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Impacts of polyethylene microplastics on the microalga, Spirulina (*Arthrospira platensis*)

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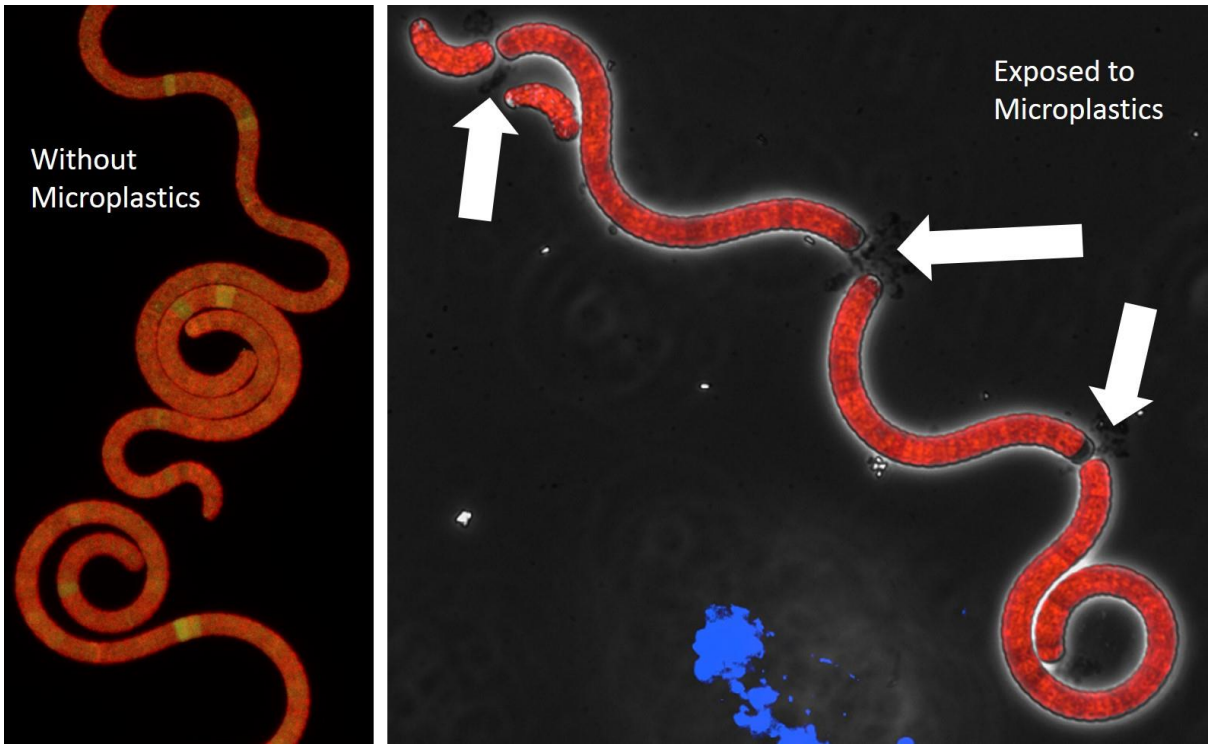
Graphical Abstract



Abstract

Microalgae play a critical role in the food web and biogeochemical cycling and produce compounds that are commercially exploited. However, their reactions and responses to microplastic contamination are not well understood. In this study, the widely distributed and commercially important cyanobacterium, Spirulina (*Arthrospira platensis*), was exposed to different concentrations (1 to 100 mg L^{-1}) of low-density polyethylene microplastics (< 5 μm) over a 20-d period. Various end-points were combined with different microscopic techniques in order to examine physiological and biochemical effects and interactions between the plastic and microalga. Growth rate and photosynthetic activity decreased with increasing microplastic concentration, and a maximum inhibition ratio of about 9% was calculated from optical density measurements. Plastic concentrations above 10 mg L^{-1} resulted in oxidative stress and the intracellular production of proline. Fragmentation and swelling of trichomes and attachment of microplastics was observed in the exposures, and microplastics appeared to adhere or aggregate around fragmented or fragmenting regions. The latter effect may indicate trichome weakening by microplastics or their concentration around cytosolic debris; nevertheless, it provides a potential mechanism for internalisation of small particles. Although unrealistically high concentrations of well-defined

35 microplastics have been employed, relatively small disruptions at the population level incurred by
36 lower concentrations could have more serious implications for ecosystem services and functioning.

