



Universiteit
Leiden
The Netherlands

Algal extracellular polymeric substances (algal-EPS) for mitigating the combined toxic effects of polystyrene nanoplastics and nano-TiO₂ in *Chlorella* sp.

Natarajan, L.; Jenifer, M.A.; Peijnenburg, W.J.G.M.; Mukherjee, A.

Citation

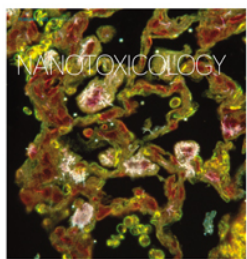
Natarajan, L., Jenifer, M. A., Peijnenburg, W. J. G. M., & Mukherjee, A. (2023). Algal extracellular polymeric substances (algal-EPS) for mitigating the combined toxic effects of polystyrene nanoplastics and nano-TiO₂ in *Chlorella* sp. *Nanotoxicology*. doi:10.1080/17435390.2023.2179438

Version: Publisher's Version

License: [Licensed under Article 25fa Copyright Act/Law \(Amendment Taverne\)](#)

Downloaded from: <https://hdl.handle.net/1887/3594569>

Note: To cite this publication please use the final published version (if applicable).



Algal extracellular polymeric substances (algal-EPS) for mitigating the combined toxic effects of polystyrene nanoplastics and nano-TiO₂ in *Chlorella* sp.

Lokeshwari Natarajan, M. Annie Jenifer, Willie J. G. M. Peijnenburg & Amitava Mukherjee

To cite this article: Lokeshwari Natarajan, M. Annie Jenifer, Willie J. G. M. Peijnenburg & Amitava Mukherjee (2023): Algal extracellular polymeric substances (algal-EPS) for mitigating the combined toxic effects of polystyrene nanoplastics and nano-TiO₂ in *Chlorella* sp., Nanotoxicology, DOI: [10.1080/17435390.2023.2179438](https://doi.org/10.1080/17435390.2023.2179438)

To link to this article: <https://doi.org/10.1080/17435390.2023.2179438>

 View supplementary material 

 Published online: 14 Feb 2023.

 Submit your article to this journal 

 Article views: 60

 View related articles 

 View Crossmark data 

ARTICLE



Algal extracellular polymeric substances (algal-EPS) for mitigating the combined toxic effects of polystyrene nanoplastics and nano-TiO₂ in *Chlorella* sp.

Lokeshwari Natarajan^a, M. Annie Jenifer^b, Willie J. G. M. Peijnenburg^{c,d} and Amitava Mukherjee^a

^aCentre for Nanobiotechnology, VIT, Vellore, Tamil Nadu, India; ^bVIT School of Agricultural Innovations and Advanced Learning, VIT, Vellore, Tamil Nadu, India; ^cInstitute of Environmental Sciences (CML), Leiden University, Leiden, The Netherlands; ^dCenter for the Safety of Substances and Products, National Institute of Public Health and the Environment, Bilthoven, The Netherlands

ABSTRACT

The continuous release of nanoparticles and nanoplastics into the marine environment necessitates the examination of their combined effects in marine organisms. Natural Organic Matter (NOM) can significantly influence the behavior of nanomaterials in the marine environment. The present study explores the effects of algal Extracellular Polymeric Substances (EPS) in reducing the combined toxic effects of three different polystyrene nanoplastics (PSNPs)—aminated (NH₂-PSNPs), carboxylated (COOH-PSNPs), and plain PSNPs—and P25 titanium dioxide nanoparticles (Nano-TiO₂) towards the marine alga, *Chlorella* sp. Two doses (0.25 and 2.5 mg/L) of nano-TiO₂ mixed with the PSNPs (1 mg/L) were employed. The COOH-PSNPs with 2.5 mg/L nano-TiO₂ exhibited higher growth inhibition toward algal cells. Addition of algal EPS to the mixture of NMs decreased the negative effect significantly. The mean hydrodynamic diameter increased significantly from 666 to 797 nm and 1248 to 3589 nm at concentrations 0.25 and 2.5 mg/L, respectively when the mixtures of nano-TiO₂ and COOH-PSNPs were incubated with the algal EPS. In comparison to the pristine NMs, the EPS-NMs were found to significantly reduce the superoxide and hydroxyl radical production. The results were further validated with the estimation of lipid peroxidation (LPO), esterase activity, photosynthetic efficiency, and membrane permeability in the cells. The major outcomes from this study highlight the role of algal EPS in significantly reducing the toxic impact of binary mixture of NMs in marine organisms.

ARTICLE HISTORY

Received 12 October 2022

Revised 7 February 2023

Accepted 8 February 2023

KEYWORDS

Eco-corona; marine alga; mixture toxicity; nano-TiO₂; polystyrene nanoplastics

1. Introduction

Globally, plastic waste is recognized as a threat to marine ecosystems (Andrady et al. 2011). One of the underexplored areas in understanding the impacts of plastic pollution is the assessment of nanoplastics (<1 µm) (NPs) and their biological effects. Since standard sampling methods and analytical techniques for the detection of plastic polymers make it difficult to isolate and quantify their nano-fraction, the amount of nanoplastics deposited in the oceans is mostly unknown (Cózar et al. 2014; Koelmans, Besseling, and Shim 2015). Recently, NPs have received increased attention due to the firm evidence suggesting their potential to be more destructive than microplastics (Rehse, Kloas, and Zarfl 2016; Kihara et al. 2021). Being resistant to

biodegradation, NPs can persist for a long time in the environment (Zaki and Aris 2022).

The marine environment is often considered a 'sink' for various pollutants. The increased usage of nanomaterials for commercial and consumer applications has raised significant concerns regarding their effects on biological systems. Among the various NPs, metal oxide nanomaterials can adversely affect marine biota (Xia et al. 2017; Yan et al. 2021). In particular, nano-TiO₂ (P25) is known to be one of the most used materials (Chen and Selloni 2014; Khalid, Aqeel, and Noman 2020). Nano-TiO₂ poses serious risk to marine organisms due to its photo-catalytic and non-biodegradable nature (Etacheri et al. 2015). The toxicity of nano-TiO₂ in *Chlorella pyrenoidosa* was studied by Al-Ammari et al. (2021). Their findings imply that the

size of the particles and their solubility are the key factors that dictate their cytotoxicity. A recent report by Baharlooieian, Kerdgari, and Shimada (2021) highlighted the negative effects of nano-TiO₂ in the algal species, *Chaetoceros muelleri*. Metzler, Erdem, and Huang (2018) demonstrated that the deposition of nano-TiO₂ on the cell surface of *Raphidocelis subcapitata* may block nutrient transport.

The effects of mixtures of nanomaterials are likely to differ from the average toxic effect of the individual components. Our recent study has indicated that hetero-aggregation of polystyrene NPs (PSNPs) with nano-TiO₂ led to the decreased bioavailability of the particles for the marine alga *Chlorella* sp. (Natarajan et al. 2021). Dong et al. (2018) investigated the combined effect of nano-TiO₂ and PSNPs in *C. elegans* and observed that chronic exposure to PSNPs exacerbated the effects of nano-TiO₂. On the contrary, Yu et al. (2016) found that the combination of nano-TiO₂ and CeO₂ NPs had an antagonistic interaction with *N. europaea*. Similarly, Ogunsuyi et al. (2019) proved the antagonistic action of AgNPs in combination with CuO NPs toward *Clarias gariepinus*.

One of the critical gaps in the current ecotoxicological studies of nanomaterials lies in ignoring the role of naturally occurring biopolymers in modulating their physico-chemical as well as biological interactions. Extracellular Polymeric Substances (EPS) are one such natural biopolymer that often functions as a barrier to pollutants. EPS constitutes a key component of microalgae. Algae produce EPS for a variety of reasons, including (a) secure attachment and improvement of the local environment, and (b) as a metabolic waste. Notably, EPS secretion boosts cell survival, metabolic efficacy, and adaptability (Decho and Gutierrez 2017). These biopolymers can easily adsorb to nanomaterials in the aquatic environment, modulating their bioactivity, cytotoxicity, and physiological features (Alimi et al. 2018). This phenomenon is often referred to as eco-corona formation, the effects of which need to be considered while formulating an experimental design for nano-ecotoxicity studies in aquatic organisms.

Thus, despite the fact that the toxicity of nanomaterials in marine ecosystems has been well documented, very few reports highlight their combined effects on marine organisms (Natarajan et al. 2021; Yu et al. 2022) and study the effect of eco-corona formation on nanoplastics (Nasser and Lynch 2016;

Saavedra, Stoll, and Slaveykova 2019; Fadare et al. 2020; Grassi et al. 2020). These reports fail to explain how the eco-corona may modify the toxicity of a mixture of nanoplastics and nanoparticles in aquatic organisms. The main objective of the present work is to investigate how the adsorption of algal EPS on the mixture of PSNPs [aminated (NH₂-PSNPs), carboxylated (COOH-PSNPs), and plain PSNPs] (each at a concentration of 1 mg/L) and nano-TiO₂ (0.25 and 2.5 mg/L) would alter their toxicity to the marine algae, *Chlorella* sp. This research involved the critical assessment of cytotoxicity markers in the algal cells like photosynthetic and metabolic activities, production of reactive oxygen species, and activities of major antioxidant enzymes.

