Supplementary Information (SI) for Environmental Science: Nano.

Supporting Information

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

Abhrajit Debroy[a], Mrudula Pulimi[a], Natarajan Chandrasekaran[a], Willie J.G.M. Peijnenburg[b,c], Amitava Mukherjee[a]*

[a]Centre for Nanobiotechnology, Vellore Institute of Technology, Vellore, Tamil Nadu, India

[b]Institute of Environmental Sciences (CML), Leiden University, Leiden, 2300, RA, the Netherlands

[c]National Institute of Public Health and the Environment, Centre for the Safety of Substances and Products, Bilthoven, 3720, BA, the Netherlands

***Corresponding author**

Dr. Amitava Mukherjee

Senior Professor & Former Director

Centre for Nanobiotechnology

Vellore Institute of Technology, Vellore – 632014, India.

Email: amit.mookerjea@gmail.com, amitav@vit.ac.in

Phone: 91 416 2202620

Materials and Methods

Methods S1: Chemicals used

Sulphuric acid (H_2SO_4), phosphoric acid (H_3PO_4), and hydrochloric acid (HCl) were purchased from Molychem Pvt. Ltd., India. Potassium persulfate ($K_2S_2O_8$), potassium permanganate ($KMnO_4$), and sodium carbonate (Na_2CO_3) were purchased from Sisco Research Laboratories Pvt. Ltd., India. Phosphorous pentoxide (P_2O_5), 2′ ,7′ -dichlorofluorescein diacetate (DCFH-DA) was purchased from Sigma Aldrich. DMSO (Dimethyl sulfoxide) and hydrogen peroxide (H_2O_2) were purchased from SDFCL (Mumbai). Trichloroacetic acid (TCA) and thiobarbituric acid (TBA) were purchased from Hi Media Pvt. Ltd (Mumbai, India).

Collected natural sea water (NSW) was filtered through blotting paper first for removing the larger particles followed by filtration with Whatman no. 1 (pore size - 11 μm) filter paper to discard any other colloidal particles. The filtered water was then subjected to sterilisation using autoclaving at 121° C for 15 min.

Methods S2: Synthesis of GFNs

Synthesis of GO

The modified Hummer's method was followed using graphite powder (>99.95% purity). The sulphuric acid and phosphoric acid were measured in the volume of 27 mL and 3 mL (9:1 ratio) and stirred continuously for 15 min. 0.225 g of graphite powder was added to the acid mix and followed by the slow addition of $KMnO_4$ (1.32 g). The solution changed to dark green after 6 h of continuous stirring. The removal of excess $KMnO_4$ was done by adding 0.675 mL of H_2O_2 in a dropwise manner, under continuous stirring for 10 min. The solution was allowed to cool down after completion of the exothermic reaction. At the end of the reaction, the material was washed with HCl (10 mL) and deionized water (30 mL) using centrifugation at 5000 rpm for 20 min. The obtained pellet was dried in a hot air oven (for 24 h at 80 °C) to achieve the powder form of GO for further studies.[1]

Synthesis of rGO

The prepared GO was diluted with milliQ water to form a suspension of 0.5 mg/mL. $NaBH_4$ (2.28 g) was added to 200 mL of GO suspension. The mixture was kept under stirring at room temperature for 12 h to obtain rGO. Next, the rGO was filtered and washed with milliQ water until neutralization.[2]

Synthesis of graphene

A single-step process was followed for the synthesis of stable graphene sheets. The raw graphite flakes were dispersed in a solution of ethanol and deionized water (1:9 volume ratio). The suspension was subjected to bath sonication with a working frequency of 40/50 Hz at room temperature for 180 min. The dispersion was centrifuged for 30 min at 1200 rpm to filter out unexfoliated graphite flakes. The supernatant was then dried at 80 °C for 24 h for complete drying.[3]

Methods S3: Extraction of EPS

Marine algal cells in the exponential growth phase were centrifuged at 7000 rpm at 4 °C for 10min. Cell pellets were then dispersed in ASW to reach the optical density of 0.5 at 610nm in colorimeter. To check the maximum production of EPS, the algal samples were placed under fluorescent visible light conditions (3000 lux) for various time intervals of 12, 24, 48, and 72h followed by centrifugation at 13,000rpm at 4 °C for 10min. The supernatant was collected and passed through a 0.45 μm filter followed by analysis of the total organic carbon content (TOC) by utilizing a TOC analyser (Model TOC-L, Shimadzu, Japan). TOC analysis revealed that the samples incubated for 12 h were able to produce the maximum yield. This extracted EPS was named as loosely bound EPS (LB-EPS). After the collection of LB-EPS, the algal pellet was further treated with 0.1M phosphate buffer and kept for 20min of incubation at 70 °C in a water bath. After cooling, the suspension was centrifuged at 13,000rpm for 15min followed by filtration of the supernatant through a 0.45 μm filter. This extracted EPS was named as tightly bound EPS (TB-EPS). Throughout the work, TB-EPS was used and named as EPS.

Methods S4: Characterization

Raman spectroscopy (acquisition at 532 nm) (Anton Paar Cora 5001, Austria) was used for confirmation of defects in the GFNs. The shape of the GFNs was precisely analysed by Transmission Electron Microscopy (TEM) and Field Emission Scanning Electron Microscopy (FESEM).[1]

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 230–680 nm whereas the emission wavelength range was 250–700 nm and the slit width was 10 nm. Increment for the excitation was kept at 50 nm.

