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Exploring biochemical responses and cellular adaptations of *Chlorella sorokiniana* to polyethylene microplastic exposure

Jyoti Rawat[1,2], Pooja Bhatnagar[1], Abhilasha Mishra[3], Manisha Nanda[4], Prateek Gururani[5], Afzal Hussain[6], Mikhail S. Vlaskin[7], Sanjay Kumar[1], Monu Verma[8], Hyunook Kim[8] & Vinod Kumar[1,9]✉

Microplastics (MPs) are a common long-lasting pollutant of aquatic ecosystems. Microalgae are primary producers of aquatic systems, and MP contamination could have a high impact on the aquatic food web. Therefore, the present study utilized polyethylene (PE) particles (0 to 150 mg/L) for investigating the half-maximal inhibitory concentrations (IC_{50}) of *Chlorella sorokiniana* and also studied their impacts on growth rate, biomass, pigments and other biochemical components of the microalgae. After 96 h of incubation, PE of 100 mg/L resulted in the half-maximum inhibition (IC_{50}). After reaching the stationary phase (14 d), harvesting was made for MP-exposed cultures to reveal a biomass production of 0.89 g/L, while it was 0.96 g/L for the control. A slight reduction in pigment and lipid contents was also observed, while the protein and carbohydrate contents were high in MP-exposed *C. sorokiniana* cells. Under the MP stress, reactive oxygen species (ROS) and phenolic levels were reduced, whereas flavonoid content increased. PE particles were characterized using Scanning Electron Microscopy-Energy Dispersive X-ray Spectroscopy (SEM-EDX) and Fourier Transform infrared spectroscopy (FT-IR) for their size, shape, chemical composition, and interaction with *C. sorokiniana*, followed by micro-Fourier Transform infrared spectroscopy (μ-FT-IR) for the mapping of MP. This research contributes to a deeper understanding of how MP contamination can disrupt aquatic food webs, guiding future ecological assessments and pollution management strategies.

Keywords Polyethylene (PE), *Chlorella sorokiniana*, Biomass content, Biochemical compounds, Reactive oxygen species (ROS)

Microplastic (MP) is a ubiquitous piece of plastic debris originating from a variety of sources. Once released into the environment, it has an adverse impact on human health and microbiota due to its high persistence in the environment[1]. Although there is room for debate, it is reported to carry chemical pollutants and can cause physical harm if accumulated by organisms. The concentration of MPs in aquatic environments is increasing at a very rapid rate because of the continuous increase in plastic production[2]. It has been reported that in 2016, about 335 million metric tonnes (MMT) of plastic products were produced, while in 2017, this number increased by 348 MMT, which indicates an annual bump of around 4% in plastic production globally[3].

According to statistics, 4,392.5 million metric tonnes (MMT) of plastic was generated between 1950 and 2020; and if present utilization trends continue, it is anticipated to extend to 17,312 MMT by 2050, globally[4].

[1]Algal Research and Bioenergy Lab, Department of Food Science and Technology, Graphic Era (Deemed to be University), Dehradun, Uttarakhand 248002, India. [2]Department of Biosciences, School of Liberal Arts and Sciences, Mody University of Science and Technology, Lakshmangarh, Sikar, Rajasthan 223211, India. [3]Department of Chemistry, Graphic Era (Deemed to be University), Dehradun, Uttarakhand 248002, India. [4]Department of Microbiology, Graphic Era (Deemed to be University), Dehradun, Uttarakhand 248002, India. [5]Department of Biotechnology, Graphic Era (Deemed to be University), Dehradun, Uttarakhand 248002, India. [6]Department of Pharmacognosy, College of Pharmacy, King Saud University, PO Box 2457, Riyadh 11451, Saudi Arabia. [7]Joint Institute for High Temperatures of the Russian Academy of Sciences, Moscow 125412, Russian Federation. [8]Water-Energy Nexus Laboratory, Department of Environmental Engineering, University of Seoul, Seoul 02504, Republic of Korea. [9]Peoples' Friendship University of Russia (RUDN University), Moscow 117198, Russian Federation. ✉email: vinodkdhatwalia@gmail.com

Consequently, nearly 8-MMT plastic waste directly flows into the aquatic ecosystem every year, and this amount is predicted to increase fourfold by 2050[2]. According to the National Oceanic and Atmospheric Administration (NOAA), MPs can be classified as plastic fragments that possess a size of less than 5 mm. Because of the inappropriate release of domestic and industrial wastewater, MPs are extensively disseminated in oceans, surface water, and stormwater[5]. Examples of commercially available plastics include polypropylene (PP), polyvinyl chloride (PVC), low-density polyethylene (LDPE), polyethylene terephthalate (PET), and high-density polyethylene (HDPE)[6]. Among various plastics, polyethylene (PE), polypropylene (PP), polystyrene (PS) and polyethylene terephthalate (PET) are the most prevalent in marine environments[7]. In the environment, MPs have various shapes, including fragments, fibres, beads, and pellets. Due to their high surface area to volume ratio, MPs can adsorb other chemical pollutants, raising concerns about their interactions with environmental contaminants. These plastic particles also can be adsorbed to other organic solids in the water because of their hydrophobic nature, and the smaller the size, the easier they can build up to a harmful concentration[1,8]. Additionally, many environmental pollutants like polybrominated diphenyl ethers (PBDEs), polychlorinated biphenyls (PCBs), 2, 2-bis (p-chlorophenyl) 1, 1, 1-trichloroethane (DDTs), and polycyclic aromatic hydrocarbons (PAHs) are very well known to be easily adsorbed onto microplastics[9].

Microplastic pollutants in aquatic environments may affect organisms at different trophic levels via adsorption, food intake, and transformation through the food chain[10,11]. The food chain transfer between tropical levels is majorly associated with their shape, size, accumulation and residence time. The longer residence time of MP in biota will facilitate their easy movement across the trophic levels, which will possess a definite effect on the overall ecosystem[11]. Due to bioaccumulation, such organic pollutants pose a consequential challenge to the aquatic environment[5]. Aquatic bodies are a huge abode of renewable sources of natural substances[12]. Microalgae (photosynthetic microbes) are primary producers that consume inorganic substances to synthesize protein, lipids and carbohydrates. Being primary producers, microalgae play a major part in preserving environmental stability. Increasing exposure to MPs could influence the internal components of microalgae and the aquatic environment. Microalgae have a short growth cycle, are easy to cultivate, and are sensitive to toxic substances, which makes them suitable for performing eco-toxicity assays[13]. Microplastics have been reported to adversely affect microalgae populations by reducing their photosynthetic efficiency and increasing reactive oxygen species (ROS) generation[14,15]. The amount of MPs in aquatic settings varies greatly; in virgin waters, it is quite low, but in contaminated places, it is much higher. In a study performed on the Zuari River in Goa, India, MPs were detected at all sampling sites with concentrations ranging from 0.01 to 1.38 particles/L[16]. These concentrations demonstrate the pervasiveness of MPs in aquatic systems, even though they are below several experimental criteria. Lang et al.[17] investigated the effect of different polystyrene (PS) concentrations (25–200 mg/L) on marine diatom *P. tricornutum* for 96 h. At lower concentrations (25–50 mg/L), diatom produced more trihalomethanes as a result of oxidative stress. However, higher concentrations (100–200 mg/L) resulted in significant cellular damage and decreased the formation and release of trihalomethanes. A similar study examined the combined toxicity of 50-mg/L MPs and six concentrations of sulfadiazine (5, 10, 20, 50, 100, and 200 mg/L) on *Chlamydomonas reinhardtii*, observing notable cellular damage and oxidative stress[18]. Previous findings indicated that MPs serve as a substrate for the attachment and proliferation of microbes[19]. The proliferation and colonization can alter microbes' diversity, interaction between species, metabolic activity and microbial composition. From this point of view, the major concern is that MP can serve as a vector for hazardous contaminants and a host for pathogenic microbes, which could spread when coming into contact with them[20,21].

Current research are still investigating the effect of MPs on creatures and ecosystem, involving their influence on ecosystem services, food webs and biodiversity. Studies have stated that MPs accumulation may lead to those of contaminants like dichloro-diphenyl-trichloroethane (DDTs), bisphenol A (BPA), polychlorinated biphenyls (PCBs) and polybrominated diphenyl ethers (PBDEs) in aquatic life that are likely included in the food web thus indirectly affecting the human health. Moreover, MP accumulation in sediments can affect benthic species and interrupt the pathways of nutrient cycling. Therefore, minimizing MP pollution is important for protecting aquatic life as they are crucial for the processes like nutrient cycling and carbon sequestration[22]. For better understanding of these interactions, advanced analytical techniques such as scanning electron microscopy with energy dispersive X-ray spectroscopy (SEM-EDX) and Fourier transform infrared spectroscopy (FT-IR) are frequently used. SEM-EDX can provide the detailed visualization of the surface and elemental composition of MPs, while FT-IR detects specific polymer types and chemical bonds, enabling accurate characterization of MPs in environmental samples[23]. A study utilizing SEM-EDX and FT-IR to examine the interaction between MPs and *Spirulina sp.*, revealed significant biochemical alterations in the microalgae when exposed to polyethylene terephthalate (PET) and polypropylene (PP) MPs[24]. Similarly, a study comparing μ-FT-IR and SEM-EDX for assessing MPs released from plastic bottles confirmed that both methods were effective[25].