2. Materials and methods

2.1. Chemicals

The details of the various chemicals used in the current work are provided in the [Supplementary Information](#) section ([Supplementary Table S1](#)).

Artificial Sea Water (ASW) was selected as the test medium to mimic the realistic environmental characteristics. [Supplementary Table S2](#) summarizes the composition of ASW. The ASW was autoclaved for 15 minutes at 121 °C before further use in all the experiments.

2.2. Culturing of *Chlorella* sp

The marine microalga, *Chlorella* sp., procured from Central Marine Fisheries Research Institute (CMFRI), Rameswaram, Tamil Nadu, was used as the model organism for toxicity assessment. The organisms were cultivated in sterilized 200-ml flasks containing ASW and the required micronutrients ([Supplementary Tables S2 and S3](#)). During the growth period, the cultures were exposed to Philips Fluorescent lamps (3000 lux) for 16 hours of light and 8 hours of darkness at 24 ± 2 °C. The culture flasks were shaken daily once or twice to reduce sedimentation (Natarajan, Jenifer, and Mukherjee 2021).

2.3. Preparing dispersions of PSNPs and nano-TiO₂

The PSNPs were dispersed in Milli-Q water for preparing a fresh stock suspension of 100 mg/L. This suspension was bath sonicated for 15 min to ensure

uniform dispersion. Similarly, the stock suspension of nano-TiO₂ (100 mg/L) in Milli-Q water was prepared and ultrasonicated for 20 min. The working doses for nano-TiO₂ were fixed at 0.25 and 2.5 mg/L and PSNPs at 1 mg/L. Notably, the EC₅₀ value of nano-TiO₂ for the same species was determined to be 6.5 mg/L (Thiagarajan et al. 2019). The working concentrations were also based on our previous study, wherein the combined toxicity of nano-TiO₂ and PSNPs was analyzed (Natarajan, Jenifer, and Mukherjee 2021). Another previous report suggests that the environmentally relevant concentrations of nano-TiO₂ and PSNPs in the marine system would fall between µg/L and mg/L (Xia et al. 2017).

2.4. Algal EPS extraction and adsorption on PSNPs and nano-TiO₂

The EPS extraction was carried out by following our previous study (Natarajan et al. 2020). At the exponential phase, well-grown algal cells were centrifuged at 7400 × g for 10 min (at 4 °C), and the cells were suspended in ASW to yield a suspension of 0.5 Optical Density (OD) at 610 nm. These samples were centrifuged at 16 575 × g for 10 min (4 °C) and incubated in light for 24 h. The abundance of organic compounds present in the extracted algal EPS was studied by performing HR-LCMS as reported in our previous study (Natarajan et al. 2020). The results are included in the [Supplementary Table S4](#). A TOC (Total Organic Carbon) analyzer was used to measure the organic carbon content in the supernatant (Model TOC-L, Shimadzu). To negate the Natural Organic Matter (NOM) already present in the exposure medium, the TOC of ASW was also measured and duly subtracted from the total TOC value.

Each suspension of nano-TiO₂ (0.25 and 2.5 mg/L) was combined with 1 mg/L of the PSNPs (COOH-PSNPs, NH₂-PSNPs, and Plain-PSNPs) (hereafter mentioned as *pristine-NMs*). To analyze the effect of algal EPS on the NMs (mixture of PSNPs and nano-TiO₂), different combinations of NMs were incubated with the algal EPS at 24 ± 2 °C under visible light conditions for 72 h. The NMs with adsorbed EPS are hereafter mentioned as *EPS-NMs*.

2.5. Characterization of pristine-NMs and EPS-NMs

2.5.1. Microscopic analysis

Transmission Electron Microscopy (TEM) was used to examine the morphological characteristics of the EPS-NMs and pristine-NMs. For this, a portion of the dispersion from (a) nano-TiO₂ (2.5 mg/L) + PSNPs (1 mg/L) + Algal EPS, and (b) nano-TiO₂ (2.5 mg/L) + PSNPs (1 mg/L) was dropped on a carbon-coated copper grid, which was subsequently imaged by TEM (HRTEM, FEI TecnaiG2 T20 S-twin).

2.5.2. Particle size analysis

The mean hydrodynamic diameter (MHD) was analyzed using DLS instrument (Dynamic Light Scattering) (90 Plus Particle Size Analyzer, Brookhaven Instruments Corp., USA). The MHD of EPS-NMs and pristine-NMs was measured at the 0th and 72nd h.

2.5.3. Sedimentation analysis

The sedimentation of nano-TiO₂ (2.5 mg/L) and PSNPs (1 mg/L) in the presence and absence of algal EPS was examined using a UV-visible spectrophotometer (Hitachi U2910, Japan). At varying time-points, 1 mL of the sample was taken from the surface of the fixed setup, and the absorbance was determined at 324 nm. The percentage sedimentation was evaluated by means of the following formula:

$$\% \text{ Sedimentation} = (1 - \text{OD}_0 / \text{OD}_i) \times 100$$

OD₀ and OD_i represent the OD₃₂₄ values of the samples at 0th h and various time-points, respectively (Yang et al. 2021).

2.6. Confirmation of EPS adsorption on NMs

Nano-TiO₂ (0.25 and 2.5 mg/L) and PSNPs (1 mg/L) in the presence of algal EPS were incubated for 72 h to determine the amount of EPS-protein (protein fraction in EPS) adsorbed on the various mixtures of NMs using the Bradford assay. After 72 h of incubation, these samples were centrifuged at 7400 × g for 10 min at 4 °C, and the filtrate was collected. Using the Bradford assay, the collected filtrate was quantified to determine the amount of unbound EPS-protein. The amount of EPS-protein adsorbed on the combination of NMs could be

resolved by subtracting the unbound protein fraction from the initial protein index (0h).

To support and reinforce the protein adsorption data, the changes in the TOC was also measured. Samples were prepared similarly as mentioned in the preceding paragraph, and the TOC was measured.

2.7. Assessing the growth inhibition effects

Well-grown algal cells from the exponential phase were collected and centrifuged in a fixed angle rotor at $7400 \times g$ for 10 min at 4°C temperature. The pellet collected was then resuspended in ASW. Using a colorimeter, the optical density of the algal suspension was adjusted to 0.1 OD. The interaction of NMs with algae was studied to assess the growth inhibition by the binary mixture of PSNPs and nano-TiO₂, without and with adsorbed EPS. Algal cells that did not undergo any of the treatments were considered as control samples. These samples were incubated for 72 h at room temperature ($25\text{--}30^\circ\text{C}$) under visible light. Growth inhibition was assessed after the incubation time by counting the healthy cells put on a hemocytometer and examined under a phase-contrast microscope. Live algal cells that are healthy can be differentiated from dead algal cells as they exist as distinct, relatively big cells whereas dead cells form clusters. The cell counts for the treatment groups were compared with the control group (Natarajan et al. 2020).

2.8. Oxidative stress analysis

2.8.1. Superoxide radical generation

In this study, dihydroethidium (DHE) was used to assess the superoxide radical generated due to its capacity to readily penetrate cell membranes and is perhaps the most sensitive dye as it targets predominantly most of the superoxide radicals. Algal cells treated with pristine-NMs and EPS-NMs for 72 h were centrifuged at $5100 \times g$ for 20 min at 4°C . The collected pellet was incubated with 10 mM DHE dye dispersed in dimethylsulfoxide and dark-incubated for 30 min. Using a spectrofluorometer, the fluorescence intensity was measured [480 nm (excitation); 570 nm (emission) (Cary Eclipse fluorescence spectrophotometer, model G9800A; Agilent Technologies, USA). The fluorescence data acquired

was compared with the fluorescence data from control algal samples, which served as a reference.

2.8.2. Hydroxyl radical generation

The APF dye (aminophenyl fluorescein) was employed to determine the hydroxyl radical generation in algal cells (Setsukinai et al. 2003). Algal cells that had interacted for 72 h were centrifuged at $5100 \times g$ for 20 min at 4°C . The collected pellet was treated with 10 mM APF dye reconstituted in dimethylformamide for 30 min (dark). The fluorescence intensity of the treated samples was measured using a spectrofluorometer at 490 nm (excitation) and 515 nm (emission) (Cary Eclipse fluorescence spectrophotometer, model G9800A; Agilent Technologies, USA). The fluorescence data acquired was compared to fluorescent data from control algal samples, which served as a reference.