Methods S5: Surface charge, hydrophobicity, adsorption

The surface charge of pristine materials and their mixtures were analyzed via the zeta potential (90 Plus Particle Size Analyzer, Brookhaven Instruments Corp., USA). The hydrophobicity/hydrophilicity of the samples were measured using a goniometer (Model: DMs-401, Kyowa Interface Science Co., Ltd.; software- FAMAS). To check the hydrophobicity, a minute drop of liquid sample was taken and kept over the glass slide for air drying. When the sample was properly air dried, the contact angle was measured. This was done with the sessile drop technique using water.[4]

Ultra-performance liquid chromatography (UPLC system, Waters Inc., Bedford, MA, US) was used to measure the concentration of TBBPA as well as for the mixture treatment groups of TBBPA with GFNs at 0, 72, and 360 h (biotic, and abiotic condition). The measurements were carried out in the presence and absence of algal cells in replicates. For UPLC, the protocol of Yang et al. (2021) was followed with slight modification.[5] Methanol:water was used at 60:40 (v/v) ratio as mobile phase. An ACQUITY UPLC BEH C18 column (2.1 mm ×100 mm, 1.7 µm, Waters) was chosen to separate the target com- pounds. The injection volume was 5 µL. The flow rate was set at 0.2 mL/min and the column was kept at 40℃. Total run time was set for 15 min.

Methods S6: Effects on cell viability and accumulation of TBBPA

The cell viability was assessed after the incubation time (72 and 360 h) by counting the healthy cells on a haemocytometer and examined under an optical microscope. Healthy algal cells can be differentiated from dead algal cells as they exist as distinct, relatively big cells, whereas dead cells form clusters. Highly damaged cells were also counted as dead cells. The cell counts for the treatment groups were compared with the control group.[6]

$$Cell\ viability\ (\%) = \left(\frac{Test}{Control} * 100\right)$$

EC_{50} was calculated with a range of concentration of 0.075 mg/L to 16 mg/L for TBBPA pristine, by following the log concentration and probit values.

For the accumulation of TBBPA, after 72, and 360 h interaction, the algal cells were collected by centrifugation at 7000 rpm for 10 min at 4 °C followed by washing with 0.5 M phosphate buffer saline (PBS). The samples were further homogenized in PBS, followed by centrifugation at 13,000 rpm for 20 min at 4 °C. The supernatant was collected and the accumulation of TBBPA was checked by following the UPLC protocol.

Methods S7: Oxidative stress - Total ROS

DCFH-DA is a non-fluorescent cell permeable dye. In reaction with ROS, this dye is oxidized to the highly fluorescent 2',7'-dichlorofluorescein, which is detected. After 72 and 360 h of interaction, 10 ml of algal cell suspension was incubated with 100 µl of DCFH-DA (100 mM) under the dark condition for 30 min. The fluorescence intensity of the samples loaded in 96 well plate (white) was measured at excitation and emission wavelengths of 485 nm and 530 nm respectively, using a spectrofluorometer. The generated ROS was calculated with reference to the algal cells without treatment.[7]

Methods S8: Oxidative stress- MDA content

After 72 and 360 h of exposure, treated samples were centrifuged at 4 °C for 10 min at 7000 rpm. The cell pellets were further treated with 0.25% TBA (prepared in a 10% TCA solution) and kept in a water bath at 95 °C for 30 min. The heated sample mixtures were quickly placed on ice to stop the reaction and centrifuged at 7000 rpm for 10 min. The supernatant's absorbance containing the released MDA enzyme was measured by subtracting the absorbance at from 532 nm to 600 nm.[8]

Methods S9: Oxidative stress- SOD, Catalase

For SOD, after 72, and 360 h interaction, the algal cells were collected by centrifugation at 7000 rpm for 10 min at 4 °C followed by washing with 0.5 M phosphate buffer saline (PBS). Samples were further homogenized in PBS followed by centrifugation at 13,000 rpm for 20 min at 4 °C. After that, the supernatant was collected, mixed with other chemicals (hydroxylamine hydrochlorides at a concentration of 20 mM, nitro blue tetrazolium chloride at a concentration of 96 mM, Na_2CO_3 buffer at a concentration of 50 mM with a pH of 10, and Triton X-100 at a concentration of 0.6%) and kept under visible light for 20 min. The reaction mixture was analyzed using a UV–Vis microplate-reader (xMARK microplate absorbance spectrophotometer, BIO-RAD) at a wavelength of 560 nm.

For catalase activity, after 72 and 360 h interaction treated algal cells were centrifuged at a temperature of 4 °C for 10 min at 7000 rpm. The collected cell pellet was treated in the similar fashion as mentioned prior for the SOD activity. Using a spectrophotometer (Thermofisher evolution 220), the absorbance decrease was recorded at 240 nm for 3 min after adding 800 µl of freshly made H_2O_2 solution (0.3% v/v) to 200 µl of supernatant. The reaction solution lacking H_2O_2 was treated as a blank.9

Methods S10: Photosynthetic parameters assessment

In this study, a photosynthesis yield analyzer (Mini PAM, Heinz Walz, Germany) was used to measure photochemical quantum yield of the PS II (Y II) system, and electron transport rate (ETR) in the treated and control algal cells. After 72 and 360 h of exposure, the control and the treated cells were placed in dark incubation for 15 min, followed by addition of 400 µl of sample into the instrument chamber and passing on the high-intensity Actinic light to record the effective quantum yield of PS II (Y II) and ETR of the samples.[10]

Methods S11: Protein and carbohydrate

For protein estimation, after 72 and 360 h of exposure, the algal cells were collected by centrifugation at 7000 rpm for 10 min at 4 °C followed by washing with 0.5 M phosphate buffer saline (PBS). The samples were further homogenized in PBS, followed by, centrifugation at 13,000 rpm for 20 min at 4 °C. The supernatant was collected and the protein content was checked using Bradford reagent using a UV–Vis microplate-reader (xMARK microplate absorbance spectrophotometer, BIO-RAD) at a wavelength of 595 nm.[11]

For carbohydrate content, algal cells exposed for 72 and 360 h were centrifuged at a temperature of 4 °C for 10 min at 7000 rpm. The collected cell pellet was treated in the same fashion as mentioned prior for the protein content. After the final round of centrifugation, supernatant was collected and carbohydrate content was measured by phenol-sulphuric acid method at a wavelength of 490 nm.[11]

