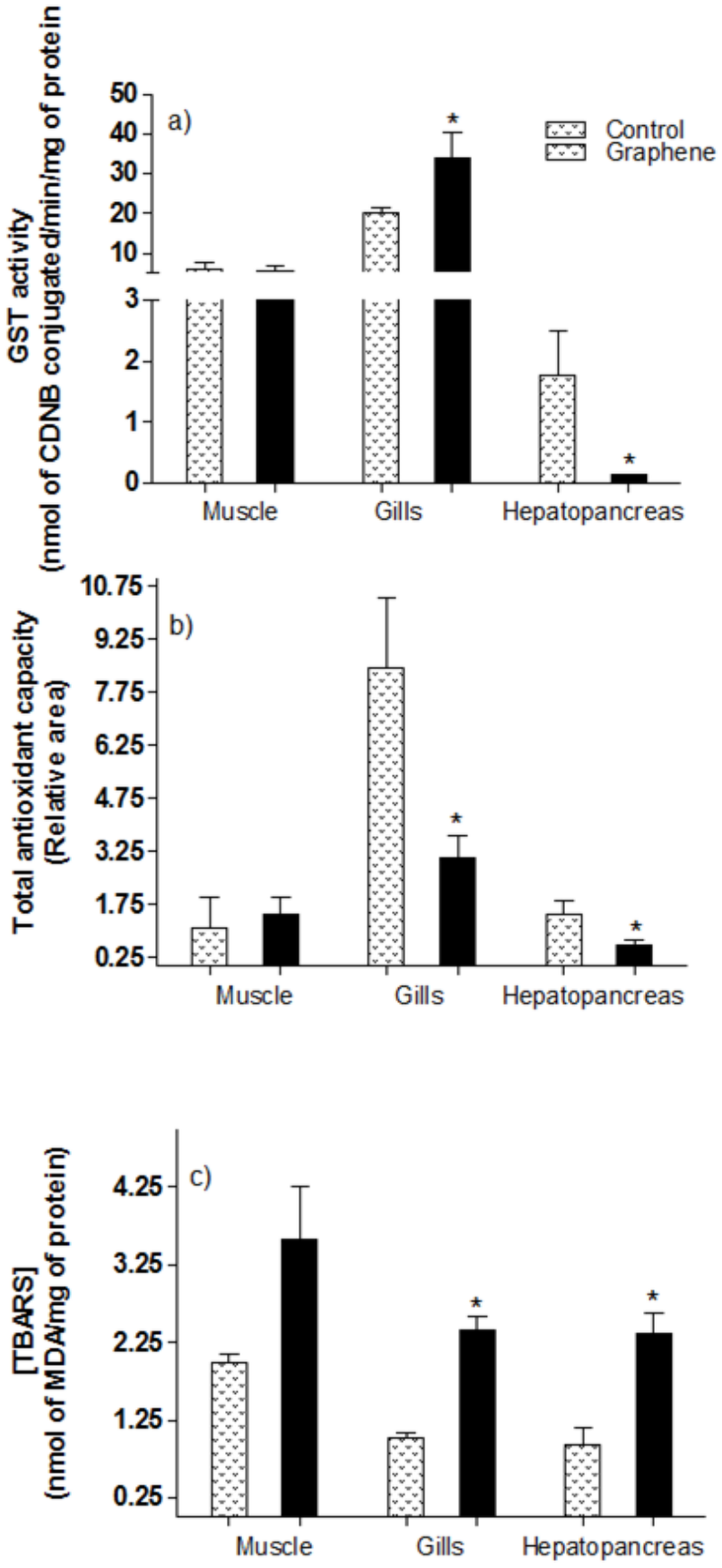

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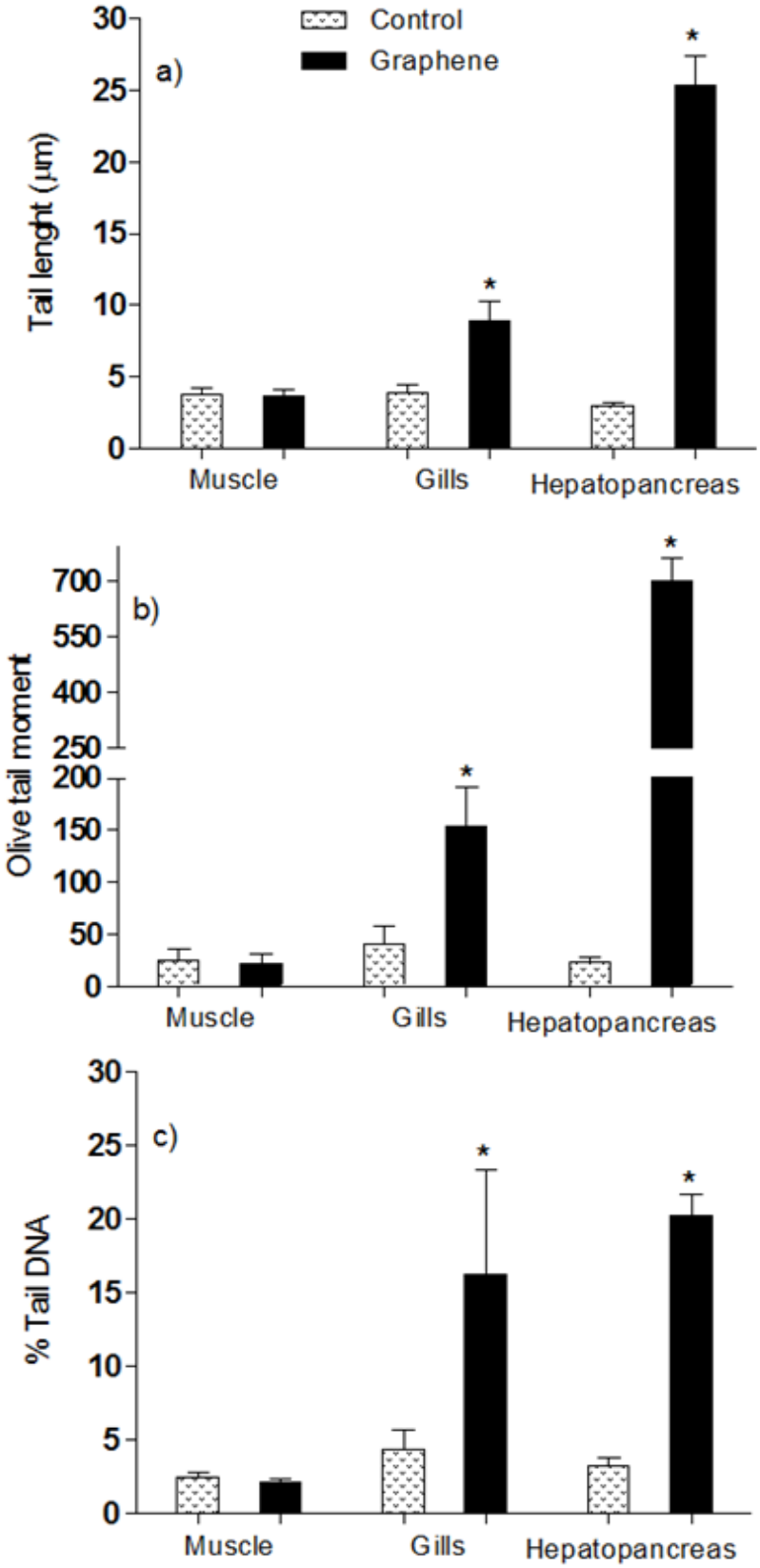

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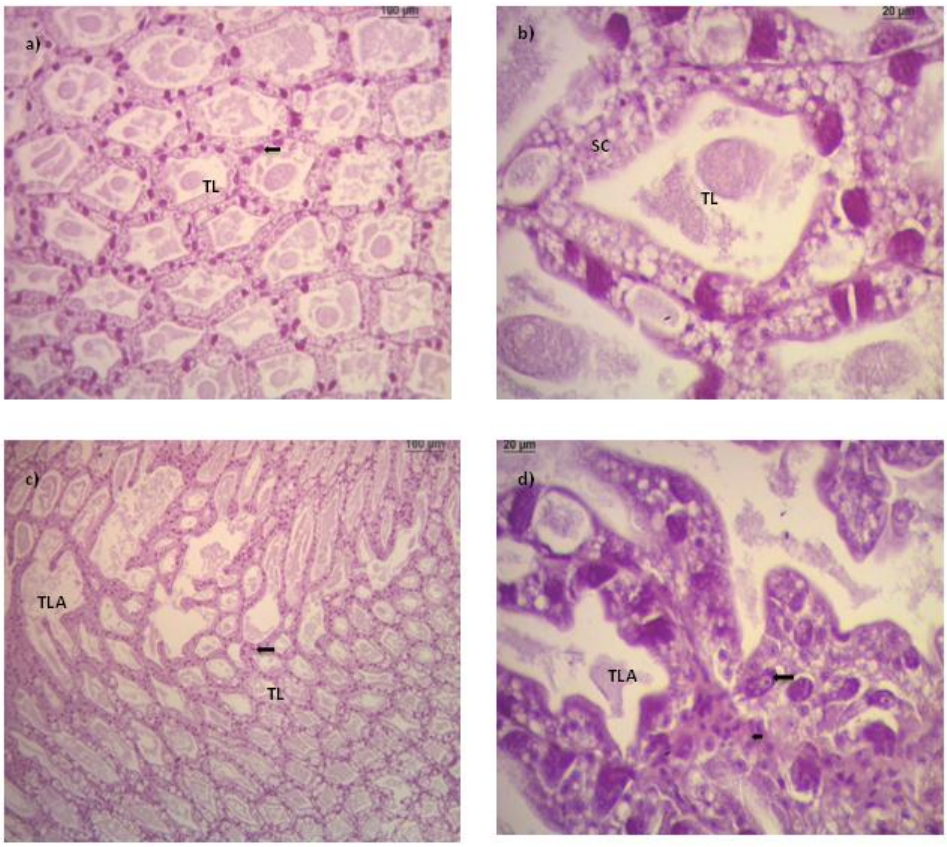
914 **Figure 5.**



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916 **Figure 6.**

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6. Capítulo 2

Evaluation of graphene effects in different tissues of *Danio rerio* (Cyprinidae): a molecular, biochemical and histological approach.

