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# EPS-corona formation on graphene family nanomaterials (GO, rGO and graphene) and its role in mitigating their toxic effects in the marine alga *Chlorella* sp.<sup>☆</sup>

Abhrajit Debroy<sup>a</sup>, Namrata Roy<sup>a</sup>, Sayani Giri<sup>a</sup>, Mrudula Pulimi<sup>a</sup>, N. Chandrasekaran<sup>a</sup>, Willie J.G.M. Peijnenburg<sup>b,c</sup>, Amitava Mukherjee<sup>a,\*</sup>

<sup>a</sup> Centre for Nanobiotechnology, Vellore Institute of Technology, Vellore, Tamil Nadu, 632014, India

<sup>b</sup> Institute of Environmental Sciences (CML), Leiden University, Leiden, 2300, RA, the Netherlands

<sup>c</sup> National Institute of Public Health and the Environment, Centre for the Safety of Substances and Products, Bilthoven, 3720, BA, the Netherlands

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## ABSTRACT

GFNs have widespread applications but can harm marine systems due to excessive use and improper disposal. Algae-secreted EPS can mitigate nanomaterial harm, but their impact on GFN toxicity is understudied. Hence, in the present study, we investigated the toxicity of three GFNs, graphene oxide (GO), reduced graphene oxide (rGO), and graphene, in pristine and EPS-adsorbed forms in the marine alga *Chlorella* sp. At an environmentally relevant concentration of 1 mgL<sup>-1</sup>, all three GFNs induced considerable oxidative stress and impeded growth and photosynthetic activity of the algae. The order of the toxic potential followed GO > rGO > graphene. The various facets of adsorption of EPS (1:1 mixture of loosely bound, and tightly bound EPS) on GFNs were investigated through microscopy, surface chemical analyses, fluorescence quenching studies, and isotherm and kinetics studies. Amongst the pristine GFNs treated with algal cells, GO was found to exert the maximum negative effects on algal growth. Upon adsorption of EPS over the GFNs, a significant decline in growth inhibition was observed compared to the respective pristine forms which strongly correlated with reduced oxidative stress and enhanced photosynthetic parameters in the cells. The formation of a layer of eco-corona after interaction of GFNs with EPS possibly caused a barrier effect which in turn diminished their toxic potential. The findings from the present investigation offer valuable insights into the environmental toxicity of GFNs and show that the eco-corona formation may lessen the risk posed by these materials in the marine environment.

## 1. Introduction

Different forms of graphene, which include graphene oxide (GO) and reduced graphene oxide (rGO), are together known as graphene family nanomaterials (GFNs). They possess excellent mechanical, hydrophilic, electrochemical, and biocompatible properties (Zhang et al., 2019). So the uses of GFNs have increased exponentially in wastewater treatment plants, catalysis, sensing, biomedicine, electronics, and energy storage in recent days (Nogueira et al., 2015; Priyadharshini et al., 2022). Since GFNs are produced in large quantities they are destined to end up in aquatic environments in various stages of their life cycles.

Algae are eukaryotic photoautotrophs and they play a crucial role in marine ecosystems as primary producers. They are continuously

exposed to various particulate matter in the environment (Malina et al., 2019). In this study, a marine microalga, *Chlorella* sp. is selected as a test organism for determining the toxic potential of GFNs. Most of the previous ecotoxicity assessments of GFNs in algae did not consider a comparison of effects among the various GFNs. The toxic effects of alternatively oxidized GO were studied in a cyanobacterium (*Synechococcus elongatus*), and a green alga (*Raphidocelis subcapitata*). The cyanobacterium displayed more sensitivity towards GO for all the concentrations used (0.39, 6.25, 25, 100, and 200 µg mL<sup>-1</sup>) (Malina et al., 2019). The difference in the toxic potential of six different nanocomposites of rGO was studied on two algal species (*Scenedesmus obliquus* and *Chlamydomonas reinhardtii*). The study suggested that *C. reinhardtii* was more sensitive to rGO with oxidative stress being the

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\* Corresponding author. Centre for Nanobiotechnology Vellore Institute of Technology, Vellore, 632014, India.

E-mail addresses: [amit.mookerjee@gmail.com](mailto:amit.mookerjee@gmail.com), [amitav@vit.ac.in](mailto:amitav@vit.ac.in) (A. Mukherjee).

major mode of action (Yin et al., 2020). A concentration-dependent increment in the toxicity of graphene was noticed in *Chlorella pyrenoidosa* (Guo et al., 2016). A comparative toxicity assessment among graphene, GO and rGO in *Chlorella pyrenoidosa* at 50 mgL<sup>-1</sup> concentration revealed rGO to be the most toxic material (Zhao et al., 2017a). Shading effects and hetero agglomeration were proposed to be the major modes of action.

Algal extracellular polymeric substances (EPS) are composed of a mixture of high molecular weight biopolymers such as carbohydrates, lipids, proteins, and nucleic acids (Xiao and Zheng, 2016). They form a major part of natural organic matter in the aquatic ecosystem and are known to modify the effects of various nanomaterials in aquatic biota. A previous study revealed that humic acid (HA) could reverse the toxic potential of GFNs in *Chlorella pyrenoidosa* in the order of rGO > GO > graphene. One of the key mechanisms for toxicity mitigation by HA was reportedly the reduction in membrane damage. This was accomplished by reducing both the oxidative stress and direct interactions between graphene family nanomaterials (GFNs) and algae. Weakened GFNs-algae hetero-aggregation (for rGO and graphene) and enhanced steric hindrance (for GO, rGO, and graphene) minimized direct interactions. A reduction in the direct contact between GFNs and algae also lessened the toxic effects (Zhao et al., 2019). Although previous studies underlined the influence of humic acid in mitigating the toxic potential of various GFNs, there have been no studies highlighting how the interactions with algal EPS would influence their toxicity on marine algal species *Chlorella* sp.

Previous research indicated that environmentally relevant concentrations of GFNs are in the range of 0.001 to more than 10 mgL<sup>-1</sup> (Ali et al., 2023; Pires et al., 2022; Xing et al., 2014). To the best of the authors' knowledge, no study has evaluated the effects of all three GFNs together at an environmentally relevant dose, despite prior research demonstrating the harmful effects of each GFNs individually at a variety of concentrations. Hence we have selected to study and compare the effects of various GFNs at an environmentally relevant dose of 1 mgL<sup>-1</sup>.

Thus, keeping the previous studies in mind, in the present study the authors hypothesized that GFNs induce toxicity to the algal cells by generating intracellular oxidative stress and reducing the photosynthetic efficiency and EPS-corona formation would alleviate their toxic effects. To the best of the authors' knowledge, this is the first-ever work that (i) extensively assesses toxicity GFNs at the environmentally relevant concentration of 1 mgL<sup>-1</sup> in marine microalga *Chlorella* sp; (ii) highlights the role of EPS-corona in mitigating the toxic effects of GFNs; (iii) considers contributions of both loosely bound and tightly bound EPS in the corona formation (instead of only one type). To closely resemble the conditions of real-world exposure artificial seawater was directly used as the interaction medium instead of a growth medium in this work. A layer of EPS-corona was formed on the GFNs by interacting them with a 1:1 mixture of loosely and tightly bound EPS. The adsorption of EPS was confirmed through microscopy, surface chemical, and fluorescence quenching studies, and isotherm and kinetics analysis. This was followed by assessing the toxic impact of pristine and EPS-GFNs (GFNs with EPS corona layer) by determining growth inhibition, oxidative stress, antioxidant enzyme activity, membrane integrity, and photosynthetic activity. The roles of shading effects, surface hydrophobicity, and surface charge were also considered.

## 2. Materials and methods

### 2.1. Chemicals and materials preparation

Details of the various chemicals required in this study are included in [Supplementary Methods S1](#). This study used artificial seawater (ASW) as the interaction medium similar to our previous studies (Natarajan et al., 2023; Thiagarajan et al., 2019). The method of preparation and composition of the same is listed in the supplementary section ([Supplementary Table S1](#)). The ASW was autoclaved and cooled down before

its utilization in experimental studies.

The synthesis of the GFNs was adapted from the previously published protocols (Chia et al., 2014; Roy et al., 2023; Singh et al., 2018). The details of the synthesis are provided in the supplementary section ([Supplementary Methods S2](#)).

