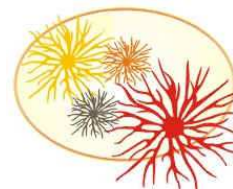**UNIVERSIDADE FEDERAL DO RIO GRANDE-FURG**
INSTITUTO DE CIÊNCIAS BIOLÓGICAS (ICB)
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS FISIOLÓGICAS-
FISIOLOGIA ANIMAL COMPARADA

Efeitos bioquímicos da exposição ao nanomaterial óxido de grafeno em diferentes tecidos do camarão branco (*Litopenaeus vannamei*; Crustacea, Decapoda), através da suplementação na ração.

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Para a minha família,
que partilha comigo
todos os momentos da vida,
a caminhada, as realizações.
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Sumário

Resumo

O desenvolvimento da nanotecnologia vem se intensificando nos últimos anos. Sendo que os NM já estão sendo utilizados em vários produtos disponíveis no mercado. Dentre os NM mais utilizados estão os compostos de carbono que embora sejam compostos somente por este elemento podem ter estruturas diferentes que refletem em suas aplicações e possivelmente em seus efeitos. Dentre os NM de carbono, o grafeno e o óxido de grafeno apresentam promissoras características que ampliam sua utilização em diversos segmentos desde eletrônicos até a distribuição de medicamentos. A intensificação da produção e utilização destes NM é acompanhada pela liberação destes nanomateriais no ambiente que pode afetar os organismos vivos, principalmente os animais aquáticos. Entretanto, pouco se sabe sobre os efeitos do óxido de grafeno em crustáceos de importância comercial como é o caso do camarão branco *Litopenaeus vannamei.* Portanto, a presente dissertação teve como objetivo avaliar os efeitos biológicos da exposição ao óxido de grafeno em diferentes tecidos do camarão.

Palavras-chaves: óxido de grafeno, estresse oxidativo, respostas antioxidantes, *Litopenaeus vannamei.*

Abstract

The development of nanotechnology has intensified in recent years. Since the NM are already being used in several products on the market. NM Among the most used are carbon compounds that although only compounds with this element can have different structures that reflect in their applications and possibly its effects. Among the NM carbon, graphene and graphene oxide show promising features that expand its use in various segments from electronics to drug delivery. Increased production and use of these NM is accompanied by the release of nanomaterials into the environment that can affect living organisms, especially aquatic animals.

However, little is known about the effects of graphene oxide in crustaceans of commercial importance such as the white shrimp *Litopenaeus vannamei*. Therefore, this thesis aimed to evaluate the biological effects of exposure to graphene oxide in different tissues of shrimp

Key-words: Graphene oxide, oxidative stress, antioxidant responses, *Litopenaeus vannamei.*

A. INTRODUÇÃO

A.1. Disposições gerais

A nanotecnologia está baseada na produção de materiais de tamanho na escala nanométrica com o intuito de produzir materiais com amplas aplicações nos mais variados segmentos (Aikten et al., 2006). Os nanomateriais (NM) podem ser definidos como partículas que possuem pelo menos uma dimensão espacial, possuindo escala nanométrica de 1 a 100 nm, onde 1 nanômetro é um bilionésimo de metro, cerca de mil vezes menor que um glóbulo vermelho (Oberdörster, 2004), podendo assumir estruturas diferentes além de formar agregados ou aglomerados (Aitken et al., 2006), devido a estas características estes NM podem ter distintas aplicações e possivelmente diferentes efeitos biológicos.

Muitos estudos se referem às nanopartículas como os materiais que devem substituir materiais empregados nas tecnologias atuais, tais como, equipamentos eletrônicos, indústria têxtil, bens de consumo, cosméticos e aplicações farmacêuticas (Aitken et al., 2006), além destas aplicações que evidentemente beneficiam a sociedade alguns NM também estão sendo empregados no gerenciamento de certos tipos de poluição ambiental (Hansen et al., 2008). Por exemplo, os NM devido as suas caracteristicas físico-químicas podem adsorver contaminantes, como por exemplo, hidrocarbonetos policíclicos aromáticos (PAHs) e alguns metais. Desta forma diminuindo a concentração destes no ambiente (Tungittiplakorn, 2005). Entretanto, existe a possibilidade de que após o tratamento com os NM ligados aos contaminantes, estes possam ser incorporados pelos organismos aquáticos podendo exercer efeitos tóxicos até mesmo superiores ao contaminante isolado, efeito esse conhecido como cavalo de tróia (Park et al., 2010).

Dentre os nanocompostos mais estudados estão os NM de carbono, que incluem os fulerenos (C_{60}, C_{70} e C_{84}), nanotubos e o mais recentemente produzido, o grafeno. O C_{60} é uma nanoforma de carbono que pode ocorrer naturalmente no ambiente (Jortner e Rao, 2002), assumindo uma estrutura semelhante a uma esfera oca com anéis hexagonais, podendo ser produzido a partir da fuligem de incêndios (Huczko e Byszewski, 1998), mas também tem sido extensivamente produzido pela nanoengenharia, visto a alta demanda destes nanomaterias, onde o lucro obtido a partir desta produção deverá até 2015 movimentar um trilhão de dólares (Aitken et al., 2006). Por outro lado, os nanotubos também são compostos somente por átomos de carbono porém mais alongados formando uma estrutura tubular, chegando a ter 0,1mm de comprimento, formado por uma única ou múltiplas camadas de átomos de carbono (Maynard et al., 2004). A **Figura 1** mostra as diferenças estruturais dos nanomateriais de carbono citados no texto.
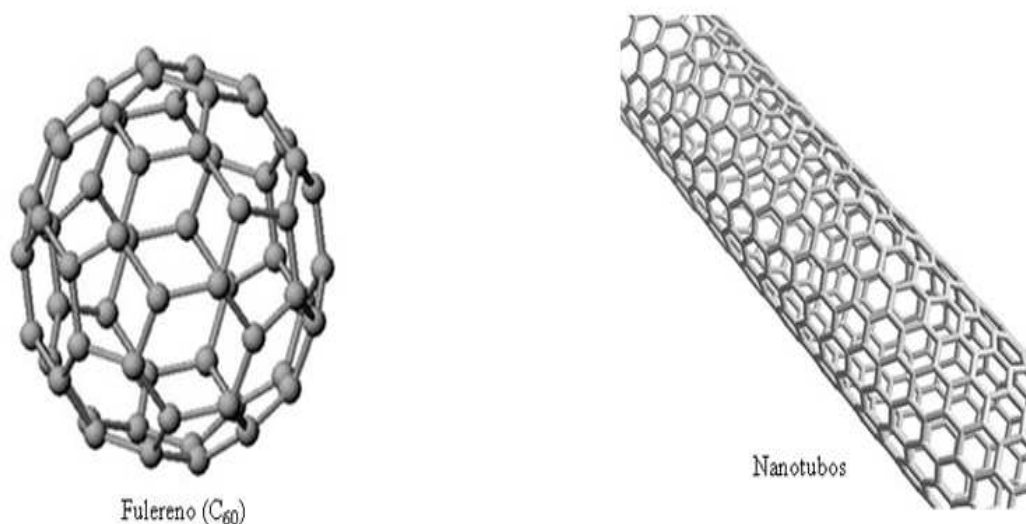

Fulereno (C_{60})

Nanotubos

Figura 1. **:** Diferentes nanomateriais de carbono. Forma esférica (fulereno; C_{60}), forma tubular (nanotubos), (Baseado em Mashino et al., 2003; Liu et al., 2011).

Cada estrutura dessas nanopartículas possui propriedades únicas, apesar da sua composição atômica idêntica, formada somente por átomos de carbono, indicando a importância da forma estrutural nas propriedades do nanomateriais (Liu et al., 2011). Estas propriedades podem inclusive afetar a capacidade de penetração em diferentes tecidos de animais aquáticos (Krysanov et al., 2009) e possivelmente a toxicidade. Por exemplo, a exposição ao C_{60} induziu a má formação em embriões de zebrafish enquanto a exposição aos nanotubos este efeito não foi observado (Cheng et al., 2007). Por outro lado, na truta arco-iris a administração de C_{60} ou nanotubos através da ração não induziu nenhuma toxicidade significativa (Fraser et al., 2011).

Outro nanomaterial de carbono que foi produzido recentemente é o grafeno e seus derivados, como o óxido de grafeno, por exemplo, que devido as suas características únicas têm atraído a atenção dos mais diversos segmentos. Devido a escassez de dados toxicológicos considerando este NM, o óxido de grafeno foi escolhido para a execução do presente projeto. As características deste NM bem como a sua aplicação serão descritas na **Seção A.2.**

A.2. Grafeno e suas características

Grafeno é um nanomaterial cuja estrutura é formada por uma única camada de átomos de carbono firmemente acondicionados em duas dimensões (2D) formando uma estrutura semelhante a uma folha (*nanosheets*) (Geim e Novoselov, 2007), o óxido de grafeno possui grupos funcionais na sua superfície como grupos epóxido, hidroxila e carboxila que conferem a molécula polaridade aumentando assim a solubilidade deste NM (Sanchez et al., 2012). A **Figura 2** ilustra a estrutura bidimensional do grafeno e do óxido de grafeno.

Grafeno   **Óxido de grafeno**
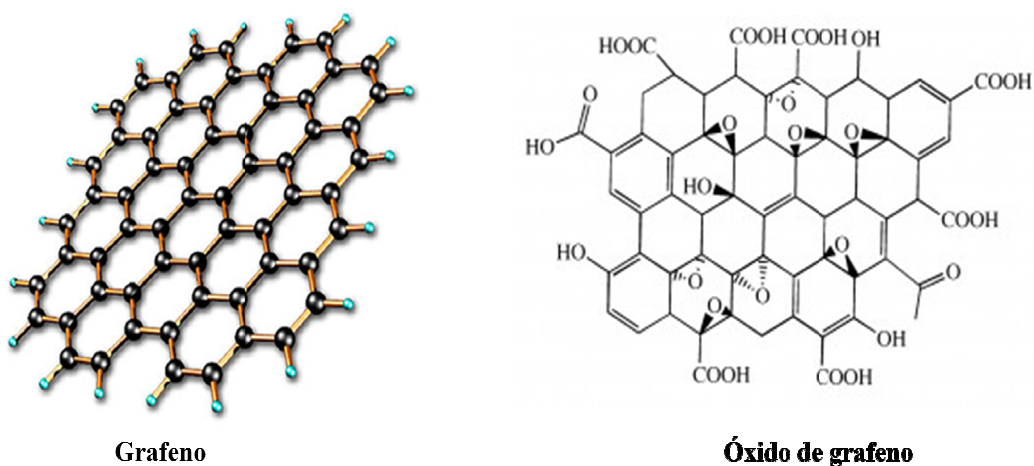
Figura 2. Estrutura do grafeno: átomos de carbono em arranjo planar com estrutura semelhante a uma folha. Óxido de grafeno com grupos carboxila, hidroxila e grupos epóxido na superfície da molécula (Baseado em Liu et al., 2011 e Sanchez et al., 2012).