The exploration of the connection between microalgae and MP warrants evaluating the potential impact of plastics on aquatic ecosystems[26]. The impact of MPs on the ecosystem and human health is a dynamic area of research, and efforts are ongoing to minimize their production and presence in the environment through regulation of manufacturing practices. Generally, many reports are available regarding interaction, colonization, and toxic effects of MPs on aquatic life and microalgae. However, very few reports concerning the characterization and its influence on biochemical compositions of microalgae have yet been documented.

This study presents a novel approach by evaluating the effects of polyethylene (PE) microplastics (MP) on the growth and biochemical parameters (biomass, lipid, pigments, carbohydrates, and proteins) of *Chlorella sorokiniana*. Contrasting with previous studies that primarily focused on MP toxicity at extremely high concentrations, this research determines the half-maximal inhibitory concentration (IC_{50}) of PE MP on *C. sorokiniana*, establishing a critical threshold for microalgal response. This study investigates the underexplored concentration-dependent effects of polyethylene microplastics on ROS dynamics and pathways in *Chlorella sorokiniana*. The comparative analysis of growth rate, biomass production, pigment content, biochemical

compounds, production of phenols and flavonoids was carried out for the control (devoid of MP) and the MP-treated *C. sorokiniana*. The interactions between MPs and microalgae were also investigated by using advanced characterization techniques like SEM-EDX, FT-IR, and μ-FT-IR thus providing detailed insights into the morphological and chemical properties of the MP. Additionally, it systematically evaluates multiple concentrations of MP, offering valuable data on the concentration-dependent effects on algal health and biochemical composition.

Materials and methods

Chemicals and reagents

All the chemicals and reagents used in the present study were purchased from Sigma-Aldrich, India. Polyethylene granules were purchased from Chemika Biochemika, India.

Preparation of PE MP

One gram PE granules were dispersed in 10-mL xylene by stirring on a magnetic stirrer at 500 rpm and 70 °C for 1 h. The prepared dispersions were cooled down at room temperature, followed by the addition of 20-mL ethanol. The mixture was further stirred for an additional 30 min to ensure proper homogenization. Microplastics were filtered and washed with ethanol and dried at room temperature. Then, the dried MP were collected in a dried zip-lock bag. Prepared MP were dispersed in distilled water and after sonication the particle size distribution, mean particle size and polydispersity index were analysed using a particle size analyzer (Zetasizer Lab, Malvern, India). An overall scheme of the present investigation is illustrated in Fig. 1.

Microalgae cultivation and experimental design

The previously isolated microalgal strain *Chlorella sorokiniana* UUIND6 (GenBank accession number KY780616) was used in the current study[27]. The culture was grown and maintained in Bold's Basal Media (BBM), under white light emitting diodes (LED) light at an intensity of around 300 μmol/(m^2 s) with continuous illumination and incubated at 25 ± 1 °C for 10 days. The cultures were gently shaken every 6 to 8 h, and growth was monitored each day by measuring optical density (OD) at 730 nm using a UV-VIS spectrophotometer (Agilent & Cary-60 UV-Vis). After 10 days incubation, the cultures were harvested and prepared inoculum was used for further experiments.

The experiments were conducted to examine the half-maximal inhibitory concentrations (IC_{50}) by utilizing different concentrations of PE MP (25–150 mg/L) (average size 1.42 μm) in BBM. The flask without PE MPwas taken as the control. After 96-hr incubation period, the growth of microalgae cultures was assessed by measuring

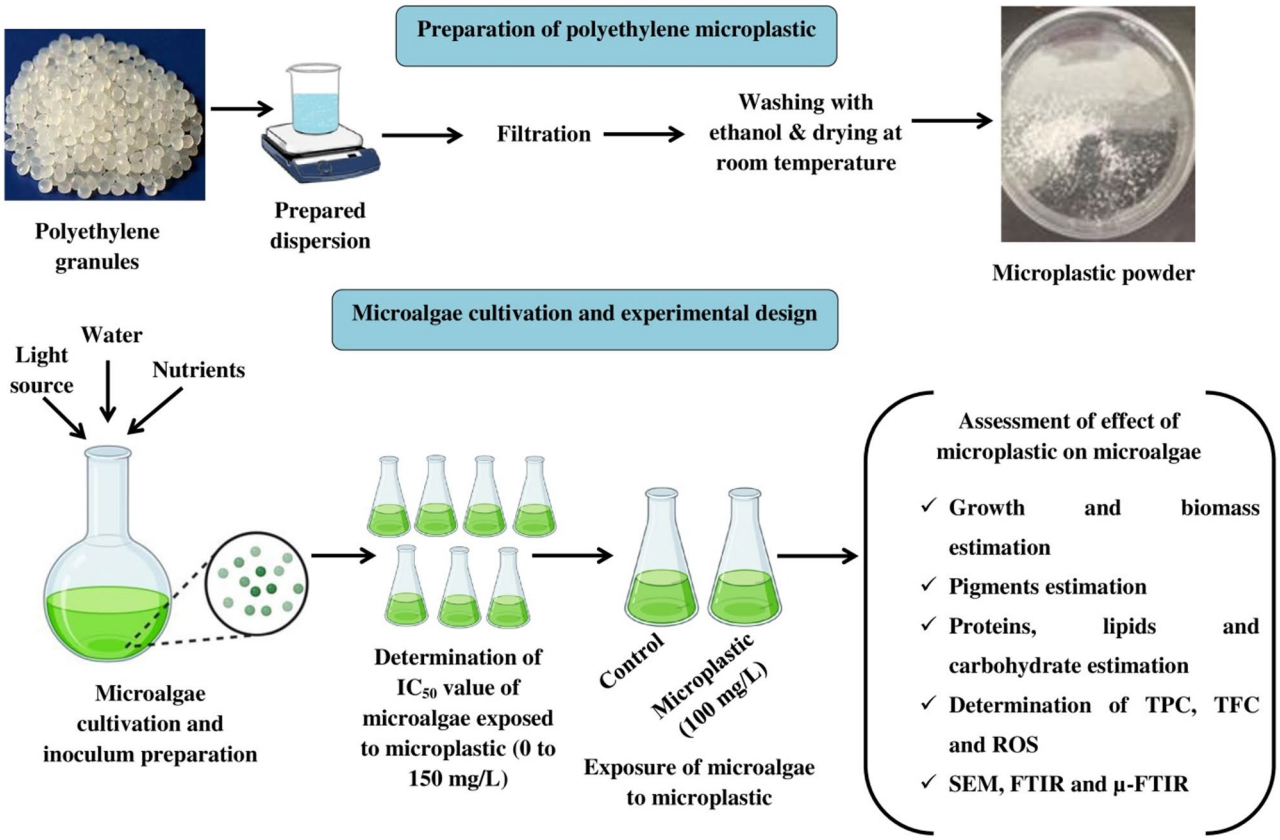


Fig. 1. Overall scheme of present investigation.

their OD at 730 nm, and percent inhibition was calculated by using Eq. 1[28]. Later on, a graph was plotted between inhibition percentage and MP concentrations. The IC_{50} value of MP concentration was utilized to study its effect on growth, biomass content and biochemical compounds of *C. sorokiniana*.

$$\%\ inhibition\ = \frac{Microalgae\ growth\ in\ control\ -\ Microalgae\ growth\ in\ treatment}{Microalgae\ growth\ in\ control} \times 100 \quad (1)$$

The IC_{50} was calculated and found to be approximately 100 mg/L, which was then selected for use in subsequent experiments to ensure consistent and physiologically relevant exposure.

Estimation of biochemical components of microalgae

Growth curve and biomass collection

The cell growth was measured by taking OD values during incubation. The culture was incubated for up to 14 d. All the cultures (control and PE-treated) were harvested after 14-d incubation by centrifugation in pre-weighted falcon tubes. The biomass thus obtained was dried at 60 °C in a hot-air oven, weighed and stored for further analysis.