2.9. Antioxidant activity

2.9.1. Superoxide dismutase activity

The technique described by Kono (1978) was used to assess the superoxide dismutase activity in the algal cells. The samples of algae that had interacted during 72 h were centrifuged ($7400 \times g$, 10 min, 4°C) and homogenized with 2 mL of 0.5 M phosphate buffer. Further, the homogenized samples were centrifuged at $16\,575 \times g$ for 10 min at 4°C . A series of chemicals were added to 100 μL of the collected supernatant such as 0.6% Triton X 100, 50 mM Na₂CO₃, 96 mM Nitro tetrazolium blue chloride, and hydroxylamine hydrochloride (20 mM) and were incubated at 37°C for 20 min under visible light. The absorbance was recorded using a UV spectrophotometer at 560-nm wavelength (Hitachi, U-2910, Japan). The absorbance data acquired was compared to the control algal samples, which served as a reference.

2.9.2. Catalase activity

The activity of catalase enzymes in *Chlorella* sp. was determined using the protocol described by Yilancioglu et al. (2014). For the analysis of catalase, 72-h interacted algal cells were prepared as described in the preceding paragraph (SOD determination), and then, the UV absorbance of 100 μL of homogenized samples were measured using a combination of chemicals consisting of 800 μL of

10.8 mM H_2O_2 solution and 100 μL of potassium phosphate buffer (pH 7) at a wavelength of 240 nm. Phosphate buffer with H_2O_2 was used as a blank. A UV-visible spectrophotometer was used to measure the absorbance at 240 nm (Hitachi U-2910 Japan). The absorbance data acquired was compared to the control algal samples, which served as a reference.

2.10. Maximum quantum yield of PS II (Φ_m) analysis

The 72-h-treated algal cells were dark incubated for 15 min. Dark-acclimatized samples were analyzed using Photosynthesis Yield Analyzer (Mini PAM). The percentage of variable fluorescence (F_v) to maximum fluorescence (F_m) was computed as per the procedure described by Sjollem et al. (2016).

2.11. Assessment of membrane integrity

2.11.1. Lipid peroxidation

Lipid peroxidation is a phenomenon in which oxidants like reactive oxygen species disrupt the lipids, specifically polyunsaturated fatty acids from the cell membrane of the algal cells. It is a measurement of malondialdehyde (MDA), which is known to be produced in the algal system under stressful situations (Piotrowska-Niczyporuk et al. 2012). To determine the MDA activity, the algal cells were treated with a reaction mixture containing 0.25% (w/v) TCA in 10% (w/v) TBA and heated in a water bath for 30 min at 95 °C. Later, the samples were centrifuged at 7400 $\times g$ for 10 min at 4 °C, and the supernatant was examined using a UV spectrophotometer at 532 and 600 nm (Hitachi, U-2910, Japan).

2.11.2. Assessment of membrane permeability

Membrane permeability in algal cells subjected to NMs was examined using SYTOX green, a fluorescent nucleic-acid-attaching dye that does not invade healthy cells. Following 72 h of treatment of algal cells with NMs, 200 μL of the interacted algal cultures were incubated with 0.1 M of the dye for 40 min (Feng et al. 2019). With excitation and emission wavelengths of 485 ± 20 and 516 ± 20 nm, respectively, the intensity of the observed green fluorescence was quantified and given in reference with the control samples.

2.12. Assessment of metabolic activity

2.12.1. Evaluation of esterase activity

To estimate the esterase activity in the algal cells treated with NMs, fluorescein diacetate (FDA) was employed. Acetone was used as the solvent to obtain an FDA stock solution of 25 mM and this stock solution was stored at 20 °C and used at a nominal working concentration of 0.96 mM (diluted with Milli-Q water). 400 μL of the algal sample was treated with 300 μL of prepared FDA solution for 7 min (room temperature) (Regel et al. 2002). The fluorescence intensity of these samples was measured at 530 nm. The fluorescence intensity for the treated samples was represented with respect to the control samples.

2.12.2. Determination of mitochondrial membrane potential ($\Delta\Psi_m$)

The mitochondrial membrane potential of algal cells treated with NMs was measured using rhodamine 123 dye (Rh123) (Machado and Soares 2015). Algal cells were pelleted out by centrifugation (12 750 $\times g$, 5 min, 4 °C) and incubated with a 2.5-mmol/L solution of Rh123 under dark conditions for 30 min (room temperature). Further, the stained cells were washed and re-suspended in PBS, and the fluorescence intensity was evaluated with a fluorescence spectrophotometer at excitation and emission wavelengths of 485 and 530 nm, respectively (Machado and Soares 2015). The data was provided with respect to the control samples.

2.13. Statistical analysis

All the tests in the current work were carried out in triplicates ($n = 3$). The results are presented in the form of mean $\pm$ standard deviation. The statistical significance was confirmed with the help of a two-way ANOVA test with Bonferroni post-test with the help of Graph Pad Prism 6 software.

3. Results

3.1. Colloidal stability of nano-TiO₂ and PSNP mixtures

The transmission electron microscopic images showed nano-TiO₂ and PSNPs in the presence (Figure 1(a–c)) and absence (Supplementary Figure S1) of algal EPS. The aggregation of NMs was visibly

enhanced in the presence of EPS. From the DLS data, it was observed that there was an increase in the hydrodynamic size of the mixtures when suspended in ASW medium (Figure 2(B)). Incubation of NMs in the presence of EPS further increased the mean hydrodynamic size by 9 and 12 folds for 0.25 mg/L nano-TiO₂ and 2.5 mg/L nano-TiO₂ mixed with PSNPs, respectively (Figure 2(C)). The results of the sedimentation analysis of nano-TiO₂ (2.5 mg/L) and PSNPs (1 mg/L) are represented in Figure 3. At different time points, both pristine-NMs and EPS-NMs were observed to have decreasing absorbances, denoting the settling of the NMs in the medium. The sedimentation of EPS-NMs was significantly enhanced compared to that of pristine-NMs.

3.2. EPS sorption on the NMs

The adsorption of EPS on NMs was confirmed by quantifying the adsorbed EPS-protein on the NMs

after 72h of incubation. Interestingly, increasing the concentration of nano-TiO₂ (from 0.25 to 2.5 mg/L) in the suspension significantly increased the protein adsorption. The highest EPS-protein adsorption was found in case of binary mixture of 2.5 mg/L nano-TiO₂ and COOH-PSNPs, which was 89.4% (Table 1). This was proven with the TOC data, which revealed that the organic content (EPS) adsorption was found to be maximum (94.w %) for the binary mixture of 2.5 mg/L nano-TiO₂ and COOH-PSNPs.

3.3. Effects on growth inhibition

The growth inhibition patterns for the mixtures of NMs are shown in Figure 4. The algal samples treated with pristine NMs demonstrated an increased growth inhibition with an increase in nano-TiO₂ concentrations in the mixture. COOH-PSNPs with 2.5 mg/L nano-TiO₂ exhibited the highest growth inhibition followed by NH₂-PSNPs and

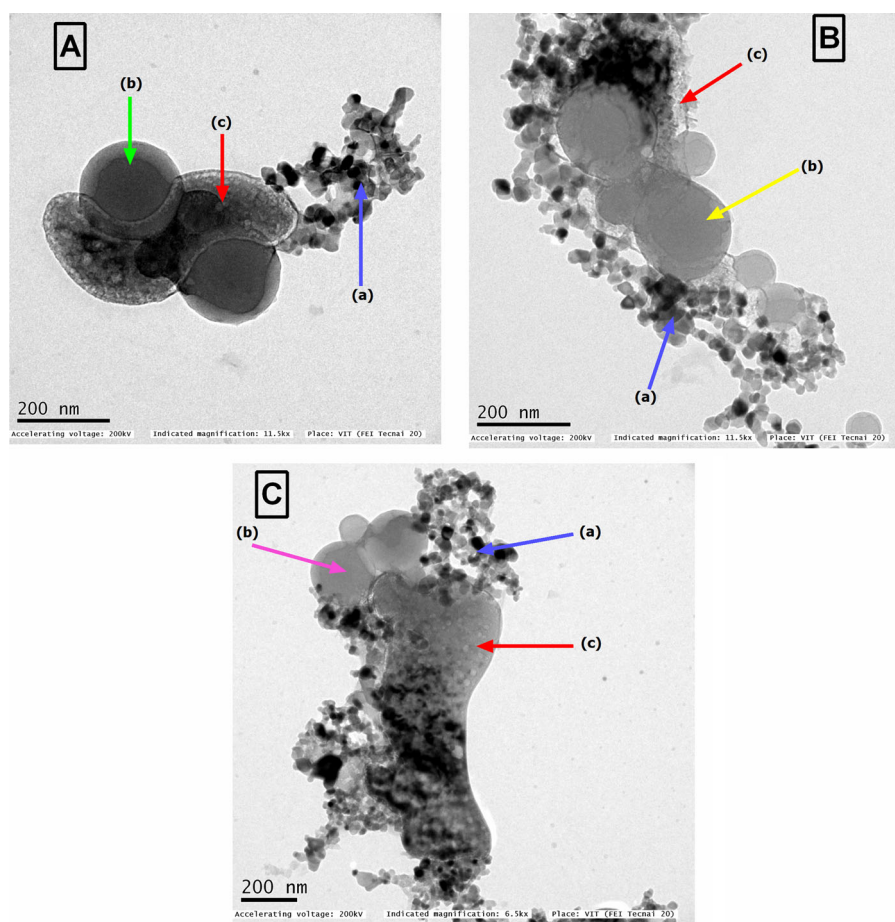


Figure 1. TEM images of nano-TiO₂ and PSNPs (binary mixture) incubated in EPS containing medium (72 h). (A) Nano-TiO₂ (a) and COOH-PSNPs (b) incubated in EPS (c); (B) Nano-TiO₂ (a) and NH₂-PSNPs (b) incubated in EPS (c); (C) Nano-TiO₂ (a) and plain-PSNPs (b) incubated in EPS (c).