Com características estruturais únicas, o grafeno e seus derivados têm mostrado propriedades físicas excepcionais, que têm atraído um interesse enorme na pesquisa em âmbito científico (Lee et al., 2008; Chen et al., 2009). Além disso, estes NM têm atraído grande interesse das indústrias devido à sua flexibilidade e transparência que são de grande importancia para a eletrônica, além disso, possibilitando sua utilização em

células de energia, produção de telas sensíveis ao toque, telas de televisores além de sensores e eletrodos (Novoselov et al., 2005; Blake et al., 2008).

Por todas estas características atribuídas aos NM de carbono, incluindo o grafeno e óxido de grafeno, ficam evidentes as vantagens da utilização destes compostos, porém quanto maior a utilização destes NM maior será a exposição dos organismos a estes por diferentes vias. No entanto, ainda não existe uma legislação que contemple um nível de segurança para a exposição dos organismos bem como a liberação destes nanocompostos o ambiente. É importante salientar que do ponto de vista toxicológico pouco se sabe sobre os efeitos dos NM em animais aquáticos, principalmente em crustáceos de possuam grande importância comercial como é o caso do *Litopenaeus vannamei*.

A.3. Efeitos toxicológicos dos nanomaterias

As formas dos NM, como o tamanho e presença de grupos funcionais nas nanopartículas de carbono influenciam as propriedades físico-química desses materiais, por exemplo, solubilidade e a produção de Espécies Ativas de Oxigênio (EAO) (Lee et al., 2008). Devido a presença de elétrons na superficie dos nanocompostos de carbono a produção de EAO pode ser facilitada (Arbogast et al., 1991), que pode vir a interagir com lipídios de membrana, estrutura de proteínas e acarretarem em danos no DNA além de modular as respostas antioxidantes.

Os nanomateriais podem ser incorporados pelos organismos através da água, dieta e também por inalação e se distribuir em diferentes órgãos podendo se acumular e causar efeitos tóxicos nos mesmos (Linhua et al., 2009, Zhang et al., 2011). Como o grafeno começou a ser produzido recentemente, poucos são os estudos considerando os efeitos destes nos sistemas biológicos, os poucos trabalhos disponíveis até o momento

são realizados em testes *in vitro*, em organismos vivos pouco se sabe sobre os efeitos da exposição a este NM.

Wang e colaboradores (2011) mostraram que o grafeno pode acumular-se em diferentes órgãos de ratos como pulmões, fígado e baço enquanto a acumulação no cérebro não foi observada, sugerindo que o grafeno não pode atravessar a barreira hematoencefálica e também não consegue ser eliminado através do sistema renal, estes resultados sugerem que a capacidade de bioacumulação pode resultar em efeitos deletérios devido ao maior tempo de contato entre os NM e os tecidos. Esta hipótese foi corroborada em um estudo realizado com pulmões de camundongo onde foi observada uma alta acumulação de grafeno após 14 dias de exposição resultando em danos neste tecido que incluem infiltrações de células inflamatórias, edema pulmonar e formação de granulomas (Zhang et al., 2011). Em testes *in vitro*, utilizando células tumorais de pulmão foi observado que o grafeno induz um aumento na produção de EAO que culminou com diminuição da viabilidade celular (Chang et al., 2011). Estes resultados sugerem que o grafeno pode ter o tecido respiratório como alvo para acumulação e toxicidade, embora mais estudos devam ser realizados para confirmar esta hipótese.

Na linhagem celular U251 (glioma) o grafeno mostrou que com a excitação de luz infravermelha o NM pode induzir apoptose nas células além de aumentar a produção de EAO causando disfunção mitocondrial que culminou com a maior produção de radical ânion superóxido ($O_2^{\cdot-}$) enquanto estes efeitos não foram observados após exposição aos nanotubos de carbono (Markovic et al., 2011). Estes resultados sugerem que o grafeno pode auxiliar na terapia fototérmica contra certos tipos de câncer, sugerindo sua aplicação farmacêutica.

B. OBJETIVOS

B.1. Objetivo Geral

O trabalho teve como objetivo avaliar os efeitos da exposição de nanopartículas de óxido de grafeno incorporadas à ração em diferentes tecidos do camarão *Litopenaeus vannamei* (Crustacea; Decapoda) considerando parâmetros bioquímicos e histológicos.

B.2. Objetivos específicos

A exposição do camarão *Litopenaeus vannamei* ao nanocomposto óxido de grafeno via suplementação da ração foram considerados os seguintes objetivos específicos:

B.2.1. Analisar os efeitos do óxido de grafeno 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 hepatopâncreas, músculo e brânquias do *L. vannamei.*

B.2.2. Quantificar a produção de EAO e níveis de peroxidação lipídica no hepatopâncreas, músculo e brânquias dos animais expostos ao óxido de grafeno na concentração de 500mg/kg de ração.

B.2.3 Verificar as possíveis alterações histológicas no hepatopâncreas, músculo e brânquias e do camarão branco após exposição ao óxido de grafeno através da ração.

C. JUSTIFICATIVAS

Como pode ser observado a partir dos resultados de estudos científicos considerando a toxicidade do grafeno e seus derivados ficam evidentes as necessidade

de se realizarem mais estudos na área considerando estudos *in vivo* relacionando com respostas bioquímicas.

   É importante ressaltar que o efeito de nanomateriais de carbono através da dieta não foi até o momento explorado em crustáceos, embora se saiba que a exposição alimentar é ambientalmente relevante visto que os nanomateriais tendem a se agregar ou ligarem-se em superfícies incluindo organismos vivos que podem ser incorporados na alimentação por estas espécies. Portanto, o presente trabalho visou avaliar através da exposição ao óxido de grafeno incorporado na ração, se este NM pode alterou os parâmetros bioquímicos e histológicos em diferentes tecidos de uma espécie de crustáceo de grande importância comercial.

D. REFERÊNCIAS BIBLIOGRÁFICAS DA INTRODUÇÃO GERAL

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Artigo

Graphene oxide: an overview from the production to the biological effects in different organs of Pacific white shrimp *Litopenaeus vannamei*.

(a ser submetido à revista *Environmental pollution*)

Graphene oxide: an overview from the production to the biological effects in different organs of Pacific white shrimp *Litopenaeus vannamei.*

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Abstract

The nanomaterials (NM) of carbon as graphene oxide has being employed in several areas such as electronic and biomedicine. Consequently, with increase of use this NM there is also the bigger release into environment aquatic. However few data are available about effect in aquatic organisms. So, the objective of present study was to evaluate the effects of exposure to graphene oxide trough of diet in the shrimp *Litopenaeus vannamei* exposed during 4 or 6 weeks. The results showed: **1)** An decrease in reactive oxygen species (ROS); **2)** increase in glutamate cysteine ligase activity (GCL); **3)** increase in reduced glutathione (GSH) levels and **4)** increase in glutathione-S-transferase (GST) activity in gills and muscle; **5)** increase in total antioxidant capacity in hepatopancreas and **6)** increase in lipidic peroxidation in muscle and decrease in gills, after 4 weeks of exposure. After 6 weeks were observed: **7)** increase in ROS in muscle and gills; **8)** increase in GCL activity in all tissues; **9)** increase in GST activity in muscle and hepatopancreas and **10)** increase in total antioxidant capacity in muscle. The results suggested that graphene oxide can interfere in antioxidant response in different tissues of *Litopenaeus vannamei*.

1. Introduction

In the last years the production of nanomaterials (NM) is increasing exponentially. The NM has unique properties as large superficial area, small size, high electric conductivity (Federici et al., 2007), these characteristics allowed the use of NM several products such as paints, cosmetics, sunscreen, electronics devices, biosensors and biological applications (Kim et al., 2010).

Graphitic NM such as nanotubes (NT) and graphene, including graphene oxide (GO) are nanostructures with possess unique features such as mechanical, electronic, chemical and physical properties that make of these NM promising in several areas including biomedicine (Yuan et. al., 2011). Both carbon nanomaterials have identical composition (only carbon), different structure and are stable due double bonds of carbon besides high bond strength, suggesting that these NM can persist in the environment. Furthermore, the π-electrons can be partially delocalized in the surface of NM and propitiate reactive oxygen species (ROS) production (Fraser et al., 2011).In fact, most of toxicological studies performed with carbon NM have suggested that oxidative stress can be one pathway of NM toxicity. However, few are know about the toxicological effects of graphene and their derivatives as graphene oxide.

Graphene is a single-atom-thick of sp^2-hibridized carbon atoms that display a high flexibility, conductivity and mechanical resistance (Matesanz et al., 2013). Graphene oxide (GO) possess high solubility due presence of carboxylate groups. Both graphene and graphene oxide are being used in biomedical field as intracellular imaging (Gollavelli and Ling, 2012). Unavoidably, the increase of production and utilization of GO is expected to be accompanied by release of this NM into the environment, where the aquatic one seems be a sink for these, representing a potential hazard for aquatic biota (Lee et al., 2009).

As both graphene and GO started to be produced recently (Geim and Novoselov, 2007). 2004) few studies has being performed considering toxicological effects in living organisms (Liu et al., 2011). In fact, in human alveolar epithelial cells (A549) exposed to GO showed an increase in the production of ROS resulting in a decrease of cellular viability (Chang et al., 2012). Also, in neuronal cells (PC12) the exposure to graphene oxide induced cytotoxicity through of increase in ROS levels, decrease in metabolic activity and induction of apoptosis pathway through caspase-3 (Zhang et al., 2010). The few data considering the GO toxicity were performed in mammals systems, but information about effects in aquatic organisms still are scarce (Lee et al., 2009).

Some studies performed with NM have showed that these compounds can modified the antioxidant system. In *Carassius auratus* exposed to fullerene (also carbon NM) there was an increase in antioxidants enzymes activity as catalase (CAT) and superoxide dismutase (SOD), decrease in reduced glutathione (GSH) levels, leading to an increase in lipid peroxidation thus characterizing an oxidative stress condition (Zhu et al., 2008). GO also showed high cytotoxicity in bacterial by induce cell membrane disruption and to alter the antioxidant status by oxidation of GSH (Liu et al., 2011). However, few it is know about effect of GO exposure in aquatic organism considering stress oxidative parameters.

Due to their high adsorbent properties, NM tend to accumulate in the sediments after their aggregation and deposition, placing in potential risk the animals that live in contact with them. Under this context, it seems reasonable to expose organism with benthic feeding behavior with NM as GO added to the diet (Fraser et al., 2011). The white pacific shrimp *Litopenaeus vannamei* (Crustacea; Decapoda) is benthonic species with remarkable commercial importance (Seabra et al., 2011). The generation of scientific knowledge about potential toxic effects of GO *in vivo* can contribute to the development

of a legislation considering the release of NM within the aquatic environment. So, the objective of present study was to evaluate the toxicological effects of GO exposure in different organs of the shrimp *L. vannamei* exposed through food during 4 or 6 weeks.

