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Toxicity and possibly Reduced Graphene Oxide cellular interaction with *Raphidoceles subcapitata*: Ultrastructural analysis

Toxicidade e possível interação celular do Óxido de Grafeno Reduzido com *Raphidoceles subcapitata*: Análise ultraestrutural

Toxicidad y possible interacción celular del Óxido de Grafeno Reducido con *Raphidoceles subcapitata*: Análisis ultrastructural

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Abstract
Reduced graphene oxide (rGO) is a nanomaterial formed by carbon, presented as a graphene oxide derivative, and due to its properties, it is used in areas such as in microelectronics, mechanics, and biomedicine. Despite a large number of tests conducted with this nanomaterial, there is not yet a consensus about its toxicity, when in the environment. The aquatic environment is usually the final destination of those compounds and, for this reason, the green algae is often used as a bioindicator. This study aimed to determine the ecotoxicity and possible interactions of the rGO nanoparticle with the green algae cell of *Raphidocelis subcapitata*. The structural changes in the algae, exposed to different concentrations the rGO, were analyzed through transmission electron microscopy (TEM) and Raman spectroscopy, the toxicity was assessed by measure the inhibition of algal biomass. The results indicate that there was no toxic effect on the studied organism, except in the higher concentration (100 mg.L -1). TEM analysis demonstrated an interaction of the nanoparticles with the algal cell, by observation of the internalization of the nanoparticles, as well as by rGO deposition on the cell membrane. Despite the absence of toxicity at low concentrations, the organisms showed sensitivity to the presence of the rGO. Those results contribute to the literature in the clarification of the behavior of carbon-based nanoparticles in the aquatic environment and may allow better care with the production and release of those nanoparticles in the environment.
Keywords: Reduced graphene oxide (rGO); *Raphidocelis subcapitata*; TEM; Ecotoxicity.

Resumo
O óxido de grafeno reduzido (rGO) é um nanomaterial formado por carbono, apresentado como um derivado do óxido de grafeno e, devido às suas propriedades, é utilizado em áreas como microeletrônica, mecânica e biomedicina. Apesar da grande quantidade de testes realizados com esse nanomaterial, ainda não há consenso sobre sua toxicidade, quando no meio ambiente. O meio aquático costuma ser o destino final desses compostos e, por isso, as algas verdes costumam ser utilizadas como bioindicador. Este estudo teve como objetivo determinar a ecotoxicidade e possíveis interações da nanopartícula de rGO com a célula de algas verdes de *Raphidocelis subcapitata*. As alterações estruturais nas algas, expostas a diferentes concentrações a rGO, foram analisadas através de microscopia eletrônica de transmissão (TEM) e espectroscopia Raman, a toxicidade foi avaliada por meio da medida de inibição da biomassa algal. Os resultados indicam que não houve efeito tóxico no organismo estudado, exceto na maior concentração (100

mg.L-1). A análise de TEM demonstrou uma interação das nanopartículas com a célula algal, pela observação da internalização das nanopartículas, bem como pela deposição de rGO na membrana celular. Apesar da ausência de toxicidade em baixas concentrações, os organismos mostraram sensibilidade à presença do rGO. Esses resultados contribuem com a literatura no esclarecimento do comportamento das nanopartículas à base de carbono no ambiente aquático e podem permitir um melhor cuidado com a produção e liberação dessas nanopartículas no meio ambiente.
Palavras-chave: Óxido de grafeno reduzido (rGO); *Raphidocelis subcapitata*; TEM; Ecotoxicidade.