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Amanda Lucena Fernandes[1,2*], Jefferson Patrício Nascimento[3], Adelina Pinheiro Santos[3], Carlos Eduardo da Rosa [1,2], Luis Alberto Romano[4], Clascídia Aparecida Furtado[3], José Marìa Monserrat[1,2,4],

Juliane Ventura-Lima[1,2*].

[1]Instituto de Ciências Biológicas (ICB), Universidade Federal do Rio Grande - FURG, Rio Grande, RS, Brasil.

[2]Programa de Pós-Graduação em Ciências Fisiológicas - FURG.

[3]Centro de Desenvolvimento da Tecnologia Nuclear – CDTN/CNEN, Belo Horizonte, MG, Brazil.

[4]Programa de Pós-Graduação em Aquacultura-FURG.

* Corresponding authors: Amanda Lucena Fernandes (E-mail: amandalfernandes@outlook.com)

Juliane Ventura-Lima (E-mail: juliane_ventura@yahoo.com.br).

Abstract

The use of graphene increased in the last years, leading to a higher chance of being released into the environment and coming into contact with organisms. Data on graphene toxicology has shown its ability to alter the antioxidant system and cause oxidative stress in marine crustaceans; however, information regarding fish toxic effects data is scarce. Based on this, the aims of this study was to evaluate the effects of graphene in different tissues of the *Danio rerio* fish, evaluating parameters of oxidative stress. Animals were exposed intraperitoneally (*i.p)* to 10 ul of two concentrations of graphene, 5 and 50 mg/L during 48 h, after they were euthanized and gills, intestine, muscle and brain dissected. It was analyzed parameters of oxidative stress, as expression of the *nrf2* and *gclc* genes, as well as the activity of the GCL (glutamate cysteine ligase) enzyme, reduced glutathione (GSH) concentration, glutathione-S-transferase (GST) activity and lipid peroxides levels. Histological analyses were also performed to observe if the exposure can induce pathological damage in different tissues. Results showed no significant difference in the expression of *gclc* and *nrf2* genes after the exposure. In contrast GCL and GST activity and GSH concentration were affected regarding SDS-control, in different tissues and different concentrations. Lipid damage was observed in gills, also pathological damage was observed after the exposure of both concentrations, excluding intestine. Overall, results indicated that graphene induced toxicological effects that depend of the analyzed organ, causing distinct pathologic effects on some, and oxidative effects on others.

Key-words: antioxidant system, carbon nanomaterials, nanotoxicology, *Danio rerio*, oxidative stress.

[1] **Abbreviations:**

[1] CNM, carbon nanomaterials; *i.p*, intraperitoneal; GCL, glutamate-cysteine-ligase; GSH, reduced glutathione; GST, glutathione-S-transferase; GR, graphene; MWCNT, multi-walled carbon nanotubes; SWCNT, single walled carbon nanotubes; NM, nanomaterials; *nrf2*, Nuclear factor [erythroid-derived 2]-like 2; PEG, peglated; SDS, sodium dodecyl sulphate.

1. Introduction

Graphene (GR) is a nanomaterial (NM) consisting of a single layer of carbon atoms with two dimensions and a one-sheet structure (nanosheets) (Mafra, 2008). Its several properties include the high electrical conductivity, thermal and chemical stability, as well as its large surface area. Due these characteristics, an increased the use of NM has been registered in different areas of sciences (Chen et al., 2010; Huang and Shi, 2012, Nguyen and Berry, 2012). Besides, graphene is resistant to degradation due to its large surface/volume ratio and morphology, because both its surface and its edges are electric active, and are great sites for the attraction of biological molecules, being so endangering for living organisms (Stankovich et al., 2006, Yang et al., 2013).

Among the effects caused by GR exposure it can be highlighted cellular membrane agglutination, induction of oxidative stress in some cell types of bacteria and mammals (Jaworski et al., 2013, Li et al., 2012, Markovic et al., 2011, Nguyen and Berry, 2012, Zhang et al., 2010). In different marine crustacean (*Artemia salina* and *Litopenaeus vannamei*) the *in vivo* exposure to graphene induced antioxidant responses and oxidative damages to macromolecules, characterizing an oxidative stress situation (Pretti et al., 2014; Fernandes et al., 2017).

Until now, data about effect of GR exposure in fishes are scarce (Yang et al., 2013). For this reason, in this study, *Danio rerio* was the selected biological model, being an organism commonly used in scientific studies due to specific characteristics such as rapid development, small size, low production cost and its already unveiled genome (Lawrence, 2007). The species is also a model for toxicity studies for display biochemical, molecular, and behavioral responses to different types of NM (Da Rocha et al., 2013, Filho et al., 2014, Webber et al., 2014).

In this way, the objective of this study was to analyze toxic effects of GR considering molecular and biochemical responses of oxidative stress, and histological alterations in different organs of zebrafish *Danio rerio*, caused by GR intraperitoneal (*i.p*) exposure. This exposure route (*i.p*) was chosen to assure that the animals receive a precise dose of this NM, since it is known that oral absorption of peglated graphene by the intestine is limited in mammals, so *i.p* exposure would be more appropriate in experiments (Yang et al., 2013). Previous studies have employed the same approach to analyze the toxic effects induced by carbon nanomaterials in zebrafish (da Rocha et al., 2013). The data obtained in this study, will certainly contribute to know part of toxic effect induced by graphene in fishes and to infer in other species that possess molecular similarity with *D. rerio*.

2. Material and Methods

2.1 Biological model and maintenance of animals

Animals were obtained from a local supplier and kept in the aquarium of the Biological Sciences Institute (ICB) of the Federal University of Rio Grande (FURG) for acclimatization for at least two weeks prior experiments in 60 L aquariums (maximum of 100 animals per aquarium). They were kept under photoperiod conditions 12 hours light/12 hours dark, temperature 28 °C, pH 7.5-8.0, and constant aeration with feed twice a day.

2.2 Obtainment and characterization of graphene

Graphene was obtained in procedure of two steps, first 3.3 g of natural graphite (Graflake 99580-supplied from Nacional do Grafite LTDA-Brazil) was added in 1.000 ml of NMP (1-methyl-2-pyrrolidinone, Sigma-Aldrich) and after, it was submitted to ultrassonic bath for 168h and centrifuged at 110 x *g* during 45 min. The supernatant then was filtered in a nylon membrane (0.2 μm of diameter), washed with 400 ml

deionized water, 200 ml of ethanol (95% P.A) and 100 ml of diethyl ether (Synth P.A) and dried during 24 h at 150 °C under vacuum. Secondarily, it was added 16 ml of NMP (24 $mg.mL^{-1}$) and sonicated during 24 h, after, the dispersion obtained was kept standing during 8 days and the supernatant was collected and filtered. Finally the material was washed and dried as described above. This methodology is based on Khan et al. (2011).

In this study was used the same sample of graphene utilized in our previous work and a broad characterization of nanomaterial was performed and described in the mentioned study (Fernandes et al., 2017).

2.3 Preparation of the solution with graphene

Two graphene solutions were prepared, one of 5 and other of 50 mg of graphene/L, the different concentrations (low and high) were chose to observe if the graphene would agglomerate and cause different effects. The concentrations chosen was based on Fernandes et al. (2017), being chosen one 10 times lower (50mg) and other lowest (5mg). The detergent sodium dodecyl sulphate (SDS) (3 g/L) was used as vehicle; SDS was diluted in MilliQ water, after graphene was added to that solution and then sonicated (ECO SONICS - Ultrasonique, 40kHz, 150VA) during 5 h. This methodology was based on Da Rocha et al. (2013) and Smith et al. (2007).

2.4 Graphene exposure

The graphene solution was administered intraperitoneally (*i.p*) for this, the animals were anesthetized by immersion on tricaine (MS-222) for about 1minute, then 10 µl of solution (graphene or vehicle) were injected into the animal. After, there were placed in a beaker with oxygenated clean water for recovery that occurred between 30 s and 1 min (Da Rocha et al. 2013). Animals were not fed 24 h prior or during the

experiment, and after 48 h fish were euthanized by immersion on 500 mg/L of tricaine, and gills, intestine, muscle and brain were removed.

Experimental design consist on: Animals were divided in four groups and pool of six fish was used to compose one sample (n=5-6 pools) in biochemical analysis, while for molecular analysis it was chosen a pool of three fishes; animals were exposed twice intraperitoneally in 48 h: one exposure at 0h and the other 24h after, to their due treatments, being killed after 48 hours of exposure. All procedures performed in this study were in accordance with the EU Directive 2010/63/EU for animal experiments. Experimental groups are described below:

Control-control group: Animals were kept in aquarium and after period of acclimatization received *i.p*. of 10 µl MilliQ water.

SDS-control group: After acclimatization the animals received *i.p*. of 10 µl of SDS detergent. SDS group was used as a second control, besides MilliQ water, because the graphene was dispersed in SDS solution, so it was tested if the detergent alone induced a toxic response.

Graphene group 1 (G1) - low concentration: After acclimatization period animals received *i.p*. of 10 µl of graphene solution of 5 mg/L.