The stock suspensions (100 mgL<sup>-1</sup>) for all the GFNs were prepared in deionized water (Lu et al., 2018) using ultrasonication (130 W, 20 kHz: Sonics, USA) for 20 min. The working stock of pristine GFNs was fixed at 1 mgL<sup>-1</sup>.

*Chlorella* sp., the marine microalgae used as a model organism for this work was acquired from CMFRI (Central Marine Fisheries Research Institute), Rameswaram, Tamil Nadu. The algal cells were cultured in Erlenmeyer flasks of 200 mL comprising NSW (natural seawater) and certain amounts of micronutrients (details are provided in [Supplementary Tables S2, S3, and S4](#)) for 15–20 days. To ensure the optimal growth of cells, the cultures were exposed to a photoperiod of 16 h illumination of 3000 lux under a white fluorescent light source (Philips TL-D Super 80, linear fluorescent lamp, India) in a temperature-controlled chamber with a temperature of 24±2 °C (Natarajan et al., 2023).

The algal cells in the exponential growth phase were centrifuged at 7000 rpm at 4 °C for 10 min. Cell pellets were then dispersed in an ASW medium to obtain the optical density of 0.5 at 610 nm. To optimize the EPS production, the algal samples were kept under fluorescent visible light conditions (3000 lux) for various time intervals of 12, 24, and 48 h followed by centrifugation at 13,000 rpm for 10 min at 4 °C (Natarajan et al., 2020). The total organic carbon (TOC) of the supernatant was measured using a TOC analyzer (Model TOC-L, Shimadzu, Japan) which showed that 12 h samples gave the maximum yield. This extracted EPS (12 h incubation) is termed loosely bound EPS (LB-EPS). For the extraction of tightly bound EPS (TB-EPS), two methods – NaCl and heating methods were followed. After the extraction of LB-EPS, deposited cells in the bottom of the tube were collected and re-suspended in 0.6% NaCl solution. The suspension was centrifuged at 13,000 rpm for 15 min and filtered with a 0.45 µm filter (Qu et al., 2012). NaCl was used here to prevent cell lysis and to maintain the osmotic equilibrium. The heating method was executed at 70 °C, using a water bath for 20 min by adding 0.1 M phosphate buffer to the cell pellet after the extraction of LB-EPS (Chu et al., 2015). After cooling, similar steps were followed as the NaCl method. TOC was quantified for both the TB-EPS and the heating method gave more yield, so this was adopted further. LB and TB-EPS were further mixed in a 1:1 ratio to obtain a homogenous mixture which was henceforth termed EPS.

### 2.2. Characterization of EPS, pristine, and EPS-GFNs

The total organic carbon content of the EPS was measured. The total carbohydrate of the EPS was measured using the phenol-sulphuric acid method (Fr/Olund et al., 1995) whereas the total protein content was assessed by the Bradford assay (Gerbersdorf et al., 2005). The functional groups were examined with the help of FT-IR (IR-Spirit Shimadzu, Japan) spectra (500–4500 cm<sup>-1</sup>) and Raman spectroscopy (acquisition at 532 nm) (Anton Paar Cora 5001, Austria). 3D-Excitation emission matrix (3D-EEM) study was performed using a fluorescence spectrophotometer (Cary Eclipse fluorescence spectrophotometer, Model – G9800A, Agilent Technologies, USA) (Zeng et al., 2023). The excitation wavelength range was 230–650 nm whereas the emission wavelength range was 250–700 nm and the slit width was 5 nm.

The details of the characterization of the pristine GFNs are provided in the supplementary section ([Supplementary Methods, S2](#)).

For the EPS-adsorbed GFNs preparation, EPS and GFNs stock was mixed at organic carbon: GFN concentration at 5:1 ratio (Grassi et al., 2020). The solution was mixed by vigorous shaking to obtain a homogeneous mixture termed as EPS-GFNs. For adsorption study EPS-GFNs were kept at room temperature (25 °C ± 2) at pH 6.8 ± 0.5 for 120 h while the samples were analyzed at 24 h interval for adsorbed carbohydrate. Samples were filtered with 0.1 µm filter and the filtrate was

collected for analysis. The amount of adsorbed carbohydrate was calculated by the subtraction of unbound carbohydrate concentration from the initial concentration (0 h) (Natarajan et al., 2023). The adsorption was further confirmed through TEM (JEOL JEM 2100 PLUS), and FESEM images (Carl Zeiss EVO/18 Research).

The kinetics of EPS carbohydrate adsorption over GFNs under pre-determined conditions were modelled using linear equations. The linear forms of the pseudo-first and second-order equations are represented as:

$$\ln(q_e - q_t) = \ln q_e - k_1 t$$

$$q_t = t / \left( \frac{1}{k_2 q_e^2} + t / q_e \right)$$

where adsorption equilibrium is represented as  $q_e$  ( $\text{mg g}^{-1}$ ) for EPS adsorbed. The EPS adsorbed at time  $t$  (min) is  $q_t$  ( $\text{mg g}^{-1}$ ) and the rate constant of the pseudo-first-order reaction is  $k_1$  ( $\text{min}^{-1}$ ). Whereas the rate constant for the pseudo-second-order reaction is  $k_2$  ( $\text{g (mg min)}^{-1}$ ) (Roy et al., 2022b).

The adsorption models of isotherms related to the study of adsorbent and adsorbate interaction under pre-determined conditions were also studied. The models of Langmuir, Freundlich, and Temkin isotherm can be calculated from the following equation:

$$\frac{1}{q_e} = \frac{1}{K_{L_{\max}}} \times \frac{1}{C_e} + 1/q_{\max}$$

$$\log q_e = \log K_f + \frac{1}{n \times \log C_e}$$

$$q_e = \frac{RT}{b_t} \times \ln A_t + \left( \frac{RT}{b_t} \right) \times \ln C_e$$

where  $K_L$  ( $\text{L mg}^{-1}$ ) is the Langmuir constant and  $q_{\max}$  ( $\text{mg g}^{-1}$ ) is the maximum adsorption capacity.  $K_f$  ( $\text{mg g}^{-1} (\text{mg L}^{-1})^{-1/n}$ ) is the constant for the Freundlich isotherm where an empirical parameter is denoted by  $n$ .  $A_t$  ( $\text{L mg}^{-1}$ ) is the isotherm constant for the Temkin model and  $b_t$  ( $\text{kJ mole}^{-1}$ ) represents the adsorption heat constant (Roy et al., 2022a).

The surface charge of pristine and EPS-GFNs was measured by zeta potential analysis (90 Plus Particle Size Analyzer, Brookhaven Instruments Corp., USA). The hydrophobicity of the samples was measured using a goniometer (Model: DMs-401, Kyowa Interface Science Co., Ltd.; software- FAMAS). Further details are given in the supplementary section (Supplementary Methods S3).

### 2.3. Mechanistic toxicity analysis

For the experiments, the algal cells were taken from the exponential growth phase and centrifuged for 10 min at 4 °C at 7000 rpm. The algal pellet was collected and resuspended in the sterile exposure media (ASW). The optical density at 610 nm by using a colorimeter was adjusted to 0.1. The interaction of GFNs and EPS-GFNs with algae was assessed with three different groups: (a) pristine GFNs, (b) EPS-GFNs, and (c) without any treatment (Control samples). Total interaction volume was maintained at 10 mL, at room temperature (25 °C ± 2) for 72 h under visible light (Philips, 3000 lux) at static conditions. The toxicity assessment of this study followed the OECD guidelines (OECD, 2002). All the experimental sets were kept in triplicates ( $n = 3$ ).

After incubation of 72 h, growth inhibition was determined by quantifying viable cells with a haemocytometer and examining them using an optical microscope (Zeiss Axiostar Microscope, USA) (Natarajan et al., 2023). The impact of the light shading on the growth inhibition of algal cells was measured (Zhao et al., 2017a). Flow cytometry and the additional details regarding these are included in the supplementary section (Supplementary Methods S4).

Total reactive oxygen species (ROS) were assessed for the control and

treated samples following the method of Thiagarajan et al., 2019. The details of the methods are added in the supplementary section (Supplementary Methods S5). The MDA content was measured following the method of Das et al. (2023). The details are included in the supplementary section (Supplementary Methods S6).