Resumen

El óxido de grafeno reducido (rGO) es un nanomaterial formado por carbono, presentado como un derivado del óxido de grafeno, y por sus propiedades se utiliza en áreas como la microelectrónica, la mecánica y la biomedicina. A pesar de un gran número de pruebas realizadas con este nanomaterial, aún no existe un consenso sobre su toxicidad, cuando se encuentra en el medio ambiente. El medio acuático suele ser el destino final de esos compuestos y, por esta razón, las algas verdes se utilizan a menudo como bioindicador. Este estudio tuvo como objetivo determinar la ecotoxicidad y las posibles interacciones de la nanopartícula rGO con la célula de alga verde de *Raphidocelis subcapitata*. Los cambios estructurales en las algas, expuestas a diferentes concentraciones de rGO, se analizaron mediante microscopía electrónica de transmisión (TEM) y espectroscopía Raman, se evaluó la toxicidad midiendo la inhibición de la biomasa algal. Los resultados indican que no hubo efecto tóxico en el organismo estudiado, excepto en la concentración más alta (100 mg.L -1). El análisis TEM demostró una interacción de las nanopartículas con la célula de las algas, mediante la observación de la internalización de las nanopartículas, así como por la deposición de rGO en la membrana celular. A pesar de la ausencia de toxicidad a bajas concentraciones, los organismos mostraron sensibilidad a la presencia de rGO. Estos resultados contribuyen a la literatura en el esclarecimiento del comportamiento de las nanopartículas a base de carbono en el medio acuático y pueden permitir un mejor cuidado de la producción y liberación de esas nanopartículas en el medio ambiente.
Palabras clave: Óxido de grafeno reducido (rGO); *Raphidocelis subcapitata*; TEM; Ecotoxicidad.

1. Introduction

The nanomaterials have growing development and important application due to their electrical, mechanical, and thermal properties (Toma, 2005, Paschoalino et al., 2010; Levin et al., 2016). Carbon nanomaterials such as fullerenes, carbon nanotubes and graphene are the most widely researched class of materials and hold immense potential to impact several scientific disciplines (Dresselhaus,et al., 1996, Geim, 2009, Lalwani & Sitharaman, 2013) such as as microelectronics, biotechnology and more recently biomedicine (Quyen Chau et al., 2015, Yang et al., 2013, Mendonça et al., 2016, Rezayi et al., 2019; de Paula, 2020).

Graphene is an allotrope of carbon that consists of a two-dimensional flat sheet heavily compressed with a thick atomic structure, hexagonal grids, and sp2 hybridization (Georgakilas et al., 2016). Graphene and its derivatives include mono-layer graphene, few-layer graphene (FLG), graphene oxide, (GO), reduced graphene oxide (rGO), graphene nanosheets (GNS), and graphene nanoribbons, etc. (Park et al., 2009). GO and rGO are the most vital chemical graphene derivatives of the graphene-family nanomaterials (GFNs), which attracts increasing attention for its potential biomedical applications, high stability after dispersion in various solvents, facilitating handling and processing of graphene-containing nanocomposites (Kim et al, 2017). Graphene-based materials usually have sizes ranging from several to hundreds of nanometer and are 1-10 nm thick (Shen et al., 2012) which is also the definition of ‘nanoparticles’ or ‘nanomaterials’

The potential widespread use of graphene-based nanomaterials for commercial products and science applications will increase their interactions with biological and environmental constituents. So, products containing nanomaterials as the rGO can generate potentially harmful waste, that when disposed of in environmental matrices can cause toxicity in the system-wide (Lam et al., 2006; Lalwani et al., 2014).

Depending of application there are a number of pathways by which graphene from these products can enter the environment, including production line (e.g. dust, wastewater), transport, direct use or disposal of used graphene-based products in landfills (Chen et al., 2013). This rapid diversification and intensive expansion in the use of graphene nanomaterials (GNMs) are increasing the repercussions and impact of their extensive use to the environment and ultimately to

human health (Oberdorster, 2010, Bacchetta et al., 2018). Unfortunately, the legislation regarding the use and safety of nanomaterials (including graphene) usually falls behind the fast development, progress and market penetration of nanomaterial-based products (Markovic et al., 2020).

Nanomaterials still lack data on its bio-toxicity, bioaccumulation, and biodegradation (Petersen & Henry, 2012, Irazusta et al., 2018, Debashish et al., 2019). To ensure the safe and sustainable development of this innovative technology, evaluation of its biological and ecological risk, as well as finding innovative solutions to mitigate the potential hazard, are essential (Kraegeloh rt al., 2018; Guiney et al., 2018).