Tables

Table S1. Composition of Artificial Sea Water per 1000 ml

Chemicals	**Grams**
Sodium Chloride	26.29
Potassium chloride	0.74
Calcium chloride	0.99
Magnesium chloride	6.09
Magnesium sulphate heptahydrate	3.94

Table S2. Preparation of stock 1 per 100 ml

Chemicals	**Grams**
Zinc chloride	2.1
Cobalt chloride	2.0
Ammonium molybdate	0.9
Copper sulphate	2.0

Table S3 Preparation of stock 2 per 100 ml

Chemicals	Milligrams
Vitamin B12	10
Vitamin B6	10

Table S4. Preparation of stock 3 per L

Chemicals	Grams
Iron chloride hexahydrate	1.3
Manganese chloride	0.36
Boric chloride	33.6
Ethylenediaminetetraacetic acid	45.0
Sodium dihydrogen phosphate dehydrate	20.0
Sodium nitrate	100

Table S5: Surface charge of TBBPA in the presence and absence of GFNs, EPS in pristine and mixture form

	0 h		72 h		360 h	
Materials	Avg (mV)	SD	Avg (mV)	SD	Avg (mV)	SD
TBBPA (Pristine)	-2.90	0.80	-2.17	0.65	-1.90	0.20
TBBPA (Pristine) +EPS	-1.83	0.70	-1.53	0.40	-1.27	0.32
GO (Pristine)	-16.77	1.81	-13.73	0.47	-9.77	0.55
GO+EPS	-11.20	0.50	-10.37	0.35	-8.10	0.30
GO+TBBPA	-12.70	0.36	-12.20	0.26	-9.13	0.49
GO+TBBPA+EPS	-11.03	0.30	-9.97	0.51	-7.77	0.15
rGO (Pristine)	-20.73	0.57	-14.57	2.10	-11.63	0.40
rGO+EPS	-10.73	0.21	-8.57	0.42	-7.53	0.38
rGO+TBBPA	-12.20	0.78	-11.87	0.15	-8.43	0.35
rGO+TBBPA+EPS	-8.30	0.66	-6.53	0.51	-5.87	0.21
Graphene (Pristine)	-10.43	0.89	-8.27	0.59	-6.33	0.40
Graphene+EPS	-6.83	0.31	-4.93	0.15	-4.13	0.49
Graphene+TBBPA	-7.07	0.47	-5.87	0.25	-5.20	0.36
Graphene+TBBPA+EPS	-4.93	0.45	-3.73	0.57	-2.90	0.50

Table S6: Wettability of TBBPA in the presence and absence of GFNs, EPS in pristine and mixture form.

	0 h		72 h		360 h	
Materials	Avg (°)	SD	Avg (°)	SD	Avg (°)	SD
TBBPA (Pristine)	11.60	0.72	9.67	0.31	7.30	0.26
TBBPA (Pristine) +EPS	10.10	0.26	8.43	0.35	6.83	0.31
GO (Pristine)	40.70	1.93	35.17	1.16	32.20	2.01
GO+EPS	35.10	1.77	26.73	2.48	22.50	0.95
GO+TBBPA	35.57	0.57	33.50	0.87	30.77	0.32
GO+TBBPA+EPS	29.17	1.43	24.37	1.38	20.40	0.62
rGO (Pristine)	44.57	3.56	39.07	1.38	36.17	1.53
rGO+EPS	27.03	1.87	22.80	1.48	18.60	0.92
rGO+TBBPA	33.33	2.16	28.63	0.75	27.43	0.55
rGO+TBBPA+EPS	20.43	0.95	16.70	0.75	13.37	1.27
Graphene (Pristine)	21.20	1.31	19.70	0.95	16.77	1.46
Graphene+EPS	13.87	0.57	12.57	0.76	9.43	0.45
Graphene+TBBPA	17.53	0.81	15.60	1.10	11.03	0.90
Graphene+TBBPA+EPS	13.30	0.85	10.83	0.31	8.17	0.21

Table S7: UPLC for TBBPA analysis in biotic, and abiotic condition

	Abiotic						Biotic (Accumulation by algal cells)			
	0 h		adsorbed at 72 h		adsorbed at 360 h		72 h		360 h	
	Avg (µg/L)	SD	Avg (µg/L)	SD	Avg (µg/L)	SD	Avg (µg/L)	SD	Avg (µg/L)	SD
TBBPA	60.788	4.394	1.706	0.523	6.544	4.157	14.077	2.390	16.736	12.275
TBBPA+EPS	63.791	5.775	5.838	5.108	17.420	5.748	14.061	2.370	13.073	5.696
GO+TBBPA	62.923	12.097	13.868	2.426	16.853	12.101	19.335	1.394	25.963	13.256
GO+TBBPA+EPS	65.262	1.398	18.973	1.398	19.199	1.397	18.904	1.959	22.227	11.476
rGO+TBBPA	68.144	11.479	21.981	11.453	22.135	11.479	19.303	1.415	25.947	13.260
rGO+TBBPA+EPS	71.885	13.255	25.696	13.229	25.860	13.259	17.819	5.776	22.203	11.478
Graphene+TBBPA	59.992	2.392	7.026	2.090	13.989	2.384	17.798	5.773	18.932	1.949
Graphene+TBBPA+EPS	64.865	1.959	18.637	1.928	18.829	1.957	14.269	4.471	16.959	12.096