The SOD activity was analyzed for the control and treated samples after incubation following the method of Kono (1978); Kono (1978). The details are provided in the supplementary section (Supplementary Methods S7). The catalase activity of the treated and control samples was measured following a previous study by Giri et al. (2023). The detailed method is added in the supplementary section (Supplementary Methods S7).

The effective quantum yield of photosystem II (Y(II)), qP (Photochemical quenching), light response curve, and the electron transport rate (ETR) values of the treated and control samples were measured following the method of Giri and Mukherjee (2021); Lee et al. (2020). Further details are included in the supplementary section (Supplementary Methods S8).

### 2.4. Statistical analysis

In this study, all the experiments were performed in triplicates ( $n = 3$ ). The information is displayed as mean ± SD (standard deviation). A normal distribution test was performed on all the data generated followed by a two-way ANOVA test with a Bonferroni post-test employed in GraphPad Prism 5 to examine the statistical significance of various test groups compared to controls. To make graphs, GraphPad Prism 5, OriginLab, and Microsoft Excel were used. To comprehend the effects of EPS adsorption over GFNs, on the physicochemical and biological parameters, principal component analysis (PCA) was performed with the results.

## 3. Results

### 3.1. characterization

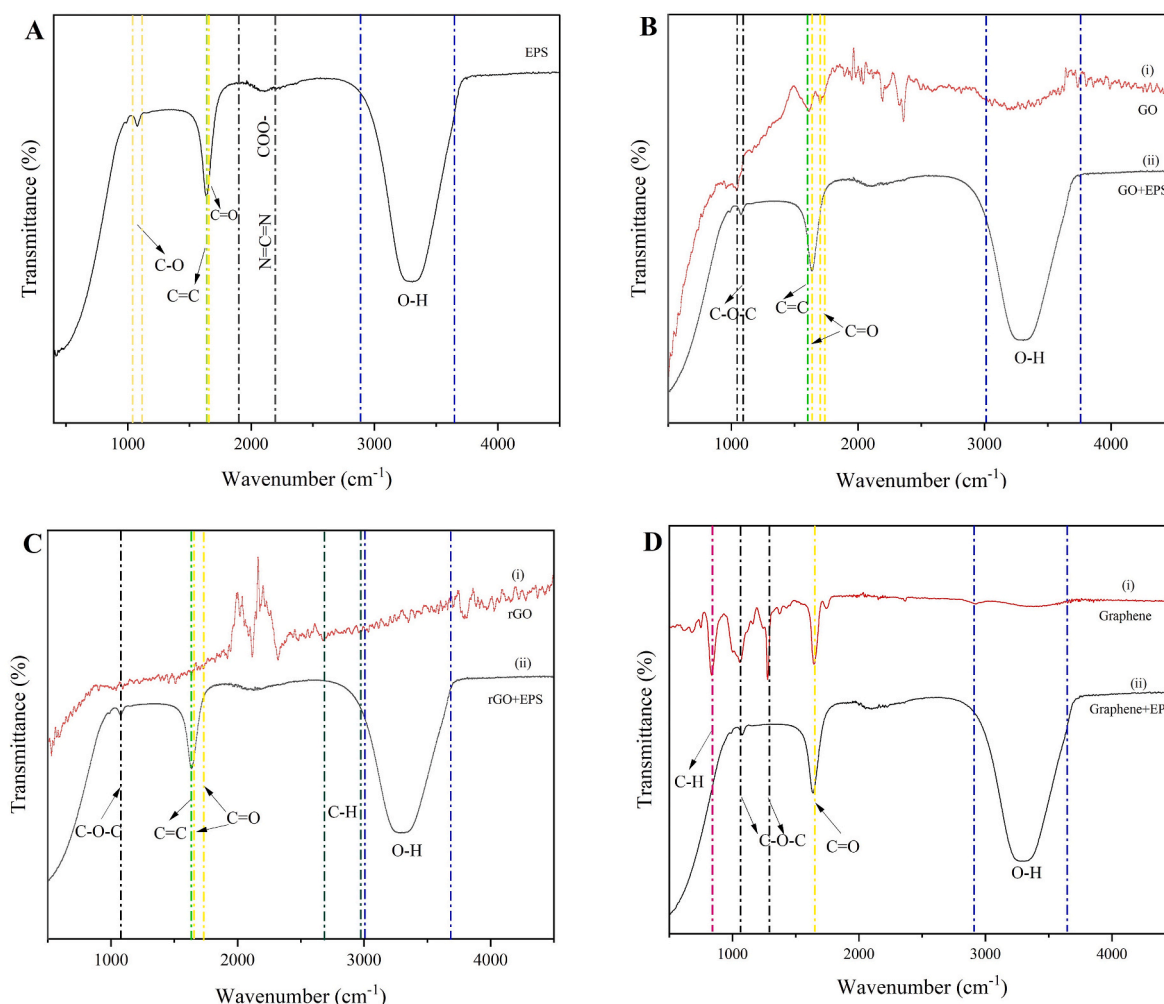
The dissolved organic carbon concentration in the algal EPS was 57.94  $\text{mg L}^{-1}$ . The carbohydrate ( $_{490 \text{ nm}}$ ) and protein ( $_{595 \text{ nm}}$ ) content in EPS were 30.85  $\text{mg L}^{-1}$  and 8.96  $\text{mg L}^{-1}$ , respectively.

The surface functional groups of EPS were analyzed using FT-IR spectral scanning (Fig. 1A). The spectra had protein peaks at 1633  $\text{cm}^{-1}$  related to C=C (amide I), and 2124-2172  $\text{cm}^{-1}$  of N=C=N stretching (carbodiimide) (Gao et al., 2019). Additionally, the peak at 1639  $\text{cm}^{-1}$  confirmed an amide I (C=O) stretching vibration (Ji et al., 2020). The glycosidic linkage of polysaccharides was confirmed by peaks at 1076  $\text{cm}^{-1}$ , and 1036  $\text{cm}^{-1}$  due to C-O vibrational stretching. The peaks at 2012-2124  $\text{cm}^{-1}$  and 2918-3604  $\text{cm}^{-1}$  were assigned to COO- symmetric stretching and O-H (polysaccharides) stretching, respectively (Zhou et al., 2016). The Raman spectra of EPS showed a distinctive peak at 1080  $\text{cm}^{-1}$  representing the C-C stretching vibrations of lipids or the C-N stretching vibrations of proteins whereas another peak at 1648  $\text{cm}^{-1}$  represented the amide I vibrations (Chen et al., 2013).

The three-dimensional excitation and emission matrix (3D-EEM) fluorescence spectra confirmed the presence of protein moieties in EPS (Fig. S2A). The region a1 (the excitation/emission wavelength (Ex/Em) of 240–250/280–300 nm) (Pan et al., 2016) was predominantly owing to the presence of aromatic protein-like substances, while the region b1 (Ex/Em = 250/460 nm) could be ascribed to humic-like substances (Lee and Hur, 2016). The surface potential of EPS,  $-2.19 \pm 0.86$  mV indicated minimal negative charge. Additionally, the contact angle of EPS was recorded at  $20.3 \pm 2.6^\circ$ .