The use of algae species for ecotoxicology studies is common due to its sensitivity, and the fact that they are primary producers and primary components of the food chain (Amengual-Morro, 2012, Farias et al., 2018). Since many species serve directly from it as a food source, changes in the composition or productivity of the algae community can induce direct or indirect structural changes in across the ecosystem (Janssen & Heijerick, 2003, Sørensen et al., 2016, Sousa et al., 2019).

This work aimed investigate the potential toxicity of an engineering nanoparticle rGO, in an aquatic toxicity assay, looking for a possible cellular interaction with green algae *Raphidocelles subcapitata* species.

2. Methodology

2.1 rGO sinthesis

The material (rGO) has been made in the nanotechnology laboratory at the Faculty of Electrical and Computer Engineering. In the following way: the main interest in developing new techniques for preparing nanostructured materials stems from the fact that the materials may have new or better properties than those in the bulk state. It was carried out a method of synthesis of carbon nanostructures from combined pyrolisis of thin-film polymers and carbon chemical vapor deposition (CVD) from camphor diluted in acetone, hydrogen and high concentration nitrogen. It was employed Copper substrates and, after cleaning the substrates have been coated with polymer(polyaniline) with low molecular weight and electrically conductive. The polyaniline was previously diluted in dimethylformamide and then dried in a hotplate in air at 373 K for 15 min. In the sequence, the samples were immersed in the reaction chamber of a hot-filament CVD system fed with mixture camphor and acetone diluted in hydrogen (15 % vol.) and nitrogen (85 % vol.). A total flow rate of about 100 sccm, regulated by precision mass flow, and a total pressure of about 20 Torr were maintained throughout. The deposition temperature was 723 K. The material morphology was characterized by Field emission scanning electron microscopy (FESEM), Transmission electronic microscopy (TEM) and Raman Spectroscopy. The equipment used for this work was Inpect F50 from the FEI company, Jeol for 200v and Renisham Invia Raman with infrared (λ= 785nm) wavelength.

2.2 Ecotoxicity Assay

Aquatic toxicity test was carried out with *Raphidocelis subcapitata* algae as a bioindicator, according to the protocol of the Environmental Canada EPS1/RM/25 (1992).

2.3 TEM Analysis

Samples of algae suspension or algae suspension with rGO (100 $mg.L^{-1}$) were pre-fixed with a 4% Osmium Tetroxide (OsO_4) solution for 2 hours in the refrigerator. After 2 hours, the samples were centrifuged, the supernatant was discarded and resuspending with 0,1 M of phosphate buffer (NaCl 0,15M; KCl 3mM; Na2HPO4; K2H2PO4 15mM, pH 7,4) for 15 minutes, and centrifuged again. The pellets of algae or algae with rGO were disposed on slides covered with 1% agarose gel layer. Then, the pellets were covered with another layer of agar like a "sandwich ". After this, the "sandwich" was cut into cubes (~5mm^2), wich were replaced into the microtubes and then, they were fixed in 4% glutaraldehyde.

The “sandwich” were dehydrated by immersion in 30% and 50% ethanol for 10 min each and then left overnight in 70% ethanol. After, the samples were placed in 90% and 100% ethanol for 20 minutes each. After dehydration, the samples were transferred to a microtubes containing 100% ethanol and propylene oxide (1:1) for 20 minutes. Then the samples were placed in pure propylene oxide for 15 minutes. After that, half of the volume was then discarded, completed with resin, homogenized, and left for 24 hours. The samples were then, placed in molds with pure resin and left to harden for 4 to 6 hours. Ultrathin slices cuts of the samples were made using an ultramicrotome.

2.4 Statistical analysis

The experimental groups were compared by ANOVA one-way test, followed by Turkey post-test, with the Prism 5.0 software, admitting 95% as confidence level.