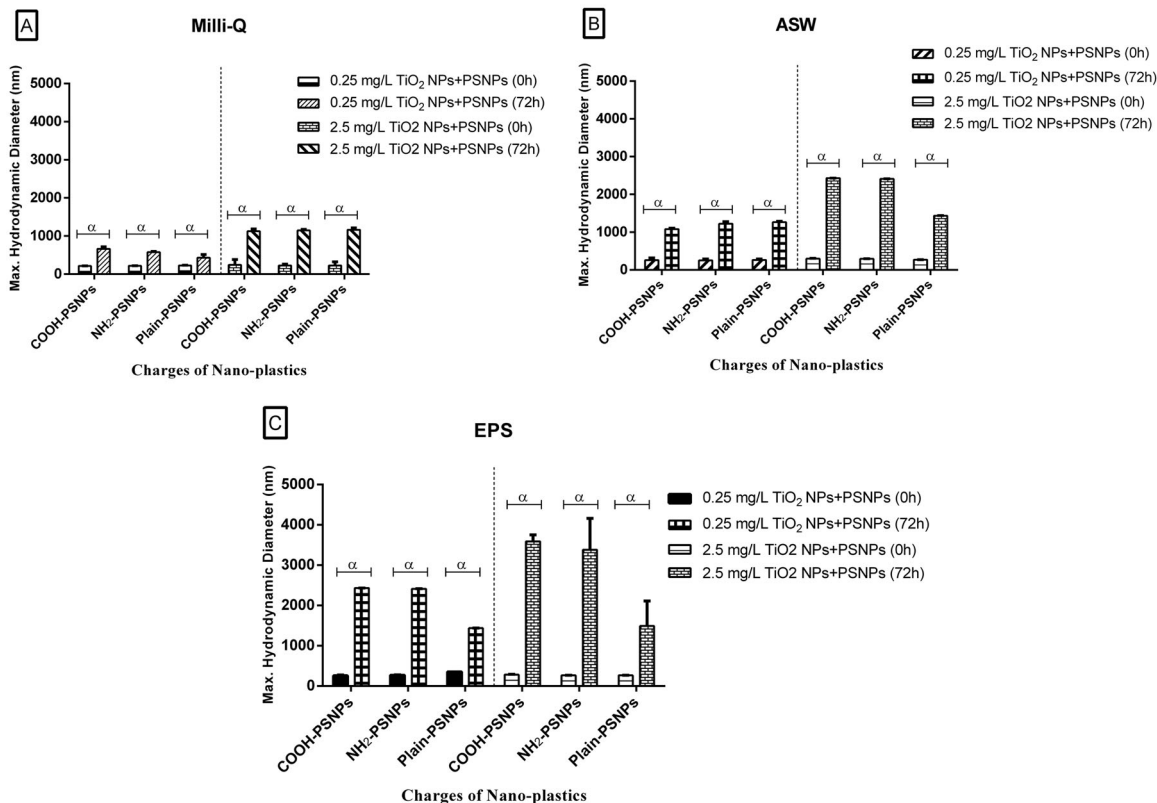


Figure 2. Mean hydrodynamic diameter of nano-TiO₂ and PSNPs dispersed in (A) milli-Q water, (B) ASW, and (C) algal EPS, measured at 0th and 72nd h. Note: 'α, δ' indicates significant difference between 0th and 72nd h ($\alpha=p < 0.001$, $\delta=p > 0.05$).

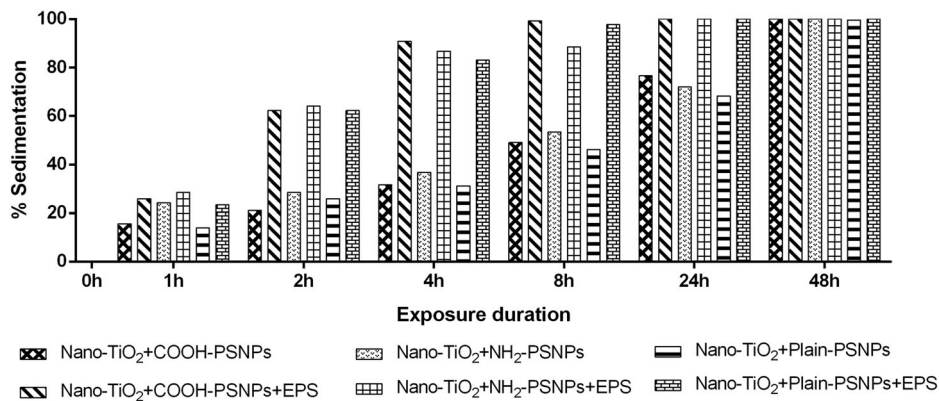


Figure 3. Percentage sedimentation of EPS-NMs and pristine-NMs.

Plain-PSNPs. A significant difference in the growth inhibition was noted between COOH-PSNPs and

Table 1. Quantification of protein and organic compounds adsorbed on the binary mixture of Nano-TiO₂ and PSNPs.

Mixture of NMs (nano-TiO ₂ +PSNPs + Algal EPS)	Bradford assay			TOC		
	0h (μg/ml)	72h (μg/ml)	Protein adsorbed on NMs (μg/ml)	0h (μg/ml)	72h (μg/ml)	Organic compounds adsorbed on NMs (μg/ml)
Nano-TiO ₂ (0.25 mg/L)+COOH-PSNPs	2.496	1.298	1.198	4.698	2.649	2.049
Nano-TiO ₂ (0.25 mg/L)+NH ₂ -PSNPs	2.421	1.347	1.074	4.615	2.606	2.009
Nano-TiO ₂ (0.25 mg/L)+Plain-PSNPs	2.083	1.256	0.826	4.441	2.743	1.685
Nano-TiO ₂ (2.5 mg/L)+COOH-PSNPs	2.495	0.264	2.231	4.986	0.291	4.695
Nano-TiO ₂ (2.5 mg/L)+NH ₂ -PSNPs	2.240	0.430	1.810	4.758	0.655	4.103
Nano-TiO ₂ (2.5 mg/L)+Plain-PSNPs	2.570	0.595	1.975	4.740	0.583	4.157

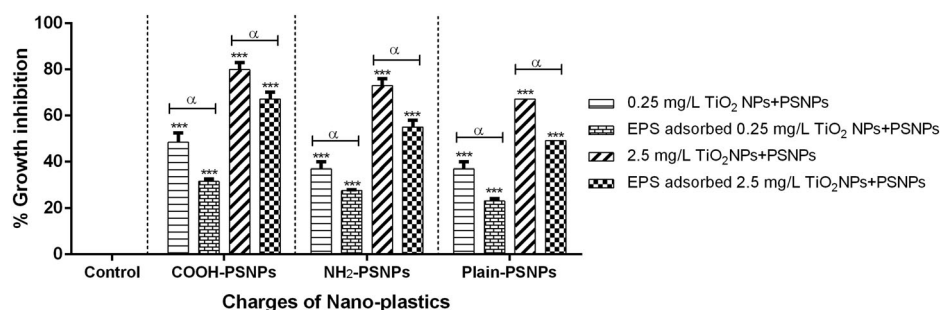


Figure 4. Percent increase of growth inhibition in the algal cells treated with EPS-NMs and pristine-NMs ($n = 3$). Note: ' α ' denotes significant difference between EPS-NMs and pristine-NMs ($\alpha = p < 0.001$).