### 3.2. GFNs characterization

An FTIR spectral scan (500-4500  $\text{cm}^{-1}$ ) of the GFNs shows the



**Fig. 1.** FTIR spectra of (A) extracted algal EPS, (B) (i) GO and (ii) GO-EPS, (C) (i) rGO, and (ii) rGO-EPS, and (D) (i) graphene and (ii) graphene-EPS.

surface functional groups in Fig. 1 (Bi, Ci, and Di). The pristine GO and rGO represented peaks at  $1041\text{ cm}^{-1}$  and  $1087\text{ cm}^{-1}$  for C–O–C epoxide vibrations. The C=C vibrational stretching at  $1613\text{ cm}^{-1}$  and  $1630\text{ cm}^{-1}$  confirmed the aromatic graphitic domains in GO and rGO (Ebrahimi Naghani et al., 2023; Singh et al., 2018). The peaks at  $1705\text{ cm}^{-1}$ ,  $1730\text{ cm}^{-1}$ , and  $1644\text{ cm}^{-1}$  were owing to C=O stretching of ketonic species (Sánchez-Campos et al., 2020). Moderately strong peaks of C–H at  $2696\text{ cm}^{-1}$ – $2965\text{ cm}^{-1}$  were observed for rGO (Al-Gaashani et al., 2019). A strong peak at  $843\text{ cm}^{-1}$  indicated a bending vibration of C–H in pristine graphene. Additionally, graphene showed peaks at  $1059\text{ cm}^{-1}$  and  $1283\text{ cm}^{-1}$  for C–O stretching and C–O–C stretching, respectively (Hayyan et al., 2015). The Raman spectra (Fig. S3) of the pristine GFNs showed two conspicuous bands, a very weak D band ( $\sim 1300\text{ cm}^{-1}$ ) and a sharp G band ( $\sim 1580\text{ cm}^{-1}$ ). These may be traced to in-phase vibrations of the graphite lattice (Smith et al., 2016; Zhang et al., 2022). The ratio of D band intensity to that of G band ( $I_D/I_G$ ) is indicative of distortions in graphitic sheet structures of GFNs. The  $I_D/I_G$  ratio of the pristine materials decreased in the order of graphene > rGO > GO in this work. Consequently, a comparison of  $I_D/I_G$  ratios revealed that both GO and rGO structures contained more defects/disorders than graphene.

The TEM images of pristine GFNs illustrated defined layers of nanosheets and rough edges (Fig. 2a–c) quite clearly. The FE-SEM images also supported similar surface features (Figs. S1(a–c) in Supplementary information).

### 3.3. Eco corona formation: adsorption of the EPS over the GFNs

#### 3.3.1. Structural and morphological changes

The interacted samples of EPS with GO, rGO, and graphene were investigated using FT-IR spectroscopy (Fig. 1 (B(ii), C(ii), and D (ii))). Compared with pristine EPS, the stretching vibration of the C=O group (amide I) at  $1726$  and  $1639\text{ cm}^{-1}$  was evident in the spectrum of the GO-EPS. The hydrogen bonds in EPS appear perturbed, as indicated by the widened peak at  $3348\text{ cm}^{-1}$  and intensity variations of the C–H stretching vibrations in the GO-EPS. The amide I band of rGO-EPS shifted from  $1639\text{ cm}^{-1}$  to  $1655\text{ cm}^{-1}$  in contrast to the GO-EPS, indicating significant interaction between the protein components and rGO. Enhanced intensities of both amide II and aromatic C=C bands were noted for the rGO-EPS and graphene-EPS. Further,  $1070\text{ cm}^{-1}$  to  $1076\text{ cm}^{-1}$  peaks in all the EPS-GFNs confirmed C–O–C glycosidic linkage (Rasoulzadeh and Namazi, 2017). The Raman spectra (Fig. S3) of the EPS-adsorbed GFNs showed an additional band at  $\sim 1076\text{ cm}^{-1}$ . Also, the spectra of the EPS-adsorbed GFNs were observed to have a significant change in the G band intensity which can be indicative of the EPS adsorption.

The 3D-EEM graph of the EPS (Fig. S2A) clearly shows the regions that represent protein and humic-like components. On adsorption of EPS over the different GFNs, the intensity of the two regions (Protein (a1 region): Ex/Em =  $240\text{--}250/280\text{--}300\text{ nm}$ ; humic (b1 region): Ex/Em =  $250/460\text{ nm}$ ) was reduced. The maximum reduction in the fluorescence intensity for protein (a1 region) was noted for GO (Fig. S2B). The reduction in the fluorescence intensity followed the order GO > rGO >

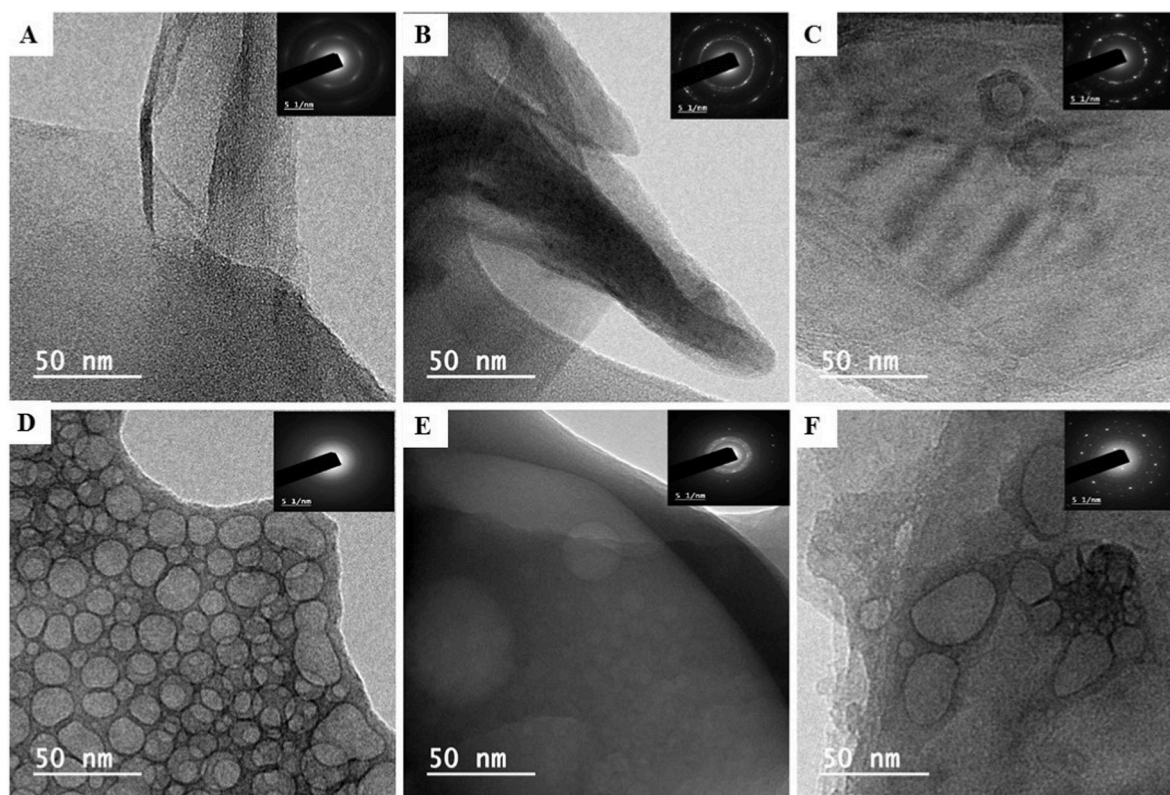


Fig. 2. TEM and SAED pattern (inset) images of (A) GO, (B) rGO, (C) graphene, (D) GO-EPS, (E) rGO-EPS, and (F) graphene-EPS.

graphene.

The TEM images showed the presence of a mixture of both wrinkles and transparent sheet structures in the case of all three GFNs. In the selective area electron diffraction (SAED) patterns of GO and rGO diffused ring structures with the interspersed bright spots confirmed the restoration of the  $\pi$  bond (Sinha et al., 2021). The crystal structure of graphene nanoplatelets was confirmed by the clearly defined diffraction spots in the case of graphene (Wang et al., 2008). In the images of the EPS-GFNs, the deposition of EPS on the graphitic sheets was visible in the form of darker patches on the transparent sheet structures. Also, these images clearly showed the clouding of the GFN sheets. A decreased number of bright spots were noticed in the SAED patterns of the EPS-GFNs. The FE-SEM imaging of EPS-GFNs showed a cloud-like structure. The layers of GFNs ( $1 \text{ mgL}^{-1}$ ) dispersed in the EPS matrix of  $5 \text{ mgL}^{-1}$  concentration showed a well-adsorbed EPS layer on the surface of GO, rGO, and graphene (Figs. S1(d–f)).

### 3.3.2. Adsorption kinetics and isotherms

Although the FTIR spectra presented confirmed the adsorption of carbohydrate as well as protein components of EPS onto the GFNs, the carbohydrate content was almost three times the protein content in the EPS. The carbohydrate component was therefore selected for studying adsorption kinetics and isotherms.

Fig. S4 (supplementary information) represents the adsorption kinetics of carbohydrates over GO, rGO, and graphene. The adsorption kinetics data fitted well with the pseudo-first-order model showing  $R^2 = 0.91, 0.99$ , and  $0.99$  for GO, rGO, and graphene, respectively.