Graphene group 2 (G2) - high concentration: After acclimatization period animals received *i.p*. of 10 µl of graphene solution in concentration of 50 mg/L.

This form of exposure has been selected to ensure that the animal is exposed to an exact concentration of graphene since when dispersed in water the characteristics of the nanomaterial can form agglomerate altering the size of the NM and possibly their the biological effects. The use of all animals in this experiment was approved by the Ethics Committee for Experimental Animal Use (CEUA) from Universidade Federal do Rio Grande (FURG), through the process number 23116001258/2015-16.

2.6 Gene expression

In order to analyze glutamate-cysteine-ligase catalytic subunit (*gclc*) and Nuclear factor [erythroid-derived 2]-like 2 (*nrf2*) genes expression, Real-time PCR were performed in samples of gills, intestine and muscle (based on Rosa *et al*. 2010. Briefly, after dissection the tissues were immediately immersed in TRIzol® reagent (Invitrogen, USA) for the extraction of total RNA according the manufacturer's instructions. Afterwards, RNA concentration was quantified by spectrophotometry (280 and 260nm), and integrity was checked using agarose gel (1%) electrophoresis. Sequentially, complementary DNA (cDNA) was prepared from the total RNA, using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) and used as a template for the amplification of *gclc* and *nrf2* genes, the specific primers are escribed in **Table 1**. The PCR reactions were performed on the Applied Biosystems 7300 Sequence Detection System, using the SYBR-Green PCR Master Mix (Applied Biosystems). The housekeeping genes *ef1α* (elongation factor 1 alpha) and *18S* (ribosomal RNA) were employed to normalize the *gclc* and *nrf2* expression in the tissues. Brains were not used to this analysis due to lack of tissue sample. The data were analyzed by the deltadelta CT method and the results expressed as relative gene expression considering the SDS-control group gene expression as 1.

2.7 Biochemical analysis

2.7.1 Preparation of homogenates

Gills, intestine, muscle and brain of *D. rerio* were homogenized (1:5; p/v) in homogenization buffer containing Tris-HCl (100 nM, pH7.75), EDTA (2 mM) and Mg^{2+} (5 mM) (Da Rocha et al., 2013). The homogenates were centrifuged at 10,000 *x g* for 20 minutes at 4°C and the supernatants were used for the dosages of the enzymatic

activities, the total proteins were quantified using a commercial kit based on Biuret method using a microplate reader (BiotekELx 800) at 550nm.

2.7.2 Enzymatic assays

To determine the GCL activity and GSH levels was employed the methodology described by White et al. (2003). This analyze is based on the ability of the compound 2,3-naphtalenedicarboxaldehyde (NDA) to react with γ-glutamylcysteine (γ-GC) and/or glutathione (GSH) forming a fluorescent cyclic compound (GC-NDA and GS-NDA, respectively). The fluorescence of this complexes were measured on a fluorometer (2 Victor, Perkin Elmer) with wavelengths of 485 and 535 nm for excitation and emission, respectively.

Glutathione-S-transferase (GST) activity consist on the analysis of using absorbance for evaluate the conjugation of 1 mM GSH (Sigma) with 1 mM of the reagent 1-chloro-2.4-dinitrobenzene (CDNB, Sigma), a reaction catalyzed by GST. The complex formed has a maximum absorbance at 340 nm. Methodology was determined according to Habig et al. (1974).

The lipid peroxidation was measured by fluorimetry of thiobarbituric acid reactive substance (TBARS) method (Oakes and Kraak 2003). The method is involves reagents like tretramethoxypropane (TMP, Sigma), and is based on a reaction of malondialdehyde (MDA), a degradation product of peroxided lipids, with thiobarbituric acid (TBA) under conditions of high temperature and acidity, resulting in a fluorescent chromogen detected in wavelength of 595 nm (emission) and 520 nm (excitation). The content of lipid peroxide was expressed as nmol of TBARS/mg of protein.

2.8 Histological analysis

The fish were euthanized (n=6) and after a cuts were made sectioning head and part of the fishes muscle leaving the entrails exposed to the fixative, after whole fish were put in cassets and fixed in 10% buffered formalin (10% formaldehyde, 90% distilled water, dibasic sodium phosphate 6.5g, monobasic sodium phosphate 4.0g) for histopathologycal evaluation. Samples were processed in an automatic tissues processor LUPE PT 05 (dehydration, rinsing, clearing and impregnation) and put in Paraplast (Sigma-Aldrich), after the tissues were sectioned in microtome (LUPETEC MRPO3) at 4μm sections. The sections were stained with hematoxylin and eosin (H&E), after staining the slides were dehydrated during 3 min in 70, 80, 90 and 96% ethanol, rinsed twice in ethanol 100% for 5 min and cleared with xylene.

3. Statistical analysis

The statistical differences were tested using one-way variance analysis (ANOVA) follow by Neuman-Keuls test as *post hoc* comparison, significance level was fixed in 5%, the normality and variance were previously checked and mathematical transformations were made when necessary (Zar, 1984).

4. Results

TEM images showed that some graphene nanosheets were agglomerated, but the majority possessed well-defined borders, approximately 10 layers of graphene and distribution of lateral sizes between 100 and 2000 nm with average value of length and width of 900 and 400 nm, respectively **(Figure 1**).

Considering the glutamate cysteine ligase catalytic subunit gene expression (*gclc)* no effects were observed in gills, intestine and muscle after the animal being exposed to the two graphene solutions ($p>0.05$; **Figure 2a**, **2b** and **2c**, respectively). Same results were obtained for *nrf2* expression (**Figure 3**; $p>0.05$).

The GSH concentration decreased after the exposure of G1 in gills compared to SDS-Control group ($p<0.05$; **Figure 4a**), the same was not observed in intestine, that showed an increased concentration in G1 when compared to SDS-control group ($p<0.05$; **Figure 4b**). In muscle the exposure does not seem to had any effect ($p>0.05$; **Figure 4c**), while in brain, both G1 and G2 showed an increase in concentration compared to SDS-control ($p<0.05$; **Figure 4d**).

Glutamate-cysteine-ligase activity was not modulated in gills ($p>0.05$; **Figure 5a),** while intestine showed an increase of activity in G1 and G2 when compared to SDS-control ($p<0.05$; **Figure 5b**). In muscle, it was not observed any difference between groups ($p>0.05$; **Figure 5c**), as for brain an increase of activity in G1 and G2 it was observed when compared with SDS-control ($p<0.05$; **Figure 5d**).

GST activity remains almost constant in gills ($p>0.05$, **Figure 6a**). A different result was observed in intestine, where the treatment with graphene on low dose induced an increase on the activity of GST when compared to SDS-control group ($p<0.05$, **Figure 6b**). In muscle no modulation of the enzyme activity was observed ($p>0.05$, **Figure 6c**). In brain, was observed that G1 induced an increase in this enzyme activity when compared to SDS-control ($p<0.05$, **Figure 6d**).

In gills it was observed an increase in lipid peroxidation in the group exposed to higher concentration of graphene (G2) when compared to SDS-control group ($p<0.05$, **Figure 7a**). A different result was observed in intestine and brain, where lipid damage was not observed after exposure to both graphene concentrations ($p>0.05$, **Figure 7b** and **7d** respectively). In muscle, both graphene concentrations showed to decrease the lipid damage levels when compared with SDS-control group ($p<0.05$, **Figure 7c**).

Histological changes were observed in gills, where cells suffered moderate and severe hyperplasia after the exposure to G1 and G2, respectively (**Figure 8a**). While in the intestine, it was not observed any tissue alterations for the different treatments (**Figure 8b**). Besides, the muscle suffered pathological damage, being observed inflammatory infiltrates between the muscular fibers in the two groups of graphene-exposed fish (**Figure 8c**). Brain showed that exposure to G1 caused ganglionic proliferations, increased microglia cells and moderate edema, while exposure to G2 increased the ganglionic proliferation leading to a larger number of astrocytes, and caused severe edema (**Figure 8d**).

5. Discussion

The use of NM, including carbon nanomaterials, had increased constantly for over the last years, due to its unique physical and chemical characteristics that allow them to be implemented in multiples scientific fields, such as biological and pharmaceutical as well as technological (Aitken et al., 2006, Oberdörster, 2004). The release of NM into aquatic environment is an important aspect to be considered, so that it is possible to evaluate the damage for the present species in this environment, that are probably being exposed to toxic effects through inhalation and/or direct contact with these nanomaterials (Park et al., 2011, Zhang et al., 2011). It was already demonstrated that GR and GO are capable of inducing oxidative stress in others aquatic species, such as the crustacean *Litopenaeus vannamei* and *Artemia salina*, activating their enzymatic antioxidant system (Fernandes et al. 2017; Pretti et al., 2014), however, few data are available about graphene toxicity in fish. In this study, it was used the fish *Danio rerio* as biological model, suitable for toxicity studies, including those dealing with environmental contaminants and NM, as for the fact that exhibit physiological responses to xenobiotics similar to that occurring in mammals, because the genome is

similar in those animals, having equivalent physiological and immunological responses (Fako and Furgeson, 2009; Froehlicher et al., 2009; Pyati et al., 2007).

Besides the biochemical responses, organisms also exhibit molecular defenses that can be activated when a pro-oxidative situation is induced. However, a response at the molecular level does not always interleaved with a biochemical response, regards the toxic being evaluated. The nuclear transcription factor NF-E2-related, factor 2 (*nrf2*), plays an important role in this system, activating the expression of several antioxidant genes, among them are phase II detoxifying enzymes, as GST, also GSH peroxidase (GPx) enzyme and glutamate cysteine ligase catalytic subunit (*gclc*) gene, the limiting enzyme for GSH production, thus being directly involved in GSH concentration (Enomoto et al., 2001; Lewis et al., 2010; Kobayashi and Yamamoto, 2005). Therefore it was the genes chosen for the present study, where a possible response in *nrf2* gene expression could also lead to a response on *gclc* expression, as shown in studies by Mishra et al. (2014) and Kowluru and Mishra (2016).