Table S8: EC_{50} values

Materials	EC_{50} (mg/L) (Short term)	EC_{50} (mg/L) (long term)	R^2 value (Short)	R^2 value (Long)
TBBPA	3.54	2.095	0.966	0.908
TBBPA + EPS	3.979	5.15	0.963	0.983
GO+TBBPA	0.905	0.26	0.822	0.796
rGO+TBBPA	1.07	0.426	0.830	0.744
Graphene+TBBPA	1.8	1.201	0.885	0.841
GO+TBBPA+EPS	1.398	0.59	0.871	0.760
rGO+TBBPA+EPS	1.58	0.704	0.897	0.793
Graphene+TBBPA+EPS	2.39	1.95	0.905	0.898

Figures

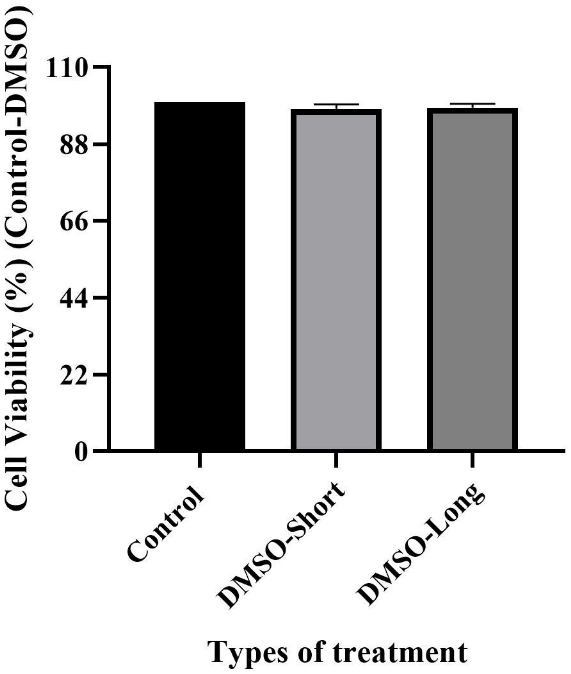


Fig S1: Control treatment with DMSO revealed that there is no significant difference in the DMSO test groups in short and long-term exposure with respect to control

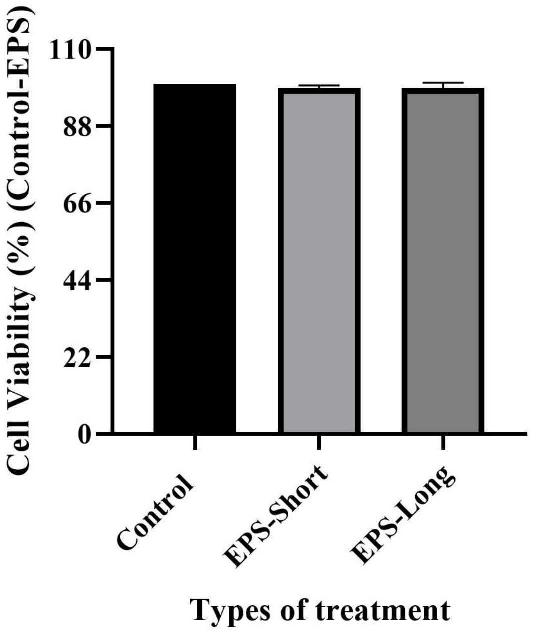


Fig S2: Control treatment with EPS revealed that there is no significant difference in the EPS test groups in short and long-term exposure with respect to control