The adsorption isotherms are presented for the interaction of EPS carbohydrates with GO, rGO, and graphene. Figs. S5a–S5c (supplementary information) illustrates the adsorption isotherm plots following Langmuir, Freundlich, and Temkin models. The sorption data for the GFNs (rGO, GO, and graphene) followed the Freundlich model with  $R^2 = 0.97, 0.94$ , and  $0.94$ , respectively. The isotherm constant ( $K_f$ ) was  $0.14, 0.15$ , and  $0.19$  ( $0 < K_f < 1$ ) for rGO, GO, and graphene,

respectively. This indicates the multi-layer adsorption of EPS carbohydrates. Furthermore, the theoretically calculated adsorption capacity ( $q_e$ ) was  $267.22 \text{ mg g}^{-1}$ ,  $250.5 \text{ mg g}^{-1}$ , and  $191.80 \text{ mg g}^{-1}$  for rGO, GO, and graphene, respectively. The quantities of EPS carbohydrates adsorbed on rGO, GO, and graphene were  $260 \text{ mg g}^{-1}$ ,  $228 \text{ mg g}^{-1}$ , and  $121 \text{ mg g}^{-1}$ , respectively.

### 3.3.3. Changes in surface charge and hydrophobicity

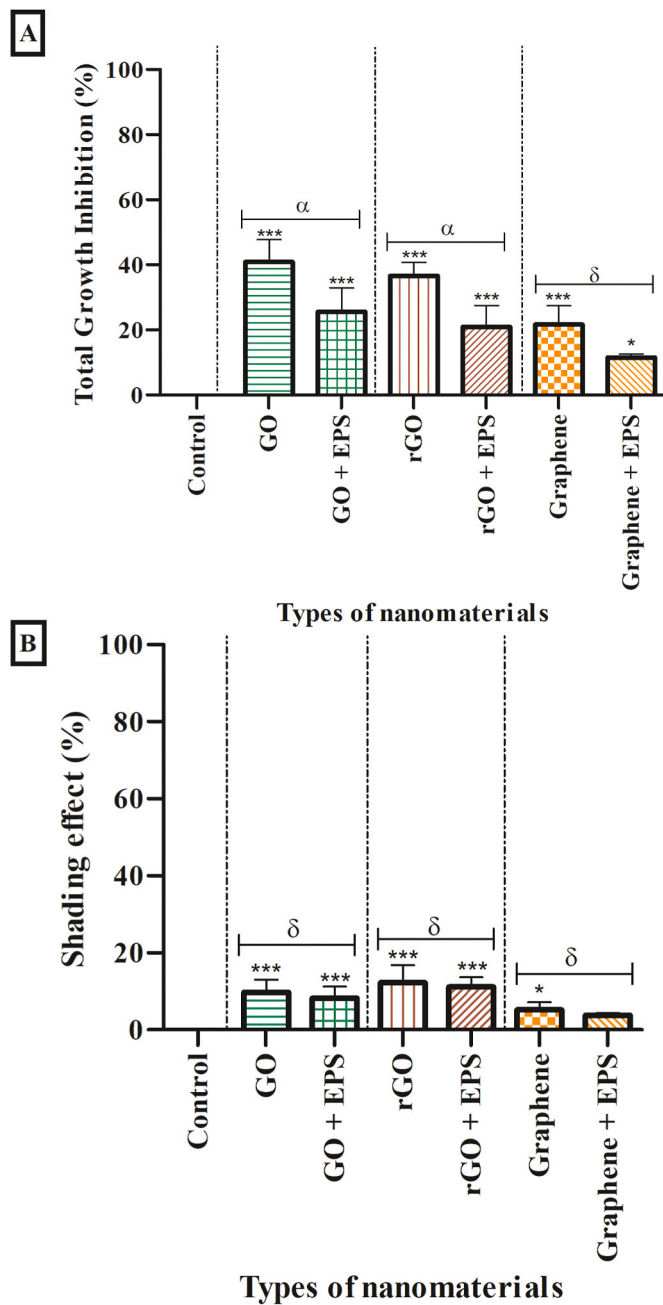
The zeta potentials of pristine GO, rGO, and graphene in deionized water were  $-17.9 \pm 1.3$ ,  $-23.1 \pm 0.4$ , and  $-22.00 \pm 0.3 \text{ mV}$ , respectively. The zeta potential of pristine GO, rGO, and graphene in the ASW medium was noted to be  $-12.3 \pm 1.1$ ,  $-8.5 \pm 2.3$ , and  $-6.1 \pm 1.4 \text{ mV}$ , respectively. After the adsorption of EPS, there was a slight decrease in the negative surface charge of GFNs to  $-10.3 \pm 0.9$ ,  $-2.7 \pm 0.4$ , and  $-4.4 \pm 0.5 \text{ mV}$  (in ASW medium) respectively.

The contact angle of marine *Chlorella* sp. was  $15.1 \pm 3.6^\circ$ . The contact angles for pristine GO, rGO, and graphene were found to be  $38.66 \pm 0.92^\circ$ ,  $32.00 \pm 2.6^\circ$ , and  $28.6 \pm 4.06^\circ$ , respectively. There was a decline in the contact angles after interaction with EPS to  $27.23 \pm 1.15^\circ$ ,  $24 \pm 2.36^\circ$ , and  $23.67 \pm 1.1^\circ$  for EPS-GO, EPS-rGO, and EPS-graphene respectively indicating enhanced hydrophilicity of the EPS-GFNs.

### 3.4. Growth inhibition

The impact of the treatment of GFNs on the direct and indirect toxicity as well as the cell viability of marine *Chlorella* sp. cells is shown in Fig. 3. Fig. 3A displays the percentage growth inhibition of the algal cells on treatment with pristine graphene, GO, and rGO respectively. The highest growth inhibition was observed in the algal cells treated with pristine GO NM which was noticed to be  $\sim 41\%$ . The order of toxicity potential based on growth inhibition followed  $\text{GO} > \text{rGO} > \text{graphene}$ .

The shading effects of the GFNs (Fig. 3B) also contributed to the growth inhibition. The contribution of shading effects towards the total



**Fig. 3.** The comparison of (A) total growth inhibition of the pristine GFNs and EPS-GFNs; (B) Shading effect of the pristine GFNs and EPS-GFNs. The level of significance for algal cells treated with GFNs with respect to control is marked with '\*\*\*\*' ( $p < 0.001$ ), ' $\alpha$ ', ' $\delta$ ' indicates a significant difference between pristine and coronated treatment groups ( $\alpha = p < 0.001$ , and  $\delta =$  no significance).

toxicity of rGO was 32.9%, the highest amongst the pristine GFNs. Indirect toxicity owing to the shading effect followed the order of rGO > graphene > GO.

When compared to the pristine treatments, all the GFNs showed a significant decline in growth inhibition upon adsorption of EPS as shown in Fig. 3A. The reduction was noted to be 9.4% for graphene, 15.3% in the case of GO and 15.7 % for rGO in comparison to their pristine counterparts. Adsorption of EPS over the GFNs also led to a decline in the shading effects (Fig. 3B).