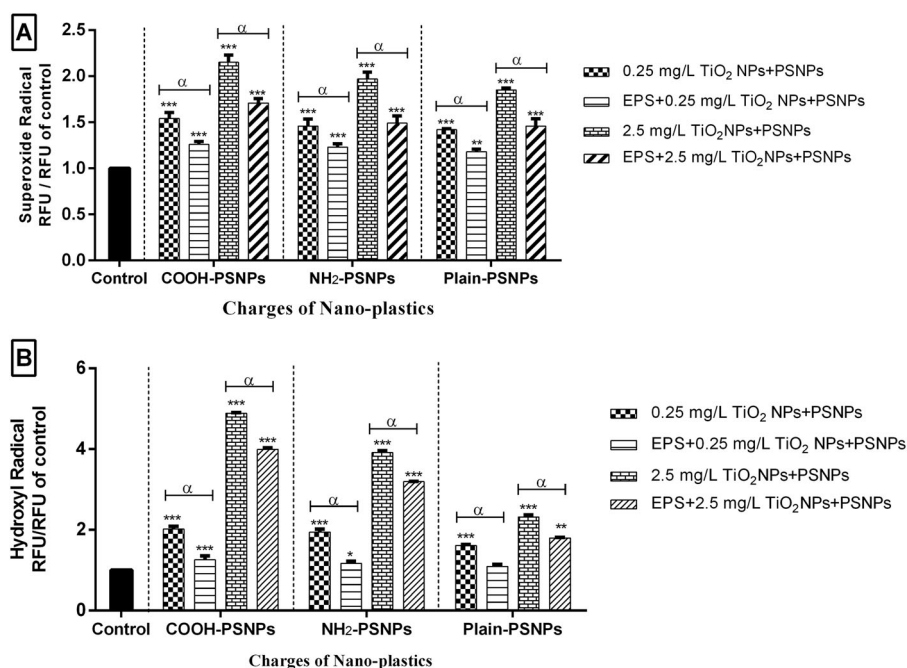


Figure 5. Ratio of (A) hydroxyl radical and (B) superoxide radical production by the algal cells treated with EPS-NMs and pristine-NMs ($n = 3$). Note: ' α ' symbolizes significant difference between EPS-NMs and pristine-NMs ($\alpha = p < 0.001$).

Plain-PSNPs (both pristine and EPS-NMs). Interestingly, when 1 mg/L algal EPS was added to the mixture of NMs, the adverse impact of these particles was significantly reduced. A noticeable decline in growth inhibition was observed for all the mixtures. This clearly shows a cushioning effect of algal EPS.

3.4. Effects on oxidative stress parameters and antioxidant enzyme activity

A significant increase in radical generation was noted in the cells treated with pristine NMs when compared to the control algal cells (Figure 5(A)). The highest radical generation was observed in the

binary mixture of 2.5 mg/L nano-TiO₂ with COOH-PSNPs. On comparing the different surface charges of the PSNPs, the highest significant difference was observed between NH₂-PSNPs and plain-PSNPs. Interestingly, interaction with the EPS-NMs induced significantly lower superoxide radical generation in the mixture of 2.5 mg/L nano-TiO₂ and COOH-PSNPs with adsorbed EPS.

The algal cells treated with the pristine-NMs were observed to generate higher hydroxyl radicals when compared to the control samples (Figure 5(B)). The highest radical generation was observed in nano-TiO₂ (2.5 mg/L) with COOH-PSNPs. However, after EPS-adsorption on the NMs, the radical generation reduced significantly. The highest drop in the radical

generation was noted for this same mixture (2.5 mg/L nano-TiO₂ and COOH-PSNPs) with adsorbed EPS.

The SOD activity in the algal cells treated with pristine NMs was found to decrease when compared with the control samples (Supplementary Figure S2A). However, after EPS sorption on the NMs, the activity of the SOD enzyme was observed to increase. The difference in SOD activity between EPS-NMs and pristine NM mixture of 0.25 mg/L nano-TiO₂+ PSNP-treated algal cells was quite larger, irrespective of the charge of the PSNPs. When compared to the pristine NMs, the highest increase in SOD activity was observed in the mixture of 2.5 mg/L nano-TiO₂ NPs and COOH-PSNPs after EPS adsorption. In contrast to other biological markers studied, no significant difference was observed between the charges of PSNPs (after EPS adsorption) when mixed with the higher concentration of nano-TiO₂ (2.5 mg/L).

The activity of the CAT enzyme in the cells treated with the mixture of pristine NMs was greater than that in the control samples (Supplementary Figure S2B). The activity of the CAT enzyme was markedly reduced in the mixture of 0.25 mg/L nano-TiO₂ and PSNPs with adsorbed EPS. Independent of the charge on the PSNPs, the difference between the EPS-adsorbed and the pristine NM mixtures of nano-TiO₂+ PSNPs-treated algal cells was significant. Similar to SOD activity, CAT activity in the algal cells treated with the EPS-PSNPs and 2.5 mg/L nano-TiO₂ showed no significant difference between the different functionalization of the PSNPs.

3.5. Effects on maximum quantum yield of PS II (Φ_m)

As the EPS-NMs reduced growth inhibition effects and oxidative stress in the algal cells, it was also necessary to determine their effects on the photosynthetic activities. So, the maximum quantum yield of PS II in the cells after treatment with the mixture of NMs was analyzed (Supplementary Figure S3). The percentage Φ_m activity in the mixture of nano-TiO₂ (0.25 and 2.5 mg/L) + PSNPs-treated algal cells (both EPS-NMs and pristine NMs) was found to decrease when compared to the untreated cells. After the EPS-adsorption on the NMs, Φ_m activity notably enhanced for both the concentrations of nano-TiO₂ (0.25 and 2.5 mg/L). Interestingly, no

significant difference is found between the groups based on the charges of PSNPs.

3.6. Changes in membrane integrity

The MDA produced in the algal cells treated with pristine NMs increased dramatically when compared to the control samples (Figure 6(A)). The maximum MDA production was observed for the mixtures of nano-TiO₂ and COOH-PSNPs. Further, EPS-adsorption on the NMs significantly reduced the MDA production. The structural changes in the cell membrane were confirmed with the help of SYTOX green dye using a membrane permeability assay. In the case of the mixture of 0.25 mg/L nano-TiO₂ and COOH-PSNPs, the fraction of cells with damaged membranes decreased maximally upon EPS adsorption on NMs (Figure 6(B)). A high significant difference was found between all the three functionalizations of PSNPs for EPS-NMs in the presence of nano-TiO₂ (0.25 and 2.5 mg/L).

3.7. Effects on metabolic activity

The esterase activity in the algal cells treated with the pristine NMs was reduced compared to the esterase activity in the control algal cells (Supplementary Figure S4A). The highest decrease in esterase activity was observed in the mixture of nano-TiO₂ and COOH-PSNPs followed by NH₂-PSNPs and Plain-PSNPs. Further, the esterase activity increased significantly in the algal cells treated with EPS-NMs. Regardless of the PSNP charge, the difference between the EPS-NMs and the pristine NMs mixtures of 0.25 mg/L nano-TiO₂+ PSNPs-treated algal cells was merely significant.

3.7.1. Mitochondrial membrane potential ($\Delta\Psi_m$)

The $\Delta\Psi_m$ activity after treating with the pristine NMs was lower than that in the untreated samples (Supplementary Figure S4B). For mixtures of nano-TiO₂+ PSNPs-treated samples, an enhancement in $\Delta\Psi_m$ activity was noticed after incubating in the EPS containing medium. The results are quite consistent with the reactive oxygen species generation and photosynthetic activities of the cells discussed in the earlier sections in detail. 2.5 mg/L nano-TiO₂ with PSNPs exhibited higher differences between the three functionalization, with COOH-

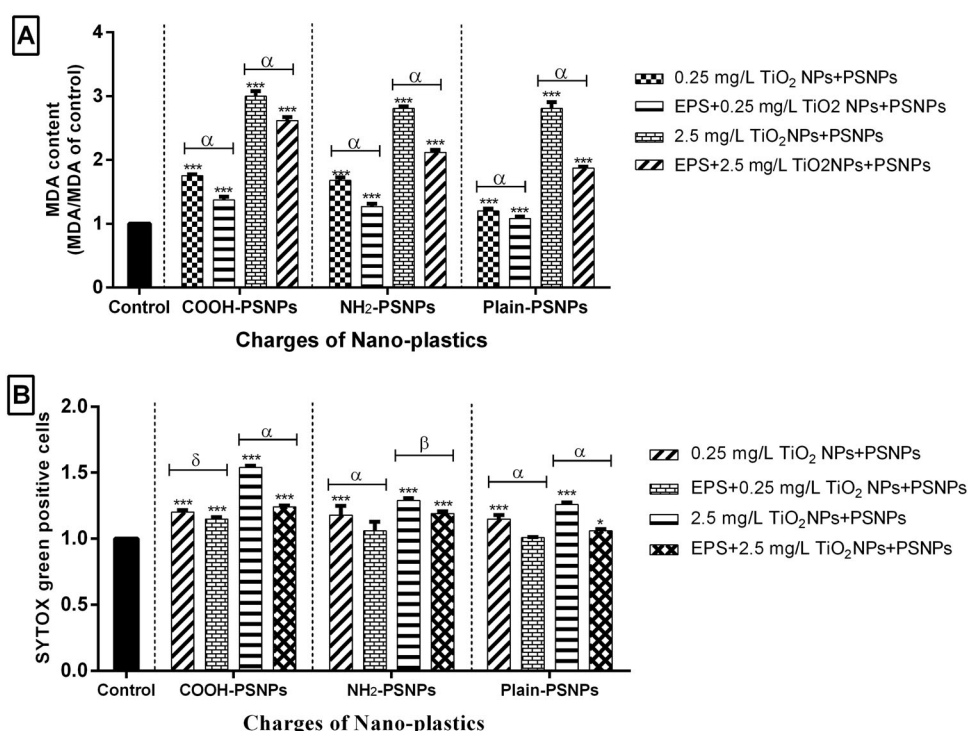


Figure 6. Ratio of (A) MDA content and (B) Membrane permeability in the algae cells treated with EPS-NMs and pristine-NMs ($n=3$). Note: ' α ', ' β ', and ' δ ' represent significant differences between EPS-NMs and pristine-NMs ($\alpha=p < 0.001$, $\beta=p > 0.001$, $\delta=p > 0.05$).