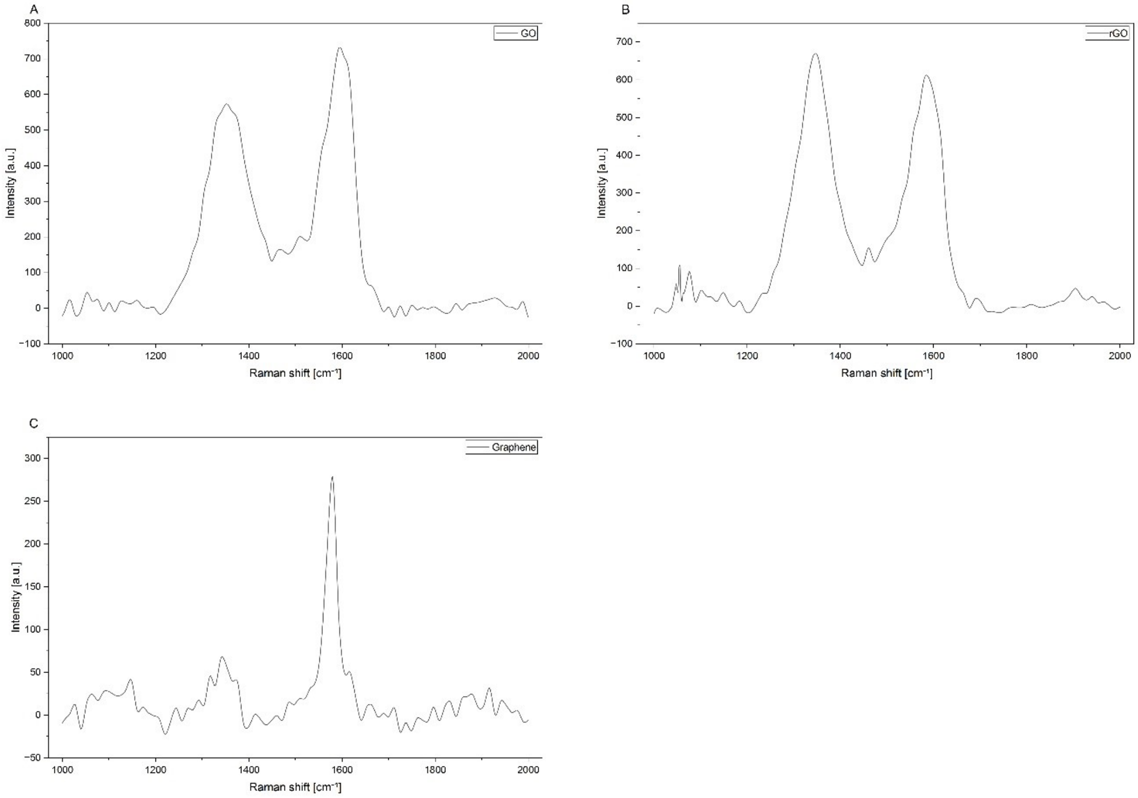


Fig S3: Raman spectra of A-GO, B-rGO, C-Graphene

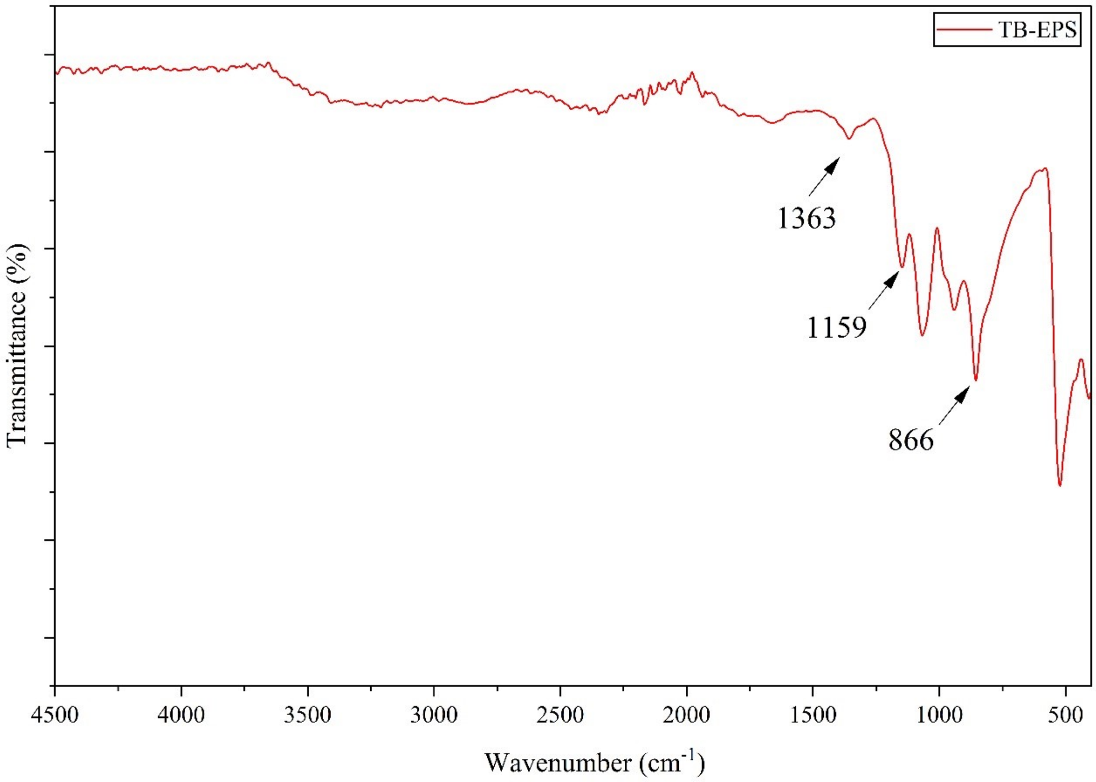


Fig S4: FTIR spectra of extracted TB-EPS (Debroy and Pulimi 2025)

