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The role of algal-EPS in modifying the short-term and long-term toxicity of binary mixtures of TBBPA and GFNs towards marine *Chlorella* sp. cellular toxicity, uptake, and environmental risk assessment

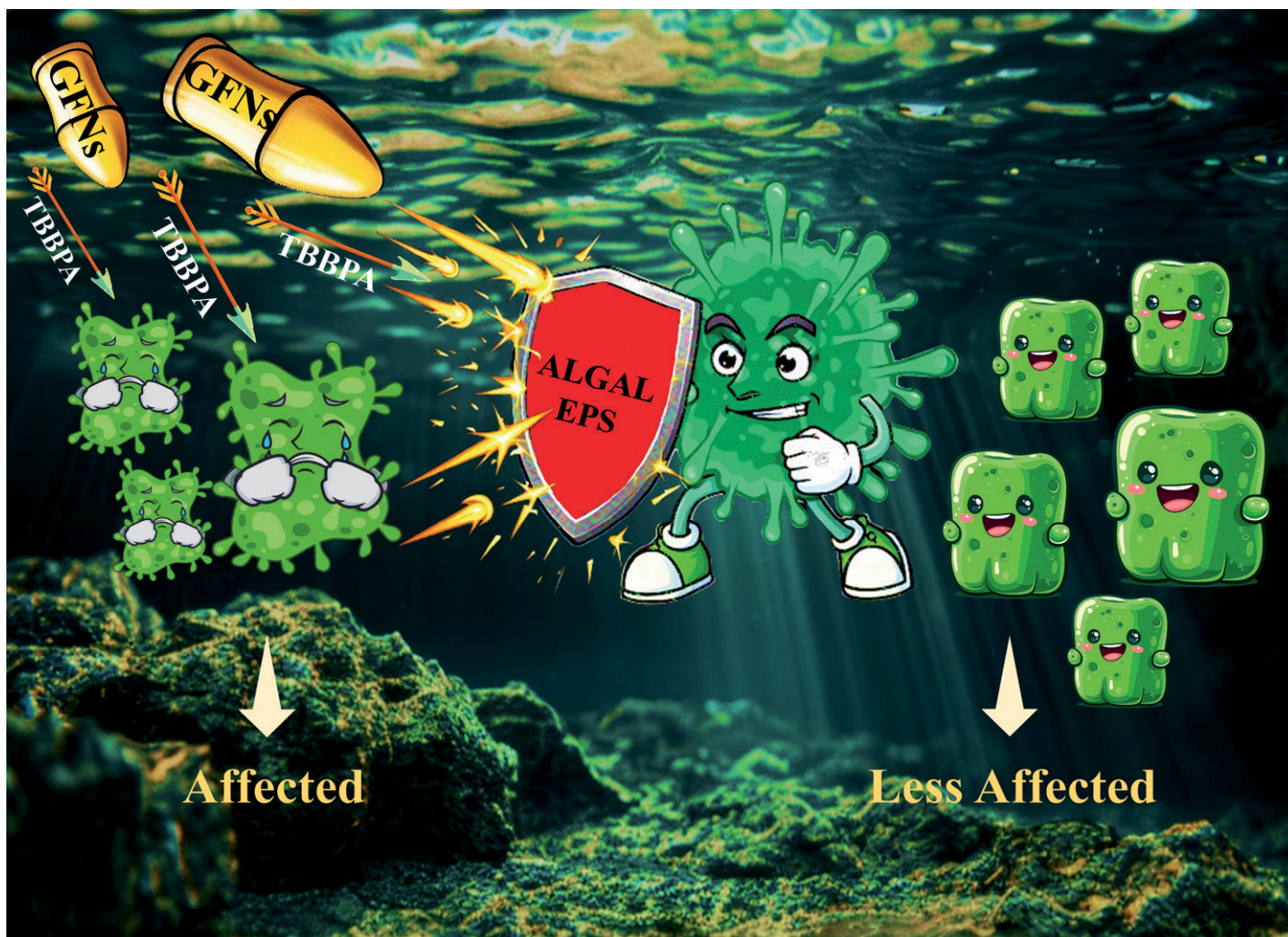
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Showcasing research from Professor Amitava Mukherjee's laboratory, Centre for Nanobiotechnology, Vellore Institute of Technology, India.

The role of algal-EPS in modifying the short-term and long-term toxicity of binary mixtures of TBBPA and GFNs towards marine *Chlorella* sp.: cellular toxicity, uptake, and environmental risk assessment

The increasing presence of graphene family nanomaterials (GFNs), including graphene, graphene oxide and reduced graphene oxide, alongside the flame retardant tetrabromobisphenol A (TBBPA), threatens marine ecosystems. These contaminants induce toxicity in algae through oxidative stress, membrane damage, and photosynthetic disruption. Our study offers the first systematic evidence that exopolysaccharides (EPS) can mediate the detoxification of GFNs, TBBPA, and their combinations in the marine alga *Chlorella* sp. over both short-term (72 h) and long-term (360 h) exposures, providing sustained protection with a single addition at the beginning.

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The role of algal-EPS in modifying the short-term and long-term toxicity of binary mixtures of TBBPA and GFNs towards marine *Chlorella* sp.: cellular toxicity, uptake, and environmental risk assessment

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The increasing occurrence of graphene family nanomaterials (GFNs) such as graphene oxide (GO), reduced graphene oxide (rGO), and graphene, along with the widespread flame retardant tetrabromobisphenol A (TBBPA), poses a growing threat to marine ecosystems. Both types of contaminants are known to induce toxicity in algae primarily through oxidative stress, membrane impairment, and photosynthetic disruption. This study provides the first systematic evidence of EPS-mediated detoxification of GFNs, TBBPA, and their combinations in the marine alga *Chlorella* sp. over both short-term (72 h) and long-term (360 h) exposures. EPS were added only once at the beginning, demonstrating their ability to provide sustained protection in a batch culture system. Detoxification was evident through reduced growth inhibition, significant suppression of reactive oxygen species (ROS) and lipid peroxidation (MDA), stabilisation of photosynthetic efficiency, and normalisation of biochemical responses, including proteins, carbohydrates, and antioxidant enzyme activities. UPLC-based uptake studies further confirmed that EPS reduced cellular accumulation of TBBPA, indicating a barrier or binding effect that limited bioavailability of TBBPA. Supporting analyses, including zeta potential, wettability, UPLC analysis, and 3D-EEM, revealed that EPS modulated surface interactions and minimised direct contaminant–cell contact. Clustered heatmap, correlation analysis, and PCA also showed the correlation. Among the GFN treatment groups, rGO revealed the highest toxicity, although in the presence of TBBPA and EPS, the toxicity was higher in the presence of GO. Ecological risk assessment highlighted the broader environmental relevance of these findings. Overall, this work establishes EPS as natural and effective detoxification agents capable of mitigating the long-term toxic impacts of emerging contaminants, thereby emphasising their potential as a sustainable ecological defense mechanism in aquatic systems.

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Environmental significance

In this study, we present the first evidence of extracellular polymeric substances (EPS) being able to successfully reduce the harmful effects of virgin GFNs, TBBPA, and their combinations in algal systems after both short- and long-term exposures. EPS significantly relieved the cytotoxicity effects of the contaminants by diminishing intracellular ROS production and inhibiting antioxidant enzyme overactivation, restoring cellular balance. The ability of EPS to bind to nanomaterials and organic contaminants demonstrates their promise as natural detoxifying agents in aquatic habitats. By establishing the protective role of EPS, this study paves the way for the development of EPS-based bioremediation technologies for sustainable ecosystem management.

1. Introduction

Flame retardants are commonly utilized in various household items such as electronics, furniture, and textiles to restrain the process of combustion and quickly limit the spread of fire. One of the most commonly used subclasses of flame retardants includes brominated fire retardants (BFRs). By virtue of their low cost and superior activity, BFRs have acquired almost 25% of the global market of flame retardants.¹

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Tetrabromobisphenol A (TBBPA), with its unique properties such as superior thermal stability and high efficiency, has extensive usage in various products, including textiles, building materials, furniture, and electronic products.² The annual consumption of TBBPA is reported to reach approximately 200 000 tons.³ Consequently, the overwhelming usage of TBBPA resulted in increased production and inevitable release into the environment. In the past decade, the concentrations of TBBPA in marine environments have significantly increased.⁴ The widespread presence and increasing concentrations of TBBPA in estuarine and coastal ecosystems have prompted serious concerns about its toxicity. TBBPA has been shown to induce acute as well as chronic harmful effects on various aquatic biota, including fish, molluscs, zooplankton, and microalgae.⁵ More specifically, TBBPA was found to inhibit the growth of the microalga *Chlorella sorokiniana* in a dose-dependent fashion at concentrations in the range of 3–15 mg L⁻¹.⁶

Graphene-family nanomaterials (GFNs) are a diverse group of structurally related materials distinguished by graphene-like topologies that vary in terms of lateral dimensions, layer count, surface functionalization, and structural flaws. Bianco *et al.* (2013) defined this category as graphene-based quantum dots, multilayer graphene, few-layer graphene, reduced graphene oxide (rGO), graphene oxide (GO), and other derivatives made utilizing graphene, GO, or comparable graphene-based precursors.⁷ Due to their excellent physicochemical properties – such as high thermal and electrical conductivity, great mechanical strength, huge specific surface area, flexibility, and low density – these materials have considerable functional advantages.⁸ GFNs can enter the environment through both deliberate uses (such as incorporation into consumer items, agricultural interventions, and environmental clean-up) and inadvertent releases that occur during the manufacture, use, or disposal processes.⁹ Whilst GFNs are primarily used as polymer composites, these composites are sensitive to breakdown in aquatic settings *via* both abiotic and biotic processes, resulting in the possible entry of GFNs into the environment. Given the heightened production and deployment of GFNs, their infiltration into the environment *via* diverse channels has prompted concerns about their possible damage to aquatic organisms, plants, and animals. Recent investigations have revealed that environmentally relevant GFN concentrations can range from 0.001 to 10 mg L⁻¹, with even higher values recorded in specific circumstances.¹⁰ A previous study investigated the acute toxicity of GO and GO quantum dots (GOQDs) to the unicellular cyanobacterium *Microcystis aeruginosa*. GOQDs demonstrated good dispersion over the pH range evaluated. The 96 hour median effective concentrations (EC₅₀) were 49.32 for GO and 22.46 mg L⁻¹ for GOQDs. Both types of nanomaterials were internalized through hetero-agglomeration and envelopment, although GOQDs caused plasmolysis, greater membrane permeability, and intracellular lipid-body formation compared to GO.¹¹ Current research has indicated that brief exposure to rGO can be toxic to organisms spanning three trophic levels,

including *Artemia franciscana*, *Brachionus calyciflorus*, and *Thamnocephalus platyurus*.¹² In addition, Wang *et al.* (2021) found that rGO exposure intensified the toxic effects of nano-zirconium oxide on the freshwater microalga *Chlorella pyrenoidosa*, leading to increased oxidative stress and cytotoxicity compared to graphene nanoplatelets.¹³ In another study, a 96 h flow-cytometric assay on *Porphyridium purpureum* quantified growth, esterase activity, membrane potential, and ROS to establish NOEL (no observed effect level), EC₁₀, and EC₅₀ values for multi-walled carbon nanotubes (CNTs), GO, graphene (Gr), and fullerene (C₆₀). Toxicity ranking by growth-rate EC₅₀ (mg L⁻¹) was CNTs (2.08) > GO (23.37) > Gr (94.88) > C₆₀ (>131), indicating CNTs as the most potent and C₆₀ as the least potent.¹⁴

Extracellular polymeric substances (EPS) released by microbes are made up of a variety of biopolymers, including lipids, humic acids, polysaccharides, uronic acids, and proteins. In marine habitats, EPS can be found in biofilms, on cell surfaces, or freely diffused, all of which contribute considerably to carbon cycle processes.¹⁵ EPS secretion as a stress response can have a major impact on the environmental fate of numerous contaminants, including heavy metals, hydrocarbons, persistent organic compounds, and microplastics, by promoting breakdown, aggregation, or sedimentation.¹⁶ Furthermore, EPS can serve as a barrier between microalgal cells and their immediate vicinity, increasing their resistance to environmental stresses.¹⁷ EPS may be considered as a subclass of dissolved organic matter (DOM) in the marine environment. According to previous studies, the aromatic and polar properties of DOM have an important role in determining the toxicity of hydrophobic organic pollutants.¹⁸ DOM can increase the apparent water solubility of TBBPA, making it more mobile and potentially affecting its bioavailability and biological impact. Under test conditions with DOM, the median effective or fatal concentrations of TBBPA decreased by at least 32% for *Chlorella pyrenoidosa*, 52% for *Daphnia magna*, and 6.6% for *Danio rerio*, indicating an overall increase in acute toxicity. As DOM levels increased, the intensity of TBBPA-induced reactive oxygen species decreased.¹⁹ Recent studies have demonstrated that humic acid (HA) can significantly reduce the toxic effects of GFNs to *Chlorella pyrenoidosa*.²⁰ The degree of mitigation follows the sequence rGO > GO > pristine graphene. HA offers this protection primarily by reducing membrane damage while simultaneously lowering oxidative stress and direct GFN–alga interactions. Zhao *et al.* (2019) found that weaker GFN–alga hetero-aggregation (for rGO and graphene) and higher steric hindrance (for GFNs) reduce physical interaction and toxicity, respectively.²⁰ Despite these findings, the effect of algal EPS on the toxicity of the mixtures of TBBPA and GFNs to marine *Chlorella* species remains relatively unclear.