Pigments estimation

Pigments content was measured following the method of Lichtenthaler[29] with slight modifications. 2-mL sample was poured into a tube and centrifuged for 5 min at 5000 rpm on 10th day for microalgae cultivation. The supernatant was removed, and 2-mL methanol was added to the pellet. The suspension was kept in a water bath at 45 °C until the pellets turned colourless. At last, the suspension was centrifuged and then supernatant was collected; the absorbance of the supernatant was measured at 665.2, 652.4, 470 and 750 nm in a UV-Vis spectrophotometer. The values obtained from 665.2, 652.4, and 470 nm were corrected by subtracting the value obtained from 750 nm. Chl-a, Chl-b and Car were calculated by using following equations (Eq. 2 to 4):

$$Chl-a\ \left(Chl-a;\ \frac{\mu g}{ml}\right) = 16.72A_{665.2} - 9.16A_{652.4} \quad (2)$$

$$Chl-b\ \left(Chl-b;\ \frac{\mu g}{ml}\right) = 34.09A_{652.4} - 15.28A_{665.2} \quad (3)$$

$$Car\ \left(\frac{\mu g}{ml}\right) = \frac{(1000A_{470} - 1.63\ Chla - 104.9\ Chlb)}{221} \quad (4)$$

Lipid extraction

The dry weight of the obtained cells was used for lipid extraction following Bligh and Dyer's method with slight modification[30]. Initially, a 3-mL mixture of chloroform and methanol (v/v = 2/1) was added to the disrupted cells. The suspension was incubated in a shaker at 180 rpm for 3 h under room temperature. After that, the mixture was centrifuged for 10 min at 5000 rpm. The upper layer was separated in a fresh tube, and the collected solution was treated with 3 mL of 0.034% $MgCl_2$ to remove polar impurities and enhance separation and again centrifuged at 5000 rpm for 10 min. Then, the upper phase was discarded, and to remove residual methanol and contaminants 3 mL of 2 N KCl was added to the lower phase and again centrifuged. Once more, the upper phase was discarded, and the lower phase was treated with 3 mL of the mixture of chloroform, methanol, and distilled water in the ratio of 3:47:48 (v/v/v), followed by centrifugation for 5 min at 5000 rpm. At last, the resulting lower phase was collected and dried at 45 °C for 6–7 h in a hot air oven to ensure complete solvent removal and obtain total lipids. The dried lipid was weighed, and then the lipid percentage was calculated by using the given equation (Eq. 5).

$$Lipid\ content\ \ (\%) = \frac{Total\ lipid\ \ (g)}{Biomass\ taken\ \ (g)} \times 100 \quad (5)$$

Total protein and carbohydrate Estimation

Elemental carbon, hydrogen and nitrogen analyzer (Thermo Fisher) was used to estimate the total nitrogen content in microalgae. The amount of crude protein was calculated by using the following equation (Eq. 6)[31].

$$Protein\ Content\ \ (\%) = Nitrogen\ content \times 4.78 \quad (6)$$

For total carbohydrate estimation, lipid-extracted microalgal biomass was hydrolyzed by 5% H_2SO_4 and autoclaved. Then, released sugar was assessed using phenol-sulfuric acid method; d-glucose was kept as reference (Eq. 7)[32] as shown in Fig. S1.

$$Carbohydrate\ content\ \ (\%) = \frac{Total\ carbohydrate\ \ (g)}{Biomass\ taken\ \ (g)} \times 100 \quad (7)$$

Determination of phytochemical compounds and ROS

Determination of total phenolic content (TPC)

For microalgae extract preparation 5 g of freeze-dried algal biomass were mixed with 100 mL of 80% methanol (v/v). The mixture was sonicated for 10 min with the temperature maintained below 40 °C. Then solution was centrifuged at 8000 rpm for 10 min. Supernatant was concentrated using rotary evaporator (Dlab RE 100-Pro,

China made), marked as methanol extract and stored at − 20 °C for further analysis. Total phenolic content of the control and the MP-treated microalgae were determined spectrophotometrically using Folin-Ciocalteu (FC) colorimetric method adopted from Bisht et al.[33] with slight modification. In the present study, gallic acid was used to obtain standard calibration curve as shown in Fig. S2. The sample solution was made by dissolving 10 mg in 100 µL of methanol. Before use, the FC reagent was mixed with distilled water in 1: 9 ratio. In a test tube, 20 µL of microalgae extract was diluted with 450 µL of distilled water to make 500 µL total volumes. To this solution, 250 µL of FC reagent was added and incubated for 1 min. Further, the solution was supplemented with 2 mL of 7.5% sodium carbonate, and the mixture was left to stand for 90 min. The absorbance against the blank was measured at 765 nm and expressed as gallic acid equivalent (GAE) per g of dry weight (DW). TPC was determined using the following equation (Eq. 8).

$$\mathrm{TPC}\left(\frac{\mathrm{mg\ GAE}}{\mathrm{g\ DW}}\right) = \frac{\mathrm{c} \times \mathrm{v}}{\mathrm{m}} \tag{8}$$

Where c is the polyphenol content from the calibration curve expressed in mg GAE/mL, v is the volume of chemical used in the test (mL), and m is the sample weight (g DW).

Determination of total flavonoid content (TFC)

Total flavonoid contents of the control and the MP-treated microalgae extract were assessed spectrophotometrically using the colorimetric technique reported by Bisht et al.[33] with minor modifications. A 5-mL volumetric flask was used to hold 20-µL sample solution. Furthermore, 2.5 mL of distilled water was added to the flask, followed by 0.15 mL of 5% $NaNO_2$ and 5 min of stirring. After that, 0.15 mL of 10% $AlCl_3$ and 1 M NaOH were added at 5 min intervals, and the volume was increased to 5 mL with distilled water. The solution was vortexed, and absorbance v/s blank was measured at 510 nm using spectrophotometer. The blank sample was made in the same way as described above. The results were determined against quercetin standard curve (Fig. S3) and represented in mg quercetin equivalent per g DW. TFC was determined using the following equation (Eq. 9).

$$\mathrm{TFC}\left(\frac{\mathrm{mg\ quercetin}}{\mathrm{g\ DW}}\right) = \frac{\mathrm{c} \times \mathrm{v}}{\mathrm{m}} \tag{9}$$

Where c is the flavonoid concentration from the calibration curve expressed in mg quercetin/mL, v is the volume of chemical used (mL), and m is the sample weight (g DW).

Determination of reactive oxygen species (ROS)

The amounts of ROS produced in the control and the MP-treated microalgae extract were measured in terms of H_2O_2 scavenging. The microalgae extract (20–100 µg/mL) was added to a 40-mM H_2O_2 solution buffered with phosphate (pH 7.4). After 10 min, absorbance was measured at 230 nm, and the scavenging percentage was calculated using the Eq. 10[34]:

$$\%\ H_2O_2\ scavenging = \frac{\mathrm{Control\ absorbance} - \mathrm{sample\ absorbance}}{\mathrm{Control\ absorbance}} \times 100 \tag{10}$$

.

Characterization of PE MP

The prepared PE MPs were characterized to check their properties by their shape, size, and elemental analysis. Before characterization, fixation of microalgal cells for scanning electron microscopic analysis (SEM; Carl Zeiss EVO 18) was done by treating them overnight in the dark at 4 °C with 2.5% glutaraldehyde solution, followed by washing with phosphate-buffered saline (PBS) to remove excess fixative. Then, microalgal cells were dehydrated by treating with 10–100% ethanol solutions for 10 min at each step. The dehydrated samples were then gold-sputter-coated to enhance conductivity for SEM analysis[35].

SEM analysis was done to perceive the changes in the surface morphology of *C. sorokiniana*, PE MP, and their interaction. Whereas, SEM coupled with EDX (Carl Zeiss EVO 18; Energy dispersive X-ray) was used to estimate the alteration in elemental composition and quantify alterations in elemental percentages during the interaction of *C. sorokiniana* with PE MP. The technique was chosen due to its ability to provide high-resolution surface imaging and elemental mapping, enabling us to understand MP interaction at the microscale. The elemental composition data obtained from the SEM-EDX analysis were cross-validated with FT-IR (Themo Scientific Summit lite) analysis to confirm changes in the organic compositions and surface chemistry of *C. sorokiniana*. Microalgal biomasses cultivated with and without (control) PE MPs were dehydrated and analyzed using the potassium bromide (KBr) pellet technique with FT-IR in the range of 4000 –400 cm^{-1}. Zeta potential was measured at room temperature on a Zeta size analyzer (Malvern Instrument, ltd, UK). Zeta potential is used to assess the reduction in repulsive forces and the formation of larger flocs before and after the coagulation process between microalgae and MPs[36]. It plays a crucial role in algal aggregation, separation and flotation efficiency[37].