2. Materials and Methods

2.1. Preparation and purification of graphene oxide

Graphene oxide (GO) was prepared through oxidation of natural nanographite (99.5 wt% of purity), supplied by the Brazilian Company Nacional de Grafite Ltda. The oxidation procedure was performed H_2SO_4, $KMnO_4$ and H_2O_2 (Hummers and Offeman, 1958). The oxidation product was first washed with HCl(10%), filtered (0,45 µM) and dried at 65°C, and then purified (for removal of oxidized carbonaceous residues) by washing with subsequently aqueous NaOH/water with pH adjusted to 7.0, ethanol and ether. Finally, the GO was filtered on a PTFE membrane with pore diameter of 0.45µm and dried for 24h at 100° C under vacuum (Marcano, 2010).

2.2. Characterization of GO

To evaluate the purity, structural quality, oxidation degree and morphology, GO samples were characterized by transmission electron microscopy (TEM), energy dispersive spectroscopy (EDS), dynamic light scattering (DLS), Raman spectroscopy, X-ray diffraction (XRD), Fourier transform infrared spectroscopy (FTIR) and acid-base potentiometric titration. For some of these characterizations, the GO was dispersed in aqueous medium (pH 12.0, 0.125 mg.mL^{-1}) by sonication in ultrasonic tip (Sonics Vibracell VCX 500 - 750 watts and 20 kHz) with an amplitude of 25% for 5 min.

TEM images were acquired in a microscope Tecnai G2-Spirit-2006-EIF operated at 80 kV. GO dispersion was dropped on copper grids covered with holey carbon film (300μm mesh) and dried under vacuum for 24 h at room temperature. DLS measurement was performed in a Zetasizer Nano ZS apparatus, equipped with a 633 nm laser.

For the measurements of Raman spectroscopy and FTIR, a film was prepared by placing a few drops of the GO dispersion (0.125 mg.mL^{-1}) on a Si/SiO$_2$ substrate and dried under vacuum for 24 h. Raman spectra were recorded from 1,000 to 4,000 cm^{-1} on a Horiba Jobin Yvon iHR 550 Raman spectrometer with a 514 nm (2.41 eV) argon ion laser using a 50 x objective. The laser power was set in 4.6 mW. Infrared spectrum in the 650-4,000 cm^{-1} wavenumber or wavelength range was collected in the transmission mode of a Centaurus microscope (magnification 10 x, observed region of 150 x 150 μm^2) attached to a FTIR Nicolet Nexus 470 spectrometer. The spectral resolution was better than 4 cm^{-1} and the spectra were averaged over 60 scans.

The potentiometric titration was performed using an automated microburette system Titroline Alpha from Schott S.A.. Potentiometric titration curves were fit by a non-linear regression program (Mesquita et al., 2006). EDS measurements were carried out in a JEOL JXA-8900RL apparatus at 15 kV and beam current of 15 nA. The XRD was performed using a diffractometer Rigaku D/max-210 equipped with a Cu anode (K$_\alpha$ = 1.54056Å). The diffraction patterns were carried out in the continuous mode with a scan speed of 0.05 °/3 seconds covering 2Θ angles between 7° to 70°, 40 kV/20mA.

The graphitic nature of the sheets and consequently the confirmation of the obtaining of GO were evaluated by Raman spectroscopy, FTIR and XRD. Was compared the Raman spectra obtained for the film produced from the GO dispersion (A) and the natural nanographite (B) **Figure 2**. The bands associated with the graphene oxide (D, G, D', G',

D and D+ G'+G) (Paredes et al., 2009) have been observed in **Figure 2b.** The G band ($\sim$1,580 cm^{-1}) is characteristic of all carbonaceous materials with *sp^2* hybridization. The D band ($\sim$1,350 cm^{-1}) arises from a double resonance phenomenon. Since the presence of structural defects is a necessary condition for the occurrenceof this process, the intensity of the D bandis used asa measure of crystallinity of graphitic materials [the ratio of the intensities of D and G bands(I_D/ I_G) increases with increasing the number of defectsor disorder]. The G' band ($\sim$2,690cm^{-1}) is a second order vibration mode of the D band. The Raman spectrum for GO(**Figure 2a)** shows an increaseof the I_D/I_Gratioin comparison to that fornatural nanographite (**Figure 2b**), indicating that the oxidation of the graphite introduced defects in the structure of graphene oxide. Consequently,there is a decrease (relative toG band) of the intensity of G' band and the appearance of D'($\sim$1,610cm^{-1} - vibration mode induced by structural defects) and the combinations D+ G ($\sim$2,930cm^{-1}) and D' + G ($\sim$3,160cm^{-1}).

2.3. Obtaining, maintaining of shrimp and experimental design

The animals were obtained from Marine Station of Aquaculture, Institute of Oceanography (IO) at the Universidade Federal do Rio Grande –FURG (Rio Grande, Southern Brazil), and transferred to the Instituto de Ciências Biológicas (ICB) of the same University and acclimated in tanks under controlled parameters (salinity 30 ‰, pH 8.0, 12L, 12D, aeration constant, feed Purina$^®$, twice per day) for at least two weeks prior to the experiment. After the acclimation period, the animals were divided into two experimental groups**: 1)** treated with GO during 4 weeks (n=10); **2)** treated with GO during 6 weeks (n=10). Controls groups (n=10) were run in parallel for each experimental group.

2.4. Preparation of diet supplemented with graphene oxide

The treated groups were feed with food enriched with graphene oxide at a concentration of 500 mg/kg of food, according to a previous study of Fraser et al. (2011). Commercial feed Purina® (macerated) containing 45% crude protein was supplemented with graphene oxide, after mixing of the food with NM it was added 3.3% bovine gelatin (Sigma-Aldrich) dissolved in MilliQ water to seal the feed. After this procedure the feed was placed in an oven at 50°C for about 4 h to dry, and then the pellets were stored in containers sealed at 4 °C. Preparation of the control diet followed the same steps cited above except the enrichment with graphene oxide. Feed was offered twice a day in an amount of 2.8% of the average weight of animals per aquarium (Fraser et al., 2011). The animals were weighted once a week for adjustments in the calculation of the quantity offered daily in each treatment. The animals were subjected to this diet during all experiment time.

2.5. Preparation of samples for histological analyses

Muscle, gills and hepatopancreas were collected and cut into smaller pieces of 1cm x 1cm (to allow the better fixation), identified and packaged in containers with fixative solution (formalin 10%). These materials were placed in bath with decreasing levels of alcohol (100-70%), then there were bathed in xylol and after submerged in paraffin. Posteriorly, paraffin blocks were cut into microtome and then these materials were placed on glasses slides and stained with hematoxylin and eosin. Samples were analyzed with light microscopy by a pathologist who did not know the experimental groups to which the samples belonged. Photographs were produced using an Olympus BX51 optic microscope and an Olympus digital camera (DSC 250).

2.6. Preparation of samples for biochemical analyses

The muscle tissues, gills and hepatopancreas were dissected and homogenized (1:4p/v) in buffer containing 100 mM Tris-HCl, 2mM EDTA, 5mM $MgCl_2$, 0,1% of cocktail inhibitor proteases (1:100, Sigma-Aldrich), pH 7.75, centrifuged at 10,000g for 20 min at 4 °C, and the supernatant was used for biochemical analysis. Biuret method was used to the measurements of total proteins, utilizing a microplate reader (BiotelELX800).For further measurements, aliquots of the supernatants were stored at-80 °C except in the ROS analysis where it was used fresh tissue.

2.7. Reactive oxygen species (ROS) measurement.

Immediately after dissection, the muscle, gills and hepatopancreas of *L. vannamei* were homogenized in cold buffer and centrifuged (10,000 x*g*, 20 min at 4 °C), the supernatant was used for the quantitation of total protein and adjusted to 2 mg/ml of extract. ROS quantification was performed according Ferreira-Cravo et al. (2007), using 2'7'-dichlorofluorscein diacetate (H_2DCF-DA) to generate a fluorochrome detected at wavelength 485/520 nm excitation and emission, respectively.

2.8. Glutathione-S-transferase (GST) activity.

The activity of GST was determined by the conjugation of 1 mM of GSHand 1 mM1-chloro-2,4-dinitrobenzene (CDNB) this conjugation was monitored spectrophotometrically at 340nm as described in the methodology of Habig and Jakoby (1981).

2.9. Glutamate cysteine ligase (GCL) activity and reduced glutathione (GSH) levels

GCL activity and baseline GSH determination were analyzed following White et al. (2003). This method is based on the reaction of naphthalene dicarboxialdehyde (NDA) with glutathione (GSH) or γ-glutamylcysteine (γ-GC) to form cyclized products that are highly fluorescent. NDA-GSH fluorescence intensity was measured (485 ex/530 em) on a fluorescence microplate reader (Victor 2,Perkin Elmer).

2.10. Antioxidant capacity against peroxyl radicals

This analysis was performed measuring thermal decomposition of ABAP (2,2'-azobis (2 methylpropionamidine) dihydrichloride) at 35ºC (Winston, 1998) using H_2DCF-DA. The esterases present in the samples cleave the H_2DCF-DA, generating a non-fluorescent compound (H_2DCF) that is oxidized by ROS forming a fluorescent compound (DCF) that is detected at wavelengths of 485 and 520 nm for excitation/emission respectively using a microplate reader (Victor 2, Perkin Elmer). Total fluorescence generation was measure along of time, adjusting the fluorescence data to a second order polynomial function. To analyze the total antioxidant capacity against peroxyl radical was considered the relative area with and without ABAP, being that high fluorescence area indicates low antioxidant competence (Amado et al., 2009).

2.11. Lipid peroxide content

The protocol described by Oakes and Kraak (2003) was used for the analysis of oxidative damage. Lipid peroxides were measured as thiobarbituric acid reactive substances (TBARS) by fluorimetric method in which the fluorescence was measured at520/580nm excitation/emission, respectively. Tetramethoxypropane (TMP) was employed as a standard and the results were expressed as nmol of TMP equivalents/mg of proteins.

2.12. Statistical analysis

Statistical differences between various parameters were analyzed through analysis of variance (ANOVA). *Pos hoc* comparisons were made using Newman-Keuls test or orthogonal contrasts. A factorial ANOVA was run for all variables taking as factors the treatments (control and graphene oxide) and time of exposure (four and six weeks). Assumptions of normality and variance homogeneity were previously analyzed and when necessary mathematical transformation were applied (Zar, 1984). In all cases significance level was fixed in 5%.