37

38 **Keywords**: cyanobacteria; growth inhibition; photosynthesis; nanoplastics; fragmentation;
39 ecosystem services

40

1. Introduction

Microalgae are small, photosynthetic, autotrophic organisms that exist individually or in chains or groups and include eukaryotes, diatoms, dinoflagellates and prokaryotic cyanobacteria. Despite ranging in size from only a few μm to several hundred μm, their abundance, efficient biological fixation of carbon and rapid growth rates mean that microalgae consume significant quantities of CO_2 and produce at least one-half of the planet's atmospheric oxygen (Gigova and Marinova, 2016). Coupled with their role in nutrient recycling, microalgae are also a critical link in the food chain.

Microalgae also produce a variety of secondary metabolites, such as pigments, dyes, antioxidants and polysaccharides, with structures and activities generally not encountered in other organisms. Consequently, microalgae have attracted commercial and industrial interest in various sectors that include animal husbandry, pharmaceuticals, cosmetics and food production (Priyadarshani and Rath, 2012; Skjånes et al., 2017). Among the most widely used microalgae in this respect are *Cyanophyceae* (blue-green algae), *Chlorophyceae*, *Bacillariophyceae* and *Chrysophyceae* (Mobin and Alam, 2017).

Microalgae are often exposed to biotic and abiotic stressors and it is critical, therefore, to understand their reactions and responses. One type of aquatic contaminant that has gained considerable interest recently is microplastics, or primary and secondary plastics below 5 mm in size. However, a review by Prata et al. (2019) concluded that the effects of microplastics on microalgae have seldom been determined and that experimental results provide no consensus. For example, the same polymer can promote, inhibit or have no effect on growth, depending on the precise experimental conditions and species employed.

Spirulina are filamentous, blue-green cyanobacteria that are found widely in soil, marshes, freshwater, seawater and thermal springs, but thrive in warm, saline, alkaline environments with high levels of insolation. *Arthrospira platensis* is one of the most common and important species in the genera (*Spirulina* and *Arthrospira*), and being easy to harvest and process and with a high nutrient content is popular in the human health food industry and as an aquaculture feed additive (Habib et al., 2008). Despite extensive documentation in scientific research and in public health and food security literature, however, its physiological or biochemical response to microplastic contamination is not well understood. Specifically, exposure to microplastics of different composition appears to inhibit the growth of *Spirulina* sp. and alter biochemical composition and promote the production of extracellular polymeric substances (Abed et al., 2021; Hadiyanto et al., 2021; 2022).

In the present study, we investigate the effects of different concentrations of microplastics constructed of polyethylene, one of the most widely used polymers, on the growth, photosynthesis and production of reactive oxygen species (ROS) of *A. platensis*. We combine established methodologies and end-points with Raman spectroscopy and microscopic imagery in order to explore possible mechanisms, morphological changes and plastic-organism interactions involved in these effects and with microalgae more generally.

2. Methods

2.1. Materials and exposure conditions

Spirulina microalgae, *Spirulina* (*Arthrospira) platensis,* were cultured in the Department of Biology at Shiraz University. All reagents were purchased from Merck or Sigma-Aldrich and irregularly-shaped polyethylene (PE) microplastics, derived from milling and sieving pure low-density polyethylene (LDPE), were sourced from Torun University, Poland. Scanning electron microscopy (see below) revealed maximum and median particle diameters of about 8 μm and 2.5 μm, respectively, meaning that particles are at the lower end of the size spectrum of microplastics (conventionally defined as 1 μm to 5 mm). Microalgae were maintained at 30 $\pm$ 2 ^{o}C under fluorescent lighting 3500 lux and with a cultivation cycle of 20 d in Zarrouks medium, prepared from ACS or analytical grade salts dissolved in distilled, sterilised water and whose pH was 9.5 and salinity was about 27.