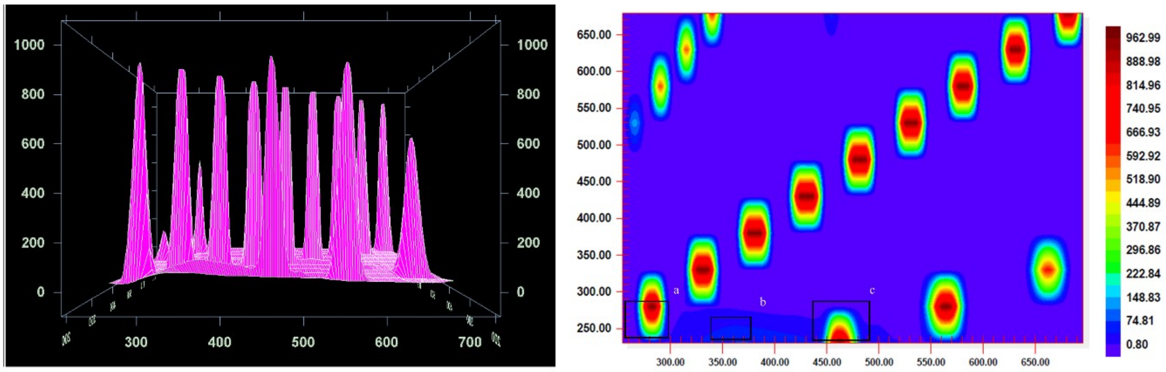


Fig S5: 3D EEM spectra of extracted algal EPS

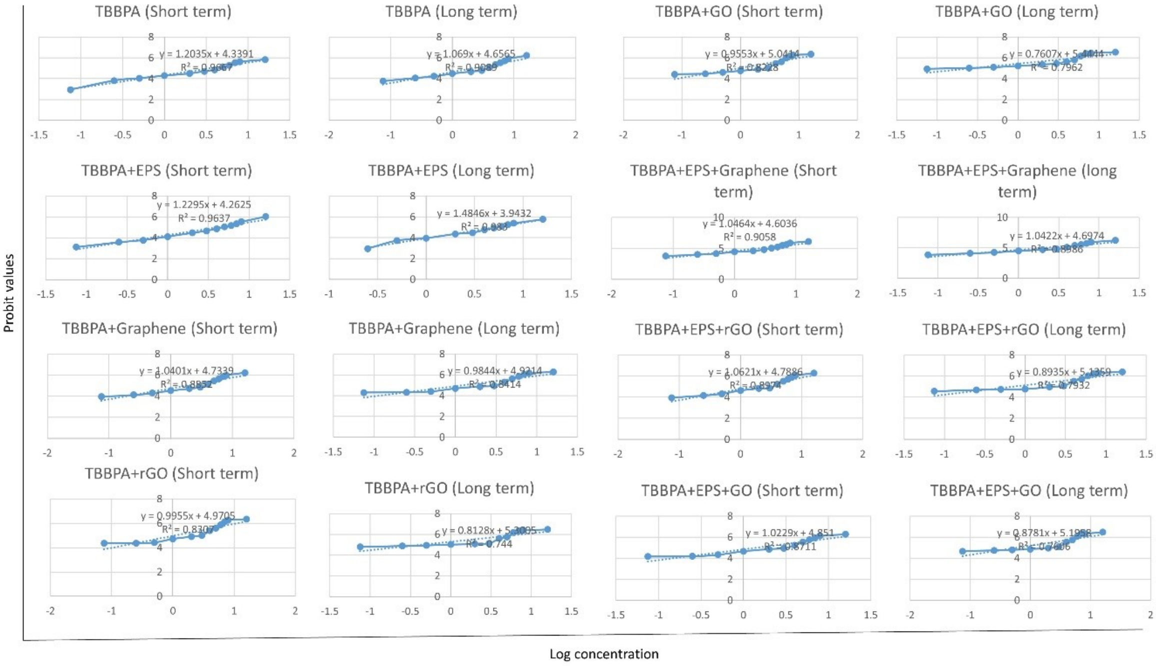


Fig S6: EC_{50} of the treatment groups

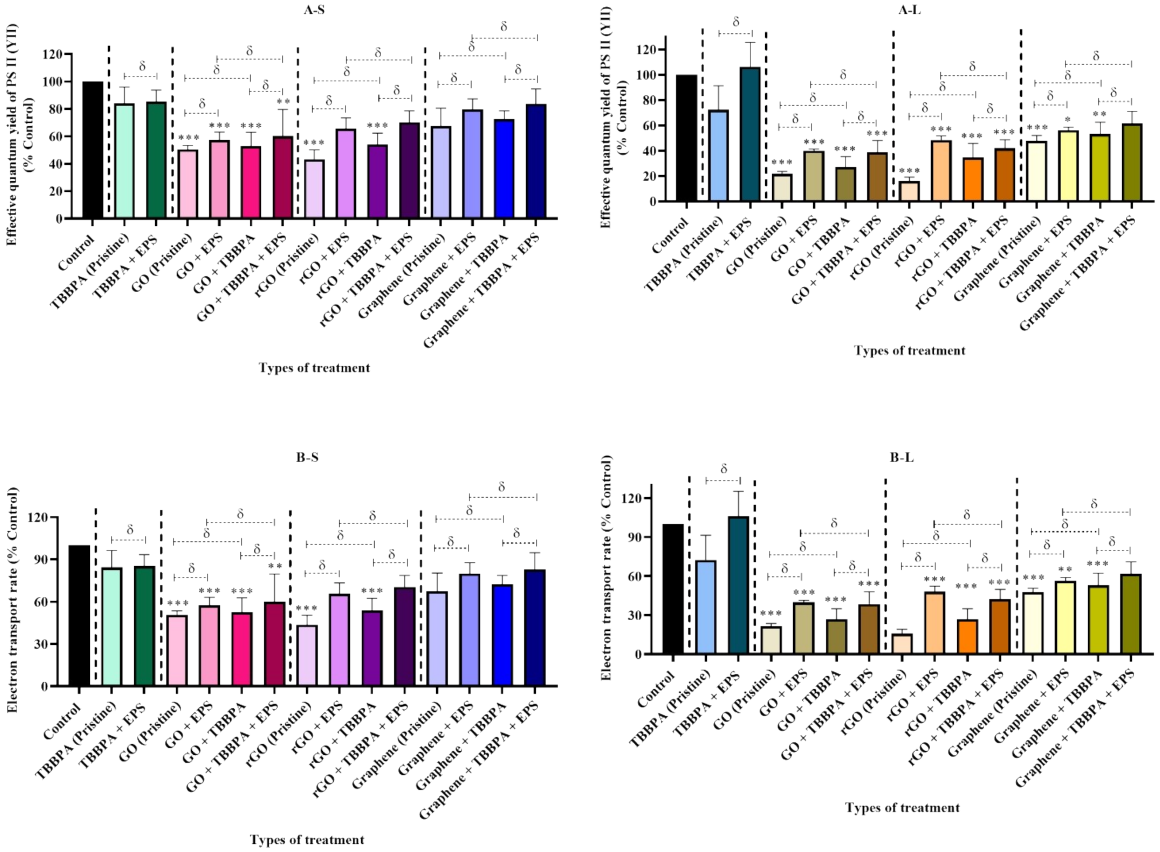
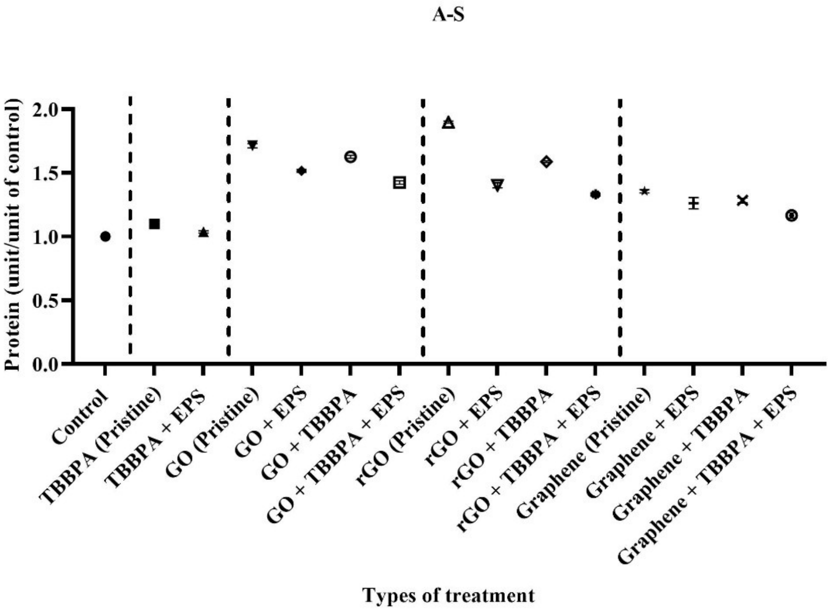