PSNPs showing the highest effects before and after EPS-adsorption.

4. Discussion

The concentration and time-dependent increase in the MHDs of the pristine-NMs in ASW medium proved the colloidal instability and aggregation of the NMs (Hakim and Kobayashi 2021). The electrostatic repulsion between the particles decreases when they are dispersed in highly salinized seawater. This can induce the aggregation between nano-TiO₂ and PSNPs (Natarajan et al. 2021). The inter-particle aggregation phenomena largely control the biological impacts of nanomaterials in aquatic environment (Wang et al. 2016). The increased aggregation of the EPS-NMs was also proven by TEM images and sedimentation analyses.

The toxic potential of the EPS-NMs will differ from that of the pristine NMs due to differences in effective size, surface charge, and surface chemical properties (Yin et al. 2022). The EPS protein adsorption results suggested that the enhanced aggregation could be correlated with adsorption of EPS on the NMs. The formation of a biomolecular layer

[bio-corona] over the NMs is facilitated by hydrogen bonds, Van der Waals forces, hydrophobic interactions, and strong chemical sorption. This increases the attachment efficiency of colliding NMs. The compression of the electric double layer around PSNPs in the EPS-containing medium could have drastically decreased the electrostatic energy barrier leading to aggregation (Zhou et al. 2013). This in turn would reduce the bioavailability and bio-reactivity of the NMs in the medium.

The cellulose, polysaccharides, and glycoprotein components of algal cell walls provide a variety of nonspecific (electrostatic, hydrogen bonding, and hydrophobic) binding sites for NMs (Poulhazan et al. 2021). Other studies also revealed that the EPS secreted by the green alga *Dunaliella tertiolecta* (Morelli et al. 2018; Corsi, Bergami, and Grassi 2020) attached to the nano-TiO₂ surface, subsequently forming a layer or corona. The trapping of the algal cell by the particle agglomerates may stop energy transductions (associated to ATP generation) as well as cause reduction reactions in the cell membrane, which may eventually result in oxidative stress (Rowenczyk et al. 2021). On the other hand, the strong aggregation of NMs with EPS may lead to

decreased bioavailability of NMs for sorption on the algal cells as the large-sized particles would settle more. Thus, decreased toxicity is expected in aquatic organisms (Bergami et al. 2017). This is in agreement with the previous reports highlighting the role of NOM in protecting aquatic organisms against various pollutants (Alimi et al. 2018). Previous work also suggests a significant reduction in the mixture toxicity of Cu nanoparticles and PS-COOH NPs in the microalga *Raphidocelis subcapitata* after interaction with EPS (Bellingeri et al. 2019).

ROS generation is considered a major mode of action (MOA) in nanoparticle-induced toxicity in algae (Bai et al. 2019). Incubating the NMs in EPS-containing medium is shown to reduce the ROS levels in the cells. This could be due to weaker interactions of the EPS-NMs with the algal cell membrane owing to the similar charge on EPS and *Chlorella* sp. (Nolte et al. 2017). According to the past findings, either EPS treatment resulted in a decrease in the attachment of the NMs or quenched the ROS generated by the NMs. The composition of the NOMs present in EPS will vary from species to species, which could probably cause substantial differences in the ROS levels. Xiao and Zheng (2016) noted that EPS from the red alga *Porphyridium* can buffer the cell against ROS by scavenging free radicals and transferring them from the cell to the medium.

Antioxidant enzymes play a crucial role in protecting cells from oxidative damage. Upon NM exposure, both CAT and SOD levels can increase to remove the excess ROS (Hu et al. 2014). Increase in the CAT activity in the algal cells treated with the nanoparticles was also reported previously (Roy et al. 2016). The same pattern was observed in *Synechococcus* sp. when it was treated with iron nanoparticles coated with fulvic acid (a form of NOM) (He et al. 2017). In response to the ROS produced in the presence of NMs, SOD could get activated in *Chlorella* sp., and convert superoxide radicals into H_2O_2 . This H_2O_2 adds up with the H_2O_2 already present in the system and thereby increases the activity of CAT, which converts the H_2O_2 to H_2O (Li et al. 2015). In the current study, increasing SOD levels and decreasing CAT levels were observed after coating the NMs with the algal EPS. When the findings in Figure 5 were compared to the data in

Figure S2B, the observations were found to follow this line of thought.

The results of the photosynthetic efficiency tests demonstrate that mixtures of NMs have an adverse effect on PS II light energy transfer. The increase in ROS concentrations upon NM treatment may disrupt the integrity of thylakoids and impede photosynthesis. Since microalgal EPS can help in scavenging free radicals, it is reasonable to hypothesize that the presence of algal EPS contributed to a general mitigation of oxidative stress, eventually decreasing adverse effects on photosynthetic parameters in the algae. In general, these results demonstrated two processes: the adsorption of biomolecules leading to EPS-NMs complexes, and the formation of NM aggregates induced by the chemistry of the medium. The aggregation-related reduced bioavailability and the free radical scavenging effect together has helped to reduce the toxicity of EPS-NMs. The outcome of these processes depends on the [EPS]:[NMs] ratio in the suspension (Surette and Nason 2019).

The results from the membrane damage experiments also follow the trend observed in the case of growth inhibition and ROS production. Since there was a notable reduction of the oxidative stress by the EPS-NMs, the effects were also visible in improved metabolic activity. In a number of previous studies, the NOMs of different origins also played a protective role in various aquatic organisms exposed to multiple types of pollutants (Von Moos and Slaveykova 2014; Alimi et al. 2018; Saavedra, Stoll, and Slaveykova 2019).

5. Conclusions

The results of this study confirm that the adsorption of algal EPS on the mixture of PSNPs and nano-TiO₂ could substantially reduce their toxicity toward the marine alga *Chlorella* sp. This reduction of harmful effects is strongly correlated with the decreased bioavailability of the aggregated EPS-NMs, lowered oxidative stress, and enhanced cellular photosynthetic yield. The findings from this study are of environmental significance since both nano-TiO₂ and PSNPs are emerging pollutants in the marine environment. Given the lack of information on biological interaction of mixed pollutants with marine algae, more studies need to be

executed with various combinations of pollutants including micro-nanoplastics and nanomaterials. The interplay between biogenic natural organics like EPS and the pollutants may play a major role in modifying their toxicity potential in marine organisms. As the current work demonstrates that EPS may have a cushioning effect in substantially reducing the toxic effects of a mixture of NMs, this will influence their overall impact in the marine food chains.

Acknowledgments

The authors would like to acknowledge the Vellore Institute Technology (VIT), Vellore, India for providing the Transmission Electron Microscopy facility used in this study.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Funding

The author(s) reported there is no funding associated with the work featured in this article.

Data availability statement

The authors confirm that the data supporting the findings of this study are available within the article [and/or] its [supplementary materials](#). All data will be made available on request.