Mapping of PE microplastic using µFT-IR

The supernatant was used for µ-FT-IR analysis of MPs in media containing PE MP after harvesting microalgal biomass. The supernatant was filtered by Anodisc filter paper. The resulting filters were analyzed using an FT-IR

microscope (Nexus 670, Thermo Electron Corporation, USA) in transition mode and spectral range of 1300–1400 cm^{-1} for mapping and followed by the procedure described by Song et al.[38]. The number of particles on one of the filters was estimated using the Particle Wizard option of profiling feature in the Omnic Picta software 9.12. For mapping, 200,000-300,000 spectra were collected using the ultrafast mapping mode with a linear array detector and 25-µm steps over a single section (~ 12 × 12 mm), for semi-automated and automated PE MP analysis on Anodisc filter papers ($n = 5$). The peaks of CO_2 (2200–2400 cm^{-1}) were removed, and the entire collected spectra were matched with reference spectra using the spectrum.

Statistical analysis

To evaluate the impact of microplastic (MP) treatment on various physiological and biochemical parameters of microalgae, statistical analyses were conducted using the online tool OPSTAT (Operational Statistics). A one-way analysis of variance (ANOVA) was employed to determine whether the differences observed between the treatment and control groups were statistically significant. The input data for the analysis consisted of experimentally measured values obtained from biological triplicates ($n = 3$) for control and MP-treated groups. The data are expressed as mean values ± standard deviation (SD). Statistical significance was set at $p < 0.05$. The detailed ANOVA results are provided in Table S1.

Results and discussion

Particle size distribution of MP

Through the particle size analysis of MP dispersion, the mean particle size was determined as 1420 nm (1.42 µm) (Fig. 2). The polydispersity index was found to be 1, which revealed a highly polydisperse PE. PE MPs are becoming more prevalent in surface water, marine environment, and wastewater as micropollutants. According to recent studies, prolonged exposure to higher PE MPs may harm the aquatic biosystem[39,40].

Cell viability and IC_{50} of *Chlorella sorokiniana* under MP exposure

C. sorokiniana was exposed to MPs with different concentrations ranging from 25 to 150 mg/L to examine its cell viability by measuring OD. After a 96-hr incubation period, a decrease in OD of microalgae cultures was seen from 50 mg/L to 150 mg/L of MP concentration. The %-inhibition was calculated by using Eq. 1 (Section "2.2") and a graph was plotted between %-inhibition and MP concentrations as shown in Fig. S4. The IC_{50} value was found to be 100 mg/L, indicating that this concentration of MP decreased the growth of *C. sorokiniana* by 50%, when exposed for 96 h. Therefore, MPs of 100 mg/L were utilized further for treating microalgae cultures and their effect on biomass content, pigments and other biochemical components was compared with that of the control.

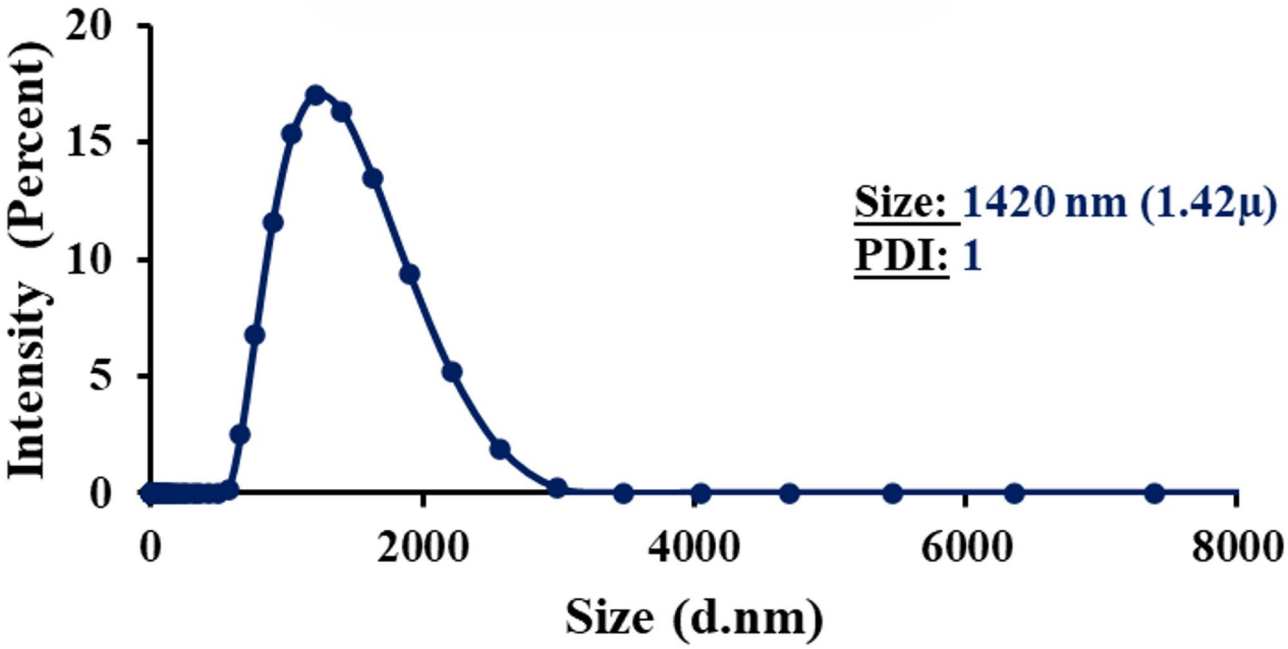
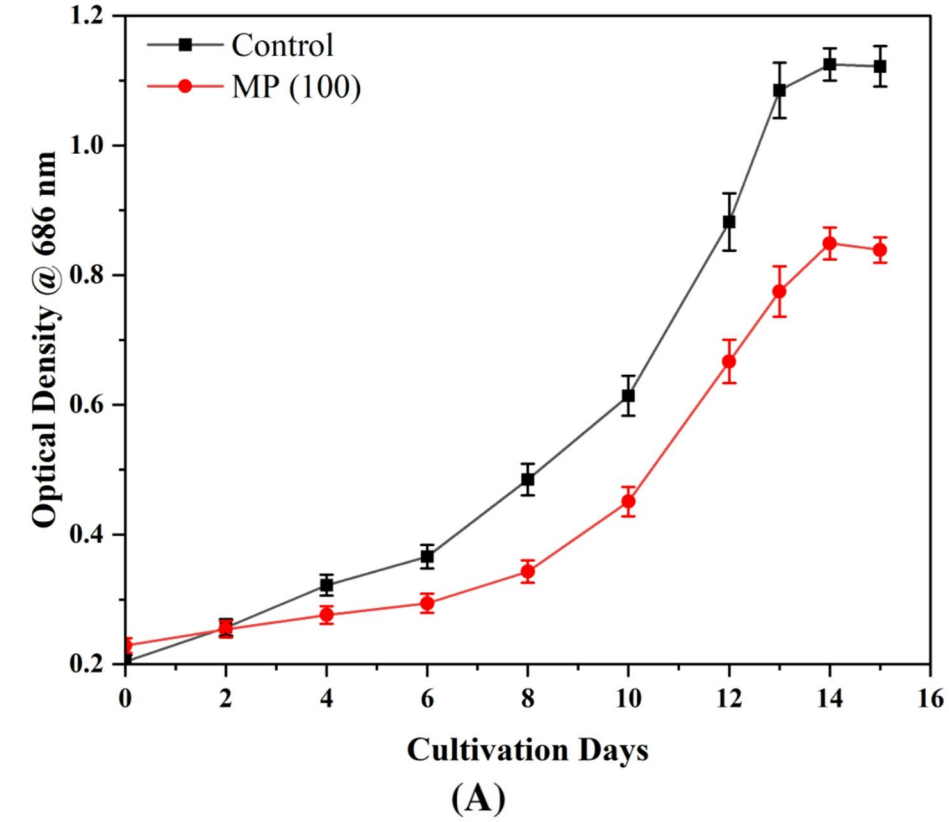


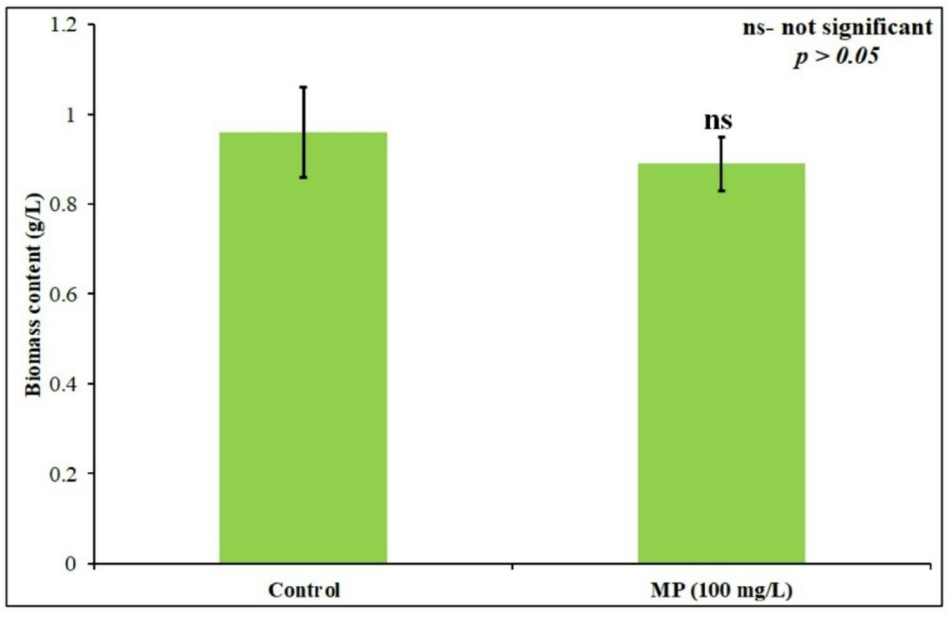
Fig. 2. Particle size distribution of microplastic dispersion.