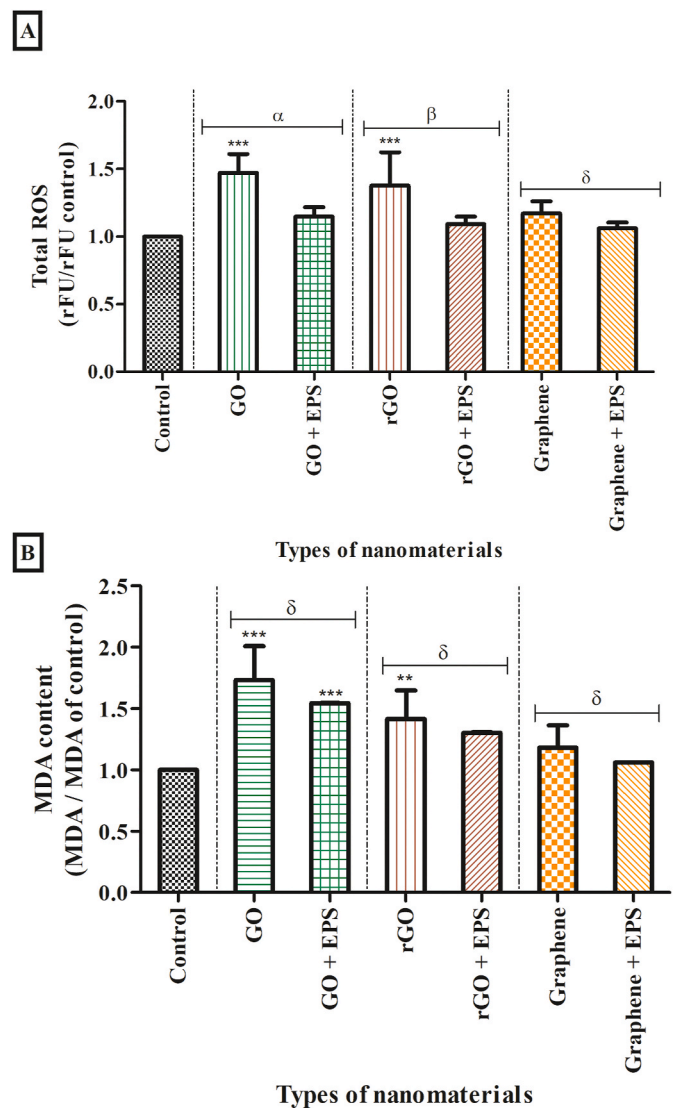
The flow cytometry results show an increase in the damage to the cell membrane of the pristine GFNs interacted cells in comparison to the control cells (0.5%) with GO having damage of 13.2%, rGO with 7.9%,

and graphene with 7.5%. Whereas upon interaction with EPS, the damage declined with EPS-interacted GO having damage of 4.5%, rGO with a damage of 3.7%, and graphene with 2.4%.

### 3.5. Oxidative stress assessment

Fig. 4A illustrates the increment in the intracellular ROS generation after treatment with pristine as well as EPS-adsorbed GFNs. The total ROS generation for the GO-treated cells was found to be the highest amongst the pristine GFNs, which was observed to be 1.47 times higher than the control. Quite similar to the growth inhibition trend, the total ROS generation of the pristine GFNs followed the order of GO > rGO > Graphene.

Upon adsorption of EPS, there was a significant decline ( $p < 0.001$ ) in the ROS generation for all the GFNs in comparison with their pristine counterparts. The decline in ROS production amongst the EPS-GFNs followed the order of GO > rGO > graphene.



**Fig. 4.** The comparison of (A) total ROS of the pristine and EPS-GFNs; and (B) MDA produced by the pristine and EPS-GFNs on the cells of the marine *Chlorella* sp.. The comparison of ROS was presented in RFU (in relative fluorescent units) of treated GFNs. The comparison of MDA was represented as a ratio of MDA of treatments/MDA of control. The level of significance for algal cells treated with pristine and coronated GFNs with respect to control is marked with '\*\*\*\*' ( $p < 0.001$ ), ' $\alpha$ ', ' $\beta$ ', ' $\delta$ ' indicates a significant difference between pristine and coronated treatment groups ( $\alpha = p < 0.001$ ,  $\beta = p < 0.01$ , and  $\delta =$  no significance).

Fig. 4B shows the changes in the MDA production after interacting with pristine and EPS-adsorbed GFNs in comparison to the control. The increase in the MDA production in the treated cells showed a similar trend to total ROS generation. Except for graphene, all the pristine GFN treatments resulted in a significant enhancement in the MDA production when compared with the control. The highest increase in the MDA production was observed for the GO-treated cells where the MDA generation was 1.73 times higher than in the case of control cells. The order of increment in the MDA generation upon treatment with pristine GFNs followed the order of GO > rGO > graphene.

The adsorption of EPS on the surface of GFNs caused a substantial decline in MDA production in the treated cells. However, the decline in the MDA generation for the EPS-adsorbed GFNs was not significant in comparison to their pristine counterparts ( $p > 0.05$ ).

### 3.6. Antioxidant enzyme activity

The changes in the antioxidant enzyme activities (SOD and catalase) upon treatment with pristine as well as EPS-adsorbed GFNs are represented graphically in Fig. S7. Like the growth inhibition and oxidative stress parameters, a general increase in the SOD activity was observed for all the GFNs treatments when compared to the control (Fig. S7A). The highest increment in the SOD activity was observed in the pristine GO-treated cells.

The comparison of the catalase activity in the algal cells treated with both pristine as well as EPS adsorbed GFNs is graphically represented in Fig. S7B. All the pristine GFNs treatments resulted in a significant increase ( $p < 0.001$ ) in the catalase activity in comparison to the control, except for the graphene. The adsorption of EPS over GO ( $p < 0.001$ ) and rGO ( $p < 0.01$ ) significantly reduced the catalase activity when compared to their pristine counterparts.

### 3.7. Photosynthetic parameters

The changes in the effective quantum yield of photosystem II (Y(II)) for the cells treated with pristine and EPS-adsorbed GFNs are represented graphically in Fig. 5A. With respect to the control samples, all the treated cells showed a significant decline ( $p < 0.001$ ) in the Y(II) value. The Y(II) value showed a considerable increase upon adsorption of EPS over the GFNs (graphene, GO, and rGO), and the increase was found to be highly significant ( $p < 0.01$ ).

The changes in ETR are graphically represented in Fig. 5B. The ETR results followed a similar trend as the Y(II) results. All the GFNs (both

pristine and EPS adsorbed) treated samples showed a decline in ETR values which was highly significant in comparison to control cells ( $p < 0.001$ ). However, the ETR values increased upon adsorption of EPS over the GFNs compared to the pristine NMs ( $p < 0.05$ ). The changes in qP along with the light response curve are represented in the [Supplementary Fig. S8](#) qP is observed to follow a similar trend with Y(II) and ETR where the cells treated with EPS-adsorbed GFNs showed an increment in qP compared to the pristine GFNs treated groups.

### 3.8. Principal component analysis

The purpose of conducting this analysis was to determine the correlation between the effects of eco-corona formation on the GFNs and their impacts on the various physicochemical parameters in the algal cells (Fig. S9). The components PC 1 and PC 2 sum up to 99.32%, which is a high level of data variance. The scatter plot collectively assembles the cell damage parameters such as growth inhibition, total ROS, and SOD in component 1. This suggests that these variables had a direct impact on the cell damage. Component 1 also includes additional parameters, such as lipid peroxidation assay (LPO) and catalase activity. Maximum photosynthetic yield and ETR were found in a different component alongside the EPS-Graphene treatment, indicating that the addition of EPS declined pristine graphene's toxic potential. Graphene and EPS-rGO treatments were situated in a separate quadrant devoid of any parameters which denoted that these treatments were not in correlation with any of the toxicity parameters.

## 4. Discussion

### 4.1. Physico-chemical characterization of GFNs and EPS-GFNs

Adsorption of carbohydrate components of the EPS followed the Freundlich isotherm model quite in agreement with previous work on algal EPS adsorption on GO and rGO (Lee and Hur, 2016). Comparing the sorption processes amongst the GFNs, the highest isotherm linearity was noticed in the case of rGO, followed by GO and graphene. A similar trend was observed for the theoretically calculated adsorption capacity. The increased adsorption capacity of EPS carbohydrates on rGO compared to GO may be attributed to the greater degree of defects in the  $sp^2$  structures and the reduced presence of oxygen-containing groups in rGO (Lee and Hur, 2016). The decreasing order of ( $K_f$ ) values (rGO > GO > graphene) confirms the presence of more heterogeneous surfaces related to the adsorption sites on rGO. Several previous studies also