Fig S7: Photosynthetic activity, A-S: Effective quantum yield of PS II (short term exposure), A-L: Effective quantum yield of PS II (long term exposure), B-S: ETR (short term exposure), B-L: Long term exposure. The experiment was performed in triplicate (n=3) for all the treatment groups and control.



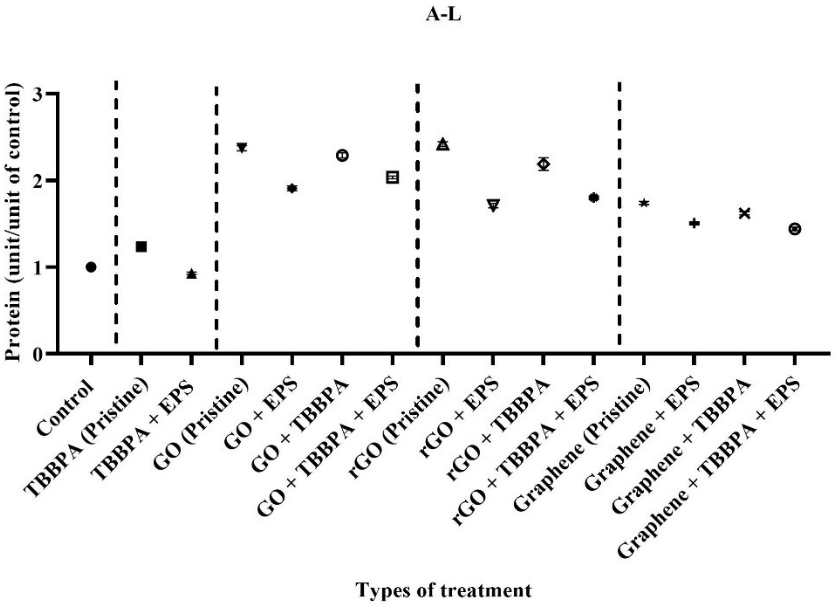
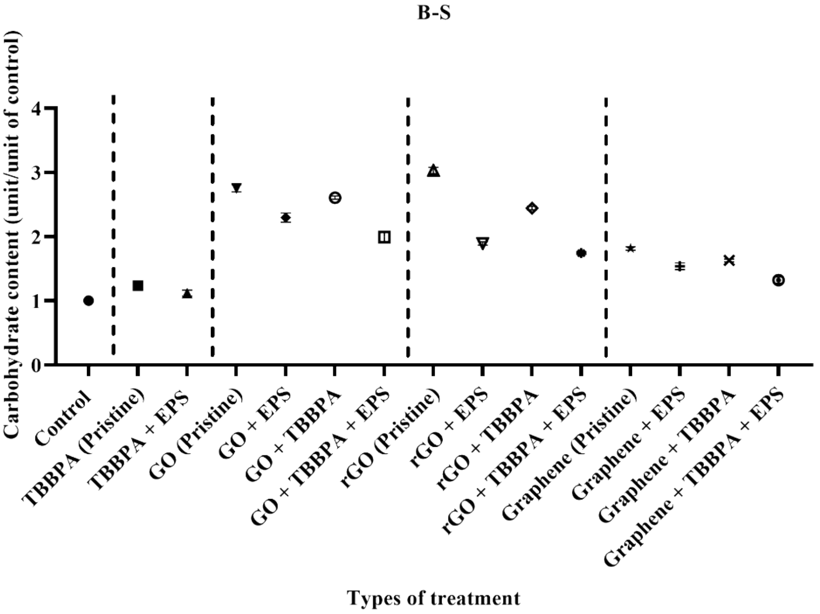


Fig S8: Protein, A-S: Short term, A-L: Long term. The experiment was performed in triplicate (n=3) for all the treatment groups and control.



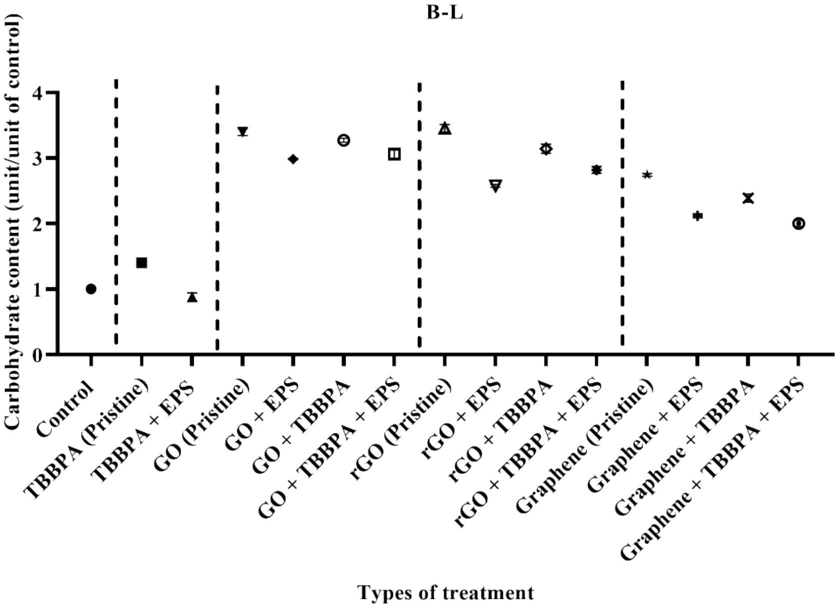


Fig S9: Generated carbohydrate, B-S: Short term, B-L: Long term exposure. The experiment was performed in triplicate (n=3) for all the treatment groups and control.