3. Results and Discussion

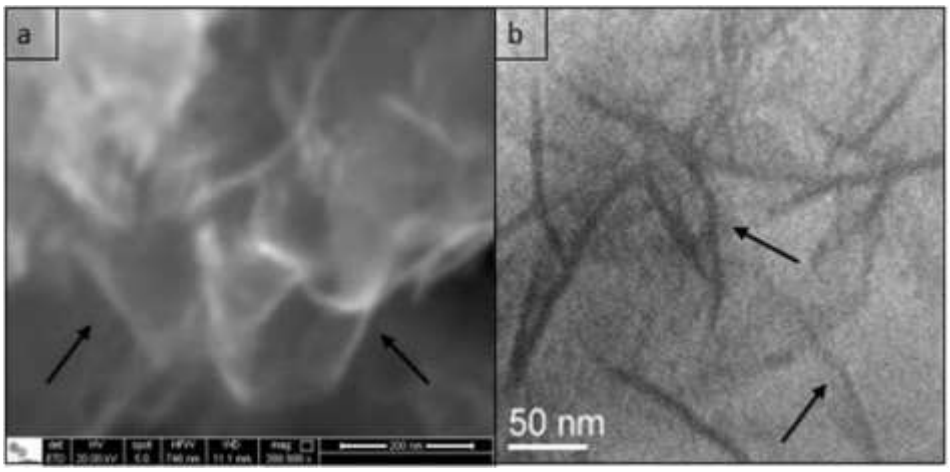
In 2004, Novoselov and Geim isolated single sheets of graphene by micromechanical cleavage of graphite or the “scotch-tape method” and characterized their quantum electrodynamics. Since then research on graphene has exploded. The number of research papers published on graphene has been increasing exponentially attracting scientists from all areas of science and technology towards the graphene studies.

Like all other pollutants, at the end of the life cycle, carbon nanomaterials are expected to end up in the environment (Nowack et al., 2013). This increases their public health and ecological risks (Petersen and Henry, 2012). The release of these

nanomaterials may eventually migrate and deposit in environmental matrices, such as aquatic environment that act as reservoirs for the pollutants, and then affect living organisms through the aquatic food chain (Begun et al., 2011, Ahmed et al., 2013, Markovic et al., 2020).

To clarify the nature of the nanomaterial, it is important its characterization by high-definition techniques, since. there are several factors which largely influence the toxicity of GFNs, such as the concentration, lateral dimension, surface structure and functionalization etc (Ou et al., 2016). The rGO morphology was verified by the TEM and SEM analyzes, presented in Figure 1 (a) and (b). The rGO showed characteristic morphology to carbon-based crystalline nanomaterials, with multilayers observing folds and wrinkles, as shown in Figure 1, this is due to the thermal synthesis process (Camargos et al., 2017; Lee et.al., 2021).

Figure 1. rGO Microscopies in (a) SEM and (b) TEM. The arrows indicate the folds and wrinkles.

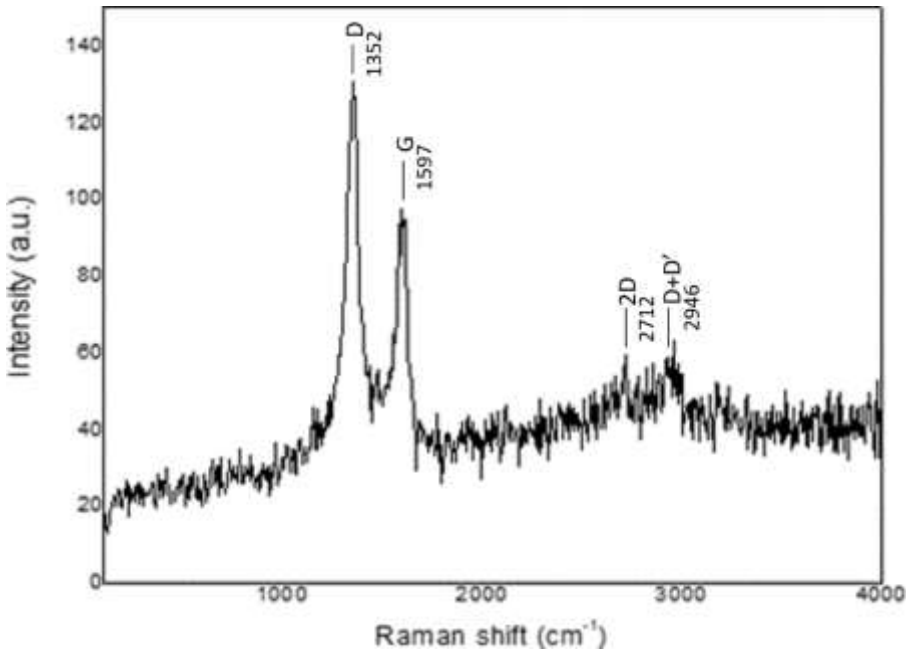


Source: Authors.