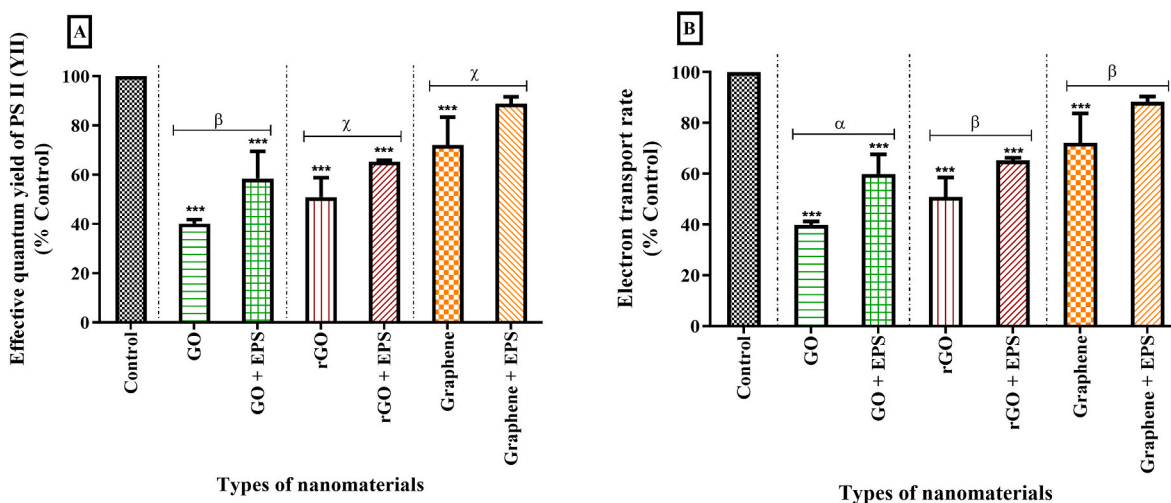


Fig. 5. Photosynthetic parameters in algal cells exposed to the GFNs were assessed. (A) The comparison of effective quantum yield of PS II (Y(II)) of pristine and EPS-GFNs. (B) The comparison of electron transport rate of pristine and EPS-GFNs. The level of significance with respect to control was represented by '\*\*\*\*' ( $p < 0.001$ ), ' $\alpha$ ' indicates a significant difference between pristine and coronated GFNs treatment groups ( $\alpha = p < 0.001$ ).

highlighted the enhanced adsorption efficiency of rGO compared to GO (Chen and Chen, 2015).

From the FTIR spectra of both GO and rGO, it is found that they contained common groups such as C–O–C, O–H, and C=O. However, rGO spectra showed an additional C–H stretch. This could influence the interactions of both the GFNs with algal cells as well as EPS. A comparison between the FTIR spectra of GFNs before and after interactions of EPS confirms the adsorption of both carbohydrate and protein components onto the NMs. The changes in the peak positions of the Amide I band, which results from the C=O stretching vibration of the peptide backbone confirm the adsorption of protein components (Lee and Hur, 2016). The differences in positions corresponding to the Amide I band between EPS-GO and EPS-rGO suggest that the chemical reduction of GO enhanced the interaction between graphene surfaces and peptide backbone structures. Enhanced intensities of both amide II and aromatic C=C bands suggest that GO reduction contributed to enhanced adsorption of certain aromatic moieties of EPS (Lee and Hur, 2016). This was further proven by the decrease in peak intensities related to protein-rich regions in 3D EEM spectra of EPS-GFNs. After adsorption, the glycosidic linkage of polysaccharides showed peaks at  $1076\text{ cm}^{-1}$ , and  $1036\text{ cm}^{-1}$  respective to C–O vibrational stretching for sugar derivatives for all the GFNs (Zhou et al., 2016) confirming carbohydrate sorption. The comparison of the Raman spectra of pristine and EPS-adsorbed GFNs reveals the addition of a new band near the D band, that was intrinsic to the EPS. After the adsorption of EPS over the GFNs the G band intensity declined considerably which influenced the  $I_D/I_G$  ratios. In a previous work decreased  $I_D/I_G$  values were noted after adsorption of humic acid on rGO and graphene owing to decreased  $sp^3$  disorders (Zhang et al., 2019). This reduction in the disordered structure of GFNs would minimize their toxic effects too (Chen et al., 2021). In the TEM images of the EPS-adsorbed GFNs, it was noticed that the wrinkles were masked by layers of EPS covering the sheets giving a cloudy appearance. The masking of the edges of GFNs possibly led to the reduction of their potential for interaction with the cells resulting in reduced toxicity (Li et al., 2022). The zeta potential of the pristine and EPS-GFNs were considerably low, which implies unstable nature of the materials in the interaction medium (Joseph and Singhvi, 2019). This suggests substantial aggregation of GFNs in the medium reducing their actual concentrations and their bio availability.

#### 4.2. Growth inhibition

The maximum growth inhibition was noticed in the cells treated with GO followed by rGO and graphene. In previous studies, rGO was found to be more toxic than GO (Zhao et al., 2019, 2017b). The purported discrepancy between the current results and previous reports may be explained by variations in the cell architecture of the different models considered. In the previous studies, freshwater algae were considered while the current work was performed with a marine strain. The exposure concentration was  $1\text{ mgL}^{-1}$  in the present work compared to  $50\text{ mg L}^{-1}$  in the previous cases. The algal cells in the current work were hydrophilic in nature. The material hydrophobicity is considered a principal factor controlling algal interactions and generally materials with higher hydrophobicity favour initial algal attachment (Tsavatopoulou and Manariotis, 2022). Initial attachment of hydrophilic algal cells to the relatively hydrophobic GFNs was therefore largely favoured. This exacerbates the possibility of mechanical damage (Girish et al., 2022). Although no substantial change in the surface charge of the GFNs was noticed after the adsorption of EPS, a significant change in surface hydrophobicity was evident for all three GFNs. EPS was found to be quite hydrophilic in nature (contact angle of  $20.30^\circ$ ). The adsorption of EPS rendered the surface of GO more hydrophilic (contact angle,  $27.23^\circ$ ) compared to their pristine counterparts. This was accompanied by a slight decrease in surface potential values. The increased hydrophilicity favoured rapid agglomeration and gravitational settling of the particles. This would lead to a declined availability of the NMs for the interactions

with the algal cells, thus lessening their cytotoxic effects. Similar observations were recorded for the other two GFNs. Additionally, decreased hydrophobicity of the GFNs would also reduce direct attachment of the NMs (Tsavatopoulou and Manariotis, 2022) thereby reducing the hetero agglomeration possibility. The flow cytometry (Fig. S6) data confirmed increased damage to the cell membranes by pristine GFNs when compared to the control. However, after adsorption of EPS over the GFNs this damage was reduced substantially. Previous reports showed that the shading effect of the GFNs played a crucial role in the toxicity of GFNs to algae (Zhao et al., 2019). However, in the current work, no noticeable changes in the shading effects of the GFNs were observed after EPS adsorption. These results are well in accordance with the changes in the morphological structures of the GFNs after EPS adsorption suggesting the positive impact of eco-corona formation.

#### 4.3. Oxidative stress generation and antioxidant enzyme activity

The  $sp^2$  hybridized electron structure of the NMs enhances intracellular glutathione oxidation or directly speeds up the electron transfer between the edges of the GFNs and biological membranes, leading to ROS accumulation in the cells (Zhao et al., 2014). GO produced the highest levels of ROS, which may be related to their better electrical conductivity and relative hydrophobicity. Increased ROS production by GO can also be related to increased direct physical contact/adhesion with the algal cells causing mechanical damage. Following interaction with EPS-sorbed GFNs the intracellular ROS levels dropped substantially. EPS is known to have a direct quenching effect on the intracellular ROS leading to a decrease in overall ROS levels in the cells (Fig. S10). An overall decrease in dispersibility and availability of the GFNs after EPS sorption would effectively reduce the intracellular ROS levels. The decreased direct contact between algal cells and GFN also contributed to the decrease in ROS production by mechanical damage (Zhao et al., 2017b). All the pristine GFNs caused considerable lipid peroxidation, and the order was similar to the ROS production ( $\text{GO} > \text{rGO} > \text{graphene}$ ) in the cells. Polyunsaturated fatty acid oxidation by ROS results in the creation of holes and membrane damage (Van Der Paal et al., 2016). EPS adsorption over GFNs essentially reduced the ROS production in the algal cells and thus lessened the membrane damage. Multiple reasons can be put forth to explain this effect. The UV-screen pigments in the EPS could effectively quench the intense ultraviolet light causing oxidative damage to the cells (Pereira et al., 2009). They may also provide consumption sites for oxidative processes outside the cell (Castenholz and Garcia-Pichel, 2012). Additionally, the GFNs may act as transporters for EPS in the medium strengthening their antioxidant capacity to scavenge ROS generated during regular physiological processes (Grassi et al., 2020).