Graphene ability to induce genes of the antioxidant system has already been showed (Chatterjee et al., 2014). However studies considering the expression of the *nrf2* gene being affected by nanomaterials are scarce until the present moment, but it is known that it can be modulated through the exposure of silver nanoparticles in human renal epithelial cells, directly influencing the GCL-GSH balance inside the cells and its consequent signaling (Kang et al., 2012). In this study, however, it was not possible to observe any effect on the expression of the *nrf2* gene in gills, intestine and muscle (**Figures 3a**, **3b**, **3c** respectively), where the exposure of the two concentrations of graphene did not alter the expression in relation to SDS-control group.

The study conducted by Usenko et al. (2008) in zebrafish, had shown that after 48 h of fullerene C_{60} exposure, the *gclc* gene had up-regulated. Similar data was observed in the study of Da Rocha et al. (2013) where *i.p*. exposure to fullerol showed an up-regulation of this gene after 48 h; however, the same was not observed when the exposure was to single-walled carbon nanotubes (SWCNT). This last result corroborates with our study, where *gclc* gene expression results had not shown statistical difference after graphene exposure in gills, intestine and muscle (**Figures 2a, 2b, 2c,** respectively) as also for the *nrf2* gene. The fact that SWCNT and graphene are similar carbon NM that differ in morphology, may explain why they exhibit similar effects despite tissue differences, while fullerol, despite being also a carbon nanomaterial, has functionality and structure (OH groups) that differ from graphene, perhaps because of this different effects had been observed in both studies (Jortner e Rao, 2002; Zhang et al., 2010).

The molecular results on this study had not shown statistical difference, and one possibility that could explain these results it is regards about exposure time. GR probably did not interfere in the expression of *gclc* and *nrf2* genes due to the short period (48 h) of exposure; which was insufficient to activate the complex machinery of antioxidant gene expression system. This hypothesis is supported by the molecular study from Wu et al. (2014), that had shown the toxic effect of graphene oxide (GO) on mRNA specific genes in *Caenorhabditis elegan*, showing that GO is able to reduce the expression of several genes and thus influencing in several pathways, including growth, describing a direct relation with mortality of the animal; this study however only had results after a long period of exposure, of about 24 days. Besides, the time statement is also corroborated by the fact that it was observed a response from the biochemical antioxidant defense system that would be the first response to a toxin due to its rapid activation (Storey, 2005).

Reduced glutathione (GSH) is the main cellular thiol antioxidant that acts as scavenger of reactive oxygen species (ROS) directly or indirectly in cells, and as co-substrate for many antioxidant phase II enzymes (Halliwell and Gutteridge, 2007; Harvey et al., 2009). It was observed that exposure to 5 mg/L of graphene caused a decrease in GSH concentration in gills (**Figure 4a**) and an increase in intestine (**Figure 4b**) when compared to SDS-control group. These results, indicates that in gills GR exposure lead to higher susceptibility to oxidative stress, with less molecules to cope against the redox alterations, and a similar result also was observed by Chatterjee et al. (2014) on HepG2 human cells and Liu et al. (2011) on bacterial cells. In the intestine, GSH levels were induced for maintenance of the redox balance against the toxicant exposure. Differently, in muscle was not observed alterations on GSH concentration after the exposure (**Figure 4c**). The same was not observed on brain thought. This organ showed that reduced glutathione levels can be altered after GR exposure on low and high concentrations (**Figure 4d**), exhibiting increase when compared to SDS-control, probably due to increased production of intracellular ROS caused by GR exposure, as was shown in Fernandes et al. (2017) and Wu et al. (2016).

The activation of enzymes responsible for GSH synthesis is one form to analyze a response to oxidative stress; the synthesis of GSH is catalyzed by two enzymes, where GCL is the pacemaker enzyme, and their activity is regulated by the GSH levels that are present on tissues (White et al., 2003). In this study it was observed that GR exposure does not seem to had any effect on the enzyme activity in gills (**Figure 5a**) and muscle (**Figure 5c**), although intestine (**Figure 5b**) and brain (**Figure 5d**) results shown an increase in GCL activity after GR exposure, on both low and high concentrations. Britto et al. (2012) showed the reduction in the activity of GCL after fullerene and fullerol exposure on gills of fish *Cyprinus carpio*. Also in gills of shrimp *L. vannamei* the

exposure to GR showed a reduction in GCL activity (Fernandes et al., 2017). Analyzing the GSH and GCL results in the intestine, at low concentration of graphene, and brain on both concentrations, they showed a regulatory mechanism, in with GR seemed to promote an oxidant environment inside cells causing the induction of the enzyme and, thus, increasing GSH levels (Halliwell and Gutteridge, 2007).

The GST enzyme is a superfamily of phase II enzymes responsible for cellular detoxification, which uses GSH as conjugant agent for the reaction process, being involved on oxidative stress responses and detoxification of several environmental toxicants (Kim et al., 2010). The modulation of GST activity in different biological models exposed to different toxicants has already been evaluated on several studies presented in scientific literature (Da Rocha et al., 2013; Ferreira et al., 2012; Pichardo et al., 2017; Weber et al., 2014), however, in exposures to CNM was evidenced the inability to modulate the enzyme. The increased levels of GST found on intestine (**Figure 6b**) and brain (**Figure 6d**), goes in encounter of the ones observed for GSH and GCL on these organs, showing the activation of the antioxidant system in front of the exposure to the low concentration of GR, where the need for more GSH molecules is being necessary for the GST enzyme to perform the detoxification process that is being caused by graphene. Similar results were observed on Marchi et al. (2017) where the exposure to multi-walled carbon nanotubes (MWCNT) in low concentrations of 0.01 and 1.00 mg/L induced the enzyme activity on two polychaete species (*Diopatra neapolitana* and *Hediste diversicolor)* and on Mesaric et al. (2015), that evaluated the enzyme activity after 48 h exposure to carbon black on *Artemia saline* larvae, showing an increase of activity at 0.01 and 1.00 mg/L concentrations. The lack of induction observed on gills (**Figure 6a**) plus the reduction of GSH levels observed indicates that GR detoxification probably does not happen via phase II enzymes such as the GST

family, as was shown on Ferreira et al. (2012) after fullerol exposure on gills of *C. carpio*; and that GSH antioxidant molecule could be acting alone against the GR toxics effects on this tissue.

This scenario on gills of *D. rerio,* probably allowed the accumulation of toxic compounds and leaving this organ more vulnerable to pro-oxidant conditions, expressed by lipid peroxidation damage. Graphene at the concentration of 50 mg/L seems to be toxic for gills as can be evidenced by the increase of peroxided lipids (**Figure 7a**). NM had already been shown to be toxic to aquatic organisms, especially in gills of *C. carpio* exposed to fullerene (Britto et al., 2012, Ferreira et al., 2012) and in polychaete *D. neapolitana* and *H. diversicolor* exposed to nanotubes, generating an increase in the levels of peroxided lipids in these animals (Mesaric et al., 2015). The similar result also was observed in gills of shrimp *L. vannamei* after graphene exposure (Fernandes et al., 2017). However, *D. rerio* antioxidant defense system in the intestine showed to be efficient against oxidative stress (**Figure 7b**), showing a better capacity for redox regulation than in human intestinal cell line Caco-2, where lipid damage was observed after the exposure to SWCNT (Pichardo et al., 2012). The brain of *D. rerio* appears to efficiently protect itself against CNM, where lipid damage was not exhibited after *i.p.* exposure to graphene (**Figure 7d**), as well other study showed that, SWCNT-PEG *i.p.* exposure did not induced oxidative damage (Weber et al., 2014).

Morphological, pathological, structural and cellular changes can lead to organ dysfunction and compromise the animal life, especially in gills, a multifunctional and complex organ that make intimate contact with the surrounding water, being responsible for osmoregulation and respiration process in fish from freshwater. In fact, histological changes were observed after 5 mg/L and 50 mg/L graphene exposure on gills, causing moderate and severe pathological hyperplasia, respectively (**Figure 8a**), while in the

intestine was not observed differences between groups (**Figure 8b**). Similar results were found on *D.rerio* exposed to MWCNT (Filho et al., 2014), being observed hyperemia, aneurism and inflammatory focus on gills, and no alterations at intestine cells, although MWCNT particles could be observed on the intestine lumen. These observations indicate how sensible gills are to the presence of CNM, and that intestine has a protection against these materials, by the presence of muscle layers that prevent the contact of the nanomaterial with intestine cells when exposed through *i.p.*, or by the mucus secreted by the epithelial cells that act as a barrier against the penetration of these materials when exposed through feed or water (Cone, 2009).

GR has already being shown to cause morphological changes on kidney of rats exposed on low and high concentrations (Patlolla et al., 2016). In this study, muscle suffered pathological damage, being observed edema and inflammatory infiltrates between the muscular fibers after exposure to both concentrations of graphene (**Figure 8c**), that could be caused by the presence and accumulation of NM in these regions after *i.p.* injections. Besides, it was observed in brain that exposure to both GR concentrations caused inflammatory responses, with ganglionic proliferations, increased microglia cells and moderate edema in G1, and increased ganglionic proliferation leading to a larger number of astrocytes, and severe edema in G2 (**Figure 8d**). These results are supported by Weber et al., (2014), that found similar responses on *D.rerio* brains after SWCNT-PEG exposure.