The Raman spectrum of the rGO material is shown in Figure 2, and it is possible to observe the bands G (1597 cm-1) and D (1352 cm-1), which are the main bands for the characterization of carbon-based materials. The G band corresponds to the high frequency E2g optical phonon, indicating the presence of sp2 graphical bonds, located around 1580 cm-1. However, the D band corresponds to the presence of defects and disorder in the carbon lattice, presented around 1340 cm-1. In this way, it is possible to characterize the material as reduced graphene oxide (rGO), considering the intensities of the bands, the D band has greater intensity that indicates the presence of disorder and defects, characteristics of the rGOs, due to its synthesis process that results in the incorporation of functional groups, thermal reduction, lattice disorder and defects (Wu et. al., 2018; Ferrari et al., 2013; Lee et. al., 2021).

Other bands also observed in the rGO spectrum were the 2D (2712 cm-1) and D + D '(2946 cm-1) bands. The 2D band is around 2700 cm-1, corresponds to the scattering of two photons, the appearance of this band is not related to the presence of defects, but related to the number of layers that the material has. The D + D' band corresponds to the carbon lattice defects (Wu et. al., 2018; Ferrari et al., 2013; Lee et. al., 2021).

Figure 2. Raman spectroscopy of rGO.



Source: Authors.

The use of graphene for various industrial and healthcare applications would lead to increased environmental exposure and its disposal into waste streams. Therefore, it is important to assess the short- and long-term environmental toxicity of graphene and graphene-based materials and develop effective strategies to minimize any potential deleterious impact to flora and fauna (Lalwani et al, 2016).

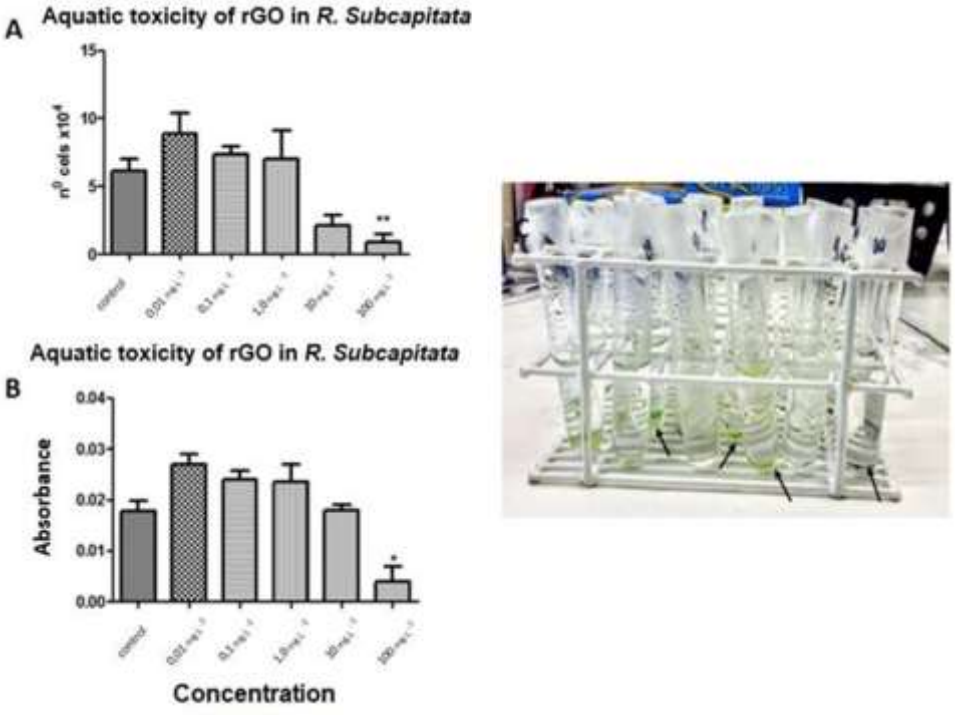
Begum et al. (2011) have investigated the phytotoxicity of graphene and its effects on root and shoot growth and shape, cell death and biomass by incubating seedlings of cabbage, tomatoes, red spinach and lettuce and observed that graphene significantly inhibited plant growth and biomass production and led to a reduction in the number and size of leaves in a dose-dependent manner, but no toxic effects were observed on lettuce at similar treatment concentrations. These results show that the phytotoxicity of graphene depends on the concentration, exposure time and plant species. Mullick Chowdhury et. al.(2014) have evaluated the post-processing effects of graphene oxide nanoribbons (GONRs) dispersed in biological buffers using various sonication steps on Medaka embryos and demonstrated precocious hatching of the embryos when exposed to