3. Results

Physical-chemical analysis

Figure 1a shows TEM images for GO after purification, illustrating the major presence of monolayer sheets and few layers with dimensions of a few micronsand good structural properties, at testing the effectiveness of the exfoliation process. Some of the GO sheets appear folded, probably due to the process of deposition on the TEM grids. The size of the sheets was confirmed by DLS results that showed a bimodal distribution

of particle sizes between 100 nm and 2,000nm and between 4,000nm and 6,000nm. The TEM imagesare representative of the whole sample. Additionally, they show the purity of the material, since almost no residue of oxidationis observed. The high purity degree was also characterized by EDS (**Figure 1b**). Besides carbon and oxygen, only traces of S (sulfur), Cl (chloro) and Na (sodium) originated from the chemical processing of expansion or oxidation and neutralization were determined.

The graphitic nature of the sheets and consequently the confirmation of the obtaining of GO was evaluated by Raman spectroscopy, FTIR and XRD. **Figure 2** comparesthe Raman spectraobtained for the filmproduced from the GO dispersion (**Figure 2a**) and thenatural nanographite (**Figure 2b**).

The band sassociated with the graphene oxide (D, G, D', G', D and D+ G'+G) (Paredes et al., 2009) are shown in **Figure 2a**. TheG band (~1,580 cm^{-1}) is characteristic of all carbonaceo us materials with sp^2 hybridization. The D band (~1,350 cm^{-1}) arises from adouble resonancephenomen on. (Saito et al., 2011). **Figure 3** shows the XRD patterns obtained fornatural nanographite (**3a**) and the solid GO (**3b**). Thepeaksin the plane (002) and (004) in **Figure 3a** correspond to the perpendicular direction (*c*-axis) to the graphite hexagonal planes (Sun et al., 2008). The presence of only one peakin the plane (001) in **Figure 3b** confirms the fulloxidation of natural nanographite into GO, as already discussed in the literature (Titelman, 2005; Wojtoniszak et al., 2012). Through Bragg's law and Sherre equationit was possible to calculate the distance between the layers in GO (d_{001}), being of 0.82 nm. This distance is increased in relation to the spacing between the graphene planes in the nanographite (d_{002}= 0.34nm, **Figure 3a**) as a result of chemical exfoliation and consequent introduction of functional groups on the surface of graphene planes during the oxidation process.

The indentification of the oxygenated groups introduced to the GO surface was performed by infrared absorption. In **Figure 4**, FTIR bands related to the vibrational modes of O-H and C-O bonds were highlighted: theintense band sat ~3,400 and 3,218cm^{-1}associated with O-H stretching of, respectively, phenolicand carboxylic groups and at ~1,395 cm^{-1}associated with O-H deformation of phenols, alcohols and carboxylic acids; other less intense bands centered at 1,708cm^{-1} (C= Ostretching of quinones and carboxylic acids), 1,220cm^{-1} (C-O stretching of carboxylic acids andphenolic groups) and 1,062cm^{-1} (C-O stretching of epoxidesand alcohols). The absorptionat ~1,570 cm^{-1} is at tributed to C= C stretching in the hexagonal structure of the graphene plane (Titelman, 2005; Vieira et al., 2007, Wojtoniszak et al., 2012).The functional groups were quantifi ed by acid-base potentio metrictitration (Mesquita et al., 2006). The results are sumarized in **Table 1**. The numbers show the presence of the majority of phenolic groups.

Biological analysis

The histological analyses not show changes after GO exposure in muscle, gills and hepatopancreas (**Figure 5a**, **5b** and **5c**, respectively).

In muscle and gills, after 4 weeks of GO treatment it was observed a decrease (p<0.05) in ROS levels when compared with control group, while in the group exposed during 6 weeks there was an increase of ROS levels (p<0.05) (**Figure 6a** and **6b**, respectively). In hepatopancreas after 4 and 6 weeks of GO exposure it was observed a decrease in ROS levels (p<0.05) when compared with controls groups (**Figure 6c**).

GCL activity was increased after 4 and 6 weeks of GO exposure in muscle, gills and hepatopancreas (p<0.05) when compared with control group (**Figure 7**)

In muscle, after 4 weeks of GO exposure there was an increase in GSH levels ($p<0.05$) when compared with the control group (**Figure 8a**). In gills it also was observed an increase in GSH levels ($p<0.05$) after 4 weeks of GO treatment while this result was not observed in the group exposed during 6 weeks ($p>0.05$) (**Figure 8b**). In hepatopancreas there was an increase in GSH levels after 4 weeks of GO treatment when compared with control group ($p<0.05$), but after 6 weeks of exposure to GO it was observed a reversal of this result ($p<0.05$) (**Figure 8c**).

In terms of GST activity, in muscle it was registered an increase of the enzyme activity after 4 and 6 weeks of GO exposure when compared with their respective control group ($p<0.05$)(**Figure 9a**). While in gills it was observed a decrease of GST activity ($p<0.05$) after 4 and 6 weeks of GO exposure (**Figure 9b**). In hepatopancreas, any change was observed in terms of GST activity after 4 weeks ($p>0.05$) of treatment, while in the group exposed during 6 weeks it showed a decrease in enzyme activity ($p<0.05$)(**Figure 9c**).

In muscle, the total antioxidant capacity against peroxyl radicals was not changed after 4 weeks of GO exposure ($p>0.05$), while after 6 weeks there was an increase of total antioxidant capacity ($p<0.05$) (**Figure 10a**). In gills, the exposure to GO during 4 or 6 weeks did not showed to alter the antioxidant capacity when compared with respective controls groups ($p>0.05$), although antioxidant competence changed over time ($p<0.05$) (**Figure 10b**). In hepatopancreas, after 4 weeks of exposure to GO there was an increase of antioxidant capacity ($p<0.05$) when compared with control group, while any change was observed after 6 weeks of exposure ($p>0.05$) (**Figure 10c**).

In terms of TBARS content, in muscle the exposure to GO during 4 weeks showed an increase in peroxidation levels ($p > 0.05$) while this result was not observed in the group exposed during 6 weeks ($p > 0.05$) (**Figure 11a**). A different result was observed in gills, where exposure to GO during 4 weeks showed a decrease in TBARS levels ($p < 0.05$), being without differences between experimental groups after 6 weeks of exposure ($p > 0.05$) (**Figure 11b**). In hepatopancreas any change in TBARS content were observed after GO exposure (**Figure 11c**).

4. Discussion

This study is one of the first to address the exposure of GO in aquatic organisms through diet. The objective was to evaluate potential toxic responses induced by GO in different organs of *L. vannamei* after 4 or 6 weeks of exposure.

Histological examination showed any changes in muscle, gills and hepatopancreas of *L. vannamei* (**Figure 5a, 5b** and **5c; respectively**), different to what found in gills of rainbow trout *Oncorhynchus mykiss,* where the exposure to nanotubes induced hyperplasia in this organ (Smith et al., 2007). The difference between results can be in part explained by structural difference between these two NM once that nanotubes posses tubular form that facilitate the penetration in cell membranes while GO displays planar form what allow that this NM is aggregated in cell membranes (Liu et al., 2011).

Most of studies performed with graphene or GO has showed that these NM can induce ROS generation in mammals system (Chang et al., 2011). Interestingly, in muscle, gills and hepatopancreas after 4 weeks of exposure it was observed a decrease in ROS levels (**Figure 6a**, **6b** and **6c**; respectively). These results can be linked to increase in glutamate cysteine ligase (GCL) activity (**Figure 7a, 7b** and **7c**) concomitantly the

increase in reduced glutathione (GSH) levels observed in the same groups (**Figure 8a**, **8b** and **8c**). The GCL is step-limiting enzyme for GSH synthesis, being this last a powered non-enzymatic antioxidant considered as the first line of defense of cells (White et al., 2003). This increase both in GCL activity and GSH levels can be contributed to decrease ROS levels once that GSH can intercept this reactive species actuating as ROS scavenger. This means that exposure to GO can alter the redox status generating a pro-oxidative condition that stimulated GSH synthesis through of GCL activity to revert this situation. Other possibility to explain the increase in GCL activity and GSH levels can be due high surface area the GO can be adsorbed to proteins inducing structural changes in the GCL that result in the GSH content (Sanchez et al., 2012) these changes can be also responsible by decrease in basal ROS levels found in this study after GO exposure during 4 weeks. However, more research is need on the possible interaction of GO with GCL and other antioxidant enzymes.

In muscle and gills exposed during 6 weeks it was observed a different result when compared with that of 4 weeks. In these groups there was an increase in ROS levels associated with a low GSH content even when GCL activity was increased. The time of exposure seem have influenced in ROS levels, suggesting that GO exposure induce a pro-oxidant situation, however in this case was not observed any changes in GSH content, in really, when compared with group exposed during 4 weeks. This increase in ROS levels in gills and muscle after 6 weeks can be related to bigger accumulation of GO that can culminate with decrease of GSH levels. In fact, the GSH levels are enough low, mostly in muscle and hepatopancreas. In this way, the GSH available was not sufficient to diminish the ROS; however, the increase in ROS levels induced an increase in GCL activity although this increase was not accompanied by increase in GSH

content, perhaps in virtue of time window that was not sufficient to allow the tripeptide synthesis.

The glutathione-S-transferase (GST) is family of enzymes involved in detoxification of several xenobiotics (Halliwell and Gutteridge, 2007). In this study, the results observed in different organs not showed any pattern of response in terms of GST activity. In muscle after 4 and 6 weeks of exposure to GO was observed an increase in the GST activity (**Figure 9a**) although this increase was not sufficient to avoid lipid peroxidation in the group exposed during 4 weeks to GO (**Figure 10a**). In gills, after 4 and 6 weeks it was observed a decrease in GST activity indicating that the exposure to GO in this organ decreases the detoxification capacity. A similar result also was observed in hepatopancreas of group exposed during 6 weeks. These results suggest that GO exposure should increase the vulnerability of gills to other pollutants, once that this NM decrease the detoxification capacity (**Figure 9b** and **9c**).

In muscle it was observed an increase in total antioxidant capacity against peroxyl radicals after 6 weeks of exposure to GO. This increase can be linked to increase of ROS levels observed in the same group. Higher ROS levels can be stimulating antioxidant responses that probably avoid oxidative damage in terms of lipid peroxidation in the same group. In gills, the treatment with GO did not induced alterations in the total antioxidant capacity; however as can be observed in the **Figure 10b,**after 6 weeks of treatment there was an increase in the antioxidant capacity suggesting that over time this NM induce a pro-oxidative situation. This increase in total antioxidant capacity observed after 6 weeks of exposure can be linked to low lipid peroxidation when compared with group exposed during 4 weeks. In hepatopancreas there was an increase in total antioxidant capacity after 4 weeks of exposure to GO (**Figure 10c**). This increase can be related with increase of GSH levels observed in the

same group; in fact the GSH contributes significantly to total antioxidant capacity against peroxyl radicals (Regoli and Winston, 2003), this favorable antioxidant capacity stimulated by GO exposure probably contributed to avoid lipid peroxidation in hepatopancreas.