Twenty-day exposures of ~ 75 mg *A. platensis* in 1 L Zarrouks medium were performed under the conditions above in a series of 1-L PET cylinders aerated and agitated with individual air stones and stirrers. Exposures (n = 16) consisted of quadruplicates of a control (no microplastics), and microplastic concentration spanning two orders of magnitude on a mass basis (1 mg L^{-1} PE, 10 mg L^{-1} PE and 100 mg L^{-1} PE).

2.2 Cell growth

At two-day intervals, air stones and stirrers were turned off, allowing PE particles to float to the surface. A 2-mL aliquot from the central part of each cylinder was then pipetted into a cuvette and absorption, as an optical density measure of cell growth (Choi and Lee, 2018), was determined at 565 nm with a UV-Vis spectrophotometer (Lambda 365, PerkinElmer). The dry mass of oven-dried

(60 °C for 24 h) pellet arising from the centrifugation (5 min at 5000 rpm) of a 4 mL aliquot sampled concurrently was determined on a five-figure balance.

2.3 End-points

At the end of the four treatments, samples were vacuum-filtered through individual 25 μm nylon filters (Hebei Reking) in order to capture remaining biomass and minimise contamination by PE microplastics, and residues were oven-dried as above.

For the determination of chlorophyll a (Chl_a), chlorophyll b (Chl_b) and carotenoids, we used a modified version of Arnon's method (Arnon, 1949). Briefly, 20 mg of dried microalgae were extracted in 80% acetone and supernatants arising from subsequent centrifugation (as above) were measured for absorption at 663 nm, 645 nm and 470 nm by UV-vis spectrophotometry. Concentrations in $\mu g\ mL^{-1}$ were derived from the following formulae:

Chlorophyll a = $12.7(A_{663})-2.69(A_{645})$

Chlorophyll b = $22.9(A_{645})-4.68(A_{663})$

Total carotenoids = $[(1000(A_{470})-1.82\ Chl_a - 85.02\ Chl_b]/198$

The concentration of the proteinogenic imino acid, proline, that has a role in ameliorating environmental stress in algae, was determined using acidic ninhydrin according to Bates et al. (1973). The reagent was prepared by dissolving 0.625 g of ninhydrin in 25 mL of a solution consisting of 15 mL glacial acetic acid and 10 mL of 6 M phosphoric acid and was stored at 4 °C. One hundred mg of dried microalgae were ground with 10 mL of 3% sulphosalicylic acid in a porcelain pestle and the resulting contents were centrifuged at 1500 rpm for 10 min. Two-mL aliquots of the extract, ninhydrin reagent and glacial acetic acid were shaken in a foil-covered test tube and heated to 100 °C for 1 h in a water bath. The test tube was then cooled on ice before 4 mL of toluene were added and the contents vortexed for 20 min before the absorbance of the upper solution was read at 520 nm by the UV-vis spectrophotometry after having been calibrated by proline standards in the range of 0 to 40 $mg\ mL^{-1}$.

Malondialdehyde (MDA) was determined as an indicator of lipid peroxidation according to Haraguchi et al. (1997). Thus, 50 mg of dried biomass were ground with 2 mL of 10% trichloroacetic acid in a porcelain pestle and mortar and the resulting suspension was centrifuged at 10,000 rpm for 10 min. One-mL of supernatant was added to 2 mL thiobarbituric acid and the contents heated in a bain-marie for 45 min at 95 °C before the cooled suspension was centrifuged at 5000 rpm for 10 min. The

absorbance of the supernatant was read at 532 nm and corrected for nonspecific absorbance at 600 nm by UV-vis spectrophotometry.

2.4. Microscopy and Raman spectroscopy

Just before the termination of the experiment, 2-mL aliquots were pipetted from each container on to individual microscope slides and the contents analysed under a Carl Zeiss binocular microscope at up to 200 X magnification and an Olympus IX51 cell culture fluorescence microscope. Additional, freeze-dried aliquots were analysed under a Tescan Vega 3 scanning electron microscope operated at 20 kV and a viewing distance of between about 5 and 10 mm, and a μ-Raman spectrometer (LabRAM HR, Horiba, Japan) employing a laser wavelength of 582 nm, a Raman shift of 400-1800 cm^{-1} and acquisition times between 20 and 30 s.