6. Conclusions

The results found in this study showed that graphene at short-time exposure induced deleterious effects in gills, muscle and brain considering histological damages. Besides, the modulation of the antioxidant system was observed in some organs, like, intestine. These results showed that direct exposure to GR modulated the antioxidant

system of zebrafish in different tissues, causing lipid damage in some and morphological alterations in others. However, the activation of key genes of the antioxidant system was not observed in a 48 h exposure, for that, a longer exposure period are suggested to evaluate these effects. Although each tissue showed different responses to GR exposure it is possible to observe, when analyzing all the results, that this NM could lead to tissue damage, compromising its function and endangering the animal welfare. Because GR use is increasingly, a detailed study on the pathway of activation of this NM in short and especially long exposure periods becomes necessary to know the systemic effect that GR exerts on the animal.

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7. Appendices

Figure 1. (a), (b) and (c) Transmission electron microscopy (TEM) images of graphene nanosheets obtained from diluted dispersion 1800x in NMP.

Figure 2. Gene expression of glutamate-cysteine-ligase catalytic subunity (*Gclc*) in gills (a), intestine (b) and muscle (c) of *D. rerio* treated with MilliQ water (control), SDS detergent, graphene suspension of 5 mg/L and graphene suspension of 50 mg/L. Data are expressed as relative gene expression considering the SDS-control group gene expression as 1. Columns follow by the same letter are not statistically different in between by the Newman-Keuls test ($p<0.05$).

Figure 3. Gene expression of transcription factor *Nrf2* in in gills (a), intestine (b) and muscle (c) of *D. rerio* treated with MilliQ water (control), SDS detergent, graphene suspension of 5 mg/L concentration and graphene suspension of 50 mg/L . Data are expressed as relative gene expression considering the SDS-control group gene expression as 1. Columns follow by the same letter are not statistically different in between by the Newman-Keuls test ($p<0.05$).

Figure 4. Glutathione concentration levels (expressed as nmol of GSH/30min/mg of protein) in gills (a), intestine (b), muscle (c) and brain (d) of *D. rerio* exposed to different treatments over 48 h, groups are detailed above. Data are expressed as average ±1 standard error (n=4-5). Columns follow by the same letter are not statistically different in between by the Newman-Keuls test ($p<0.05$).

Figure 5. Levels of glutamate-cysteine-ligase activity (expressed as nmol of GSH/30min/mg of protein) standardized by glutathione concentration in gills (a), intestine (b), muscle (c) and brain (d) of *D. rerio* exposed to different treatments over 48 h. Control: fish that were injected with MilliQ water; SDS: fish that were exposed to SDS detergente; G1: fish that were exposed to graphene solution with concentration of 5 mg/L; G2: fish that were exposed to graphene solution with concentration of 50mg. Data are expressed as average ±1 standard error (n=4-5). Columns follow by the same letter are not statistically different in between by the Newman-Keuls test ($p<0.05$).

Figure 6. Activity of glutathione-S-transferase (expressed as nmol of CDNB conjugated/min/mg of protein) in gills (a), intestine (b), muscle (c) and brain (d) of *D. rerio* exposed to different treatments over 48 h, groups are detailed above. Data are expressed as average ±1 standard error (n=4-5). Columns follow by the same letter are not statistically different in between by the Newman-Keuls test ($p<0.05$). CDNB: 1-chloro-2.4-dinitrobenzene

Figure 7. Thiobarbituric reactive substances acid **(**TBARS) levels (expressed as nmol od MDA/mg of protein) in gills (a), intestine (b), muscle (c) and brain (d) of *D. rerio* exposed to different treatments over 48h, groups are detailed above. Data are expressed as average ±1 standard error (n=4-5). Columns follow by the same letter are not statistically different in between by the Newman-Keuls test ($p<0.05$). MDA: malondialdehyde.

Figure 8. Histological analyses of gills (a), intestine (b), muscle (c) and brain (d) of *D. rerio*. Experimental groups are indicated by control (injected with MilliQ), SDS

(injected with SDS detergent), G1 (injected with graphene solution on a concentration of 5mg) and G2 (injected with a graphene solution on concentration of 50mg), n=6.

Table 1. *Danio rerio* gene-specific primers used for quantitative polymerase chain reaction expression (RT-Pcr) analysis.

Figures

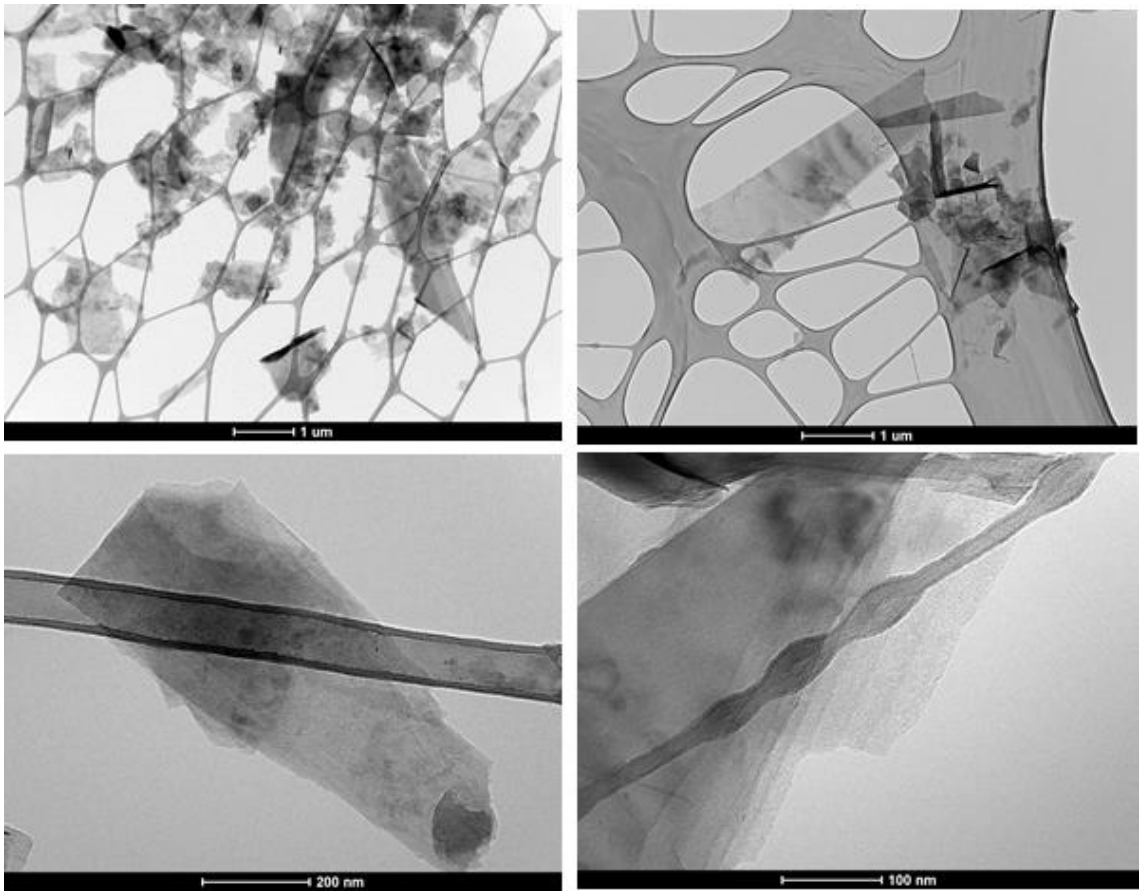
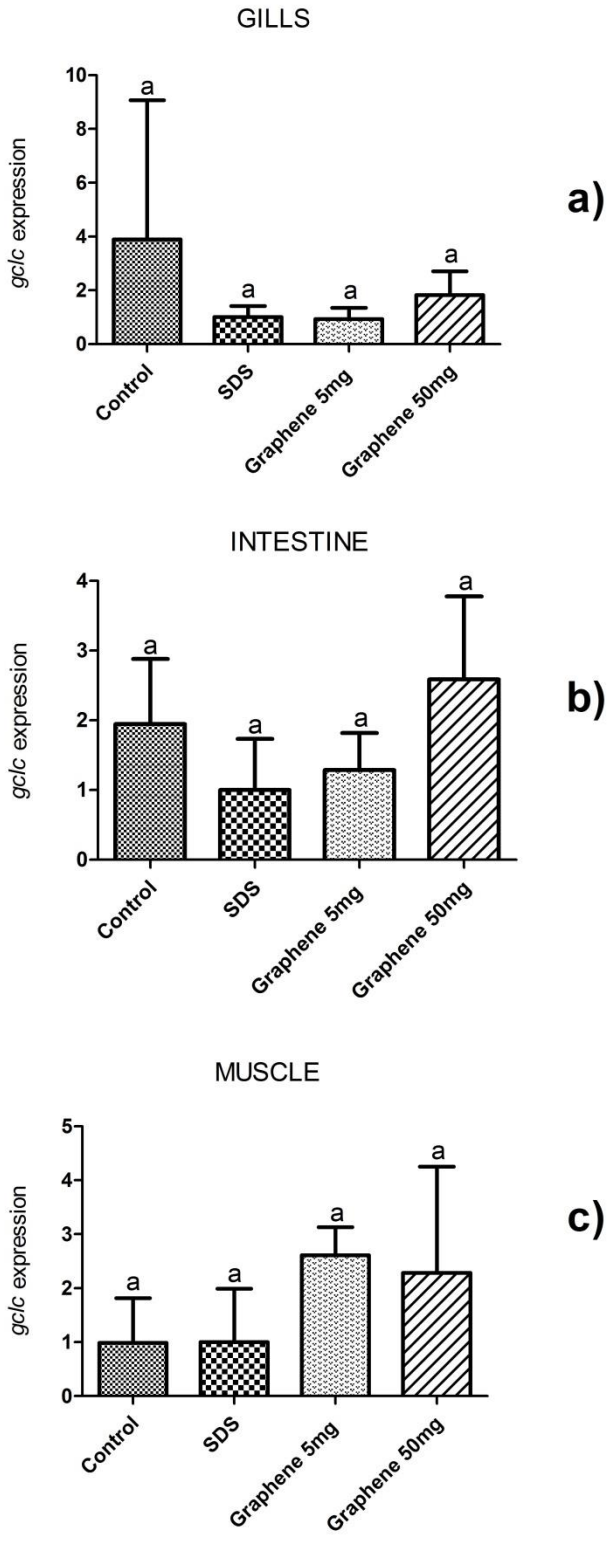
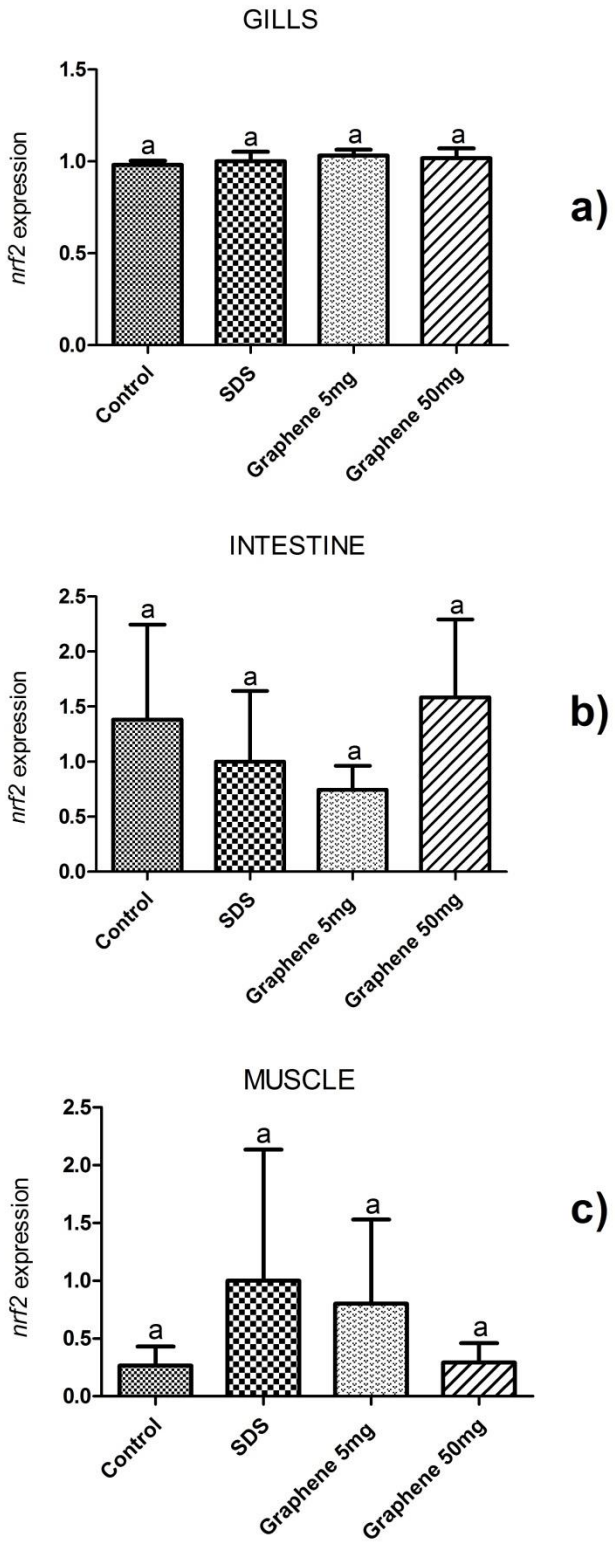