5. Conclusions

As general conclusion can be suggested that graphene oxide can modulated the antioxidant responses in different organs of *Litopenaeus vannamei* probably altering the redox state of cells favoring a pro-oxidant situation inducing an increase in GCL activity and GSH levels avoiding oxidative damage except in muscle exposed during 4 weeks where was characterized a stress oxidative situation due to increase in lipid peroxidation. Other possibility to explain the responses observed in this study, mostly after 4 weeks of exposure, are physical-chemical characteristics of GO that can interacted with protein involved in the antioxidant system increasing the antioxidant response resulting in the decrease of basal ROS levels.

Anyway, these results suggest that this NM can affect aquatic organisms, so representing a threat to environment and consequently aquatic biota.

6. References

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7. Figure Legends

Figure 1. Purification and characterization of graphene oxide. **(a)** Transmission electron microscopy (TEM). **(b)** Elemental composition of GO characterized by Energy Dispersive Spectrocopy (EDS).

Figure 2. Graphitic nature of the sheets. Raman spectra in the region between 1000 and 4000 cm^{-1} obtained for GO **(a)** and natural nanographite **(b)**. ($\lambda = 514$ nm).

Figure 3. Diffractograms for natural nanographite **(a)** and GO **(b).**

Figure 4. Infrared spectra for GO in the region between 700 and 4000 cm^{-1}.

Figure 5. Cross section of the abdominal muscle **(a)** of Litopenaeus vannamei at 400x magnification. The image shows the connective tissue (CT) between the bundles of muscle fibers. The black arrow indicates the position of muscle fibers cut transversely, with peripheral nuclei, without any apparent histopathological changes.

Cut in histologic 10X magnification, showing the arches with the filament gills **(b)** of Litopenaeus vannamei not hove gill histopathological changes highlighted, and the chlorine cells, mucous cells and cells pillars showed no change.

Hepatopancreas **(c)** of *Litopenaeus vannamei* in cross section in increased 00X, without evident histopathological changes of the tubules. It is observed connective tissue between the tubules (CT), and appears normally tubule lumen (L), with a red.

Figure 6. Reactive Oxygen Species (ROS) concentration (expressed by area). **(a)** Muscle; **(b)** Gills and **(c)** Hepatopancreas. Different letters indicate significant differences ($p < 0.05$) compared to control group. Asterisks (*) indicate significantly difference ($p < 0.05$) between treatment connected by solid lines. Data are expressed as mean ± 1 standard error (n=4-6).

Figure 7. Glutamate cysteine ligase (GCL) activity (nM GSH/mg of proteins). **(a)** Muscle, **(b)** Gills and **(c)** Hepatopancreas. Different letter indicate significant differences ($p < 0.05$) between means of different treatments. Data are expressed as mean $_+ 1$ standard error (n= 4-6).

Figure 8. Glutathione reduced (GSH) content (expressed as nmol GSH/mg of protein). **(a)** Muscle; **(b)** Hepatopancreas. Different letters indicate significant differences ($p < 0.05$) compared to control group. Asterisks (*) indicate significantly difference ($p < 0.05$) between treatment connected by solid lines. Data are expressed as mean ± 1 standard error (n=4-6).

Figure 9. Glutathione-S-transferase (GST) activity (expressed by nmol conjugated CDNB/min/mg of protein). **(a)** Muscle; **(b)** Gills and **(c)** Hepatopancreas. Different letters indicate significant differences ($p < 0.05$) compared to control group. Asterisks (*)

indicate significantly difference (p<0.05) between treatment connected by solid lines. Data are expressed as mean ±1 standard error (n=4-6).

Figure 10. Antioxidant Competence Against Peroxyl Radicals (expressed by relative area). **(a)** Muscle; **(b)** Gills and **(c)** Hepatopancreas. Different letters indicate significant differences (p<0.05) compared to control group. Data are expressed as mean ±1 standard error (n=4-6).

Figure 11. TBARS content (expressed as nmol of MDA/mg of protein). **(a)** Muscle; **(b)** Gills and **(c)** Hepatopancreas. Different letters indicate significant differences (p<0.05) compared to control group. Data are expressed as mean ±1 standard error (n=4-6).

Table 1. Quantification by acid-base potentiometric titration of the functional groups found on the surface of the GO.

pKa	Oxygenate groups	Quantity (mmol.g^{-1})
$4 < pKa \leq 6$	Carboxylic	0,39
$6 < pKa \leq 7$	Lactones	0,25
$pKa > 7$	Phenolic	7,85