GONR solutions prepared by bath sonication. These results suggest that post-processing steps of graphene such as high-energy sonication may lead to variable environmental toxicity.

Ahmed et. al. (2013) have investigated the effects of graphene oxide on the microbial community present in wastewater, and results showed a dose-dependent toxicity with significant reduction in bacterial metabolic activity, viability, and their capacity to effectively remove nutrients such as organics, phosphorous and nitrogen from activated sludge in the presence of GO and a reduction in the concentration of nitrifying bacteria. The presence of GO in wastewater led to deterioration of the quality of final wastewater effluent (increased turbidity was observed).

In this study, the algal growth was determined by count in the Neubauer chamber and by measure the absorbance at 620 nm. The results showed a inhibition in the algal growth, only by the higher rGO concentration. The 100 mg.L-1 concentration inhibited the algal biomass growth ($p<0,05$), confirmed by the manual count (Figure 3 A) and spectrophotometric absorbances measures (Figure 3B). Visually it was observed the green biomass in the bottom of the tubes with 0.01, 0.1, 1, and 10 mg.L-1 of rGO, but not with the 100 mg.L-1 concentration as shown in Figure 3 C.

Figure 3. Figure shows the count of the cells by use of Chamber of Neubauer in a microscopy with 400x magnification (A) and by spectrophotometry at 620 nm (B) showing inhibition of biomass growth only by 100 mg.L-1 concentration. It can be see the biomass in the botton of the tubes in all, but 100 mg.L-1 (C, arrows).



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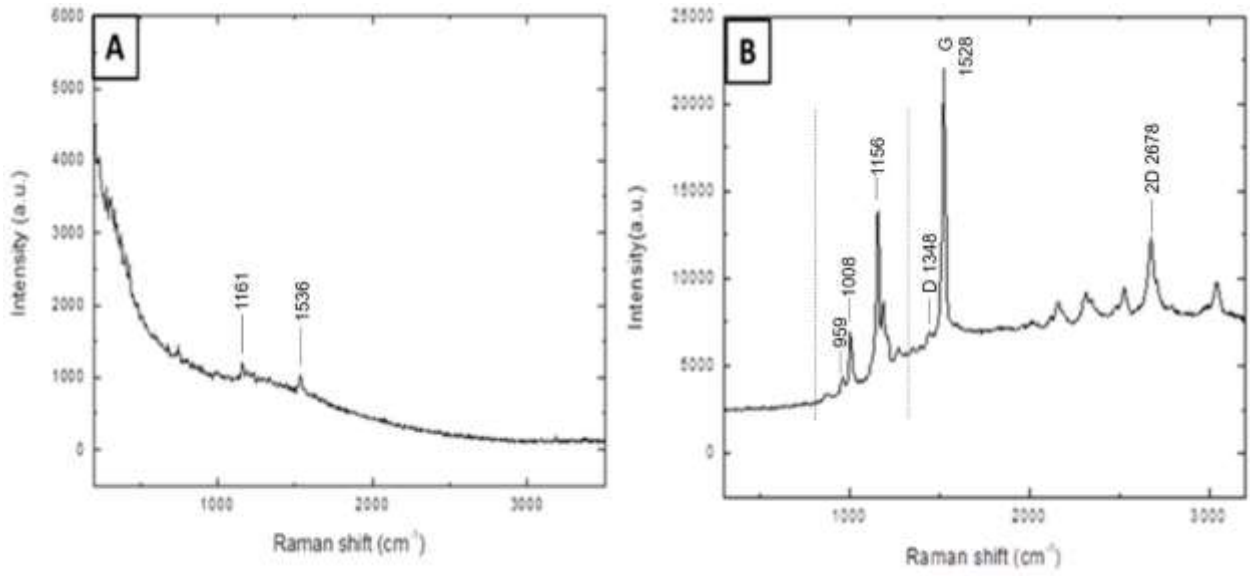
Costa et al. (2008) pointed that the high sensitivity of green algae is widely known, including for nanotoxicology studies. Recent work evaluated the hybrid metal/carbon nanotubes effects green algae *Chlamydomonas reinhardtian* (Intrchom et al., 2018), showing reduction of toxicity of Ag^+ by its association with carbon nanotubes and highlighting the usefulness of this ecotoxicological model. The microalgae bioassay has been singled out in others studies (Schwab et al. 2011; Gomes et al., 2018) who have investigated the effects of nanoparticles, like those of carbon nanotube in *Chlorella vulgaris* and *Raphidocelles subcapitata*, and they found no toxic effects of carbon nanotubes in environmental relevant concentrations, corroborating the findings of this study with the carbon nanoparticles of rGO. The number of cells/mL proved to be a reliable