Effect of MP exposure on growth rate and biomass content of *C. sorokiniana*

The effect of microplastic (MP) exposure (100 mg/L) on the growth of *Chlorella sorokiniana* was assessed by measuring optical density at 686 nm over a 14-day cultivation period (Fig. 3A). Both control and MP-treated cultures showed an initial increase in cell density; however, a visible lag in growth was observed in MP-treated cultures starting from day 4. Notably, while the MP-treated group exhibited slower growth, particularly up to 96 h



(A)



(B)

Fig. 3. Effect of MP-exposure on (**A**) growth and (**B**) biomass content of *Chlorella sorokiniana*; MP concentration: 100 mg/L.

consistent with the inhibitory concentration (IC_{50}) of 100 mg/L this difference was not statistically significant when comparing the overall biomass accumulation between the groups.

According to the one-way ANOVA analysis (Table S1), the F-calculated value for biomass content was 0.637 with a p-value of 0.51, indicating no significant difference ($p > 0.05$) in biomass between control and MP-exposed cultures by the end of the cultivation period. These results suggest that while MP exposure temporarily suppressed growth kinetics during early stages, it did not cause a statistically significant reduction in final biomass yield under the tested conditions.

The slight reduction in growth rate can be due to the adsorption of MPs onto algal cells can obstruct light from reaching their photosynthetic apparatus, potentially diminishing photosynthetic efficiency[41,42]. Moreover, MPs can carry toxic additives (like copper, bromium & titanium) into the medium, which damage the cell membranes of microalgal cells, hence reducing their growth rate[43]. The observed results were in accordance with previous studies that had also noticed a reduction in microalgal growth rate upon treatment with MPs[42–45].

Biomass did not show statistically significant changes ($p > 0.05$), suggesting minimal or variable responses under the MP stress. Biomass content was also observed in the MP treatment, i.e., 0.89 g/L, as compared to that in control groups, i.e., 0.96 g/L (Fig. 3B). In another study, Cunha et al.[46] also stated that *Phaeodactylum tricornutum* cultures exposed to MPs (polymethyl methacrylate and PS) showed a low biomass yield as compared to control ones. Nguyen et al.[47] also found that high concentrations of MPs can negatively impact the growth and biomass content of microalgae. Hence, the results indicate that the presence of MPs might cause light scattering, preventing microalgal cells from absorbing enough light, thus adversely influencing their photosynthetic efficacy and growth[46].

Effect of MP exposure on pigments and biochemical compounds of *C. sorokiniana*

In plants and algae, chlorophyll is considered a crucial pigment participating in photosynthesis, which absorbs light energy. Therefore, photosynthetic action of microalgal cells is proportional to the chlorophyll content[48]. Moreover, carotenoids are bioactive compounds, which possess a crucial role in bioavailability mechanisms, regulation of gene transcription and the activity of pro-vitamin A[49]. *C. sorokiniana* exhibits various adaptive responses when exposed to microplastics, highlighting the resilience of microalgae in polluted environments. It shows significant adaptations in its photosynthetic machinery when exposed to microplastics. These adaptations include modifications in chlorophyll florescence, adjustments in photosynthetic efficiency and changes in energy transfer within photosystems[50]. Hence, examining the effect of MPs on microalgal pigments is of great importance. As revealed by experimental data, the content of the pigments (i.e., Chl a, Chl b and car) of the MP-treatment was lower than that of control groups. The contents of Chl a, Chl b and car of the MP-treatment were 3.99, 2.01 and 1.47 µg/mL, respectively, while those of the control were 5.06, 2.60 and 1.64 µg/mL, respectively (Fig. 4A). Hence, it has been suggested that MP may negatively impact the primarily photosynthetic pigment while not strongly affecting Chl b and carotenoids. Reduced contents of chlorophyll in MP-treated groups might be due to hindrance in photosynthetic activity. MPs might block pores for cellular respiration in the cells, resulting in cellular injury and then impeding chlorophyll synthesis[4]. These findings were consistent with Xu et al.[51] studies that reported a reduction in chlorophyll content of *Skeletonema costatum* by 20% when exposed to 50 mg/L of PVC MPs. Similarly, Jiang et al.[52] also noticed that exposure to PET MPs reduced the accumulation of Chl a, Chl b and car within *Scenedesmus* sp. as compared to a control group. However, the factors like exposure time, MP types and microalgae species greatly influence the effect of MP exposure on photosynthetic activities of microalgae[51,53]. Another study examining the complex interaction between microalgae and MPs revealed that exposure to MPs led to a reduction in Chl-a content. This exposure disrupts electron transfer within the photosynthetic system, damaging electron transport and oxidative stress. Major biological functions like energy production, carbon fixation, lipid metabolism, and nucleic acid metabolism may also be impacted by these effects[54].

Moreover, biochemical analysis revealed that, as compared to the control, the lipid content was decreased, while the protein and carbohydrate contents were increased in MP-treated groups. In particular, the lipid, protein and carbohydrate contents in control were 35.7, 32.2 and 22.3%, whereas in MP-treated groups, they were 26.7, 37.6, 24.4%, respectively (Fig. 4B). As shown in the ANOVA table, parameters such as chlorophyll "a" ($p = 0.04$), lipid content ($p = 0.004$), and carbohydrate content ($p = 0.000$) exhibited statistically significant differences upon MP exposure, indicating that microplastics had a notable impact on these traits. In contrast, biomass, chlorophyll "b," carotenoids, and protein content did not show statistically significant changes ($p > 0.05$), suggesting minimal or variable responses under the same conditions. In contrast, Jiang et al.[52] observed a decrease in intracellular carbohydrates and proteins in *Scenedesmus* sp. exposed to PET MPs. Similar inhibitory effects of MPs (i.e., PVC, PP, HDPE) were observed by Ansari et al.[45] on the protein yield of *Acutodesmus obliquus*. However, culture conditions largely influence the accumulation of different metabolites in microalgal biomass[55]. For example, Ansari et al.[45] observed that PP and HDPE MPs increased the carbohydrate yield in *A. obliquus* at a concentration of 50–150 mg/L, while a decreasing trend was observed beyond 150 mg/L. Similarly, Li et al.[56] stated that PS with a concentration of 50 mg/L downregulated the carbohydrate metabolism in *Skeletonema costatum*. The heterocoagulation of microalgae and MPs can obstruct the matter and energy transfer, thus delaying carbon fixation at the time of photosynthesis and carbohydrate production[57]. In addition, amino acids are essential raw components for the synthesis of proteins, and MPs can cause a shift of energy in microalgae from photosynthetic carbon integration to the amino acid catabolism pathway. Thus, the improvement in amino acid catabolic pathways might consequently influence the buildup of carbohydrates and proteins[51,58]. Moreover, a decrease in lipid content might be due to MP stress disrupting physiological homeostasis in microalgae and the generation of ROS and intracellular oxygen-free radicals results in cellular oxidative injury. Production of ROS can lead to lipid peroxidation by damaging the polyunsaturated fatty acids (PUFAs) of microalgae. Malondialdehydes