8. Figures

Figure 1.
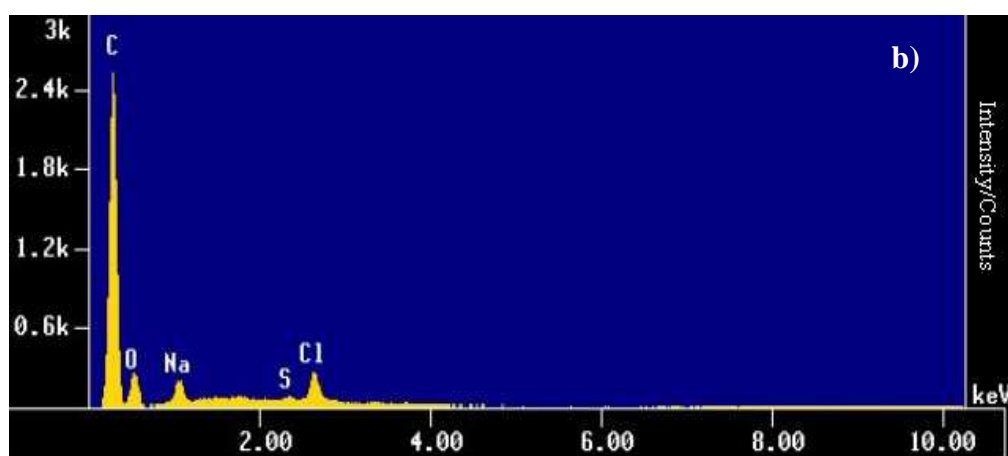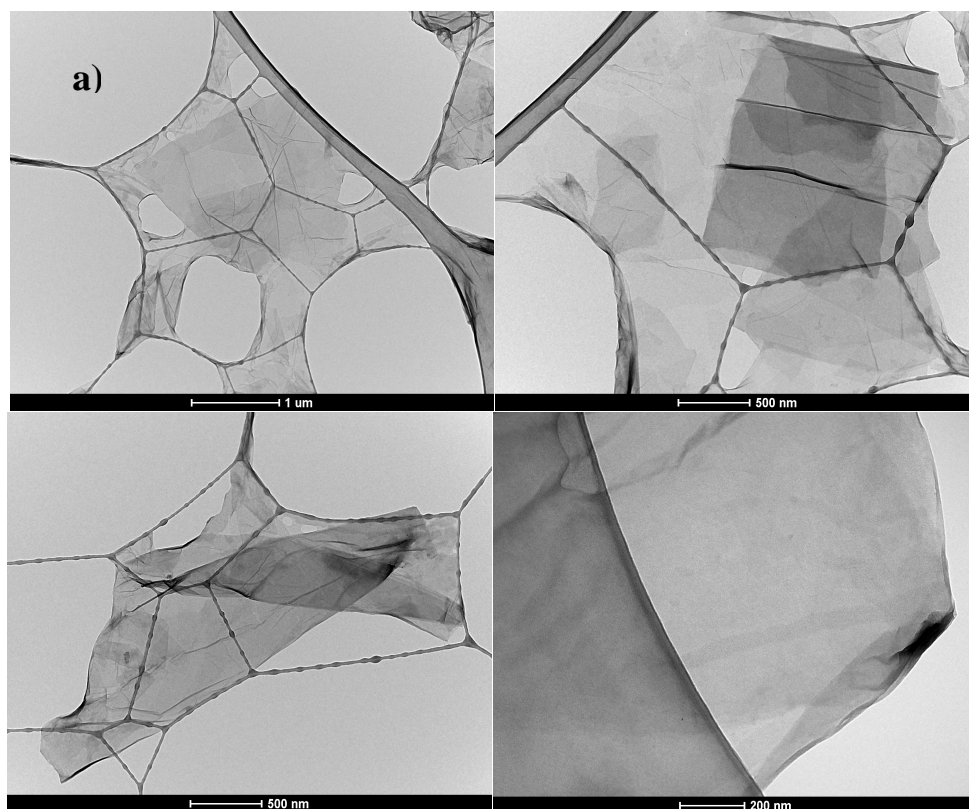
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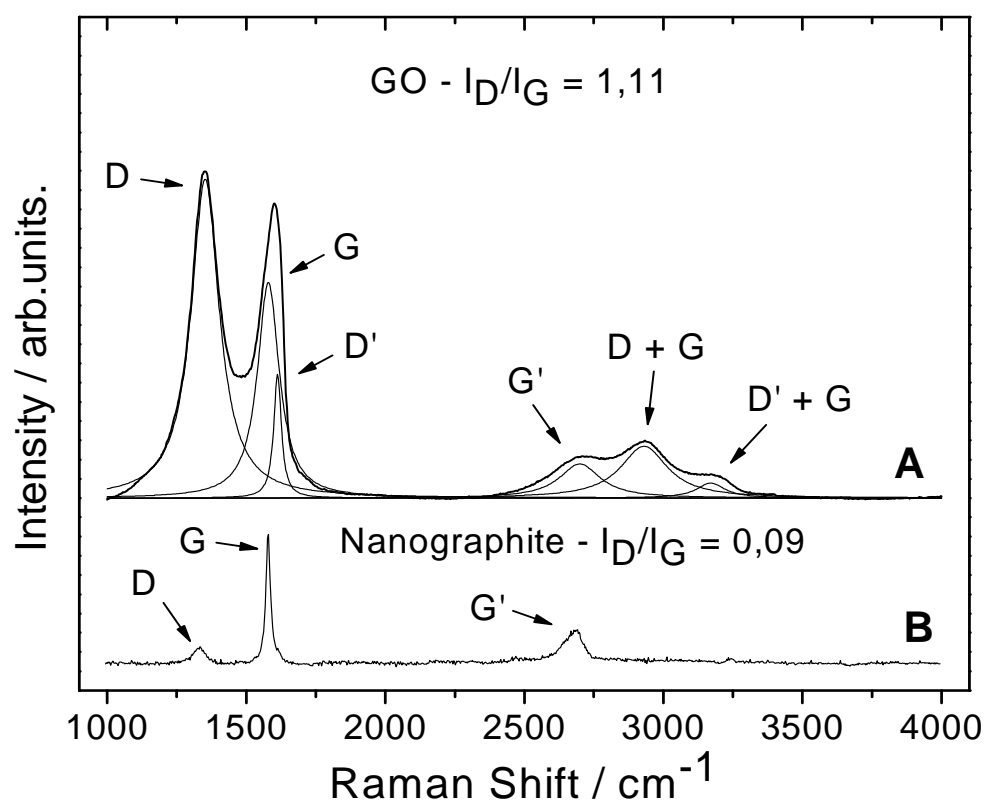
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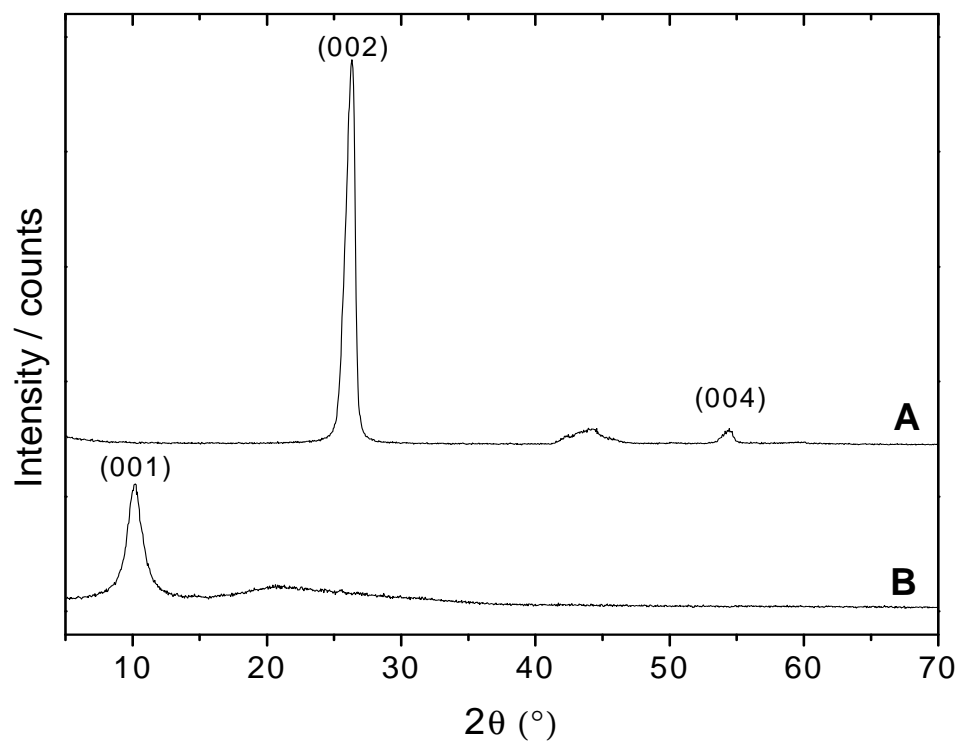
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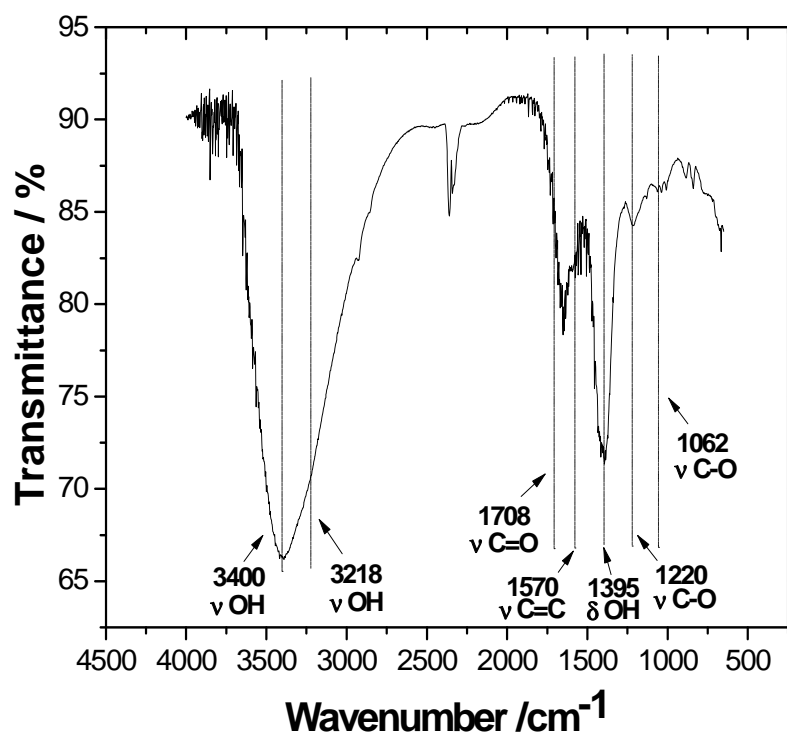