Microalgae are efficient biological indicators of eutrophication and are commonly used to measure water quality.²¹ *Chlorella* species are extensively characterised unicellular green microalgae that can be found in

waterbodies. They play an important part in aquatic food webs and are considered useful biological indicators due to their sensitivity to contaminants, ability to withstand high temperatures, and capability to flourish in nutrient-low environments.²² Furthermore, previous studies have confirmed the occurrence of GFNs and TBBPA in the marine environment. If these substances are entering the marine ecosystem, then their combination can also exist in the natural marine environment.^{23,24} In the current work, *Chlorella* sp. was selected as the model organism to assess the effect of algal EPS on the toxicity of GFNs, TBBPA, and their combined exposure at environmentally relevant doses.

A critical survey of the literature revealed very little information on how TBBPA and GFNs jointly affect marine microalgae, in short-term (72 h) and long-term (360 h) exposure. It would also be worthwhile investigating whether EPS mitigate the toxicity potential of these mixtures. To fill this gap, the present work investigates the individual and combined toxicity of GO, rGO, and pristine graphene (0.75 mg L⁻¹ each) together with environmentally relevant TBBPA (75 µg L⁻¹) toward *Chlorella* sp. in artificial seawater (ASW). In addition, there are only a handful of reports on the long-term effects of these pollutants towards marine microalgae. Therefore, the comparison between the short and long-term effects of the mixtures of GFNs, TBBPA, and EPS, after one-point addition of the contaminants, remains a major highlight of the current work. Furthermore, short- and long-term exposures were assessed on the premise that TBBPA impairs algal cells chiefly *via* oxidative stress and depressed photosynthetic efficiency. Additionally, GFNs, which are now ubiquitous in aquatic systems, may influence this toxicity by modifying the solubility and bioreactivity of the flame retardant. The accumulation of TBBPA within algal cells, as well as the protein and carbohydrate contents, and the antioxidant enzyme activities were measured. Surface charge, hydrophobicity measurements, and ultra-performance liquid chromatography (UPLC) were utilized for the quantification of the interactions between GFNs and TBBPA, confirming TBBPA adsorption at both 72 and 360 h. The toxicity was subsequently linked to these physicochemical changes through various endpoints, including cell viability, accumulation of TBBPA, production of reactive oxygen species (ROS), generation of proteins and carbohydrates, and the superoxide dismutase (SOD) and catalase (CAT) activities, in addition to the assessments of photosynthetic performance. Overall, the study clarifies the impact of algal-EPS in influencing the fate and mixture toxicity of GFNs and TBBPA under both acute and chronic exposure conditions.

2. Methodology

2.1 Chemicals, media, and synthesis of GFNs

The details of the chemicals employed in the study are provided in the SI Methods S1. In this research, artificial seawater (ASW) was employed as the interacting medium, drawn from our prior studies.²⁵ The preparation method for

ASW adheres to the procedures outlined in our earlier publications.²⁶ Comprehensive details regarding the ASW preparation protocol and the synthesis of the GFNs^{27–29} are included in the SI Table S1 and Methods S2. TBBPA was sourced from Sigma-Aldrich.

2.2 Stock solution preparation and extraction of algal-EPS

The stock solution of GFNs was made at a concentration of 50 mg L⁻¹ using Milli-Q (deionized) water with the help of a probe sonicator (130 W power, 20 kHz frequency, Sonics, USA) for 20 minutes, following the procedure described by Lu *et al.* (2018).³⁰ The working concentration for GFNs was fixed at 750 µg L⁻¹, based on the environmentally relevant concentration and EC₅₀ values. Additionally, the TBBPA solution was prepared at 500 mg L⁻¹ concentration using dimethyl sulfoxide (DMSO) and preserved at 4 °C,³¹ by following the published protocol. The selected concentration for TBBPA was determined to be 75 µg L⁻¹, which is based on previous research indicating its environmental relevance and the EC₅₀ values for the organisms being studied. Extraction of algal-EPS from *Chlorella variabilis* was performed by following our optimised and published protocol.^{32,33} The procedure is detailed in the SI, Methods S3.

2.3 Characterisation of GFNs, TBBPA, and EPS

Transmission electron microscopy (TEM) was utilized to examine the surface characteristics of GFNs. Raman spectroscopy was utilized to analyse the composition and defects of GFNs. In addition, the zeta potential and the wettability of the samples were quantified for the pristine and mixed forms in the presence and absence of the EPS. UPLC was carried out to quantify the TBBPA concentration. The detailed procedure is given in the SI Methods S3 and S4. In addition, the EPS were characterised by 3D-EEM (three-dimensional excitation–emission matrix). More information is available in the SI Methods S4. The surface charge and wettability were checked for the extracted EPS, along with the protein, carbohydrate, and total organic carbon (TOC) contents.²⁶ Details are briefed in the SI Methods S5.

2.4 Test species and experimental design

In this study, the marine microalgae *Chlorella* sp. served as the primary model organism, sourced from the Central Marine Fisheries Research Institute (CMFRI) located in Rameswaram, Tamil Nadu, India. The algal subculture was nurtured in sterile natural seawater (NSW) supplemented with specific micronutrients (details can be found in SI Tables S2–S4) for a duration of 20–25 days in graduated narrow mouth Erlenmeyer flasks (Borosil, capacity 250 ml, neck size: 34 mm, diameter: 85 mm). To promote optimal growth, the algal cultures were maintained under a photoperiod of 16 hours, utilising white, fluorescent lighting (3000 lux, Philips TL-D Super 80 linear fluorescent lamp, India) with a consistent temperature of 23 ± 2 °C.²⁶

Healthy algal cells in the exponential growth phase were chosen. The cells were subjected to centrifugation at 7000 rpm at 4 °C for 10 min. Following centrifugation, the resultant cell pellets were retrieved and subsequently re-suspended in the ASW medium. The optical density (OD) of the resulting suspension was calibrated to 0.1 at 610 nm in the ASW medium. The algae, TBBPA, GFNs, and extracted algal EPS were allowed to interact in the following experimental groups for 72 (short term exposure) and 360 h (long term exposure): (i) TBBPA in the presence and absence of EPS; (ii) GFNs in the presence and absence of EPS; (iii) TBBPA + GFNs in the presence and absence of EPS; (iv) without any treatments (control group). To check the inhibitory effect of extracted EPS and DMSO, the cell viability experiment was performed as a control study, and no significant promotion was noted (Fig. S1 and S2). Hence, these two control groups were not carried forward for further studies. The interaction setups were kept at a temperature of 23 ± 2 °C, with an overall volume of 10 mL for the duration of 72 and 360 h. All the experiments were performed under visible light conditions (3000 lux, Philips). The toxicological evaluation in this study was carried out following the guidelines set by the OECD,³⁴ in triplicate ($n = 3$).

2.5 Toxicological evaluations

2.5.1 Determination of EC₅₀ and cell viability. After a 72 and 360 h incubation period, the evaluation of cell viability was performed by counting the live cells using a hemocytometer with the help of an optical microscope (Zeiss Axiostar Microscope, USA) as outlined by Debroy *et al.* 2025.²⁵ Further details of the methodology can be found in the SI Methods S5. The EC₅₀ values of TBBPA and GFNs + TBBPA were measured in the presence and absence of EPS for both of the exposure periods of 72 h and 360 h. More information regarding the procedures can be deduced from the SI Methods S6.

2.5.2 Oxidative stress assessment. To measure the total ROS and malondialdehyde (MDA) produced, the methods outlined in the study by Rex *et al.* (2024) were followed.³⁵ The activities of antioxidant enzymes such as SOD and CAT were assessed according to the protocol described by Giri *et al.* (2023).³⁶ Additional details are given in the SI Methods S7–S9.

2.5.3 Evaluation of photosynthetic activity. The methodology outlined by Giri and Mukherjee (2021) and Lee *et al.* (2020) was employed to measure the electron transport rate (ETR) and the effective quantum yield of photosystem II (Y(II)).^{37–39} More description can be found in the SI Methods S10.

2.5.4 Estimation of carbohydrates, proteins, and antioxidant enzymes. The carbohydrate and protein levels were determined using the phenol-sulfuric acid method and the Bradford assay, as described by Gerbersdorf *et al.* (2005) and Frølund *et al.* (1995).^{40–42} Further details can be found in the SI Methods S11.

2.6 Ecological risk assessment

The present study uses the risk quotient (RQ) to evaluate the environmental hazards associated with TBBPA in the marine environment. The RQ method is a standard method for assessing the risk posed by pollutants in aquatic ecosystems. The RQ for TBBPA was calculated using specific equations.

$$\text{PNEC} = \frac{\text{EC}_{50}}{\text{AF}} \quad (\text{i})$$

$$\text{RQ} = \frac{\text{MEC}}{\text{PNEC}} \quad (\text{ii})$$

In this study, the measured environmental concentrations (MECs) were adopted from the available literature. The predicted no-effect concentration (PNEC) was determined using the EC₅₀ values along with an assessment factor (AF). For this work, the AF was set at 1000, in accordance with the guidelines from the European Chemicals Agency during the evaluation of acute toxicity.^{6,19,40} The EC₅₀ values of TBBPA were determined in the presence and absence of GFNs. Furthermore, the EC₅₀ values were quantified in the presence and absence of EPS. More information about the assessment are given in the SI Methods S10.

2.7 Statistical analysis

All toxicity assessments were conducted in triplicate ($n = 3$). The results represent the mean $\pm$ SD (standard deviation). A normality evaluation was performed on all data sets, followed by a two-way ANOVA test with the Bonferroni post-test using GraphPad Prism 8 to evaluate the significance level between different treatments and the control. Graphical representations were created using GraphPad Prism 8 and OriginPRO 2025b software. In biological parameters, to understand the effect of TBBPA on GFNs, in the presence and absence of EPS, a cluster heatmap and Pearson correlation matrix analysis were performed⁴³ along with principal component analysis (PCA) by using OriginPRO 2025b.

2.8 Quality assurance

All chemicals employed in the current work were of analytical grade. For the preparation of stock solutions and ASW, ultrapure water (resistivity ≥ 18.2 M Ω cm) was used throughout. To minimise the risk of contamination, stock solutions were prepared in sealed containers to avoid contamination. All glassware used during the study was subjected to acid-washing (10% HNO₃), followed by thorough rinsing with ultrapure water, and was air-dried to remove any residual impurities. Experimental procedures were carried out using sterilised equipment. The material stock suspensions were always freshly prepared and subjected to sonication, prior to exposure experiments. The same batch of samples were utilised throughout this study. To reduce the risk of contamination, laboratory coats and nitrile gloves were worn at all times during handling of the samples.