References

- Al-Ammari, A., L. Zhang, J. Yang, F. Wei, C. Chen, and D. Sun. 2021. "Toxicity Assessment of Synthesized Titanium Dioxide Nanoparticles in Fresh Water Algae *Chlorella Pyrenoidosa* and a Zebrafish Liver Cell Line." *Ecotoxicology and Environmental Safety* 211: 111948. doi:10.1016/j.ecoenv.2021.111948.
- Alimi, O. S., J. Farner Budarz, L. M. Hernandez, and N. Tufenkji. 2018. "Microplastics and Nanoplastics in Aquatic Environments: Aggregation, Deposition, and Enhanced Contaminant Transport." *Environmental Science & Technology* 52 (4): 1704–1724. doi:10.1021/acs.est.7b05559.
- Andrady, A.L., 2011. "Microplastics in the Marine Environment." *Marine Pollution Bulletin* 62: 1596–1605. doi: 10.1016/j.marpolbul.2011.05.030.
- Baharlooiean, M., M. Kerdgari, and Y. Shimada. 2021. "Ecotoxicological Effects of TiO₂ Nanoparticulates and Bulk Ti on Microalgae *Chaetoceros Muelleri*." *Environmental Technology & Innovation* 23: 101720. doi:10.1016/j.eti.2021.101720.
- Bai, D., B. H. K. Yip, G. C. Windham, A. Sourander, R. Francis, R. Yoffe, E. Glasson, et al. 2019. "Association of Genetic and Environmental Factors with Autism in a 5-Country Cohort." *JAMA Psychiatry* 76 (10): 1035–1043. doi:10.1001/jamapsychiatry.2019.1411.
- Bellingeri, A., E. Bergami, G. Grassi, C. Faleri, P. Redondo-Hasselerharm, A. A. Koelmans, and I. Corsi. 2019. "Combined Effects of Nanoplastics and Copper on the Freshwater Alga *Raphidocelis Subcapitata*." *Aquatic Toxicology* 210 (November 2018): 179–187. doi:10.1016/j.aquatox.2019.02.022.
- Bergami, E., S. Pugnali, M. L. Vannuccini, L. Manfra, C. Faleri, F. Savorelli, K. A. Dawson, and I. Corsi. 2017. "Long-Term Toxicity of Surface-Charged Polystyrene Nanoplastics to Marine Planktonic Species *Dunaliella tertiolecta* and *Artemia Franciscana* Long-Term Toxicity of Surface-Charged Polystyrene Nanoplastics to Marine Planktonic Species *Dunaliella tertiolecta*." *Aquatic Toxicology* 189 (June): 159–169.
- Chen, X., and A. Selloni. 2014. "Introduction: Titanium Dioxide (TiO₂) Nanomaterials." *Chemical Reviews* 114 (19): 9281–9282. doi:10.1021/cr500422r.
- Corsi, I., E. Bergami, and G. Grassi. 2020. "Behavior and Bio-Interactions of Anthropogenic Particles in Marine Environment for a More Realistic Ecological Risk Assessment." *Frontiers in Environmental Science* 8 (June): 1–21. doi:10.3389/fenvs.2020.00060.
- Cózar, A., F. Echevarría, J. I. González-Gordillo, X. Irigoien, B. Úbeda, S. Hernández-León, Á. T. Palma, et al. 2014. "Plastic Debris in the Open Ocean." *Proceedings of the National Academy of Sciences of the United States of America* 111 (28): 10239–10244. doi:10.1073/pnas.1314705111.
- Decho, A. W., and T. Gutierrez. 2017. "Microbial Extracellular Polymeric Substances (EPSs) in Ocean Systems." *Frontiers in Microbiology* 8 (May): 1–28.
- Dong, S., M. Qu, Q. Rui, and D. Wang. 2018. "Combinational Effect of Titanium Dioxide Nanoparticles and Nanopolystyrene Particles at Environmentally Relevant Concentrations on Nematode *Caenorhabditis elegans*." *Ecotoxicology and Environmental Safety* 161 (April): 444–450. doi:10.1016/j.ecoenv.2018.06.021.
- Etacheri, V., C. Di Valentin, J. Schneider, D. Bahnemann, and S. C. Pillai. 2015. "Visible-Light Activation of TiO₂ Photocatalysts: Advances in Theory and Experiments." *Journal of Photochemistry and Photobiology C: Photochemistry Reviews* 25: 1–29. doi:10.1016/j.jphotochemrev.2015.08.003.
- Fadare, O. O., B. Wan, K. Liu, Y. Yang, L. Zhao, and L. H. Guo. 2020. "Eco-Corona vs Protein Corona: Effects of Humic Substances on Corona Formation and Nanoplastic Particle Toxicity in *Daphnia Magna*." *Environmental Science & Technology* 54 (13): 8001–8009. doi:10.1021/acs.est.0c00615.

- Feng, L., J. Li, E. G. Xu, X. Sun, F. Zhu, Z. Ding, H. Tian, S. Dong, P. Xia, and X. Yuan. 2019. Short-term Exposure to Positively Charged Polystyrene Nanoparticles Causes Oxidative Stress and Membrane destruction in Cyanobacteria. *Environmental Science: Nano* 6: 3072–3079. doi:10.1039/C9EN00807A.
- Grassi, G., E. Gabellieri, P. Cioni, E. Paccagnini, C. Faleri, P. Lupetti, I. Corsi, E. Morelli. 2020. Interplay Between Extracellular Polymeric Substances (EPS) From a Marine Diatom and Model Nanoplastic Through Eco-corona Formation. *Science of The Total Environment* 725, 138457. doi:10.1016/j.scitotenv.2020.138457.
- Hakim, A., and M. Kobayashi. 2021. "Aggregation and Aggregate Strength of Microscale Plastic Particles in the Presence of Natural Organic Matter: Effects of Ionic Valence." *Journal of Polymers and the Environment* 29 (6): 1921–1929. doi:10.1007/s10924-020-01985-4.
- He, M., Y. Chen, Y. Yan, S. Zhou, and C. Wang. 2017. "Influence of Interaction between A-Fe₂O₃ Nanoparticles and Dissolved Fulvic Acid on the Physiological Responses in *Synechococcus* sp." *Bulletin of Environmental Contamination and Toxicology* 99 (6): 719–727. doi:10.1007/s00128-017-2199-y.
- Hu, C., X. Liu, X. Li, and Y. Zhao. 2014. Evaluation of Growth and Biochemical Indicators of *Salvinia natans* Exposed to Zinc Oxide Nanoparticles and Zinc Accumulation in Plants. *Environmental Science and Pollution Research* 21: 732–739. doi:10.1007/s11356-013-1970-9.
- Khalid, N., M. Aqeel, and A. Noman. 2020. "Microplastics Could Be a Threat to Plants in Terrestrial Systems Directly or Indirectly." *Environmental Pollution* 267: 115653. doi:10.1016/j.envpol.2020.115653.
- Kihara, S., A. Ashenden, M. Kaur, J. Glasson, S. Ghosh, N. van der Heijden, A. E. S. Brooks, et al. 2021. "Cellular Interactions with Polystyrene Nanoplastics—the Role of Particle Size and Protein Corona." *Biointerphases* 16 (4): 041001. doi:10.1116/6.0001124.
- Koelmans, A. A., E. Besseling, and W. J. Shim. 2015. "Nanoplastics in the Aquatic Environment." *Critical Review BT - Marine Anthropogenic Litter*. In: Bergmann, M., Gutow, L., Klages, M. (Eds.). Springer International Publishing, Cham, pp. 325–340. doi:10.1007/978-3-319-16510-3_12.
- Kono, Y. 1978. "Generation of Superoxide Radical during Autoxidation of Hydroxylamine and an Assay for Superoxide Dismutase." *Archives of Biochemistry and Biophysics* 186 (1): 189–195.
- Li, F., Z. Liang, X. Zheng, W. Zhao, M. Wu, and Z. Wang. 2015. "Toxicity of nano-TiO₂ on Algae and the Site of Reactive Oxygen Species Production." *Aquatic Toxicology* 158: 1–13. doi:10.1016/j.aquatox.2014.10.014.
- Machado, M. D., and E. V. Soares. 2015. Use of a Fluorescence-Based Approach to Assess Short-Term Responses of the Alga *Pseudokirchneriella subcapitata* to Metal Stress. *Journal of Applied Phycology* 27: 805–813. doi:10.1007/s10811-014-0351-1.
- Metzler, D. M., A. Erdem, and C. P. Huang. 2018. "Influence of Algae Age and Population on the Response to TiO₂ Nanoparticles." *International Journal of Environmental Research and Public Health* 15 (4): 585. doi:10.3390/ijerph15040585.
- Morelli, E., E. Gabellieri, A. Bonomini, D. Tognotti, G. Grassi, and I. Corsi. 2018. "TiO₂ Nanoparticles in Seawater: Aggregation and Interactions with the Green Alga *Dunaliella tertiolecta*." *Ecotoxicology and Environmental Safety* 148 (February 2017): 184–193. doi:10.1016/j.ecoenv.2017.10.024.
- Nasser, F., and I. Lynch. 2016. "Secreted Protein Eco-Corona Mediates Uptake and Impacts of Polystyrene Nanoparticles on *Daphnia Magna*." *Journal of Proteomics* 137: 45–51. doi:10.1016/j.jprot.2015.09.005.
- Natarajan, L., M. A. Jenifer, N. Chandrasekaran, G. K. Suraishkumar, and A. Mukherjee. 2021. "Polystyrene Nanoplastics Diminish the Toxic Effects of Nano-TiO₂ in Marine Algae *Chlorella* sp." *Environmental Research* 204 (November): 112400.
- Natarajan, L., M. A. Jenifer, and A. Mukherjee. 2021. "Eco-Corona Formation on the Nanomaterials in the Aquatic Systems Lessens Their Toxic Impact: A Comprehensive Review." *Environmental Research* 194 (September 2020): 110669. doi:10.1016/j.envres.2020.110669.
- Natarajan, L., S. Omer, N. Jetly, M. A. Jenifer, N. Chandrasekaran, G. K. Suraishkumar, and A. Mukherjee. 2020. "Eco-Corona Formation Lessens the Toxic Effects of Polystyrene Nanoplastics towards Marine Microalgae *Chlorella* sp." *Environmental Research* 188 (June): 109842. doi:10.1016/j.envres.2020.109842.
- Nolte, T. M., N. B. Hartmann, J. M. Kleijn, J. Garnæs, D. van de Meent, A. Jan Hendriks, and A. Baun. 2017. "The Toxicity of Plastic Nanoparticles to Green Algae as Influenced by Surface Modification, Medium Hardness and Cellular Adsorption." *Aquatic Toxicology* 183: 11–20. doi:10.1016/j.aquatox.2016.12.005.
- Ogunsuyi, O. I., O. M. Fadoju, O. O. Akanni, O. A. Alabi, C. G. Alimba, S. Cambier, S. Eswara, A. C. Gutleb, O. A. Adaramoye, and A. A. Bakare. 2019. "Genetic and Systemic Toxicity Induced by Silver and Copper Oxide Nanoparticles, and Their Mixture in *Clarias Gariepinus* (Burchell, 1822)." *Environmental Science and Pollution Research* 26 (26): 27470–27481. doi:10.1007/s11356-019-05958-6.
- Piotrowska-Niczyporuk, A., A. Bajguz, E. Zambrzycka, and B. Godlewska-Żyłkiewicz. 2012. "Phytohormones as Regulators of Heavy Metal Biosorption and Toxicity in Green Alga *Chlorella Vulgaris* (Chlorophyceae)." *Plant Physiology and Biochemistry* 52: 52–65. doi:10.1016/j.pla-phy.2011.11.009.
- Poulhazan, A., M. Widanage, A. Muszyński, A. Arnold, D. Warschawski, P. Azadi, I. Marcotte, and T. Wang. 2021. "Identification and Quantification of Glycans in Whole Cells: Architecture of Microalgal Polysaccharides Described by Solid-State Nuclear Magnetic Resonance." *Journal of the American Chemical Society*. doi:10.1021/jacs.1c07429.