endpoint to determine an effect of rGO on the growth of algae, corroborating others studies (Gomes et al. 2018, Markovic et al., 2020). These studies also showed the importance and application of those organisms for nanotoxicology assays, since changes are reflected in different carbon and nitrogen abundances in periphyton (Wängberg & Blanck, 1988). Alterations in the nutritional value of algae may impact productivity of higher-level consumers (Ozkaleli & Erden, 2018).

Despite these considerations Raman and TEM are necessary to confirm the internalization of rGO nanofraction. The use of osmium helped to avoid bias and gave a reasonably good images of algae sections, like technic proposed by Markovic et al. (2020). The Raman spectrum of algae culture without the presence of rGO is shown in Figure 4(A), which is observed the presence of characteristic bands of algae cells, such as the band around 1536 cm-1 corresponding to the clear carotenoid which is present in any plant in its cellular structure (Jehlicka et al., 2019a,b; Andreeva and Velitchkova, 2014; Reynolds et. al., 2021). Another band observed was around 1161 cm-1, which corresponds to another carotenoid common in plant and algae cells that act as a protector of chlorophyll (Gall et al., 2015; Parab and Tomar, 2012; Reynolds et. al., 2021). The Raman spectrum of the algae culture with the presence of rGO is shown in Figure 4 (B), which shows the presence of characteristic bands of the alga and rGO. The observed bands referring to the algae culture are present in the region of 959 cm-1 to 1274 cm-1, which are characteristic for the algae species (Parb and Tomar, 2012). The bands referring to the rGO are also observed, as in 1528 cm-1 corresponding to the G band, which presented a displacement for smaller wavenumbers. However, the D band (1348 cm-1) showed a decrease in intensity, which indicates a reduction in the structural defects of the rGO, and the 2D band is more intense and broader, indicating the stacking of the layers (Wu et. al., 2018; Ferrari et al., 2013; Lee et. al., 2021). Thus, one can verify that changes have occurred in raman spectrum of algae exposed to rGO, sice there was an increase in peaks produced by the algae cell, and at the same time, it can be observed characteristic peaks of the rGO.

Figure 4. Raman Spectra of *Raphidoceles subcapitata* algae culture (A) and *Raphidoceles subcapitata* algae culture exposed to rGO (B).