3. Results

3.1 Physicochemical characterization

In Fig. 1, the images of HR-TEM distinctly illustrate the structural characteristics of the graphene-based nanomaterials (GFNs). GO (Fig. 1A) displayed sheet-like structures comprised of several layers of partially oxidized graphite oxide. In contrast, the rGO (Fig. 1B) sheets were observed to have a stacked arrangement, demonstrating both folds and wrinkles. The graphene (Fig. 1C) nano-sheets showed a similar sheet-like morphology with aggregated structures. The Raman spectra indicated that among the pristine GFNs, rGO exhibited the highest I_D/I_G ratio, followed by GO, and then graphene (Fig. S3). For the EPS, the measured protein, carbohydrate, and total organic carbon concentrations, surface charge, and wettability values were noted to be $4.2 \pm 0.8 \text{ mg L}^{-1}$, $14.1 \pm 1.8 \text{ mg L}^{-1}$, $82.5 \pm 2.1 \text{ mg L}^{-1}$, $-14.2 \pm 2.4 \text{ mV}$, and $7.2 \pm 1.1^\circ$ respectively.

Fig. S4 displays the FTIR spectrum of EPS, which shows peaks at 1363 cm^{-1} (stretching vibrations of C–O–C), 1159 cm^{-1} (stretching vibrations of C–O linked to amino acid residues containing hydroxyl groups), and 866 cm^{-1} (carbonate ions in protein structures). Fig. S5 reveals the 3D-EEM spectra of extracted algal EPS as well. The 3D-EEM spectra of extracted EPS clearly revealed about the presence of proteinaceous materials (region 'a'), tryptophan-like fluorophores (region 'b'), and humic-like organic matter (region 'c') in the EPS.

The 3D-EEM spectra (Fig. 2) revealed distinct fluorescence signatures corresponding to proteinaceous components, humic-like substances, and amino acids. Specifically, regions 'a', 'b', and 'c' corresponded to proteinaceous materials, tryptophan-like fluorophores, and humic-like organic matter, respectively. The 3D-EEM spectra provided clear evidence of EPS adsorption onto the surface of the GFNs. Among the GFNs, rGO exhibited the highest level of EPS adsorption for 72 h (Fig. 2E and E-i), as indicated by the most intense drop in the fluorescence signals. For the 360 h exposure, the

adsorption of EPS over the rGO was observed to be higher both in the presence of TBBPA (Fig. 2H and H-i) and in its absence (Fig. 2G and G-i), although this increment in the adsorption was not so significant. The experimental setup with GO (Fig. 2A and A-i for 72 hours, and Fig. 2C and C-i for 360 hours) and its combination with TBBPA (Fig. 2B and B-i for 72 hours, and Fig. 2D and D-i for 360 hours) revealed significant EPS adsorption, although the fluorescence intensity was notably lower compared to the case of rGO. In contrast, graphene displayed the weakest interaction with EPS (Fig. 2I and I-i for 72 hours, and Fig. 2K and K-i for 360 hours), indicating less surface binding when compared to its oxidized variants. Additionally, the mixture of graphene with TBBPA demonstrated a similar trend to that seen with pristine graphene (Fig. 2J and J-i for 72 hours, and Fig. 2L and L-i for 360 hours). A clear temporal difference was also noticed, and adsorption was more pronounced after 360 h of exposure compared to 72 h of interaction, demonstrating the progressive accumulation and stabilization of EPS interactions with nanomaterials over time. Overall, the 3D-EEM analysis confirms the preferential binding of EPS, particularly with rGO and its mixtures, and highlights the greater extent of adsorption under long-term exposure, which likely underpins the detoxification effects observed in biological assays.

The zeta potential values of the GFNs were measured in the interaction (ASW) medium (Table S5) at 0, 72, and 360 h. Among the GFNs, rGO revealed the lowest zeta potential ($-20.73 \pm 0.57 \text{ mV}$). Upon the addition of the EPS, a reduction in the zeta potential values was noted for the GFNs. In addition, the materials became more unstable at the 360 h time point, compared to 72 h. Notably, among the mixtures of TBBPA and GFNs, GO showed the lowest zeta potential value ($-12.7 \pm 0.36 \text{ mV}$). The zeta potential became more unstable at 360 h, with respect to 72 h. The mixture of TBBPA and GO showed the lowest zeta potential values in the presence of EPS as well, for both time points indicating more instability.

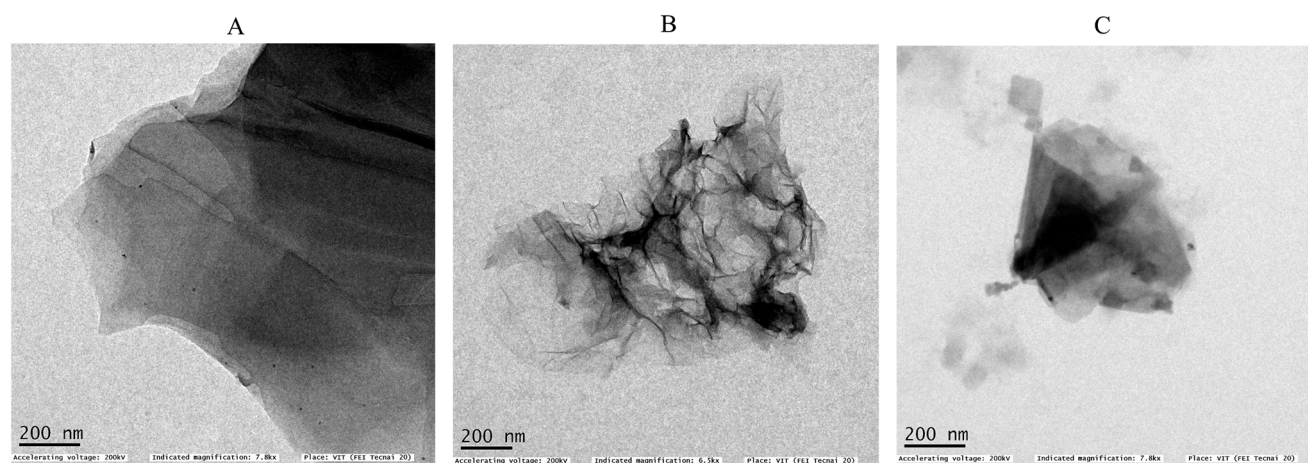


Fig. 1 HR-TEM images illustrating the morphological characteristics of (A) graphene oxide, (B) reduced graphene oxide, and (C) pristine graphene.

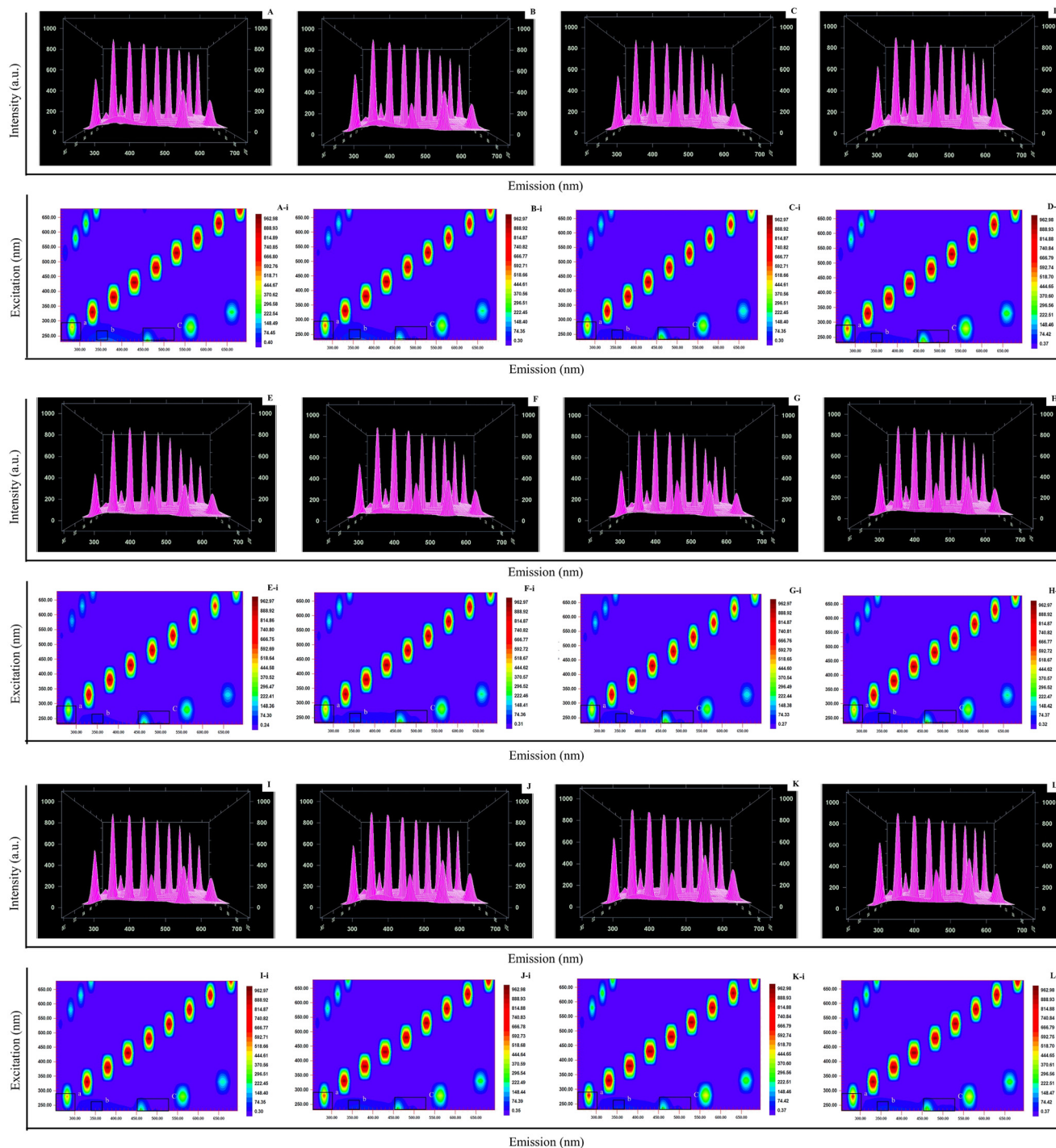


Fig. 2 Three-dimensional excitation-emission matrix (3D EEM) fluorescence spectra illustrating the adsorption behaviour of extracellular polymeric substances (EPS) on GFNs in the presence and absence of TBBPA at different exposure durations. A and A-i: GO + EPS at 72 h; B and B-i: GO + TBBPA + EPS at 72 h; C and C-i: GO + EPS at 360 h; D and D-i: GO + TBBPA + EPS at 360 h; E and E-i: rGO + EPS at 72 h; F and F-i: rGO + TBBPA + EPS at 72 h; G and G-i: rGO + EPS at 360 h; H and H-i: rGO + TBBPA + EPS at 360 h; I and I-i: graphene + EPS at 72 h; J and J-i: graphene + TBBPA + EPS at 72 h; K and K-i: graphene + EPS at 360 h; L and L-i: graphene + TBBPA + EPS at 360 h.

A similar trend was noted for the wettability measurements. Pristine TBBPA was more hydrophilic at the 72 h ($9.67 \pm 0.31^\circ$) and 360 h ($7.30 \pm 0.26^\circ$) time points with respect to 0 h ($11.6 \pm 0.72^\circ$). Upon the addition of the EPS, the hydrophilicity was increased. Among the GFNs, rGO

showed the highest value, while the lowest value was noted for graphene. For the mixtures of TBBPA and GFNs, graphene + TBBPA revealed the highest hydrophilicity, followed by rGO + TBBPA and GO + TBBPA, compared to the pristine materials. Upon addition of EPS to the mixtures, the

hydrophilicity was notably increased. Among the mixture groups, the highest hydrophilicity was noted for the graphene-treated groups. The contact angle in this case was $17.53 \pm 0.81^\circ$ (0 h), and it reached $13.30 \pm 0.85^\circ$ (0 h) upon the addition of EPS. Furthermore, among the mixture treatments, the lowest hydrophilic nature was noted for the GO-TBBPA group with a contact angle of $5.57 \pm 0.57^\circ$ (0 h), which reduced to $29.17 \pm 1.43^\circ$ (0 h) in the presence of EPS. Among the mixture groups, at 360 h, the most hydrophilic treatment group was graphene + TBBPA + EPS ($8.17 \pm 0.21^\circ$), and the GO + TBBPA + EPS group revealed the lowest hydrophilicity at $20.40 \pm 0.62^\circ$.