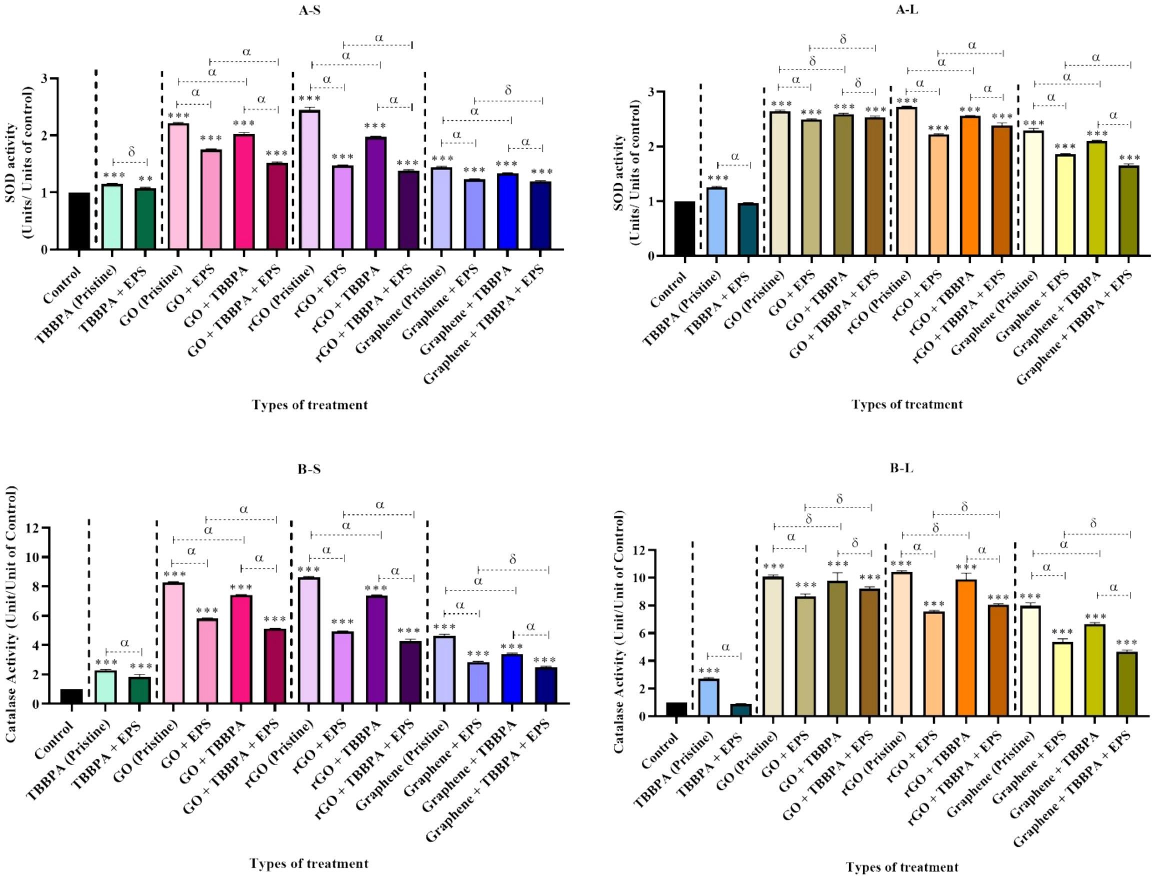


Fig S10: A-S: SOD activity at short term exposure, A-L: SOD activity at long term exposure, B-S: Catalase activity at short term exposure, B-L: Catalase activity at long term exposure. The experiment was performed in triplicate (n=3) for all the treatment groups and control.

References

1 N. Roy, K. Kannabiran and A. Mukherjee, .

2 Z. Z. Yang, Q. Bin Zheng, H. X. Qiu, J. Li and J. H. Yang, *Xinxing Tan Cailiao/New Carbon Materials*, 2015, 30, 41–47.

3 J. S. Y. Chia, M. T. T. Tan, P. S. Khiew, J. K. Chin, H. Lee, D. C. S. Bien and C. W. Siong, *Chemical Engineering Journal*, 2014, 249, 270–278.

4 J. Drelich, *Surf Innov*, 2013, 1, 248–254.

5 H. Yang, X. J. Luo, Y. H. Zeng and B. X. Mai, *J Chromatogr A*, 2021, 1647, 462152.

6 L. Natarajan, M. Annie Jenifer, W. J. G. M. Peijnenburg and A. Mukherjee, *Nanotoxicology*, 2023, 0, 1–14.

7 V. Thiagarajan, P. M., A. S., S. R., C. N., S. G.K. and A. Mukherjee, *Chemosphere*, 2019, 233, 363–372.

8 S. Das, S. Giri, G. Wadhwa, M. Pulimi, S. Anand, N. Chandrasekaran, S. A. Johari, P. K. Rai and A. Mukherjee, *Environmental Science and Pollution Research*, 2023, 70246–70259.

9 S. Giri, A. C. Christudoss, N. Chandrasekaran, W. J. G. M. Peijnenburg and A. Mukherjee, *Plant Physiology and Biochemistry*, 2023, 197, 107664.

10 S. Giri and A. Mukherjee, *J Environ Chem Eng*, 2021, 9, 105978.

11 A. Debroy, M. J. Nirmala, M. Pulimi, W. J. G. M. Peijnenburg and A. Mukherjee, *Chemosphere*, DOI:10.1016/j.chemosphere.2024.142491.