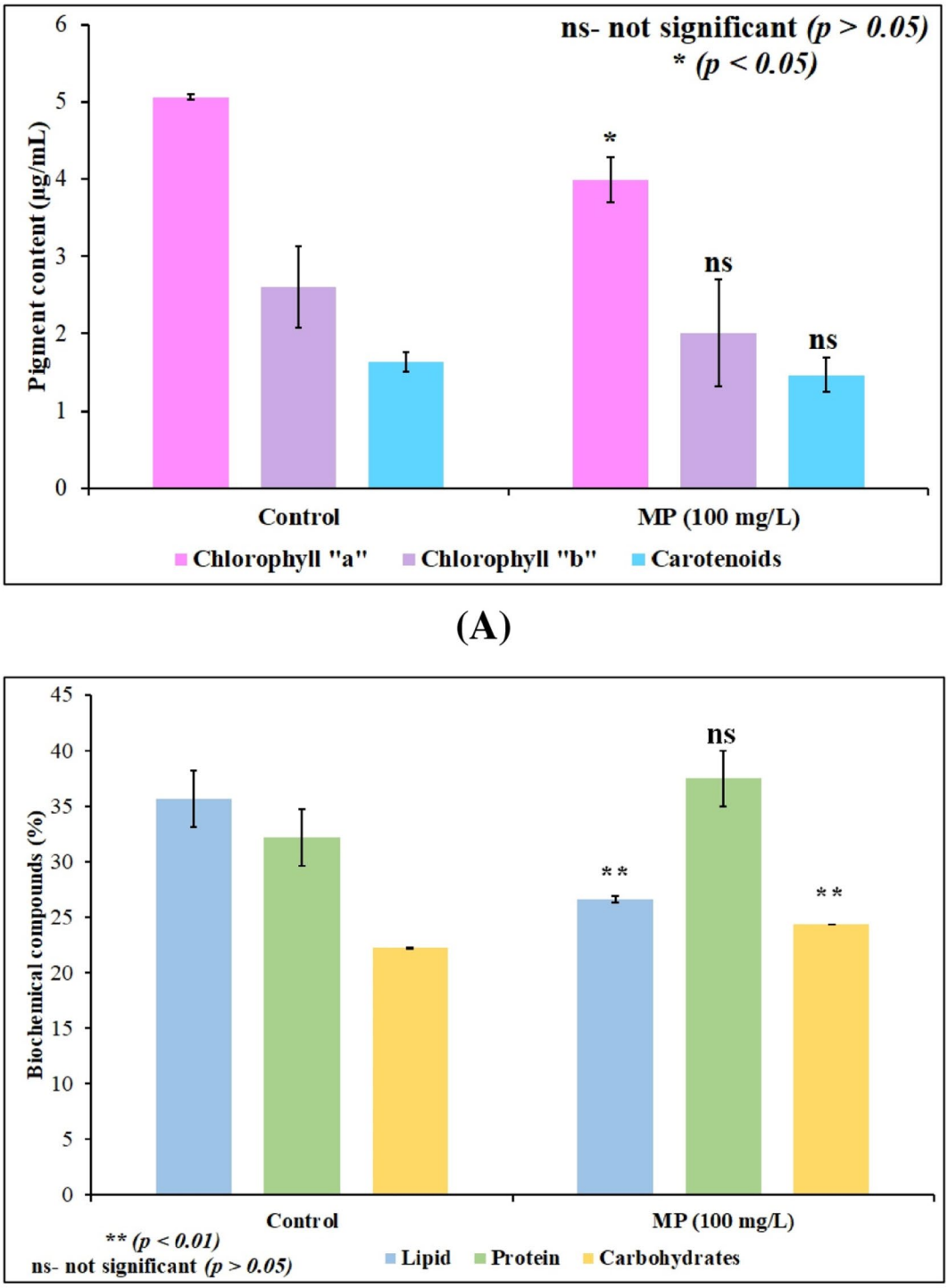


Fig. 4. Effect of MP exposure on (**A**) pigments (Chl a, Chl b, and car) and (**B**) lipids, proteins, and carbohydrates of *Chlorella sorokiniana*; MP concentration: 100 mg/L.

(MDA) are the ultimate product of membrane peroxidation, which can describe the oxidation rate and lipid damage in microalgae exposed to environmental stress[59].

Effect of MP exposure on zeta potential and size of *C. sorokiniana*

Exposure to microplastics induce notable changes in *C. sorokiniana* including alterations in cell size and zeta potential which play a crucial role to its adaptation in microplastic pollution. The size of *C. sorokiniana* in the

control was estimated as 2.7 µm and decreased to 2.2 µm after exposed to PE MP. In the control condition, the estimated zeta potential was −11.3 mV and −31.8 mV in the MP-treatment, indicating the zeta potential of the MP-treatment would be more stable. Changes in zeta potential suggest modifications in membrane properties potentially influencing nutrient uptake and stress response mechanisms. According to our findings, it was hypothesized that *C. sorokiniana* and PE MP interaction would promote cell dormancy. Recently, it was reported that the zeta potential of PE MP was 29.9 mV, which decreased to -25.8 mV over time[60]. In a previous report, it was shown that the initial zeta potentials of *Chlorella* and *Scenedesmus* were −26.1 and −11.3 mV, respectively. However, after they were exposed to +PS beads, the zeta potentials increased to 85.8 mV and 56.3 mV, respectively. However, with PS beads, the zeta potential decreased to 9.0 mV and 14.6 mV. These results proposed that positively charged PS beads have more binding affinity to *Chlorella* and *Scenedesmus* than negatively charged ones[61].

Effect of MP exposure on phenolics, flavonoids and ROS of *C. sorokiniana*

The study examined the effect of PE MP exposure on phenolics, flavonoids and ROS in *C. sorokiniana*. The experimental data revealed that the total phenolics, flavonoids and ROS levels in the control were 15.3 mg GAE/g sample, 9.3 mg QUERCETIN/g sample, and 41.8%, respectively. In contrast, MP-treated samples exhibited a significantly reduced ROS (31.0%) and phenolic content (11.4 mg GAE/g). However, the flavonoid content increased to 12.2 mg QUERCETIN/g sample (Table 1). Hence, the results clearly stated that PE MP exposure would alter the oxidative balance and secondary metabolite production in *C. sorokiniana*.

In contrast to other MP forms, such as PET, which haven known to cause a larger accumulation of ROS in microalgae, this decrease in ROS content after MP exposure suggests a reduced oxidative stress response[4,52]. Microplastics typically cause oxidative stress which results in excessive electron buildup and the production of ROS by interfering with the electron transport chain[44]. Overproduction of ROS can cause oxidative damage to cellular organelles such as nuclei, mitochondria, and chloroplasts, which can subsequently limit photosynthesis, cause genetic instability, and decrease biomass[14,62,63].

Since ROS levels were lower in PE-exposed cultures, the demand for phenolic-based antioxidant defenses was also reduced. In contrast, the increase in flavonoid content suggests an alternative non-enzymatic antioxidant defense mechanism. Flavonoids are well-known for their radical-scavenging abilities, chelation of metal ions, and DNA protection against oxidative damages[64]. The elevated flovonoid levels in *C. sorokiniana* exposed to PE MPs may indicate a compensatory response to a stress. The reduction in phenolic content in MP-treated *C. sorokiniana* aligns with previous reports suggesting that phenolic compounds are synthesised as part of microalgal responses to an oxidative stress[65]. A recent study explored the response of PS-MPs and perfluorooctanoic acid (PFOA) on *C. sorokiniana*. It was identified that PS-MPs primarily inhibited photosynthesis through shading effects, while PFOA induced an oxidative stress via ROS generation[50]. While this study focused on biochemical and oxidative stress responses, other approaches, such as molecular analysis, could provide deeper insights into regulatory pathways influenced by MP exposure. Further advanced techniques such as proteomics and metabolomics could help to identify key genes, proteins and metabolic pathways involved in the responses to an MP-stress.

SEM-EDX analysis

SEM is considered an authentic analysis for studying the surface morphology of a wide range of micro- and nano-particulate materials[66]. It also provides the chemical compositions of solids when equipped with the EDX detector[67]. Figure 5A, B and C shows SEM images and EDX spectra of MP, microalgae, and microalgae + MP. SEM image (B) revealed that *C. sorokiniana* has spherical structures, whereas SEM image (C) clearly shows morphological changes in *C. sorokiniana* cells after interaction with PE-MP. This shows that the surface of PE-MP may be positively charged, because they tend to bind the surface of the cell membrane of *C. sorokiniana* and form an aggregation, which plays a good role in the interaction with *C. sorokiniana* and supports its survival. There were no significant changes observed in the growth of *C. sorokiniana* exposed to PE-MP as compared to the control. However, during the experiment, it was noticed that when the biomass was harvested on the final day of the incubation period and subjected to centrifugation, small particles of PE-MP with slight shear force appeared in the supernatant after centrifugation. The interaction also hypothesized how long *C. sorokiniana* interacted with PE-MP before separating. It can be suggested that exposure to low concentrations of PE-MP did not result in significant interaction but did affect the growing environment. Conversely, higher concentrations of PE-MPs interacted more with *C. sorokiniana*, leading to aggregation. In Fig. 5, shading of *C. sorokiniana* is seen while the species interact with PE-MP. In a previous study, it was documented that microalgal biomass showed homo- and hetero-aggregation of PE-MPs with its surface[68]. They also demonstrated that PE-MPs could create physical blockage or shading effects that would ultimately hinder light intensity and availability of nutrients to the microalgae. Subsequently, this could create a photooxidative damage of PS-II[69]. Besides, previous reports found that PE-MPs could serve as a substrate for algal growth and enhance microalgal growth[70,71].