TBBPA concentrations in the exposure medium were determined at 0, 72 and 360 h time points (Table S7). The TBBPA concentration (in the absence of GFNs) was initially noted at $\sim 61 \mu\text{g L}^{-1}$. After 72 h and 360 h, there were no significant changes noted in the case of pristine TBBPA, although upon the addition of the EPS, a change was observed in the TBBPA concentration. The decrease in concentration of the TBBPA due to adsorption over EPS at 72 h was $\sim 6 \mu\text{g L}^{-1}$ and at 360 h was $\sim 17 \mu\text{g L}^{-1}$. Upon addition of the GFNs, changes in the TBBPA concentration were noted for the samples incubated for 72 and 360 h, with respect to the initial values. The highest reduction was noted in the presence of rGO ($21.9 \mu\text{g L}^{-1}$ at 72 h and $22.1 \mu\text{g L}^{-1}$ at 360 h). Interestingly, upon the addition of EPS to the mixture of rGO and TBBPA, the reduction in the TBBPA concentration was observed to be $25.7 \mu\text{g L}^{-1}$ at 72 h and $25.9 \mu\text{g L}^{-1}$ at 360 h, respectively.

3.2 Cell viability

3.2.1 EC₅₀ determination. The EC₅₀ concentrations (Table S8, Fig. S6) of TBBPA were assessed in the presence and absence of GFNs, and in their mixtures in the presence and absence of EPS at 72 h and 360 h. For the pristine TBBPA,

the EC₅₀ value decreased when the algal cells were exposed for 360 h (2.10 mg L^{-1}), compared to 72 h (3.54 mg L^{-1}). Upon addition of EPS, the EC₅₀ value increased to 3.979 mg L^{-1} from 3.54 mg L^{-1} at a 72 h interval, while after 360 h of exposure, the EC₅₀ value increased from 2.095 mg L^{-1} to 5.15 mg L^{-1} . A trend of reduction in the EC₅₀ values for the long-term exposure time point was noted for the mixtures of TBBPA and GFNs, both in the presence and absence of EPS.

3.2.2 Cell viability. Fig. 3 reveals the effect of GFNs, pristine TBBPA, and the combinations of GFNs and TBBPA on the cell viability of the treated algal cells compared to the control cells. The graph labelled A-S represents the short-term exposure effects to the contaminants on the algal cells for a short-term exposure of 72 h. In contrast, the graph labelled A-L depicts the effects of long-term exposure to contaminants for a period of 360 h. Both diagrams also portray the influence of adding algal EPS to the exposure mixture for both the short and long-term exposure scenarios. The pristine GFN treatment revealed significant growth inhibition compared to the control cells; the pattern was consistent for both long- and short-term exposures ($p < 0.001$). The pristine GFNs showed a toxicity trend in the order of rGO, followed by GO, and then graphene. This indicated that rGO exerted the most substantial adverse effects. For the short-term exposure, the decline in cell viability was not statistically significant for the treatment of pristine TBBPA. However, the decline was significantly lower for the long-term exposure compared to the control cells ($p < 0.001$).

Incorporating GFNs into the TBBPA-containing exposure medium led to a pronounced decrement in algal cell viability in comparison with the pristine TBBPA treatment. This decrement was most significant in the rGO-TBBPA treatment group ($p < 0.001$). Contrastingly, combinations with GO or graphene showed no statistically significant difference ($p > 0.05$) relative to TBBPA alone during short-term exposure. A similar trend was evident under long-term exposure too, with

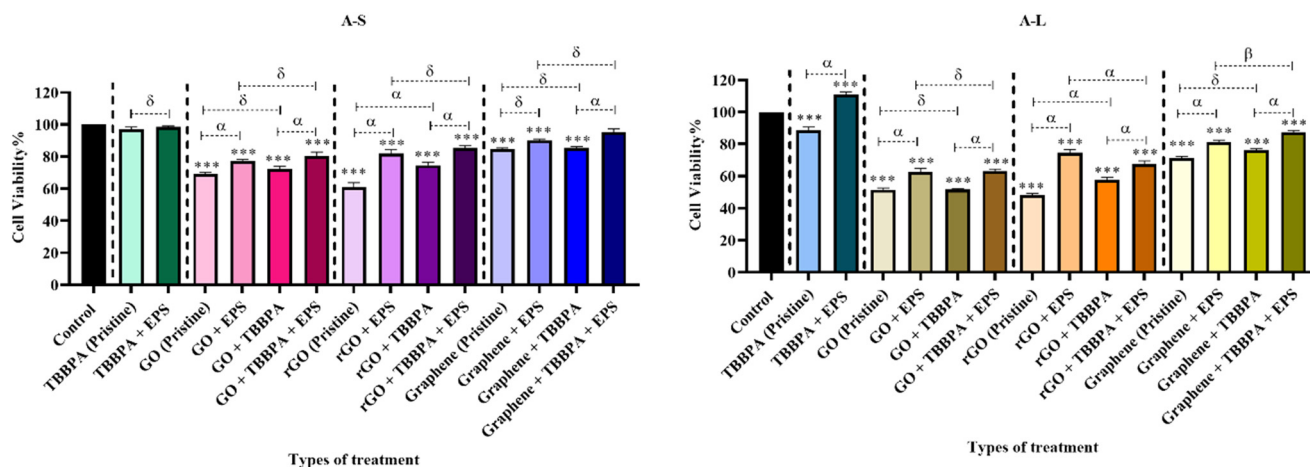


Fig. 3 Differences in the cell viability upon exposure to the various pristine GFNs, pristine TBBPA, and the mixtures of GFNs and TBBPA, in the presence and absence of EPS, A-S: short-term exposure and A-L: long-term exposure. ‘****’ shows the level of significance ($p < 0.001$) with respect to the control. Greek letters suggest the difference between the test groups ($\alpha = p < 0.001$, $\beta = p < 0.001$, and $\delta =$ no significance). The experiment was performed in triplicate ($n = 3$) for all the treatment groups and control.

the rGO-TBBPA combination consistently inducing a markedly greater loss of cell viability ($p < 0.001$) than the pristine TBBPA test groups.

Relative to the pristine treatments, the incorporation of EPS led to a marked enhancement in cell viability across all GFN and TBBPA exposures, as illustrated in the graph. This improvement was consistent for all the pristine treatments and was statistically significant ($p < 0.001$) in comparison with their pristine counterparts. In the test groups containing GFNs and TBBPA, the addition of EPS resulted in a pronounced increase in cell viability ($p < 0.001$) compared to the mixtures without EPS. This enhancement was statistically significant across all the combinations and was consistently observed for both the short- and long-term exposures, highlighting the protective role of EPS in mitigating mixture-induced toxicity.

3.2.3 Accumulation of TBBPA by the algal cells. Table S7 reveals the accumulation of TBBPA by the cells. For the pristine TBBPA, compared to 72 h ($\sim 14 \mu\text{g L}^{-1}$), the

accumulation of TBBPA by the algal cells was higher at 360 h ($\sim 17 \mu\text{g L}^{-1}$). The changes in the accumulation in the presence of GFNs were noticeable. Among the treatment groups containing GFNs and TBBPA, the accumulation was the highest in the case of GO + TBBPA ($\sim 19 \mu\text{g L}^{-1}$) at 72 h, and it was increased to $\sim 26 \mu\text{g L}^{-1}$ at 360 h. Interestingly, in the presence of EPS, the accumulation was decreased to $\sim 18 \mu\text{g L}^{-1}$ at 72 h, and $\sim 22 \mu\text{g L}^{-1}$ at the 360 h time point. In addition, the lowest accumulation among the mixtures of the GFNs and TBBPA was noted for graphene ($\sim 18 \mu\text{g L}^{-1}$) at 72 h, and it was increased to $\sim 19 \mu\text{g L}^{-1}$ at 360 h. Upon the addition of the EPS, the accumulation was decreased to $\sim 14 \mu\text{g L}^{-1}$ at 72 h and $\sim 16 \mu\text{g L}^{-1}$ at 360 h.

3.3 Oxidative stress

3.3.1 Total ROS production. Fig. 4 shows the effects of the contaminants both in their pristine forms and their mixtures, during short-term (A-S) and long-term exposure (A-L) periods.

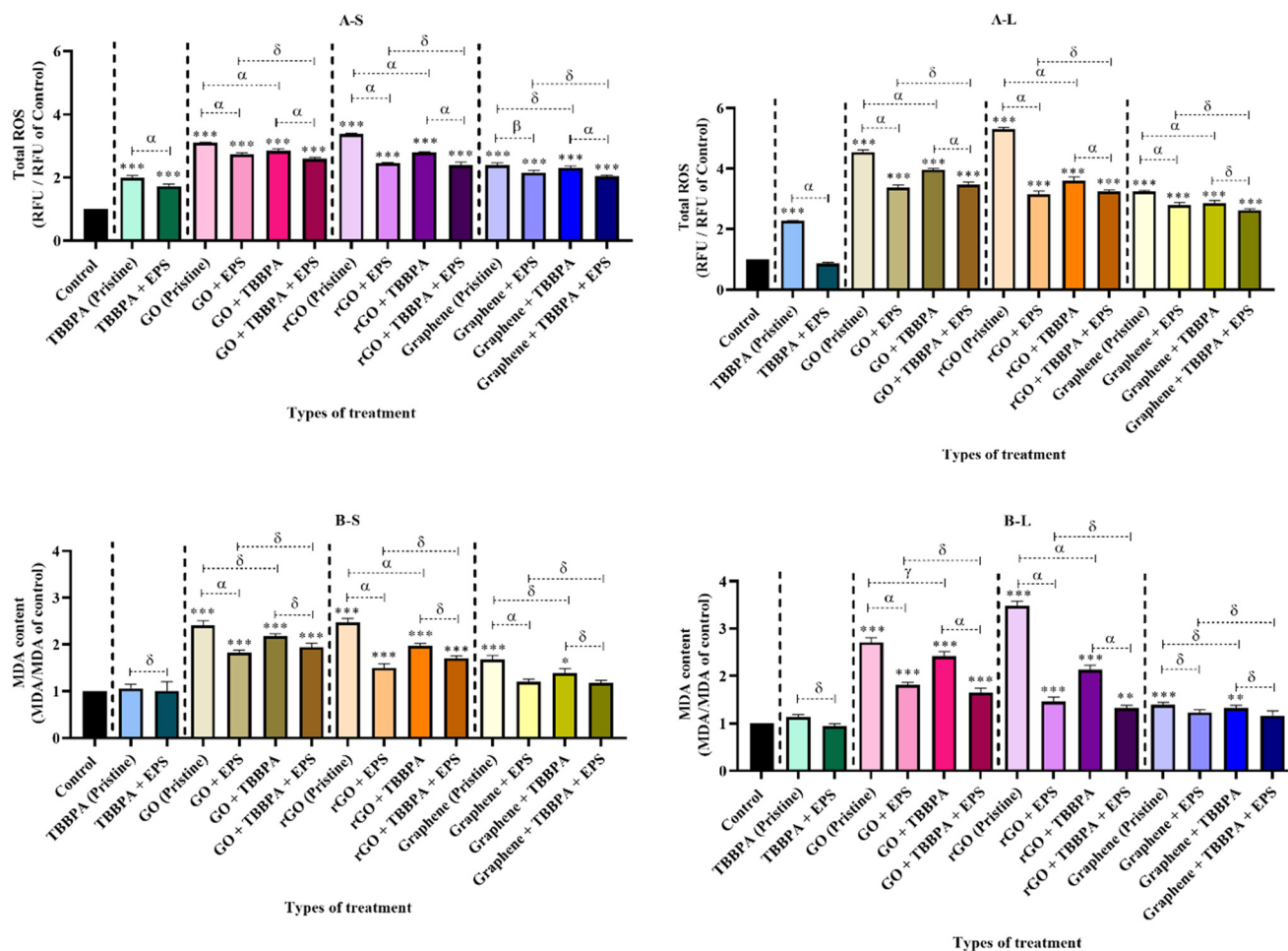


Fig. 4 Differences in the total ROS upon exposure to the various pristine GFNs, pristine TBBPA, and the mixtures of GFNs and TBBPA, in the presence and absence of EPS, A-S: short-term exposure and A-L: long-term exposure. Differences in the MDA generation, B-S: short-term exposure and B-L: long-term exposure. '****' shows the level of significance ($p < 0.001$) with respect to the control. Greek letters suggest the difference between the test groups ($\alpha = p < 0.001$, and $\delta =$ no significance). The experiment was performed in triplicate ($n = 3$) for all the treatment groups and control.