Figure 1.



1533

1534 **Figure 2.**



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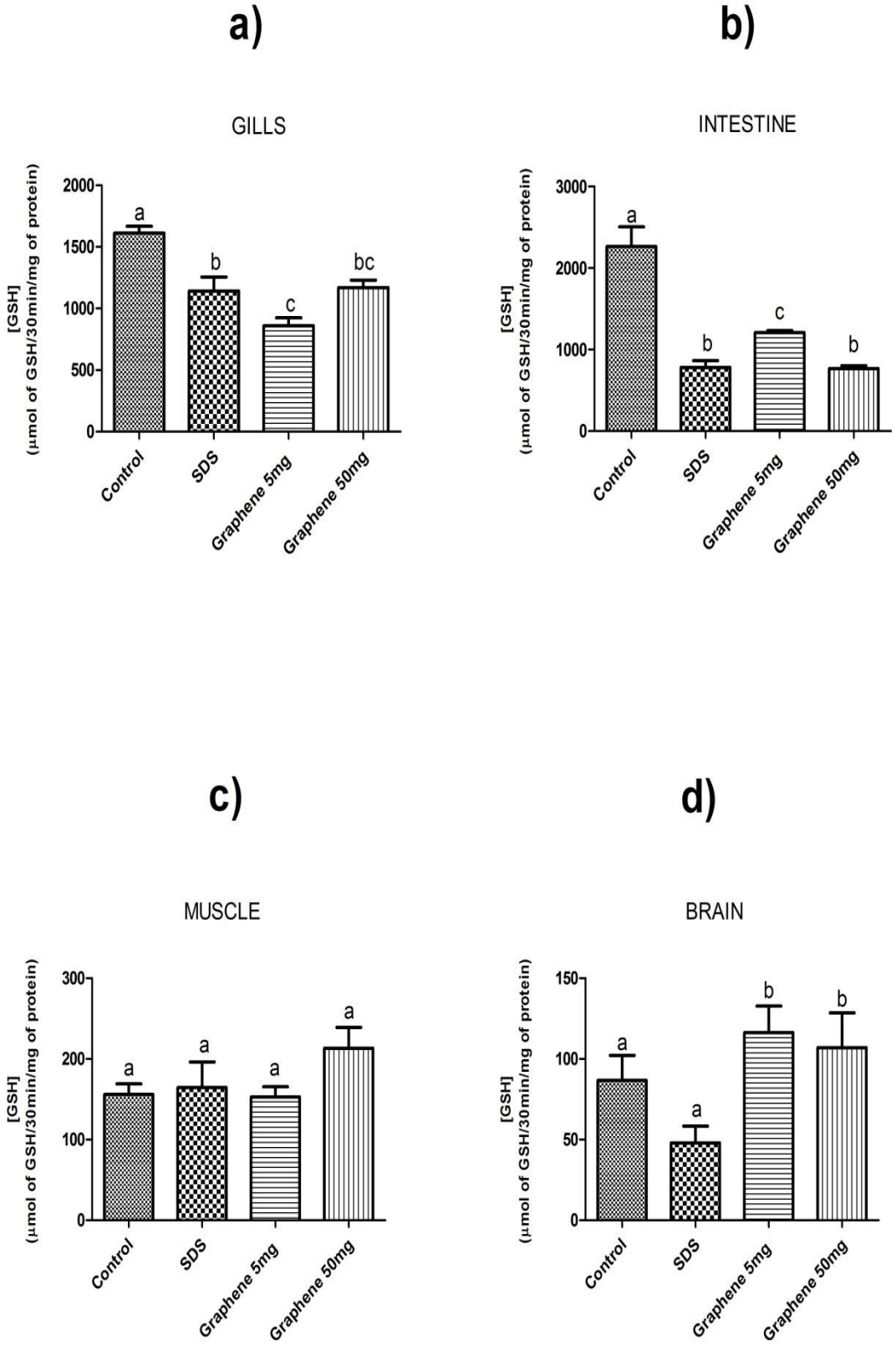