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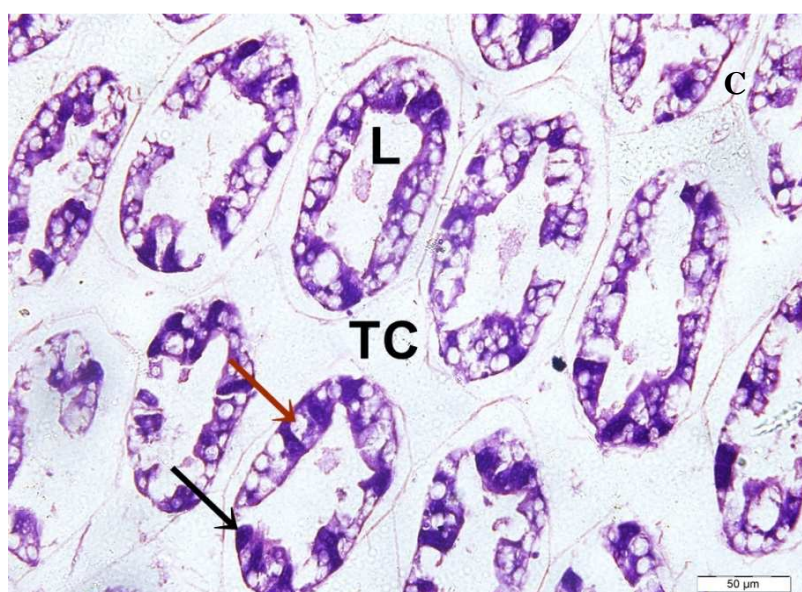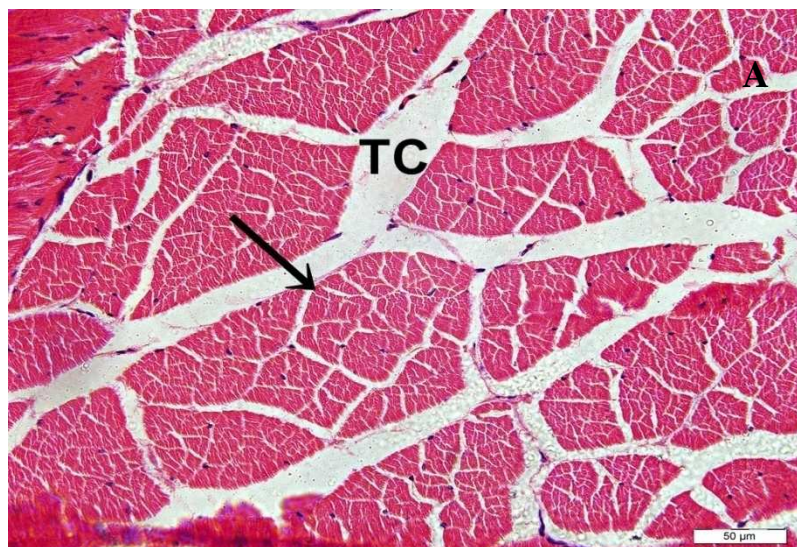
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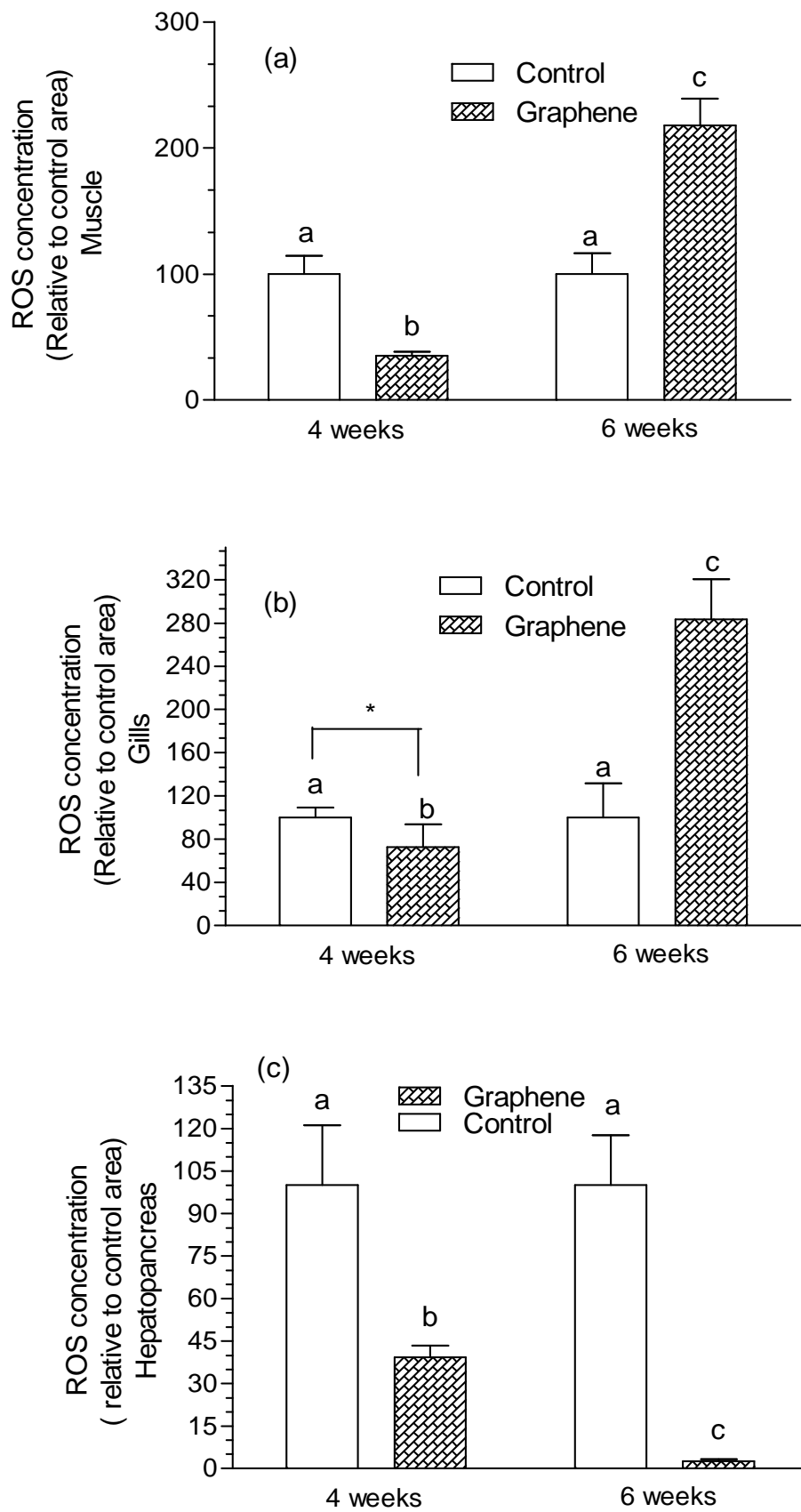
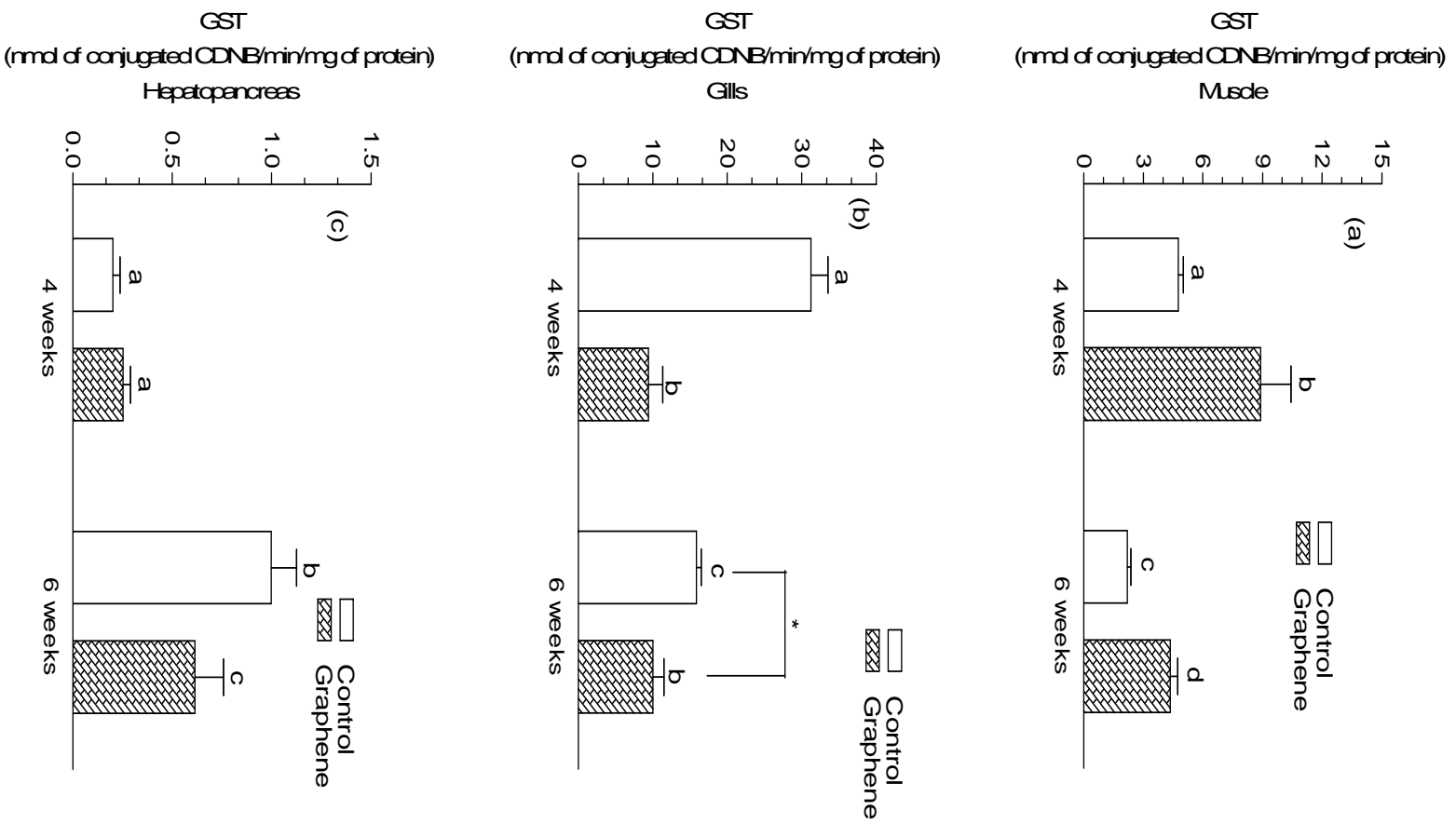**Figure 7.**
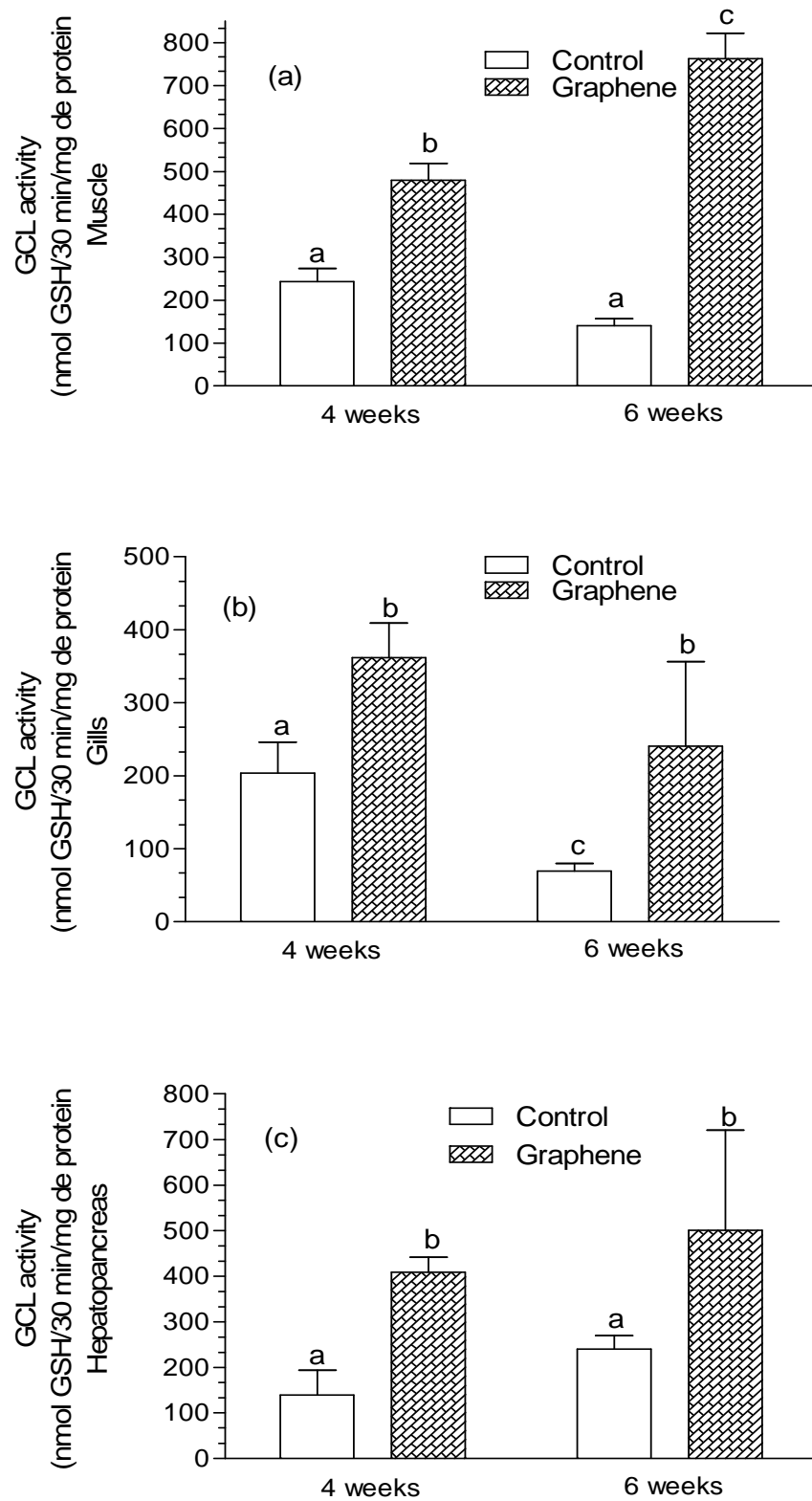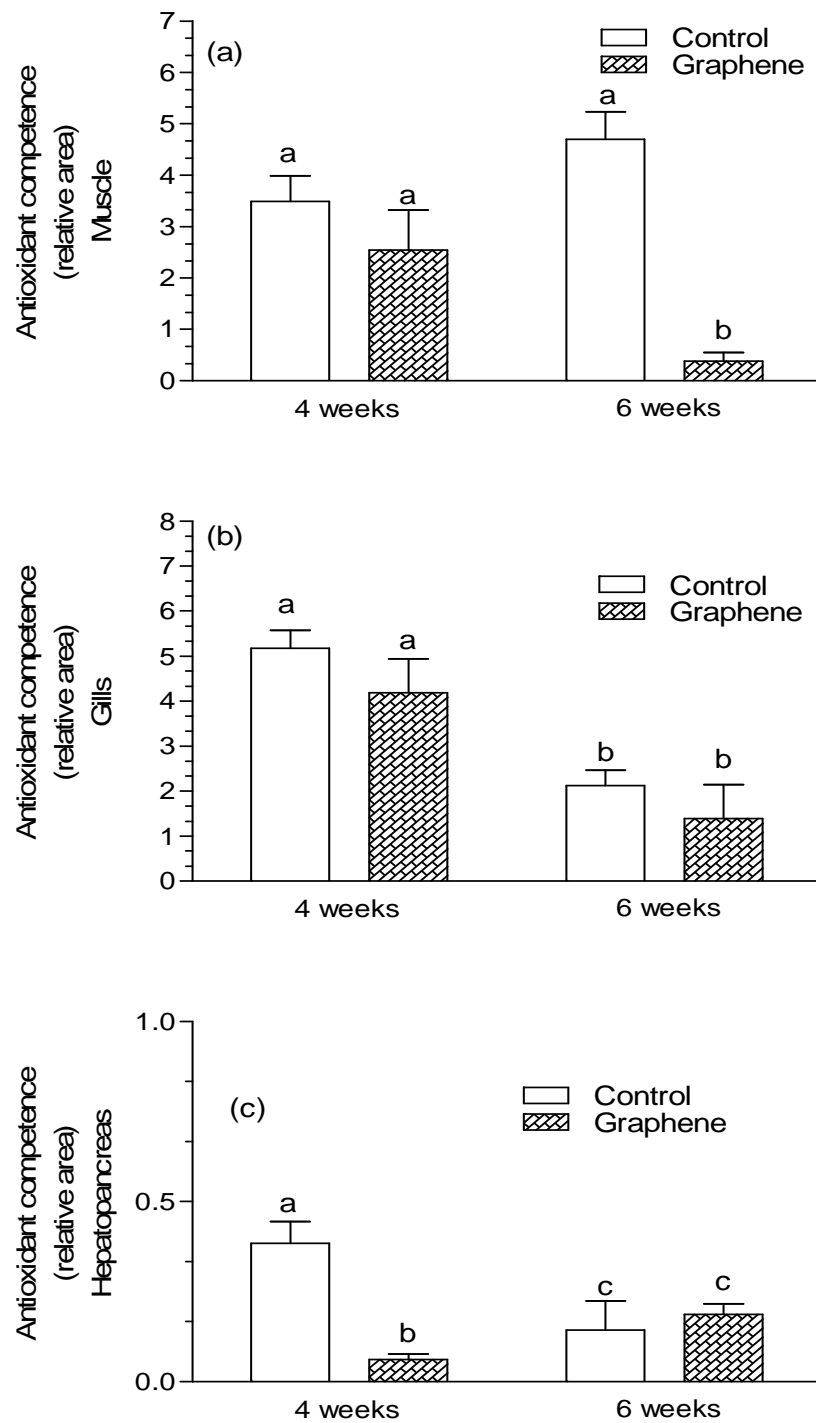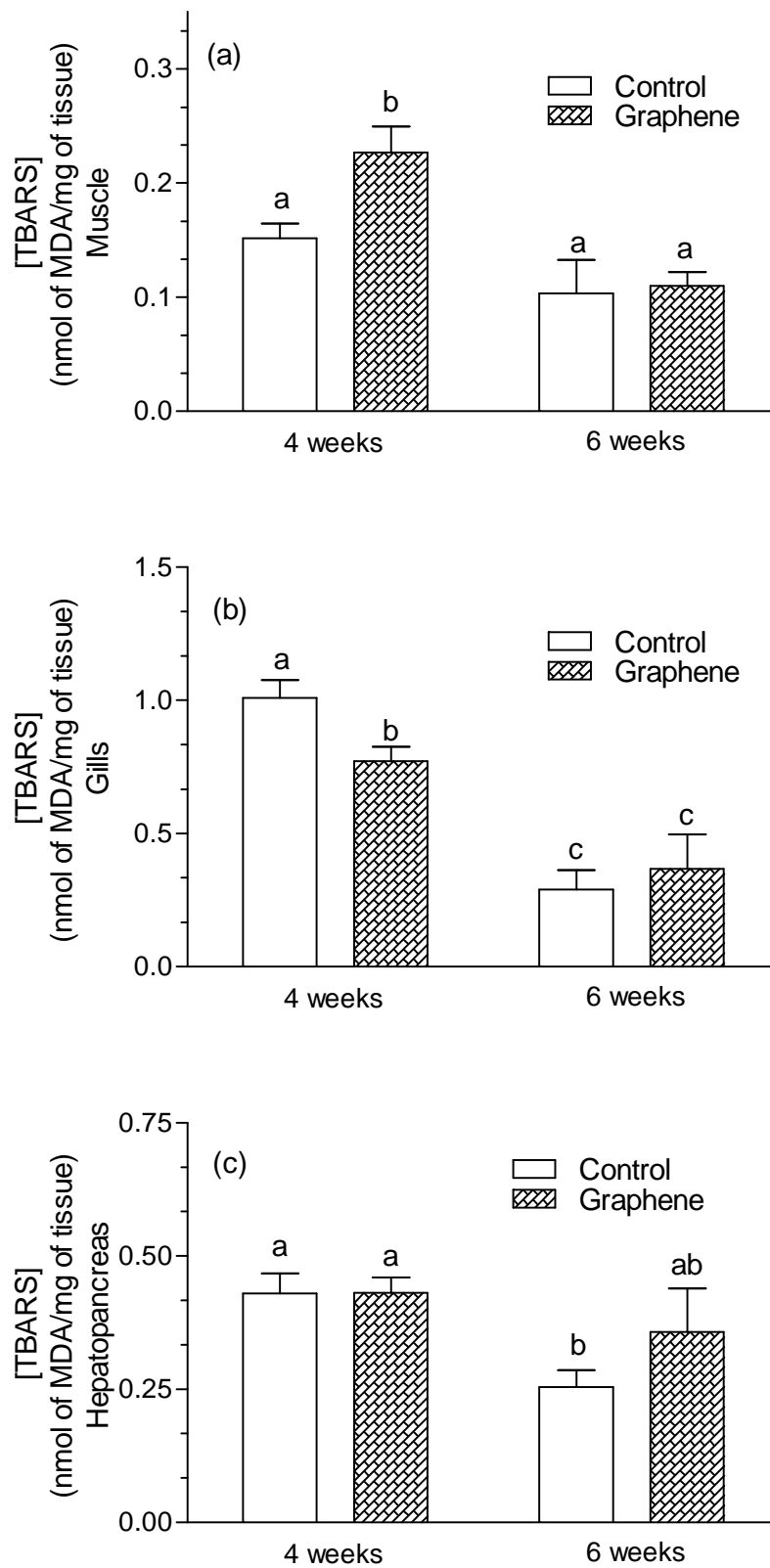
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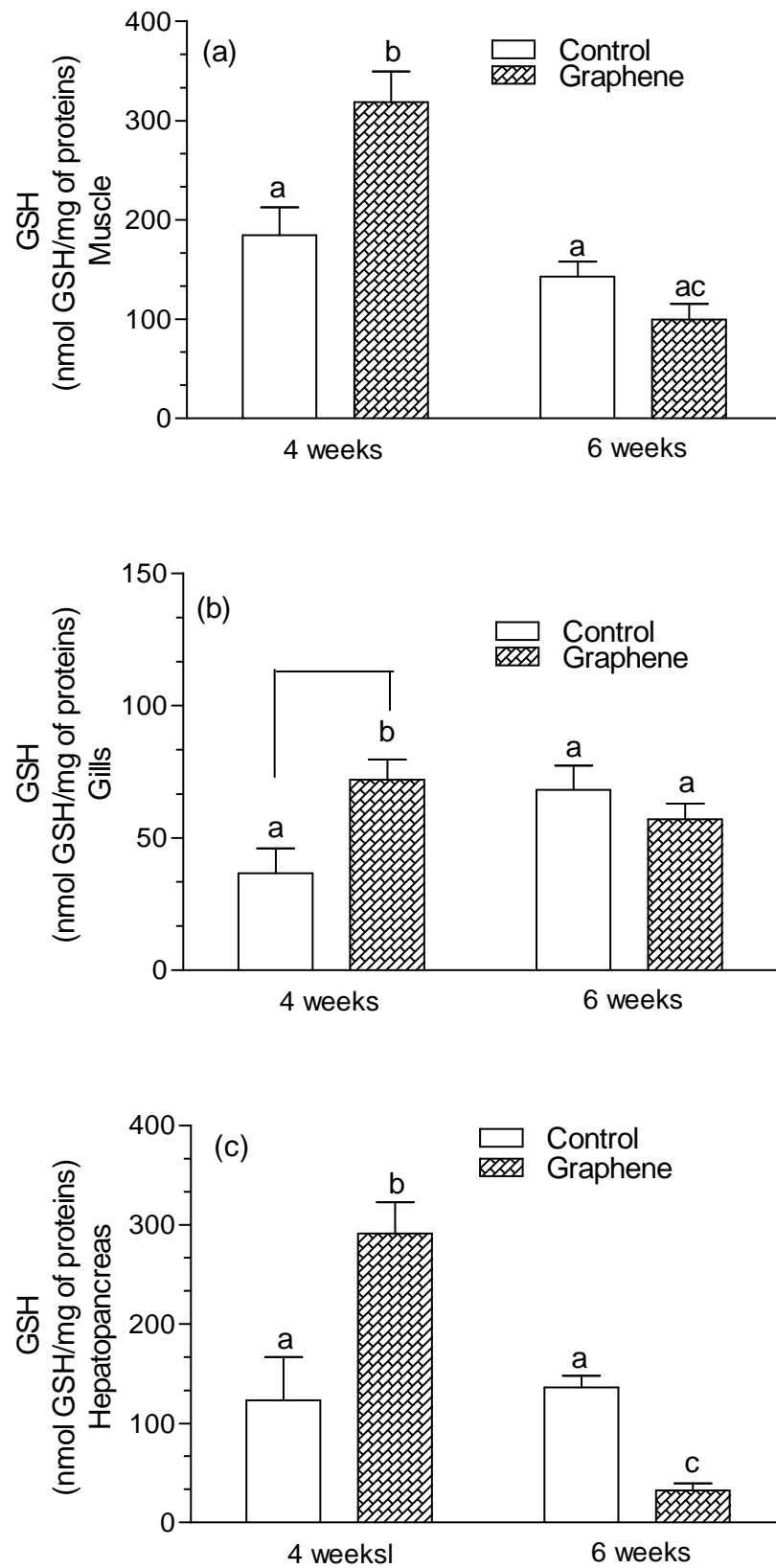
Figure 9.