The generation of total ROS was noted to be statistically higher ($p < 0.001$) for all the pristine GFNs in comparison with the control cells in both short-term and long-term exposures. Among the pristine GFN treatments, rGO showed the maximum increment in total ROS production, followed by GO, while the least ROS formation was noticed in the case of graphene-treated cells. Following the same trend as cell viability, the total ROS generation was also observed to be significantly higher ($p < 0.001$) for the pristine TBBPA treatment for both the short and long-term exposures compared to the control.

The incorporation of GFNs into TBBPA-containing media led to a marked increase in the total ROS production relative to the pristine TBBPA treatment group. The most pronounced effect was observed in the rGO-TBBPA mixture, which showed the most significant increment ($p < 0.001$) (~80% increase) for the short-term exposure and long-term exposure (~133% increase) relative to the pristine TBBPA treatment. A comparable effect was noted for the mixture comprising GO-TBBPA, where the increase in the total ROS concentration upon exposure was found to be highly significant ($p < 0.001$) for the short-term exposure (~85% increase) and long-term exposure (~169% increase) when compared to the pristine TBBPA test group. However, for the case of the treatment mixture of graphene-TBBPA, the total ROS generation was noted to be higher than that of the pristine TBBPA test group, but the increase was observed to be insignificant ($p > 0.05$) for the short-term exposure. However, for the long-term exposure to the mixture, the same increase in the total ROS content was statistically significant ($p < 0.001$) when compared to the pristine TBBPA treatment, highlighting the enhanced cytotoxic potential of GFNs in binary mixtures, especially for long-term exposures.

Relative to the pristine exposures, the incorporation of EPS markedly decreased the total ROS generation within the algal cells across all tested conditions involving GFNs and TBBPA. In the pristine treatments, EPS supplementation consistently conferred a significant protective effect, with total ROS values remaining substantially lower than in the case of their respective pristine treatments ($p < 0.001$). The beneficial influence of EPS was even more pronounced in the binary mixtures of GFNs and TBBPA. In these mixtures, where a considerable increase in the total ROS was observed in the absence of EPS, the addition of EPS led to a statistically significant decline of total ROS within the cells ($p < 0.001$) across all combinations tested. This decline was consistent irrespective of the type of GFN present, with a notable reduction of ROS generation in both short-term and long-term exposures. Importantly, the trend was not restricted to any single mixture but was uniformly observed in GO-TBBPA, graphene-TBBPA, and rGO-TBBPA combinations, thereby demonstrating the broad-spectrum protective efficacy of EPS. Collectively, these results highlight that EPS supplementation mitigates the toxic effects of both pristine contaminants and their mixtures, significantly enhancing algal tolerance under prolonged stress conditions.

3.3.2 Total MDA content. Lipid peroxidation, as measured by MDA production, was notably elevated in all pristine GFN and TBBPA treatments relative to the control, which aligned with the observed patterns in cell viability and total ROS generation, as shown in Fig. 4 (B-S for short-term and B-L for long-term exposure). In the case of the mixtures, a pronounced effect was recorded for rGO-TBBPA, where short-term exposure resulted in an ~90% increase ($p < 0.001$) in MDA levels compared with pristine TBBPA. In contrast, the mixture of GO-TBBPA and graphene-TBBPA did not produce statistically significant increases ($p > 0.05$) in MDA production under short-term conditions when compared with pristine TBBPA. A similar trend was observed under long-term exposure too, with the rGO-TBBPA mixture exhibiting the most pronounced effect, showing a nearly 100% increase in MDA production relative to pristine TBBPA ($p < 0.001$). For the GO-TBBPA mixture, the elevation in MDA generation was modest yet statistically significant ($p < 0.05$), while the graphene-TBBPA mixture continued to show no significant increase ($p > 0.05$) compared with pristine TBBPA.

Compared to the pristine exposures, the presence of EPS resulted in a pronounced reduction in MDA accumulation within algal cells for all the test groups. In treatments with pristine materials, EPS consistently exerted a significant protective effect, as evidenced by markedly lower MDA levels relative to the corresponding controls ($p < 0.001$). For TBBPA (for both short and long-term exposures) and graphene (long-term exposure), the decline in the MDA production was noted to be statistically insignificant ($p > 0.05$). This protective influence was further amplified in the binary mixtures of GFNs and TBBPA, where pristine treatments typically induced a strong elevation in MDA production. Upon EPS supplementation, however, a statistically significant decline in MDA generation was observed across all combinations for long-term exposure ($p < 0.001$), except for graphene-TBBPA mixtures, which showed a statistically insignificant decline in MDA generation ($p > 0.05$) compared with the pristine mixtures. Notably, the protective effect of EPS was not confined to any specific mixture, but was consistently evident across GO-TBBPA, graphene-TBBPA, and rGO-TBBPA systems.

3.3.3 Antioxidant enzyme activities. Fig. S8 illustrates the effects of pristine GFNs, TBBPA, and their combinations on antioxidant enzyme activities, specifically SOD (SOD: A-S for short-term and A-L for long-term exposure) and CAT (CAT: B-S for short-term and B-L for long-term exposure). A pronounced elevation in both the enzyme activities was noted in pristine treatments, which was significantly higher relative to the control cells ($p < 0.001$). Among the GFNs, the order of enzyme induction followed the trend rGO > GO > graphene, which is in line with the patterns observed for growth inhibition and other biochemical endpoints. In the case of binary mixtures of GFNs with TBBPA, enzyme activities were consistently higher than those induced by pristine TBBPA alone ($p < 0.001$), irrespective of exposure duration.

However, the incorporation of EPS into the exposure medium was noted to exhibit a marked reduction in SOD and CAT activities for both pristine GFN and TBBPA treatments. This reduction was statistically significant ($p < 0.001$) and evident in both short- and long-term exposure treatment groups. A similar suppressive effect was recorded in the case of the combinations of GFNs with TBBPA, where EPS addition significantly lowered the enzyme activities in comparison with their pristine mixture counterparts ($p < 0.001$).

3.4 Photosynthetic activities

Fig. 5 and S7 depict the effects of individual GFNs, TBBPA, and their binary combinations on the quantum yield of PSII [Y(II)] (A-S for short-term and A-L for long-term exposure) and ETR (B-S for short-term and B-L for long-term exposure) in the algal system for both short and long-term exposures. Both Y(II) and ETR exhibited a marked reduction under exposures to pristine GFNs and TBBPA. Among the GFNs, the magnitude of decline followed the trend: rGO > GO > graphene, a pattern in line with the previously observed

trends in growth inhibition, ROS accumulation, and MDA production. Interestingly, the test groups involving GFNs with TBBPA induced a further decrement in Y(II) and ETR relative to the pristine TBBPA treatment, across both short- and long-term exposures. The most pronounced decline was noted in the rGO-TBBPA combination. However, despite these observed decreases, the statistical analysis revealed that the changes in Y(II) and ETR values for graphene-, GO-, and rGO-containing mixtures were not significant with respect to the control values ($p > 0.05$).

In comparison with the pristine exposure groups, the presence of EPS markedly enhanced PSII (Y(II)) and ETR in algal cells under all tested conditions involving GFNs and TBBPA. Following EPS supplementation, elevations in Y(II) and ETR were noted across all the mixtures during both the short- and long-term exposures. This improvement was relatively uniform across the different GFNs, with enhancements observed irrespective of the exposure duration. Importantly, the protective influence of EPS was not restricted to a particular mixture but was consistently evident in GO-TBBPA, graphene-TBBPA, and rGO-TBBPA systems, although the decline in the values

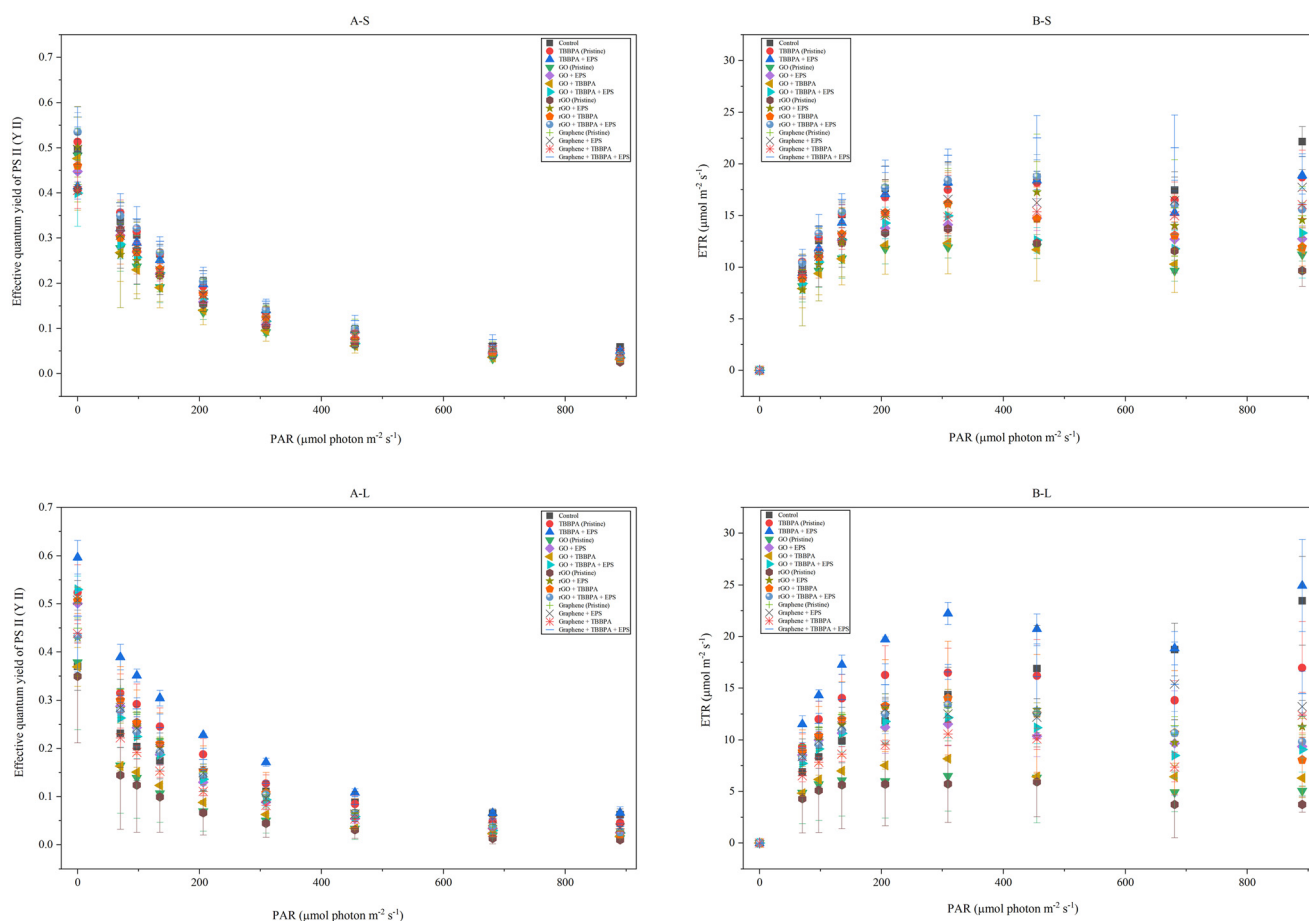


Fig. 5 Differences in the effective quantum yield of PS(II) (Y(II)) upon exposure to the various pristine GFNs, pristine TBBPA, and the mixtures of GFNs and TBBPA, in the presence and absence of EPS, A-S: short-term exposure and A-L: long-term exposure. Differences in the electron transport rate (ETR), B-S: short-term exposure and B-L: long-term exposure. The experiment was performed in triplicate ($n = 3$) for all the treatment groups and control.

was insignificant ($p > 0.05$) in comparison with the mixtures without EPS.