Since it can intercept and destroy ROS, the antioxidant system is the primary defense system against environmental stress (Du et al., 2016). SOD primarily mediates the conversion of superoxide anions to hydrogen peroxide ( $\text{H}_2\text{O}_2$ ), and the  $\text{H}_2\text{O}_2$  produced is reduced by the catalase enzyme (Chakraborty et al., 2021). In the current study, SOD and catalase activity followed similar patterns as ROS generation and MDA production. The increment in the ROS generation resulted in the subsequent increase in the SOD and the catalase activities to maintain a homeostasis state within the algal cells. However, the activity of these enzymes in the cells substantially decreased after the adsorption of EPS over the GFNs, which is consistent with the observed decrease in reactive species levels. Decreased antioxidant enzyme activity for EPS-GFNs correlates well with diminished levels of intracellular oxidative stress and cytotoxicity.

#### 4.4. Impact on photosynthetic efficacy

Photosynthetic efficiency is considered as the fraction of the light energy that gets converted into chemical energy through the photosynthesis process by photoautotrophs like algae (Lau et al., 2022). A

strong correlation exists between microalgal growth and photosynthesis (Cheah et al., 2020). The effective quantum yield of photosystem II (Y (II)) and the ETR were lower in the pristine GFNs-treated algal cells than in the control group. The observed decline in the maximum quantum yield confirms that photoinhibition of PSII reaction centers was one of the major modes of action of the GFN toxicity. Additionally, a decreased ETR would signify reduced metabolic activity in the cells since photosynthetic energy is the sole energy source for cellular metabolism in algae (Cruces et al., 2021). An essential component of how plants regulate their photosynthetic quantum efficiency is fluorescence quenching. The parameter  $qP$  of the chlorophyll fluorescence is particularly significant because it represents the reduction state of QA, the main electron acceptor in PSII, which is created via QA reoxidation (Wang et al., 2020). The decline in  $qP$  upon interaction with pristine GFNs thus signifies the reduction in the photosynthetic activity. However, the interaction of EPS with GFNs leads to an increment in the  $qP$  in comparison to the pristine GFN-treated cells. A notable improvement in most of the photosynthetic parameters was observed in the treatment groups with the EPS-GFNs compared to the pristine materials indicating a recovery effect. This can be attributed to the ROS scavenging effect of EPS that causes a decline in the accumulation of reactive oxygen species within the cells. This is supported by results of the oxidative stress generation and antioxidant enzyme activities discussed in the preceding section (Gao et al., 2015; Jiang et al., 2014).

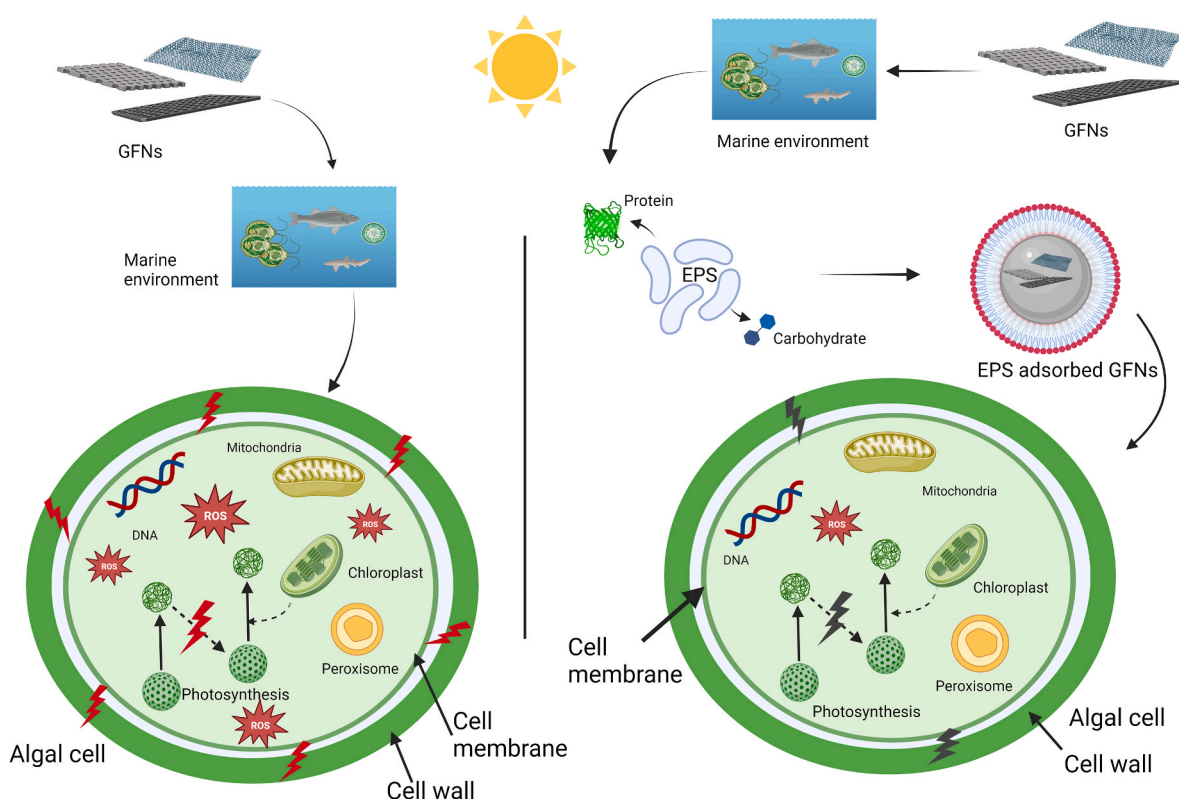
Summarizing the major modes of action (MoAs) of GFN toxicity in algae include excessive oxidative stress generation and a decline in photosynthetic efficiency. The role of EPS corona in mitigating these effects is summarized with the help of a schematic diagram (Fig. 6).

## 5. Conclusions and environmental implications

EPS exists in the marine environment ubiquitously and is known to interact readily with nanomaterials, thereby affecting their interactions with aquatic organisms. The present study provides a 'proof of concept' approach to the beneficial effects of algal EPS in mitigating the toxic potential of GFNs in marine algae. The findings suggest that GFNs are quite potent in inducing toxicity (even at  $1 \text{ mgL}^{-1}$ ) through inducing oxidative stress and photoinhibition. A layer of EPS corona formed through adsorption significantly reduced the toxic impact of the GFNs. It is to be noted that the experiments were mostly performed in a controlled system while the marine ecosystem is quite complex, comprising numerous nanomaterials, co-pollutants (dissolved and particulate forms), and natural organics. The availability of other nanomaterials, co-pollutants, and natural colloidal particles may change the way the eco-corona forms over the GFNs and interacts with the biota. Similarly, the properties of the GFNs may also vary, based on multiple synthesis routes, functionalization processes, and their end-uses. Therefore, more detailed experimental studies and theoretical understanding are required to enhance our understanding of EPS corona formation over GFNs and how they modulate their effects in marine ecosystems.

## Credit authors statement

Abhrajit Debroy: Investigation, Methodology, Visualization, Formal analysis, Writing-Original Draft; Namrata Roy: Methodology, Writing-Original Draft; Sayani Giri: Writing-Original Draft; Mrudula Pulimi: Conceptualization, Formal analysis; N. Chandrasekaran: Formal analysis, resources; Willie J. G. M. Peijnenburg: Formal analysis, Writing-Review and editing; Amitava Mukherjee: Conceptualization,



**Fig. 6.** Schematic diagram showing the mechanism of toxicity generation by pristine GFNs and how EPS addition modifies it. The red lightning strikes in the left cell of the image signify excessive accumulation of oxidative stress induced by pristine GFNs, which may result in damaging the membrane, as well as their bonding with the photosystems, which might impair the process of photosynthesis. The black lightning strike on the right cell of the image however shows a mild accumulation of oxidative stress-generating radicals, which may signify a reduction in the damage to the cell caused by EPS adsorption over the GFNs. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Methodology, Formal analysis, Supervision, Project administration, Writing- Review and editing.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

Data will be made available on request.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.envpol.2023.123015>.

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