2.5. Statistics

Mean values were compared by one-way ANOVA using SPSS. A Duncan's multiple range post-hoc test used to identify statistical differences ($\alpha = 0.05$) between specific pairs of means.

3. Results

3.1. Cell growth

Cell growth of *A. platensis* is shown as a function of exposure time for the four different treatments in Figure 1. Mean optical density (Figure 1a) is about 0.04 at the beginning of all treatment and, in the control, increases to 1.0 at 20 h. After 12 h, mean optical densities in the presence of microplastics PE are lower than the corresponding values for the control. However, the reduction is only significant ($p < 0.05$), and in the range of about 10 to 15%, at the highest concentration of PE employed.

Mean dry biomass (Figure 1b) is about 0.07 mg mL^{-1} at the beginning of all treatments and, in the control, increases to about 1.2 mg mL^{-1} after 14 h. Increasing concentration of PE microplastics is accompanied by a progressive reduction in mean dry weight after 6 h. Reduced dry weight is significant relative to the control for microplastic concentrations of 10 mg L^{-1} and 100 mg L^{-1} after 8 h, and beyond 12 h dry weight is significantly lower at 100 mg L^{-1} than at 10 mg L^{-1}.

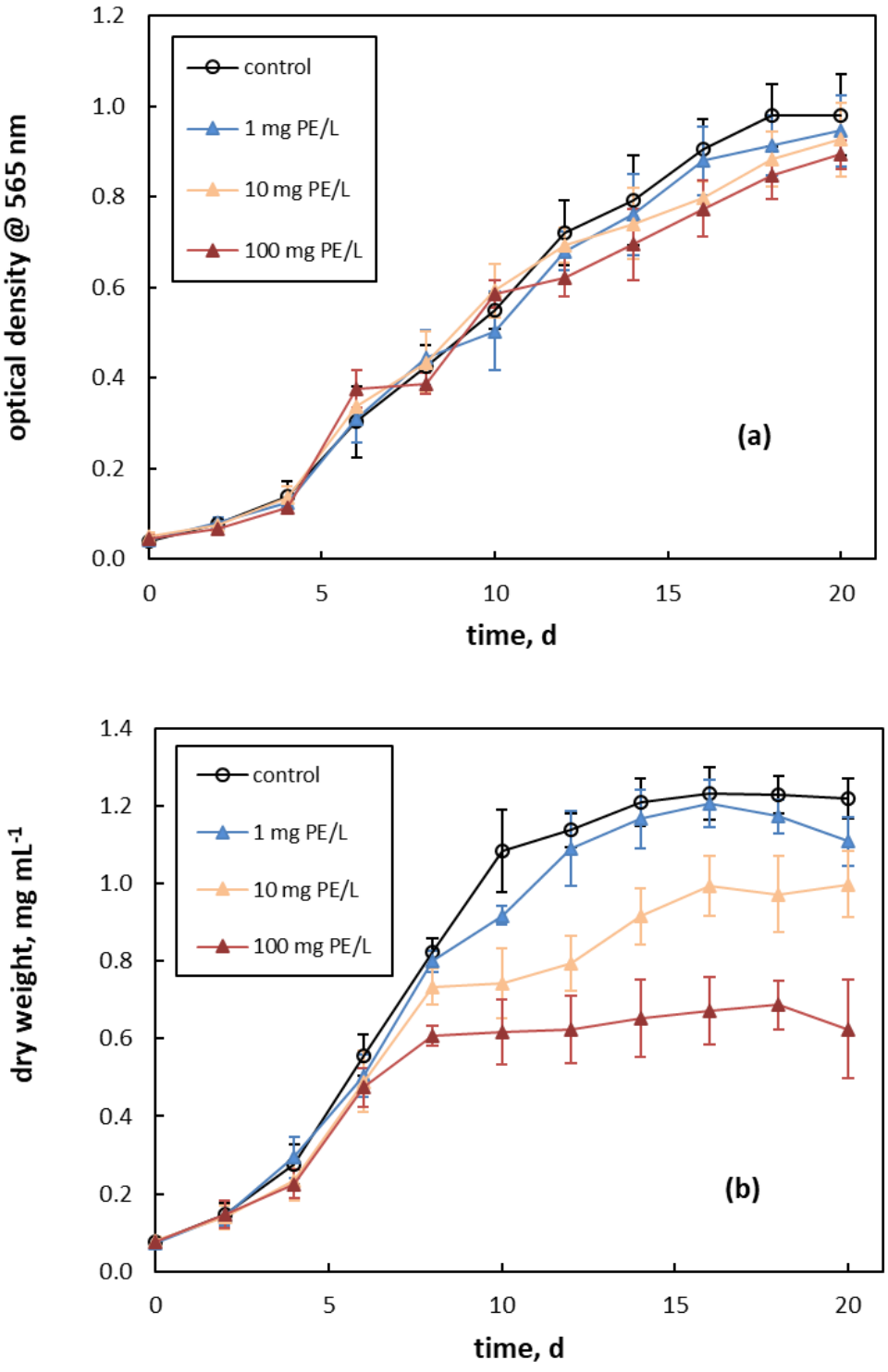


Figure 1: Cell growth of *A. platensis* as (a) optical density and (b) dry biomass as a function of exposure time for the four treatments. Errors are one standard deviation about the mean of four measurements.