- Regel, R. H., J. M. Ferris, G. G. Ganf, and J. D. Brookes. 2002. "Algal Esterase Activity as a Biomeasure of Environmental Degradation in a Freshwater Creek." *Aquatic Toxicology* 59 (3–4): 209–223. doi:10.1016/S0166-445X(01)00254-5.
- Rehse, S., W. Kloas, and C. Zarfl. 2016. "Short-Term Exposure with High Concentrations of Pristine Microplastic Particles Leads to Immobilisation of *Daphnia Magna*." *Chemosphere* 153: 91–99. doi:10.1016/j.chemosphere.2016.02.133.
- Rowenczyk, L., J. Leflaive, F. Clergeaud, A. Minet, J. Ferriol, L. Gauthier, J. Gigault, et al. 2021. "Heteroaggregates of Polystyrene Nanospheres and Organic Matter: Preparation, Characterization and Evaluation of Their Toxicity to Algae in Environmentally Relevant Conditions." *Nanomaterials* 11 (2): 415–482. doi:10.3390/nano11020482.
- Roy, R., A. Parashar, M. Bhuvaneshwari, N. Chandrasekaran, and A. Mukherjee. 2016. "Differential Effects of P25 TiO₂ Nanoparticles on Freshwater Green Microalgae: *Chlorella* and *Scenedesmus* Species." *Aquatic Toxicology* 176: 161–171. doi:10.1016/j.aquatox.2016.04.021.
- Saavedra, J., S. Stoll, and V. I. Slaveykova. 2019. "Influence of Nanoplastic Surface Charge on Eco-Corona Formation, Aggregation and Toxicity to Freshwater Zooplankton *." *Environmental Pollution* 252: 715–722. doi:10.1016/j.envpol.2019.05.135.
- Setsukinai, K., Y. Urano, K. Kakinuma, H. J. Majima, and T. Nagano. 2003. Development of Novel Fluorescence Probes That Can Reliably Detect Reactive Oxygen Species and Distinguish Specific Species. *Journal of Biological Chemistry* 278, 3170–3175. doi:10.1074/jbc.M209264200.
- Sjollema, S. B., P. Redondo-Hasselerharm, H. A. Leslie, M. H. S. Kraak, and A. D. Vethaak. 2016. "Do Plastic Particles Affect Microalgal Photosynthesis and Growth?" *Aquatic Toxicology* 170: 259–261. doi:10.1016/j.aquatox.2015.12.002.
- Surette, M. C., and J. A. Nason. 2019. "Nanoparticle Aggregation in a Freshwater River: The Role of Engineered Surface Coatings." *Environmental Science: Nano* 6 (2): 540–553.
- Thiagarajan, V., M. Pavani, S. Archanaa, R. Seenivasan, N. Chandrasekaran, G. K. Suraishkumar, and A. Mukherjee. 2019. "Diminishing Bioavailability and Toxicity of P25 TiO₂ NPs during Continuous Exposure to Marine Algae *Chlorella* sp." *Chemosphere* 233: 363–372. doi:10.1016/j.chemosphere.2019.05.270.
- Von Moos, N., and V. I. Slaveykova. 2014. "Oxidative Stress Induced by Inorganic Nanoparticles in Bacteria and Aquatic microalgae – State of the Art and Knowledge Gaps." *Nanotoxicology* 8 (6): 605–630. doi:10.3109/17435390.2013.809810.
- Wang, J., Z. Tan, J. Peng, Q. Qiu, and M. Li. 2016. "The Behaviors of Microplastics in the Marine Environment." *Marine Environmental Research* 113: 7–17. doi:10.1016/j.marenvres.2015.10.014.
- Xia, B., L. Zhu, Q. Han, X. Sun, B. Chen, and K. Qu. 2017. "Effects of TiO₂ Nanoparticles at Predicted Environmental Relevant Concentration on the Marine Scallop *Chlamys farreri*: An Integrated Biomarker Approach." *Environmental Toxicology and Pharmacology* 50: 128–135. doi:10.1016/j.etap.2017.01.016.
- Xiao, R., and Y. Zheng. 2016. Overview of microalgal extracellular polymeric substances (EPS) and their applications. *Biotechnology Advances* 34, 1225–1244. doi:10.1016/j.biotechadv.2016.08.004.
- Yan, Z., L. Xu, W. Zhang, G. Yang, Z. Zhao, Y. Wang, and X. Li. 2021. "Comparative Toxic Effects of Microplastics and Nanoplastics on *Chlamydomonas reinhardtii*: Growth Inhibition, Oxidative Stress, and Cell Morphology." *Journal of Water Process Engineering* 43 (August): 102291. doi:10.1016/j.jwpe.2021.102291.
- Yang, W., P. Gao, Y. Nie, J. Huang, Y. Wu, L. Wan, H. Ding, and W. Zhang. 2021. "Comparison of the Effects of Continuous and Accumulative Exposure to Nanoplastics on Microalga *Chlorella Pyrenoidosa* During Chronic Toxicity." *The Science of the Total Environment* 788: 147934.
- Yilancioglu, K., M. Cokol, I. Pastirmaci, B. Erman, and S. Cetiner. 2014. "Oxidative Stress is a Mediator for Increased Lipid Accumulation in a Newly Isolated *Dunaliella Salina* Strain." *PLOS One* 9 (3). e91957.
- Yin, J., G. Huang, C. An, and R. Feng. 2022. "Nanocellulose Enhances the Dispersion and Toxicity of ZnO NPs to Green Algae *Eremosphaera Viridis*." *Environmental Science* 9 (1): 393–405.
- Yu, Q., Z. Wang, G. Wang, W. J. G. M. Peijnenburg, and M. G. Vijver. 2022. "Effects of Natural Organic Matter on the Joint Toxicity and Accumulation of Cu Nanoparticles and ZnO Nanoparticles in *Daphnia Magna*." *Environmental Pollution* 292 (Pt B): 118413. doi:10.1016/j.envpol.2021.118413.
- Yu, R., J. Wu, M. Liu, G. Zhu, L. Chen, Y. Chang, and H. Lu. 2016. "Toxicity of Binary Mixtures of Metal Oxide Nanoparticles to *Nitrosomonas europaea*." *Chemosphere* 153: 187–197. doi:10.1016/j.chemosphere.2016.03.065.
- Zaki, M. R. M., and A. Z. Aris. 2022. "An Overview of the Effects of Nanoplastics on Marine Organisms." *The Science of the Total Environment* 831: 154757. doi:10.1016/j.scitotenv.2022.154757.
- Zhou, J., Z. Lu, X. Zhu, X. Wang, Y. Liao, Z. Ma, and F. Li. 2013. "NIR Photothermal Therapy Using Polyaniline Nanoparticles." *Biomaterials* 34 (37): 9584–9592. doi:10.1016/j.biomaterials.2013.08.075.