Treatments	Phenolics (mg GAE/g)	Flavonoids (mg QUERCETIN/g)	ROS (%)
Control	15.3 ± 0.2	9.3 ± 1.0	41.8 ± 0.5
MP (100 mg/L)	11.4 ± 0.0	12.2 ± 0.1	31.0 ± 1.1

Table 1. Effect of MP exposure on phenolics (mg gae/g sample), flavonoids (mg quercetin/g sample) and ROS (%) of *C. sorokiniana*.

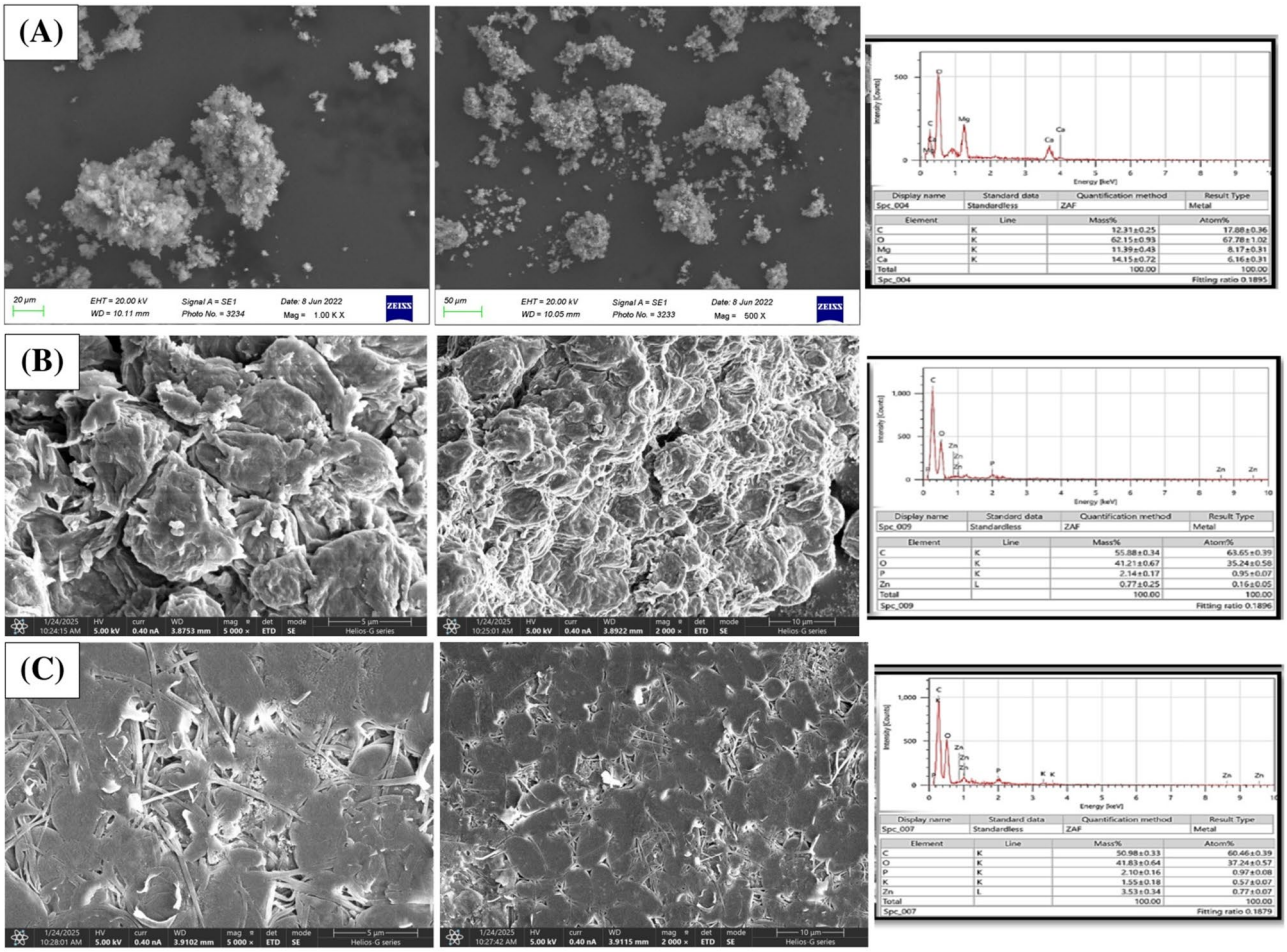


Fig. 5. SEM image and EDX spectra presenting the surface morphology of (**A**) MP; (**B**) microalgae; (**C**) interaction of microalgae with MP.

EDX analysis revealed elemental composition of PE-MP, *C. sorokiniana*, and PE-MP with *C. sorokiniana*. The results of EDX showed a reduction in the percentage of carbon in the PE-MP + *C. sorokiniana* sample compared to the pure *C. sorokiniana* sample. This shows that PE-MP created stress in the environment and influenced *C. sorokiniana*. In previous studies, SEM-EDX analysis was done to reveal the presence of all superior (C, O, N, and Ca) constituents in microalgae (*Stigonematales* sp.)[72] and Spirulina sp[73]. The information on the elemental composition is useful in identifying carbon-dominant plastics over other types of materials[74].

FT-IR analysis

In FT-IR analysis, the sharp peaks at 2917 cm^{-1}, 2842 cm^{-1}, 1458 cm^{-1}, and 708 cm^{-1} were observed in the PE-MP treatment (Fig. 6). The presence of peaks at 2950 cm^{-1} to 2800 cm^{-1} show the presence of an alkyl group which indicates that the PE-MPs contained the CH_2 polymers that form polymer chains[75,76]. SEM and FT-IR analyses of *C. sorokiniana* biomass exposed to PE-MP elucidated the surface alteration of cells and depletion of amine groups from *C. sorokiniana*, which are obvious from the spectral peaks at 1238 cm^{-1} and 2854 cm^{-1}. Therefore, based on the FT-IR analysis, it can be predicted that *C. sorokiniana* cells treated with PE-MPs have not created toxicity in terms of biomass and lipid content. The band of 1400 –1076 cm^{-1} was noticed in the IR spectrum of the *C. sorokiniana* sample, which is shown in Fig. 6. According to El-Naggar et al.[77], the bands at 1300–900 cm^{-1} denote carbohydrates/polysaccharides biomolecules such as alkanes, carboxylic acid, CH_3 linked to O or N, CH and CO stretching mode or CH_2 asymmetric stretching vibration mode. Sadiq et al.[78] and Clement et al.[79] investigated how TiO_2, a PE-MP additive, would interact with *Chlorella* sp. and *Scenedesmus* sp. They observed peak alterations in the carbonyl, amine, hydroxyl, and carboxyl groups after interaction with TiO_2. A similar study was conducted by Dmytryk et al.[80], who investigated the interaction of *Spirullina* sp. with copper, another additive in PE-MPs and observed peak shift at wavelengths 1300 to 1000 cm^{-1}. The shift indicates the presence of -OH group, suggesting an interaction between the hydroxyl and metal ions because hydroxyl is one of the binding sites on the surface of the biomass.

Further, Fig. 7 shows the μ-FT-IR spectra-display map of PE-MP. Because the technique involves minimum sample preparation and is used to detect PE-MP straight on membrane filters[81]. The spectra were collected