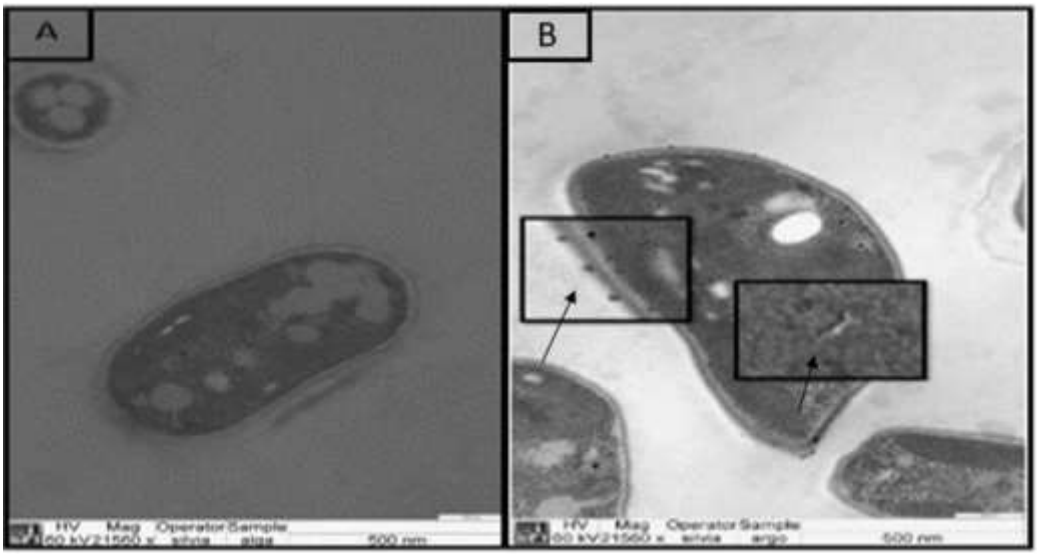


Source: Authors.

TEM analysis shows the internalization of the nanoparticle into the cell cytoplasm and also it can be see grouped particles in the membrane surface of the algal cell, as pointed by arrows in detail (Figure 5B). These characteristics are not seen in the alga cell notexposed (Figura 5A). The internalization of rGO nanoparticles was demonstrated in previous ecotoxicological studies where carbon nanotubes (Gomes et al., 2018). and graphene oxide (GO) (Markovic et al., 2020) were

it was demonstrated that these nanoparticles could be seen inside algae cells of the species R. subcapitata. Because of the different surface oxidation states of GO and rGO, GO possessing distinct hydrophilicity might be internalized and taken up by cells easily. On the contrary, rGO with evident hydrophobicity, could be adsorbed and aggregated at cell surfaces without (or with lower) uptake (Chatterjee & Choi, 2014). In a plant model with *Allium cepa*, we also have demonstrated Internalization by root cells exposed to carbon nanotubes at 10mg.L-1 (Andrade et al., 2014).

Figure 5. TEM analysis of algae cells exposed to rGO. In (A) the cell of the algal control group, in (B) the arrow in detail shows the internalization of the nanoparticle into the cell cytoplasm. In detail the arrow point grouped particles in membrane surface of the cell.



Source: Authors.

The environmental and health risks of graphene and its related particles attract considerable attention because of its wide-ranging applications in various fields and, investigations on the effects of these nanoparticles in aquatic environment are crucial, since they are released in this compartment. Recent study demonstrated that graphene oxide nanoparticles promoted toxic effects, including metabolic changes, in aquatic species at predicted environmental concentrations (ng.L-1 to µg/L-1)(Zhang et al., 2017). The present study showed no toxic effect or cellular interactions of rGO in concentrations considered environmental relevant and corroborate with the statement in previous prospective studies (Gottschalk et al., 2009, Nowack et al., 2013, Coll et al., 2016). Previous studies demonstrating internalization of rGo by *R. subcapitata* cells also highlights the need to point out that there are several factors which largely influence the toxicity of GFNs, such as the concentration, lateral dimension, surface structure and functionalization (Ou et al., 2016) and different responses can also can be attributed to changes in the nanoparticle structure that may occur in the environment (Zhao et al., 2021).

4. Conclusion

In the present study we demonstrated absence of aquatic toxicity of the rGO nanoparticle at environmental relevant concentrations. Despite this fact, we demonstrate that *R. subcapitata* cells are able to internalize the rGO and suffer biochemical alterations. Considering the possible deposition of these nanomaterials in environmental matrices, especially in the aquatic environment and considering that there are still conflicting data in the literature, one must adopt the criterion of caution in the handling of these carbon-based nanoparticles from its production process and throughout its life cycles.

Besides it is necessary more studies proposing challenges and padronization of methods in investigations of GFNs,

with the aim of completing the toxicology mechanisms, and providing suggestions to improve the biological safety of GFNs and validate their wide application.

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