3.5 Levels of proteins and carbohydrates in the cells

Fig. S8–S10 illustrate the impact of three different GFNs, TBBPA, and their combinations on cellular protein (Fig. S8: A-S for short-term and A-L for long-term exposure), carbohydrate (Fig. S9: B-S for short-term and B-L for long-term exposure), SOD, and catalase levels (Fig. S10: A-S and B-S for short-term and A-L and B-L for long-term exposure). After exposure to pristine contaminants, both protein and carbohydrate concentrations exhibited a marked increase, and this pattern was consistent across both short-term and long-term treatments. In contrast, when EPS were introduced in combination with GFNs and TBBPA, the cellular protein and carbohydrate contents were lower than those observed under pristine TBBPA exposure. This reduction in biomolecule accumulation was evident in all binary mixtures involving GFNs, and the trend persisted across both short- and long-term exposures. Such outcomes suggest that EPS supplementation effectively mitigates the stress responses otherwise induced by the pristine contaminants, thereby indicating a reduction in the overall toxicity potential of GFNs and TBBPA when present in a complex with EPS.

4. Discussion

4.1 Interactions between GFNs and TBBPA

The TEM micrographs (Fig. 1) of the GFNs reveal their layered and flaky structure typical of pristine GFNs. The GO (Fig. 1A) presents thin sheets that are folded and wrinkled, indicative of the damage resulting from oxidation. In contrast, the rGO sheets exhibit even more wrinkles and a greater number of folds compared to the GO, because of the removal of bulky, oxygen-containing functional groups during the reduction process.⁴⁴ The graphene sheets display a smooth, multi-layered configuration without any noticeable folds or wrinkles.^{45,46} rGO demonstrates a greater capacity to adsorb TBBPA compared to GO and graphene. This can be attributed to several factors: its high surface area, the remaining oxygen functional groups that enable various adsorption mechanisms besides simple π - π interactions, and structural vacancy defects.⁴⁷ GO, with its abundant oxygen-containing groups, offers strong acidic and basic functional sites that enhance the adsorption of ionic and polar pollutants. In contrast, pristine graphene has its extended π - π conjugated surface, making it more effective in capturing aromatic or hydrophobic molecules. rGO combines features of both, retaining some oxygen groups while regaining a largely conjugated surface, thus creating a versatile platform capable of adsorbing a wider range of pollutants through multiple interaction mechanisms.⁴⁸ rGO retains some functional groups while recovering much of graphene's extensive surface area and structural imperfections, resulting in a more intricate and effective adsorption capacity for organic molecules like TBBPA. The adsorption measurements

validated the presence of a consistent adsorption trend. In addition, the presence of EPS significantly enhanced the adsorption of TBBPA for 72 and 360 h. Notably, the higher adsorption at 360 h suggests that EPS, rich in proteins, carbohydrates, and humic-like substances, have strong adsorption potential. The presence of proteins and humic-like substances, as confirmed through 3D-EEM, also reveals the higher adsorption behaviour between the mixtures of TBBPA and GFNs with EPS.

Zeta potential indicates the surface charge of particles suspended in a solution. Upon introduction of GFNs into the solution, TBBPA molecules adsorbed onto their surface, leading to a reduction in zeta potential. This decrease suggests reduced colloidal stability within the mixture. Furthermore, the addition of EPS led to an even greater reduction in zeta potential, indicating that EPS made the solution more unstable. The solution exhibited increased instability at 360 h compared to 72 h. A comparable trend was observed for the wettability test: the value decreased at the 72 hour mark and further decreased at the 360 h mark. The increased hydrophilicity and decreased colloidal stability contributed to the agglomeration and gravitational settling of GFNs, particularly when the concentrations of TBBPA in the mixtures were higher. This, in turn, likely reduced the GFNs' direct availability for interaction with cells.⁴⁹ Upon addition of EPS, the hydrophilicity of the GFNs increases drastically, resulting in their rapid agglomeration, which facilitates gravitational settling of these particles. EPS become adsorbed on the surface of GFNs, thereby masking their sharp edges and providing a cushioning surface that shields the interacting cells against the harmful effects of the pristine GFNs. These findings are consistent with the observed morphological alterations in the GFNs following EPS adsorption, indicating the beneficial role of eco-corona formation.

In the presence of GFNs, TBBPA is progressively adsorbed onto their surface, and this adsorption is significantly enhanced – by several fold – when EPS are present. At 72 h, the presence of EPS facilitates gravitational settling of the TBBPA–GFN–EPS complexes, thereby reducing the effective availability of TBBPA to algal cells. This sedimentation effect becomes even more pronounced at 360 h, further limiting pollutant exposure. These trends are consistently supported by multiple analytical results, including contact angle measurements, adsorption quantified through UPLC, and variations in zeta potential. In addition, the degradation of TBBPA may impact its toxic effects on the algal cells, as some by-products of this degradation, especially lower-brominated bisphenol A (BPA) derivatives, can reveal toxic potential as well.^{50,51}

4.2 Toxicity of contaminants towards algal cells

The study results show that both the contaminants, *i.e.*, TBBPA and GFNs, led to a decline in cell viability at both the short-term and long-term exposures. The decline was

significantly reversed upon exposure to EPS. A previous investigation looked at the effects of TBBPA, Cd(II), and their combination on the growth of *Chlorella sorokiniana* over 10 days. The results demonstrated that TBBPA inhibited algal growth in a concentration-dependent manner. Cd(II) alone exhibited no significant effect up to 1 mg L^{-1} , but at 2 mg L^{-1} it significantly inhibited development, reducing the algal dry weight from 0.34 to 0.22 g L^{-1} . The combination of Cd(II) (2 mg L^{-1}) and TBBPA (1.5 mg L^{-1}) inhibited the growth by $\sim 47.1\%$, whereas the growth inhibition rate by the pristine TBBPA was noted to be $\sim 27.1\%$.⁵² Similarly, another study investigated the cytotoxicity responses of the mixtures of nano-zirconia (nZrO_2) and two graphene-based nanomaterials – graphene nanoplatelets (GNPs) and rGO – in the freshwater microalga *Chlorella pyrenoidosa*. GNPs and/or rGO were observed to significantly increase the cytotoxic effects of nZrO_2 . This increased toxic effect was primarily due to heightened intracellular oxidative stress and changes in cellular membrane functioning.¹³ In the current study, the mixture of GFNs and TBBPA also led to a decline in the cell viability of the interacted cells, both in the short- and long-term exposures to the contaminants. However, the toxic impacts of the pollutants, i.e., TBBPA and GFNs alone and in combination, were reduced upon adding EPS into the exposure medium. A similar trend was noted for the accumulation of TBBPA as well. In the presence of GFNs, the accumulation of TBBPA within the algal cells was higher at 360 h as compared to the accumulation after 72 h. The accumulation was less upon the addition of the EPS, and this might be because of the adsorption of TBBPA over the GFNs, and EPS. Similar results were observed in our previous study, where the toxic potential of the flame retardant triphenyl phosphate in combination with PSNPs was found to be significantly reduced after their exposure to algal EPS, as observed within the interacted algal cells.⁵³

Long-term exposure studies of algal cells with GFNs and TBBPA are scarce in the literature. Among them, a previous study showed that the 96 hour growth inhibition levels resulting from 10 mg L^{-1} GO exposure were ranked as follows: *S. obliquus* > *C. vulgaris* > *M. aeruginosa* > *Cyclotella* sp. > *C. reinhardtii*.⁵⁴ Similarly, the findings of another study revealed that the EC_{50} of TBBPA on *T. pseudonana* ranged from 1.67 to 3.22 mg L^{-1} over a period of 24 to 96 hours.⁵⁵ In another investigation, a high concentration (10 mg L^{-1}) of green fluorescently labelled nanoplastic beads comprising a polyamide–polymethyl methacrylate blend (21:4 w/w, 200 nm) resulted in a 42.1% loss in viability after a 12 day exposure, and an increase in the cell density of *Porphyridium cruentum*. This enhancement was associated with decreased ROS production, reduced chlorophyll degradation, and alleviation of photoinhibition. Within five hours of exposure, an increase in surface-bound sticky exopolysaccharides (b-EPS) on the algal cells facilitated nanoplastic adsorption. This interaction was supported by $-\text{CH}_3$ bonding between the polysaccharides of b-EPS and membrane lipids (phospholipids/glycolipids), contributing to

photoprotection.⁵⁶ Similar results were observed in the current study, where exposure to algal EPS resulted in an increment in the cell viability for both the short and long-term exposures to the contaminants. This could be attributed to increased agglomeration (lower bioavailability) of the GFNs and TBBPA upon interaction with algal EPS. At 360 h, EPS-coated GFNs adsorbed more TBBPA and settled rapidly, thereby lowering direct exposure to algal cells. This protective effect resulted in a significant improvement in cell viability compared to the 72 h exposure time period.

4.3 Oxidative stress and antioxidant enzyme activity

Under environmental stress conditions, excessive ROS generation can disrupt cellular redox homeostasis, leading to oxidative damage. The sp^2 -hybridised configuration of GFNs may enhance intracellular glutathione oxidation or promote electron transfer between the cellular membranes and GFNs.⁵⁷ Among these materials, GO is known to possess the highest degree of oxidation relative to graphene and rGO.⁵⁸ Interestingly, in the current study, rGO induced the highest level of oxidative stress in the algal cells. This effect could be attributed to the increased density of structural defects within its sp^2 -hybridised carbon network.⁵⁹ Graphene, which consists primarily of planar sp^2 carbon layers with minimal oxygen-containing functional groups, exhibited the lowest intracellular ROS levels. Malondialdehyde, which is a key biomarker for membrane lipid peroxidation, was employed to evaluate the degree of oxidative damage to the algal cell membranes.⁶⁰ In addition to growth suppression, pristine TBBPA-treated cells produced more MDA and total ROS. A separate study indicated that increased levels of three categories of flame retardants (FRs) resulted in increased MDA levels, implying both physiological and structural deficiencies in *C. sorokiniana*. At lower doses of TBBPA (0.125 mg L^{-1} and 0.25 mg L^{-1}), the combination of GFNs resulted in significantly higher levels of ROS and MDA production in comparison with the individual treatments. A comparable trend was reported in an earlier study, where co-exposure to binary ZnO–TBBPA led to greater ROS and MDA accumulation in *Chlorella vulgaris* than observed with ZnO nanoparticles or TBBPA alone.⁶ Similar results were obtained in the present study, where the levels of total ROS and MDA increased upon exposure to a mixture of GFNs with TBBPA, following the trend of cell viability, in both short and long-term exposure scenarios. The incorporation of EPS resulted in a marked reduction in overall oxidative stress, as evidenced by decreased levels of both total ROS and MDA in the exposed algal cells. The presence of EPS in the interaction medium facilitated the rapid agglomeration of GFNs, thereby limiting their direct contact with the algal cells. This reduction in particle–cell interaction could contribute to the diminished intracellular oxidative stress induced by the contaminants. Additionally, EPS further alleviated oxidative stress through their intrinsic ROS-quenching capacity, offering protective benefits to the algal cells.⁶¹ Furthermore,

the shielding effect of EPS may have stabilised the cellular membrane integrity, reducing ROS generation at the site of exposure. This suggests a dual protective mechanism of EPS involving both physical sequestration of contaminants and biochemical stress alleviation within algal systems. All treatment groups increased the oxidative stress on cells at long-term exposure as compared to short-term exposure. Consequently, total ROS levels were markedly suppressed in the EPS-treated systems for the two timepoints considered in this study.

Superoxide dismutase (SOD) is a metalloprotein that serves as the primary line of defense against oxidative stress by catalyzing the dismutation of superoxide radicals into molecular oxygen and hydrogen peroxide. CAT, a heme-containing tetrameric enzyme, plays a crucial role in detoxifying hydrogen peroxide by converting it into water and oxygen, thereby preventing oxidative damage within the cell.⁶² The pattern of SOD activity observed in *Chlorella sorokiniana* mirrored the relative intensity of ROS upon treatment with 0.3, 0.9, and 1.5 mg L⁻¹ concentrations of TBBPA. This indicates that the microalgae activated an antioxidant response by upregulating SOD activity to counteract elevated intracellular ROS levels. Concurrently, transcriptomic analysis revealed the upregulation of genes associated with protein synthesis in cells exposed to Cd(II) and TBBPA, suggesting enhanced translation of SOD proteins, which contributed to the increased enzymatic activity.⁵² Similarly, in the present work, the interaction of GFNs with TBBPA led to exacerbated levels of both the antioxidant enzymes, *i.e.*, SOD and catalase, in the treated algal cells, following the same trend as total ROS and MDA. The incorporation of EPS into the contaminant mixture led to a significant reduction in the activity of antioxidant enzymes during both short- and long-term exposures.