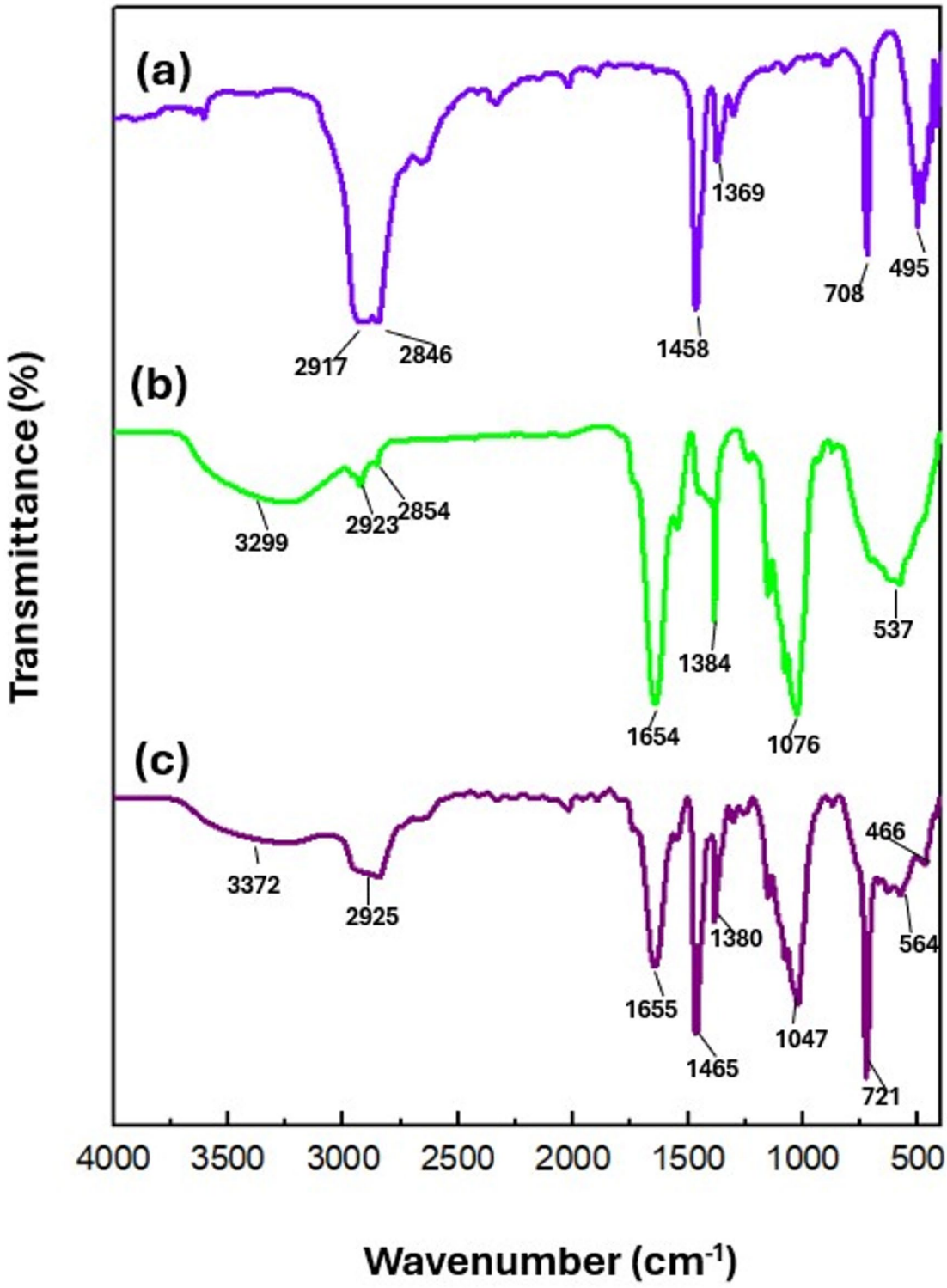
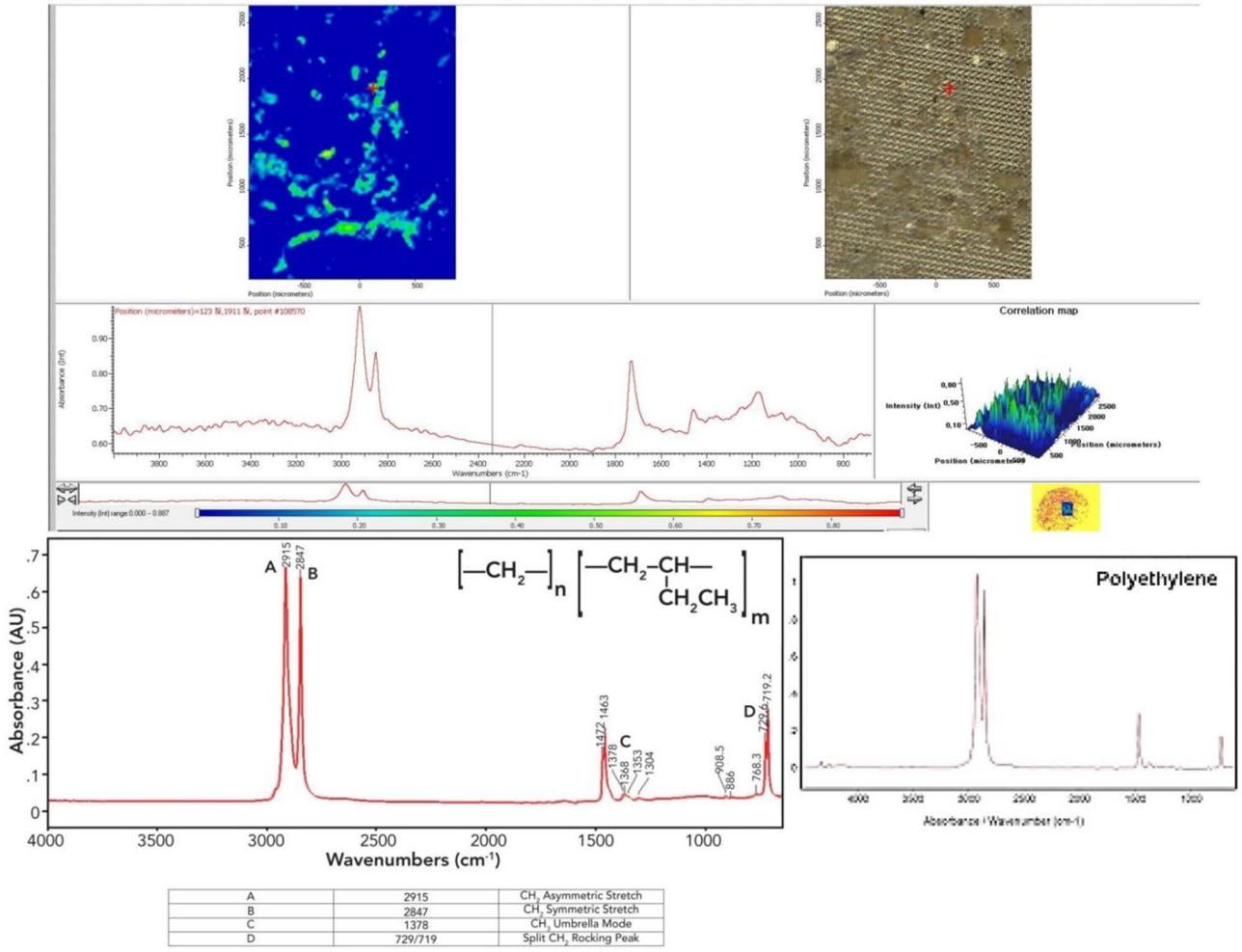


Fig. 6. FT-IR spectra showing peaks at different wavelengths (**A**) PE-MP, (**B**) *C. sorokiniana*, (**C**) PE-MP + *C. sorokiniana* samples.

in transmission mode and used for profiling. Being non-destructive and reliable, μ-FT-IR has been the latest technique used for polymer characterization of PE-MP of > 10 μm[82].

Conclusion

The present study shows the tolerance of *C. sorokiniana* to PE-MP stress. Synergistically, PE IC_{50} revealed an increase in protein, carbohydrate and total flavonoid contents, while a slight reduction was observed in the



A	2915	CH_2 Asymmetric Stretch
B	2847	CH_2 Symmetric Stretch
C	1378	CH_3 Umbrella Mode
D	729/719	Split CH_2 Rocking Peak

Fig. 7. μ-FT-IR spectra of PE using Omnic Picta software 9.12.

biomass and total lipid content. Notably, rather than inducing an oxidative stress, MP exposure appeared to reorient the microalgal biosynthetic activity, as indicated by a reduction in ROS and total phenol content compared to the control. SEM-EDX analysis showed morphological changes in *C. sorokiniana* cells after interaction with PE-MP. The elemental analysis of the cells confirmed the alterations in carbon and oxygen content, indicating environmental stress on the microalgae. Additionally, FT-IR analysis revealed the destruction and loss of functional groups in microalgal cells after exposure to PE-MP. , Thus, further research is still required to determine species-specific responses and broader ecological implications of PE-MP exposure.

Data availability

Data is provided within the manuscript or supplementary information.

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Author contributions

Jyoti Rawat: Methodology; Data curation; Writing – original draft. Pooja Bhatnagar: Writing – review & editing. Abhilasha Mishra: Conceptualization; Data curation; Writing – review & editing. Manisha Nanda: Data curation, Writing – review & editing. Prateek Gururani: Writing – review & editing. Afzal Hussain: Data curation; Writing – review & editing. Mikhail S. Vlaskin: Data curation, Writing – review & editing. Sanjay Kumar: Data curation, Writing – review & editing. Monu Verma: Writing – review & editing, Analysis. Hyunook Kim: Conceptualization, Writing – review & editing. Vinod Kumar: Conceptualization; Supervision; Writing – review & editing.

Declarations

Competing interests

The authors declare no competing interests.

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Correspondence and requests for materials should be addressed to V.K.

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