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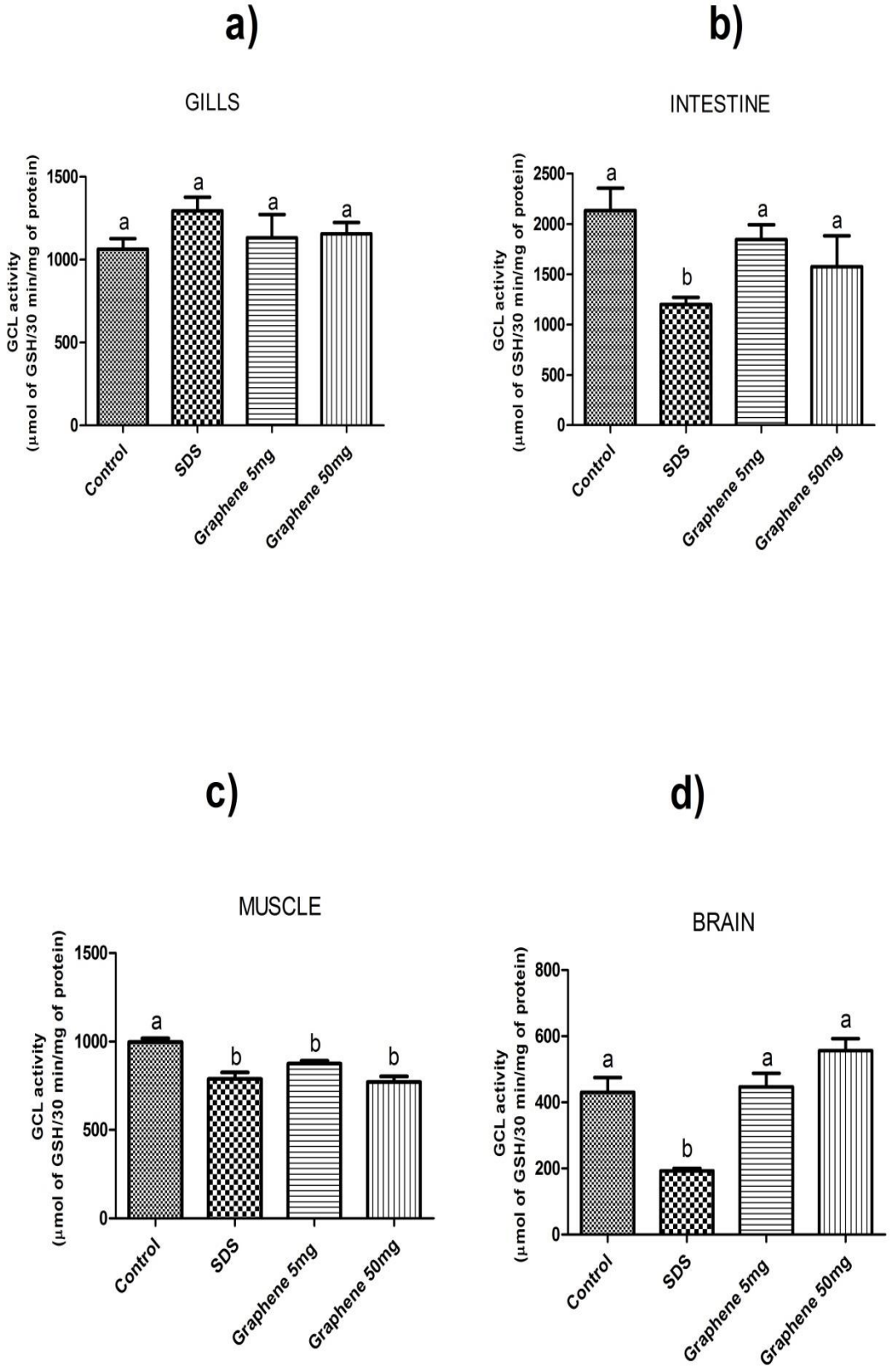

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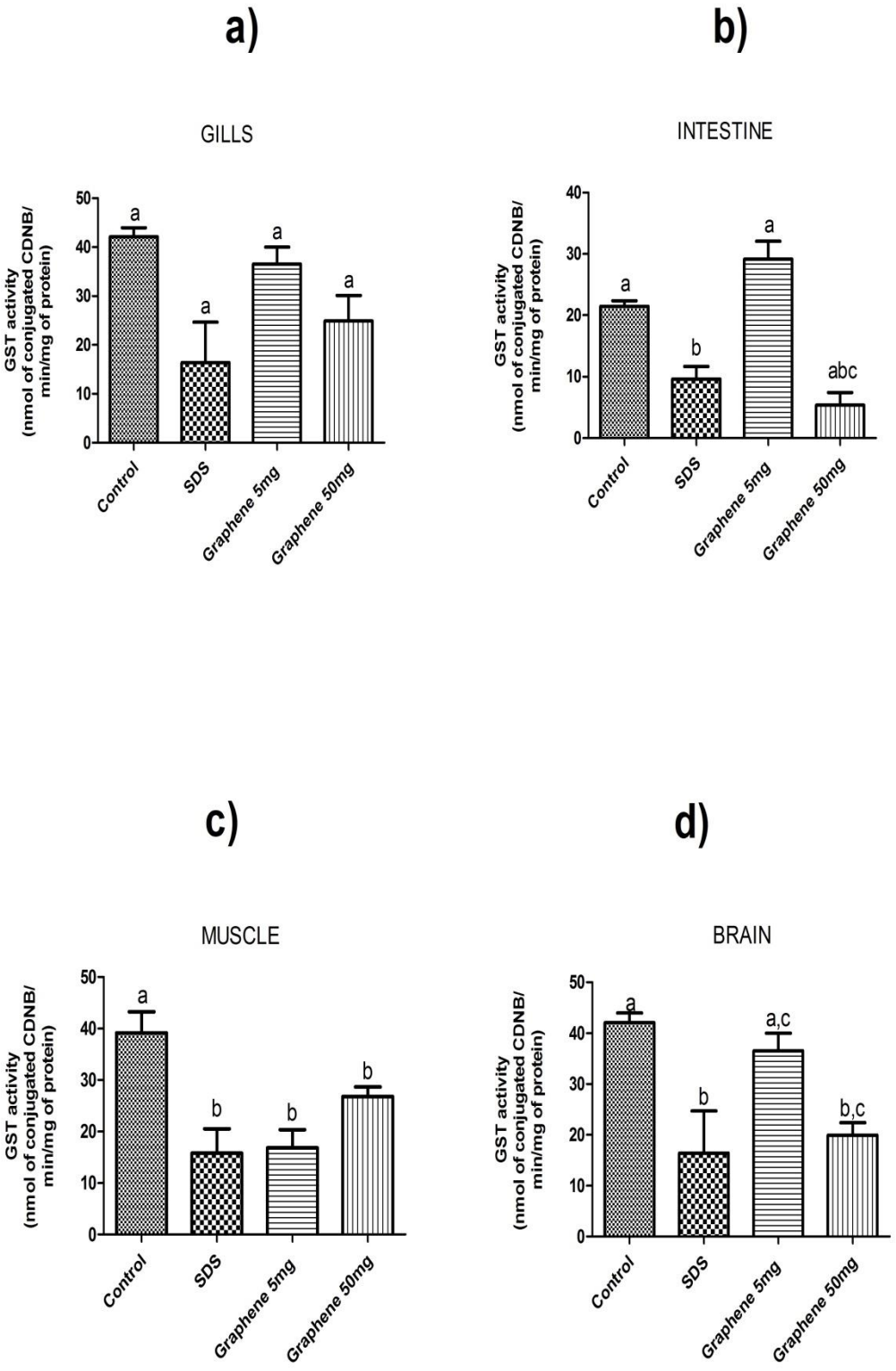

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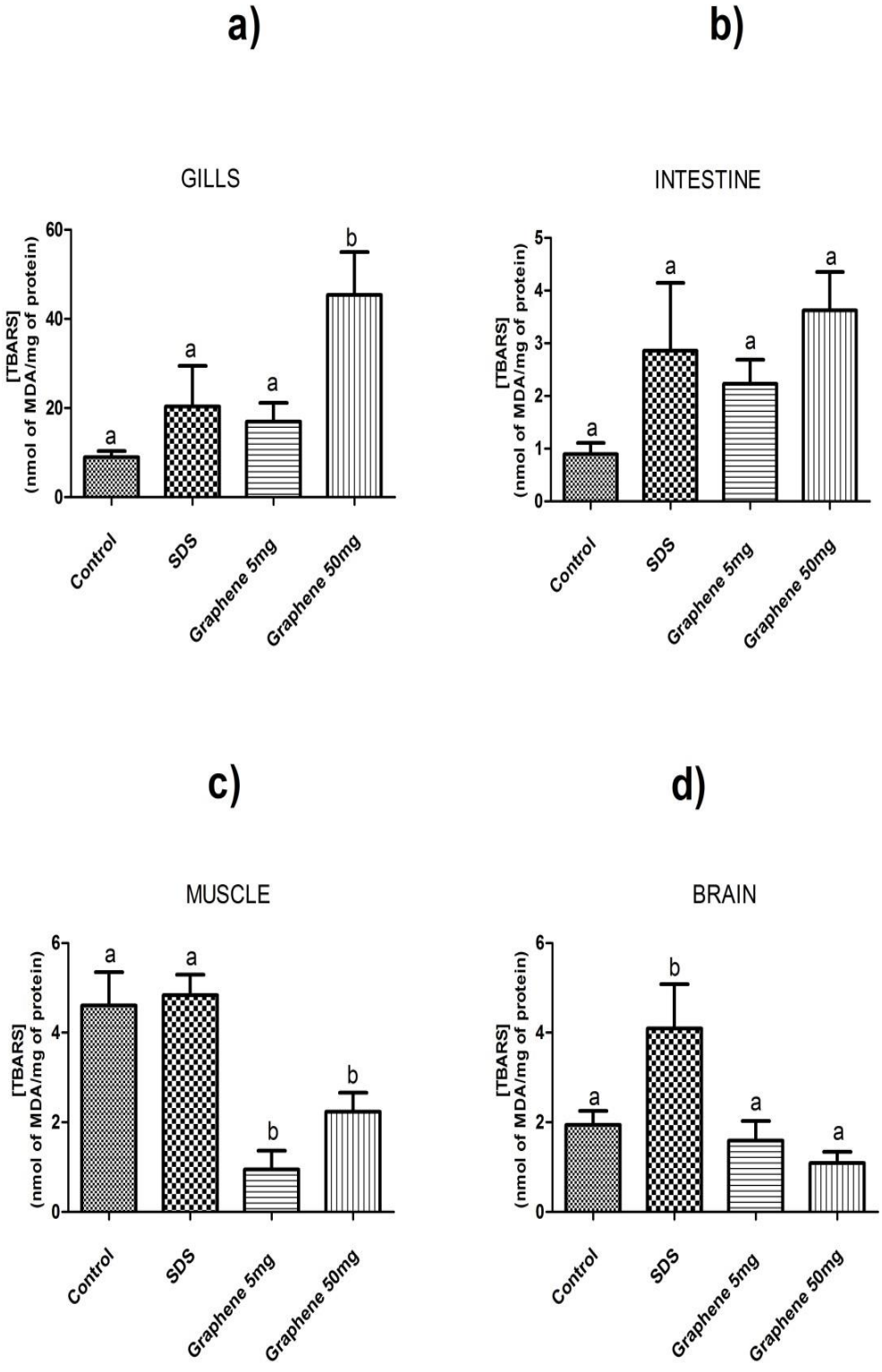
1542 **Figure 6.**