4.4 Photosynthetic parameters

GFNs interfered with algal photosynthesis primarily through a shading effect, which restricts light penetration and subsequently diminishes photosynthetic efficiency.⁶³ This impairment of the photosynthetic apparatus contributes to their overall toxicity toward algal cells. Variations in chlorophyll fluorescence can therefore serve as sensitive indicators of algal responses to environmental stress. Consistent with the trends in growth inhibition, ROS accumulation, and MDA levels, photosynthetic parameters such as Y(II) and ETR also demonstrated a concentration-dependent decline under pristine exposure. Moreover, disturbances that inactivate photosystem II (PSII) or induce sustained non-photochemical quenching led to reductions in Fv/Fm and relative ETR, reflecting compromised photosynthetic performance. In previous work, the combination of GFNs with lower concentrations of TBBPA (0.125 and 0.25 mg L⁻¹) produced a more marked decline in photosynthetic parameters – Y(II), ETR, and the light response curve – compared to pristine TBBPA exposure alone.

This suggests a possible synergistic interaction at lower contaminant levels, thereby intensifying the stress response in the algal cells. This leads to enhanced impairment of the photosynthetic electron transport chain, causing further suppression of Y(II) and a consequent decline in ETR.⁴² Following the addition of EPS to the contaminant mixtures, a noticeable improvement was observed in the photosynthetic performance of the algal cells, reflected by an increase in photosynthetic parameters under both short- and long-term exposure conditions. This alleviation of oxidative damage and restoration of electron transport efficiency contributed to the recovery of parameters such as Y(II) and ETR, and reduced oxidative stress generation. Furthermore, the protective role of EPS may also be linked to their ROS-quenching capacity, which reduces oxidative pressure on the photosystems and supports sustained photosynthetic activity. Collectively, these findings highlight the dual role of EPS in shielding algal cells from contaminant-induced stress and enhancing their physiological resilience. In addition, at long-term exposure, the observed damage in the electron transport chain and in Y(II) revealed the higher toxicity at long-term exposure, compared to the short-term treatment. EPS significantly minimized TBBPA–GFN toxicity at long-term exposure, preserving Y(II) and electron transport.

4.5 Clustered heatmap, Pearson correlation, PCA, and risk assessment

The heatmaps in Fig. 6A–S (short-term) and Fig. 6A–L (long-term) provide an integrated overview of how GFNs, TBBPA, and their mixtures influence multiple biological responses in *Chlorella* sp., and how EPS modulate these effects. In short-term exposures (A–S), pristine GFNs and TBBPA induced elevated ROS, MDA, and antioxidant enzyme activities, which corresponded with reduced cell viability and impaired photosynthetic efficiency (YII, ETR), with rGO and GO exerting the strongest effects. Binary mixtures, particularly GO–TBBPA and rGO–TBBPA, clustered at the high-toxicity end of the heatmap, indicating synergistic stress effects relative to individual components. EPS addition markedly attenuated these responses, reducing oxidative stress and enzyme over-activation while restoring photosynthetic performance and viability, with EPS-containing mixtures clustering closer to less toxic treatments. In long-term exposures (A–L), the toxic effects of pristine GFNs and mixtures became more pronounced, with intensified oxidative and biochemical stress alongside further declines in photosynthetic performance. Yet, EPS supplementation sustained a protective effect, consistently shifting clusters away from non-EPS groups and mitigating long-term damage, highlighting both its immediate shielding capacity and sustained detoxification ability. Across both time scales, oxidative stress (ROS, MDA) and antioxidant enzyme activity (SOD, catalase) emerged as central drivers of toxicity, while EPS effectively suppressed these pathways and enhanced recovery. Collectively, the

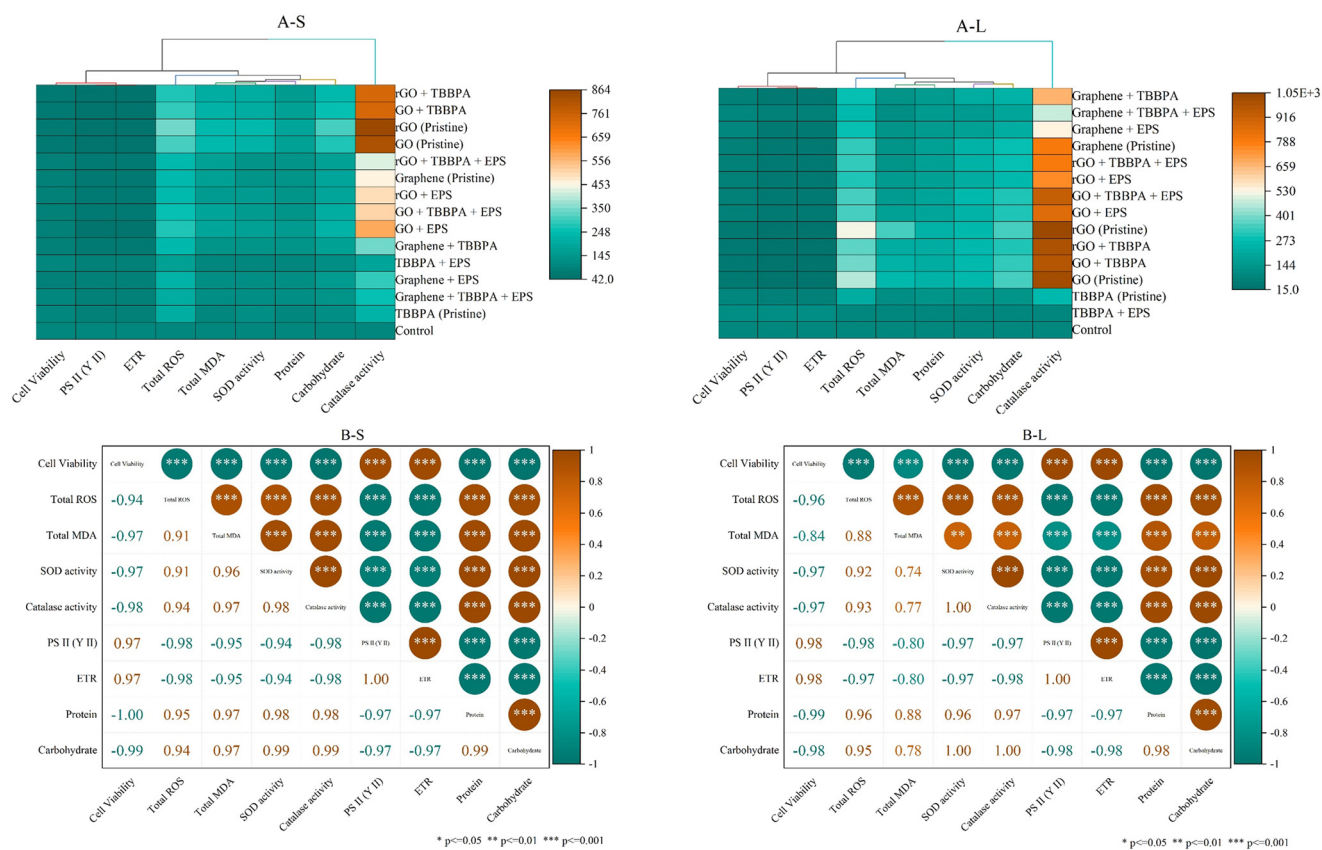


Fig. 6 The cluster heat maps (A-S: short-term exposure, A-L: long-term exposure) and correlation analysis plots (B-S: short-term exposure, B-L: long-term exposure). “*” shows statistical significance with respect to the control treatment ($p < 0.05$).

heatmaps emphasise 'the broad-spectrum and enduring protective role of EPS against GFNs, TBBPA, and their mixtures, underscoring their ecological significance as a natural detoxification mechanism in aquatic environments.

The correlation analysis revealed two distinct clusters of positively associated parameters. On the one hand, the growth rate, chlorophyll fluorescence indices (Fv/Fm, Y(II)), and electron transport rate (ETR) exhibited strong positive correlations with each other. This indicates that optimal growth of *Chlorella* sp. is tightly coupled with efficient photosynthetic performance, which was evident in the control and EPS-treated groups where these parameters were maximised. On the other hand, ROS and MDA were strongly and positively correlated, reflecting the direct linkage between reactive oxygen species generation and lipid peroxidation as markers of oxidative stress. Importantly, these two clusters were negatively correlated with one another. Elevated ROS and MDA were inversely related to photosynthetic parameters, suggesting that higher oxidative stress coincides with impaired photosynthetic machinery and reduced growth. This trend was most pronounced in pristine GFN and TBBPA exposures, where oxidative damage aligned with sharp declines in photosynthetic indices. In mixtures with GFNs and TBBPA, the strength of the negative correlations was amplified, reinforcing the synergistic effect of co-exposure on cellular stress. By contrast, in mixtures in the presence of EPS, the associations between oxidative

stress markers and photosynthetic declines weakened slightly, consistent with attenuation of toxicity due to agglomeration. EPS addition notably altered the correlation landscape: it weakened the positive linkage between ROS and MDA while restoring the positive correlations among photosynthetic parameters and growth. This shift indicates that EPS effectively disrupted the feedback loop between oxidative stress and photosynthetic inhibition, allowing algal cells to maintain physiological stability even under contaminant exposure.

The PCA (Fig. 7) biplot demonstrated a clear separation of treatments, with PC1 and PC2 together explaining the majority of the variance in physiological and biochemical responses of algal cells. The control group clustered in the positive quadrant of PC1, strongly associated with favourable parameters such as higher Y(II), Fv/Fm, and ETR, reflecting optimal photosynthetic efficiency and cellular homeostasis. In contrast, pristine TBBPA and GFNs occupied the opposite quadrants, aligning closely with stress-associated markers, including elevated ROS and MDA, which are indicative of oxidative damage and reduced photosynthetic performance. Binary mixtures with low concentrations of TBBPA (0.125 and 0.25 mg L⁻¹) shifted further away from the control cluster, particularly along PC2, underscoring synergistic toxic effects characterized by amplified ROS accumulation and pronounced inhibition of electron transport. In the presence of EPS, the mixtures clustered closer to the pristine TBBPA groups, suggesting an

attenuation of toxicity, possibly due to agglomeration effects. Importantly, EPS-supplemented treatments were positioned nearer to the control cluster, with vectors pointing toward photosynthetic efficiency parameters, highlighting their role in reducing oxidative stress and restoring algal physiological performance. Collectively, the PCA underscores three major patterns: (i) distinct stress signatures for individual contaminants, (ii) synergistic toxicity at mixture levels, and (iii) the protective influence of EPS, which shifts algal responses toward a more balanced physiological state.

Table 1 reveals the evaluated RQ of TBBPA on marine microalgae *Chlorella* sp. in the presence of GFNs and EPS. The RQ method serves as an effective tool for evaluating the ecological risks associated with TBBPA in aquatic organisms. Previous research indicates that TBBPA is frequently found in marine environments at concentrations ranging from ng L^{-1} to $\mu\text{g L}^{-1}$ levels. In this study, RQs were calculated based on acute toxicity assessments for marine *Chlorella* sp. Additionally, existing data on TBBPA levels in marine ecosystems were sourced from various studies.^{24,64,65} The RQ values are indicative of the ecological risk levels: $\text{RQ} > 1$ denotes high risk, $0.1 < \text{RQ} < 1$ indicates medium risk, and $\text{RQ} < 0.1$ suggests low risk. The findings summarised in Table 1 show that all seawater samples had RQ values below 1. However, in certain instances, the RQ values for TBBPA surpassed 0.1, suggesting that its presence could represent a moderate ecological risk to marine life. Our research involving marine algae indicated that the combination of GFNs and TBBPA in the presence and absence of EPS at 72 h and 360 h duration resulted in a reduced ecological risk compared to using TBBPA alone.