Figure 10.

Figure 11.

9. Conclusões Gerais da dissertação

- O efeito da exposição ao óxido de grafeno através da ração em *Litopenaeus vannamei* mostrou efeitos diferenciados quando o tempo é lavado em consideração. Sendo que em quatro semanas de exposição houve uma diminuição dos níveis de espécies ativas de oxigênio, aumento da atividade da enzima glutamato cisteína ligase, níveis de glutationa reduzida. Estes resultados podem representar uma dualidade de interpretação de resultados visto que existe a possibilidade de a exposição ao óxido grafeno ter alterado o estado redox das células favorecendo uma situação pró-oxidante estimulando o sistema antioxidante que resultou na diminuição significativa de espécies ativas de oxigênio. Outra possibilidade (embora seja ainda especulativa) é que a interação deste nanomaterial (devido as suas propriedades fisico-químicas) com proteínas como, por exemplo, a GCL que resultou no aumento dos níveis de GSH diminuindo a produção de EAO basal, apresentando neste último caso um papel antioxidante.

- No caso da exposição durante seis semanas o aumento da atividade da GCL não resultou no aumento da GSH, também neste mesmo grupo foi observado um aumento dos níveis de EAO, sugerindo uma situação pró-oxidante. Isto pode ser devido a maior acumulação deste NM que, por conseguinte estimula a produção destas espécies.

- Embora se necessitem mais estudos para diferenciar o possível papel pró ou anti-oxidante do óxido de grafeno em *L. vannamei*, os resultados sugerem que este NM afeta o organismo do animal podendo representar um risco a homeostasia destes. Por exemplo, o aumento da atividade da GCL leva a um aumento da síntese de GSH que é

custosa energeticamente para as células, este aumento na utilização de ATP para a síntese do tripeptídeo pode levar a redução de outros processos fisiológicos que utilizam ATP como síntese de outras proteínas bem como o crescimento do animal.