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1544 **Figure 7.**

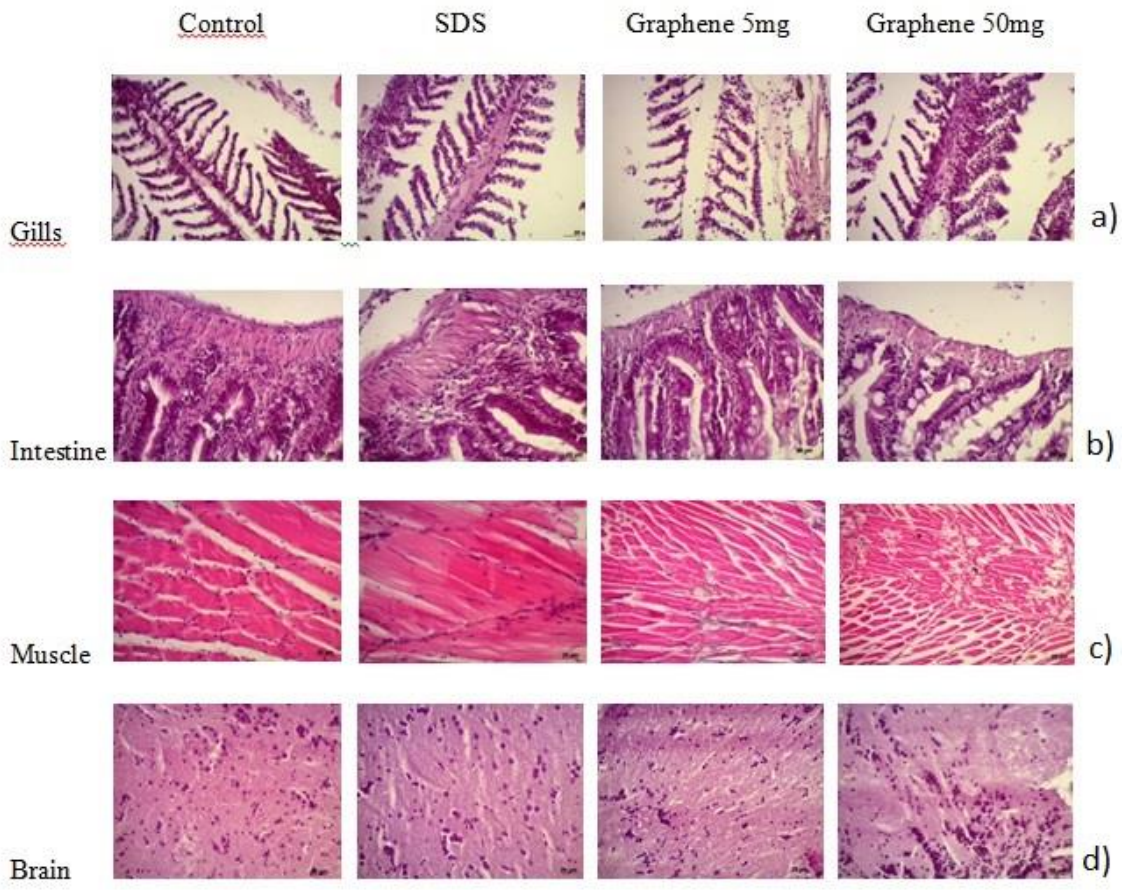

Figure 8.

Gene	*Primer sequence*	*GenBank accession nº*
nrf2	F: 5'-TGTTGGTTCGGAGGCTCTTAA- 3' R: 5'-AGGCCATGTCCACACGTACA- 3'	**NM_182889.1**
gclc	F: 5'-AGGGGATTCCCCAGGTTAG- 3' R: 5'-TTTTCAACAGGTGTGGGTTTGT- 3'	**NM_199277.2**
ef1a	F: 5'-ACATCAAGAAGATCGGCTACAAC- 3' R: 5'-GACCCACAGGTACAGTTCCAATA- 3'	**NM_131263.1**
18S	F: 5'-TGCATGGCCGTTCTTAGTTG- 3' R: 5'-AGTCTCGTTCGTTATCGGAATGA- 3'	**NM_001098396**

Table 1.

7. Discussão Geral

Nesta dissertação estão inclusos dois trabalhos, no primeiro trabalho o foco se manteve em avaliar a capacidade do grafeno em induzir um cenário oxidativo em diferentes tecidos de *Litopenaeaus vannamei*. O segundo trabalho, foi centralizado nos mecanismos de defesa e efeitos que a exposição ao grafeno pode causar aos tecidos de *Danio rerio*. Foram escolhidas diferentes concentrações, pois no primeiro estudo a exposição ocorreu juntamente à ração, que foi oferecida 2x por dia durante um mês, recriando desta forma uma situação ambiental, pois, se sabe que o grafeno não é solúvel em água porem possui a capacidade de aderir ao substrato; o camarão sendo uma espécie bentônica estaria em contato direto com o substrato no ambiente e poderia acabar facilmente ingerindo estes nanomateriais junto à alimentação. Diferentemente, no segundo estudo já havíamos observado que o grafeno possui a capacidade de induzir estresse oxidativo, portanto, escolhemos realizar uma exposição intraperitoneal para assegurar a dose exata a que estávamos expondo o animal. Neste caso, foram escolhidas diferentes concentrações, baixa e alta (5 e 50 mg/L, respectivamente), para testar se produziriam efeitos tóxicos diferentes considerando o comportamento de agregação de partículas quando o grafeno está em grande quantidade, e quais mecanismos de defesa seriam ativados frente à exposição.

No 1º trabalho, o efeito da exposição ao grafeno ficou caracterizado pela modulação do sistema antioxidante enzimático do animal, com aumento da produção de ROS e na síntese de glutationa reduzida, tanto nas brânquias quanto no hepatopâncreas. Também foi observada uma diminuição na atividade da enzima GCL em brânquias e aumento no hepatopâncreas, enquanto que a atividade da GST diminuiu no hepatopâncreas e aumentou em brânquias. Estes resultados mostram que o dano no hepatopâncreas foi significativo, onde uma grande concentração de GSH foi necessária

para agir contra a alta quantidade de ROS neste tecido. Porém, com a redução da atividade da GST as chances do crustáceo se detoxificar ficaram diminuídas; enquanto que nas brânquias a regulação ocorreu de modo que aumentasse a atividade da GST, desta forma não se tornando necessária tantas moléculas de GSH e por isso a atividade baixa de GCL, indicando que a brânquia possui melhor capacidade de detoxificação que o hepatopâncreas considerando a atividade da GST.

Além disso, a capacidade antioxidante total aumentou, em brânquias e hepatopâncreas, provavelmente devido a grande concentração de GSH, entretanto o dano lipídico não foi evitado nestes tecidos, onde um aumento nos níveis de TBARS (substâncias reativas ao tiobarbitúrico) foi evidenciado. Também foi observado o efeito do grafeno em induzir genotoxicidade nestes tecidos, gerando dano de DNA em brânquias e hepatopâncreas. Por fim, foi possível observar alterações histopatológicas nas células do hepatopâncreas, sendo este o tecido mais lesionado com a exposição. Vale lembrar que o hepatopâncreas é o órgão responsável pela metabolização de todos os compostos que o animal entra em contato, então danos nesse tecido comprometem a capacidade de sobrevivência do animal. Nenhum resultado foi observado em músculo do camarão após a exposição. Os resultados obtidos com esse estudo corroboram com os que estão presentes na literatura atual, mostrando a capacidade do grafeno em induzir estresse oxidativo em células de mamíferos, células bacterianas e outras espécies de crustáceo, como *Artemia salina*.

No 2º trabalho a exposição ao grafeno não foi capaz de modular o sistema antioxidante do animal em nível molecular, e os genes marcadores de estresse oxidativo *nrf2* e *gclc* não sofreram alteração em sua expressão. Entretanto, o sistema enzimático foi ativado, após a exposição a 5 mg/L a concentração de GSH em brânquias diminuiu, aumentou no intestino e no cérebro, que também aumentou após a exposição a 50mg/L.

A atividade da GCL aumentou após a exposição às duas concentrações no intestino e cérebro, enquanto que a atividade da GST aumentou somente após a exposição de 5mg/L nestes mesmos tecidos. Em outros estudos, a ativação destas enzimas já tinha sido testada frente à exposição de outros nanomateriais, entretanto, este estudo é o primeiro a mostrar alterações na atividade destas enzimas. A falta de ativação da GST e pouca concentração de GSH em brânquias associado à exposição gerou um aumento no nível de peroxidação lipídica após a exposição à menor concentração. Estudos com outros nanomateriais de carbono também mostram induzir dano oxidativo em brânquias de peixes, como *D. rerio* e *C. carpio*, evidenciando-se a fragilidade das brânquias frente a esse nanomateriais. Alterações morfológicas também foram observadas em brânquias, cérebro e músculo com histopatologias de grau moderado a severo, resultados que corroboram com os disponíveis na literatura referente a diferentes tipos de grafeno e outros nanomateriais de carbono.

Apesar nas diferenças em exposição dos dois trabalhos, a capacidade do grafeno em ativar defesas antioxidantes e provocar alterações histopatológicas em tecidos de espécies aquáticas se comprovou em ambos, entretanto, o curto tempo de exposição do 2º trabalho pareceu ser o limitante para observar efeitos de biomarcadores moleculares de estresse oxidativo.

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