4.6 Mode of action

The possible toxicity mechanisms of pristine GFNs and TBBPA against *Chlorella* sp. may stem from direct damage to cells and heightened ROS generation, owing to their inherent photocatalytic properties. As a result, exposure to pristine

TBBPA and pristine GO resulted in a significant rise in MDA levels. Importantly, pristine GFNs displayed a higher level of toxicity compared to pristine TBBPA, accompanied by a reduction in both the maximum quantum yield and ETR. This variation may be attributed to the sheet-like structure of GO, which potentially obstructs light penetration to the algal cells, unlike TBBPA. In the mixture groups, the toxicity mechanism affecting algal cells is influenced by the concentration of the pollutants. In the current work, the short term exposure revealed less toxicity when compared to the long term exposure which is mainly because of the more cellular damage and higher ROS generation over time. In addition, in the presence of GFNs, TBBPA was adsorbed onto the surface of GFNs, and this adsorption was significantly enhanced in the presence of EPS. The discrepancy in the toxicity could result from the distinct modes of action of the pollutants and their nature, where in the presence of EPS, the physical damage to algal cells of marine *Chlorella* sp. was reduced. As a limitation of the current work, we can conclude that more molecular studies are required to clearly differentiate the toxicity between the pristine and mixture treatment groups in the presence and absence of EPS.

5. Conclusions

The current study shows that pristine GFNs, TBBPA, and their mixtures had pronounced toxic effects on algal cells, primarily through oxidative stress, as evidenced by increased antioxidant enzyme activity (SOD and CAT), decreased cellular macromolecules (proteins and carbohydrates), and a significant decrease in cell viability. These reactions were consistent across both short- and long-term exposure scenarios, with the degree of toxicity following the sequence $\text{rGO} > \text{GO} > \text{graphene}$ among GFNs. Furthermore, combined exposures with TBBPA increased stress responses compared to individual treatments, indicating a synergistic effect of co-contaminants on algal physiology. The introduction of EPS in the exposure medium significantly reduced the harmful

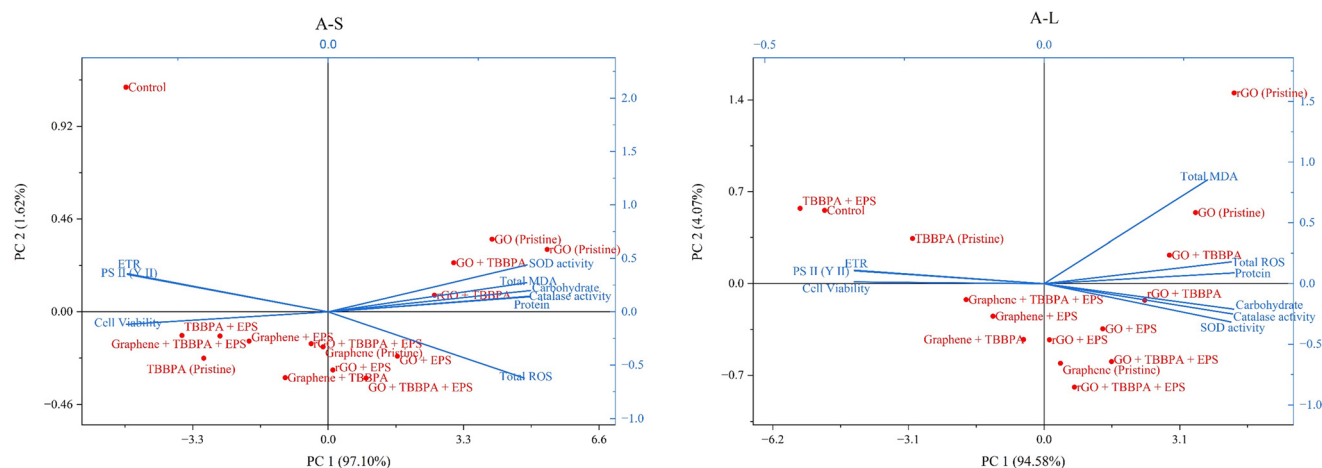


Fig. 7 Principal component analysis (PCA) of short-term (A-S) and long-term (A-L) exposures.

Table 1 Evaluated RQ of TBBPA on marine microalgae *Chlorella* sp. in the presence of GFNs and EPS

Effect type	Area	MEC & RQ	TBBPA	TBBPA + EPS	GO + TBBPA	GO + TBBPA + EPS	rGO + TBBPA	rGO + TBBPA + EPS	Graphene + TBBPA	Graphene + TBBPA + EPS
Short term effect	Estuary to Bohai Sea	MEC (lower) (ng L ⁻¹)	1.13 × 10 ²	1.13 × 10 ²	1.13 × 10 ²	1.13 × 10 ²	1.13 × 10 ²	1.13 × 10 ²	1.13 × 10 ²	1.13 × 10 ²
		MEC (higher) (ng L ⁻¹)	6	6	6	6	6	6	6	6
		RQ (lower)	0.032	0.028	0.125	0.081	0.106	0.072	0.063	0.047
		RQ (higher)	0.169	0.151	0.663	0.429	0.561	0.380	0.333	0.251
	South Yellow Sea (in autumn)	MEC (lower) (μg L ⁻¹)	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16
		MEC (higher) (μg L ⁻¹)	1.49	1.49	1.49	1.49	1.49	1.49	1.49	1.49
		RQ (lower)	0.045	0.040	0.177	0.114	0.150	0.101	0.089	0.067
		RQ (higher)	0.421	0.374	1.646	1.066	1.393	0.943	0.828	0.623
	South Yellow Sea (in summer)	MEC (lower) (μg L ⁻¹)	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16
		MEC (higher) (μg L ⁻¹)	1.58	1.58	1.58	1.58	1.58	1.58	1.58	1.58
		RQ (lower)	0.045	0.040	0.177	0.114	0.150	0.101	0.089	0.067
		RQ (higher)	0.446	0.397	1.746	1.130	1.477	1.000	0.878	0.661
	Yellow Sea	MEC (lower) (μg L ⁻¹)	ND	ND	ND	ND	ND	ND	ND	ND
		MEC (higher) (μg L ⁻¹)	0.46	0.46	0.46	0.46	0.46	0.46	0.46	0.46
		RQ (lower)	ND	ND	ND	ND	ND	ND	ND	ND
		RQ (higher)	0.130	0.116	0.508	0.329	0.430	0.291	0.256	0.192
	Southern coast of Qingdao	MEC (lower) (μg L ⁻¹)	ND	ND	ND	ND	ND	ND	ND	ND
		MEC (higher) (μg L ⁻¹)	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.8
		RQ (lower)	ND	ND	ND	ND	ND	ND	ND	ND
		RQ (higher)	0.508	0.452	1.989	1.288	1.682	1.139	1.000	0.753
Long term effect	Estuary to Bohai Sea	MEC (lower) (ng L ⁻¹)	1.13 × 10 ²	1.13 × 10 ²	1.13 × 10 ²	1.13 × 10 ²	1.13 × 10 ²	1.13 × 10 ²	1.13 × 10 ²	1.13 × 10 ²
		MEC (higher) (ng L ⁻¹)	6	6	6	6	6	6	6	6
		RQ (lower)	0.054	0.022	0.435	0.192	0.265	0.161	0.094	0.058
		RQ (higher)	0.286	0.117	2.308	1.017	1.408	0.852	0.500	0.308
	South Yellow Sea (in autumn)	MEC (lower) (μg L ⁻¹)	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16
		MEC (higher) (μg L ⁻¹)	1.49	1.49	1.49	1.49	1.49	1.49	1.49	1.49
		RQ (lower)	0.076	0.031	0.615	0.271	0.376	0.227	0.133	0.082
		RQ (higher)	0.711	0.289	5.731	2.525	3.498	2.116	1.241	0.764
	South Yellow Sea (in summer)	MEC (lower) (μg L ⁻¹)	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16
		MEC (higher) (μg L ⁻¹)	1.58	1.58	1.58	1.58	1.58	1.58	1.58	1.58
		RQ (lower)	0.076	0.031	0.615	0.271	0.376	0.227	0.133	0.082
		RQ (higher)	0.754	0.307	6.077	2.678	3.709	2.244	1.316	0.810
	Yellow Sea	MEC (lower) (μg L ⁻¹)	ND	ND	ND	ND	ND	ND	ND	ND
		MEC (higher) (μg L ⁻¹)	0.46	0.46	0.46	0.46	0.46	0.46	0.46	0.46
		RQ (lower)	ND	ND	ND	ND	ND	ND	ND	ND
		RQ (higher)	0.220	0.089	1.769	0.780	1.080	0.653	0.383	0.236
	Southern coast of Qingdao	MEC (lower) (μg L ⁻¹)	ND	ND	ND	ND	ND	ND	ND	ND
		MEC (higher) (μg L ⁻¹)	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.8
		RQ (lower)	ND	ND	ND	ND	ND	ND	ND	ND
		RQ (higher)	0.859	0.350	6.923	3.051	4.225	2.557	1.499	0.923

The concentrations of TBBPA used for the evaluation of RQ in Table 1 are cited from published literature studies (ref. 24, 42 and 64–66).

responses of the algae. EPS therapy reduced over-activation of antioxidant enzymes, restored protein and carbohydrate contents, and improved cell viability in all treatment groups. This protective effect was observed in both short- and long-

term exposures, implying that EPS functioned as shielding and detoxifying agents, most likely *via* surface binding, steric hindrance, and free radical scavenging. By controlling the bioavailability and reactivity of GFNs and TBBPA, EPS significantly reduced oxidative stress and restored cellular homeostasis. Together, our findings confirm oxidative stress as the primary mechanism of toxicity for GFNs, TBBPA, and their combinations, while also highlighting the promising function of EPS in detoxification. The findings provide mechanistic insight into how natural biomolecules might operate as ecological defenders against new pollutants, highlighting their potential in the development of green, sustainable techniques for reducing nanomaterial and organic pollutant toxicity in aquatic settings. Our findings also indicate that the toxicity is eliminated after a specific exposure period.

Author contributions

Abhrajit Debroy: conceptualization, investigation, methodology, visualization, formal analysis, writing – original draft; Mrudula Pulimi: conceptualization, formal analysis; Natarajan Chandrasekaran: methodology, formal analysis; Willie J. G. M. Peijnenburg: formal analysis, writing – review and editing; Amitava Mukherjee: conceptualization, methodology, formal analysis, supervision, project administration, writing – review and editing.

Conflicts of interest

The authors declare no competing financial interest.

Data availability

The data will be available on request.

Supplementary information (SI): Methods S1: chemicals used. Methods S2: synthesis of GFNs. Methods S3: extraction of EPS. Methods S4: characterization. Methods S5: surface charge, hydrophobicity, and adsorption. Methods S6: effects on cell viability and accumulation of TBBPA. Methods S7: oxidative stress – total ROS. Methods S8: oxidative stress – MDA content. Methods S9: oxidative stress – SOD and catalase. Methods S10: photosynthetic parameter assessment. Methods S11: proteins and carbohydrates. Table S1: composition of artificial seawater per 1000 ml. Table S2: preparation of stock 1 per 100 ml. Table S3: preparation of stock 2 per 100 ml. Table S4: preparation of stock 3 per L. Table S5: surface charge of TBBPA in the presence and absence of GFNs and EPS in pristine and mixture form. Table S6: wettability of TBBPA in the presence and absence of GFNs and EPS in pristine and mixture form. Table S7: UPLC for TBBPA analysis under biotic and abiotic conditions. Table S8: EC₅₀ values. Fig. S1: control treatment with DMSO revealed that there is no significant difference in the DMSO test groups in short and long-term exposure with respect to the control. Fig. S2: control treatment with EPS revealed that there is no significant difference in the EPS test groups in short and long-term exposure with respect to the

control. Fig. S3: Raman spectra of A-GO, B-rGO, and C-graphene. Fig. S4: FTIR spectra of extracted TB-EPS. Fig. S5: 3D EEM spectra of extracted algal EPS. Fig. S6: EC₅₀ of the treatment groups. Fig. S7: photosynthetic activity, A-S: effective quantum yield of PS II (short term exposure), A-L: effective quantum yield of PS II (long term exposure), B-S: ETR (short term exposure), B-L: long term exposure. Fig. S8: proteins, A-S: short term, A-L: long term. Fig. S9: generated carbohydrates, B-S: short term, B-L: long term exposure. Fig. S10: A-S: SOD activity at short term exposure, A-L: SOD activity at long term exposure, B-S: catalase activity at short term exposure, B-L: catalase activity at long term exposure. See DOI: <https://doi.org/10.1039/d5en01078k>.

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