Figure 2 shows the mean concentrations of chlorophyll a, chlorophyll b and total carotenoids in the biomass of *A. platensis* arising from the four exposures. Chlorophyll a exhibits a significant, progressive decrease with increasing concentration of PE microplastics, while chlorophyll b and carotenoids exhibit significant reductions relative to the corresponding controls are only observed at particle concentrations of 10 mg L^{-1} and 100 mg L^{-1}. Overall, mean concentrations of chlorophyll a, chlorophyll b and total carotenoids decrease by about 70%, 50% and 40%, respectively, from the control to the highest concentration of microplastics added.

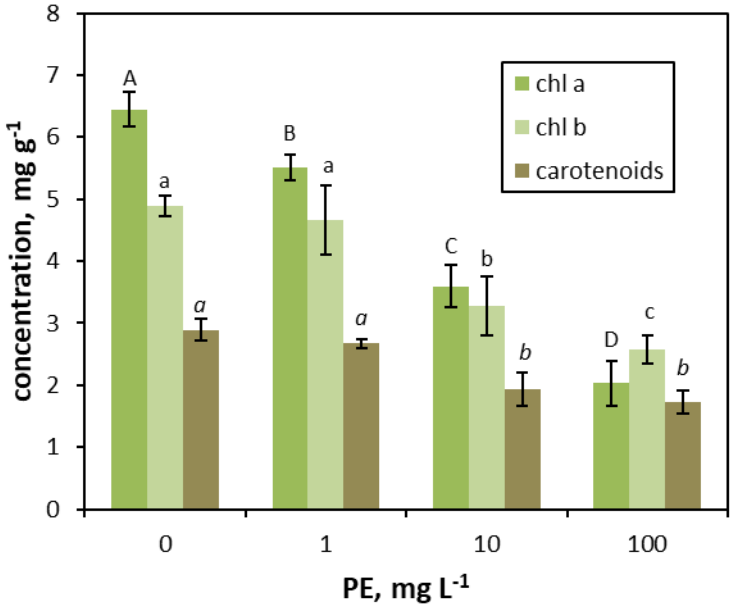


Figure 2: Concentrations of chlorophyll a, chlorophyll b and total carotenoids, normalised to dry weight of *A. platensis*, in the four treatments. Error bars represent one standard deviation about the mean (n = 4) and, for each component (and letter style), different letters indicate a significant differences ($p < 0.05$).

Figure 3 shows the mean proline concentration of *A. platensis* in the different treatments. Addition of 1 mg L^{-1} of PE microplastics results in a proline concentration that is not significantly different to the control (about 7.5 μmol g^{-1}), but 10 mg PE L^{-1} and 100 mg PE L^{-1} result in progressive increases that are significant, with a mean proline concentration of about 32 μmol g^{-1} in the highest exposure.

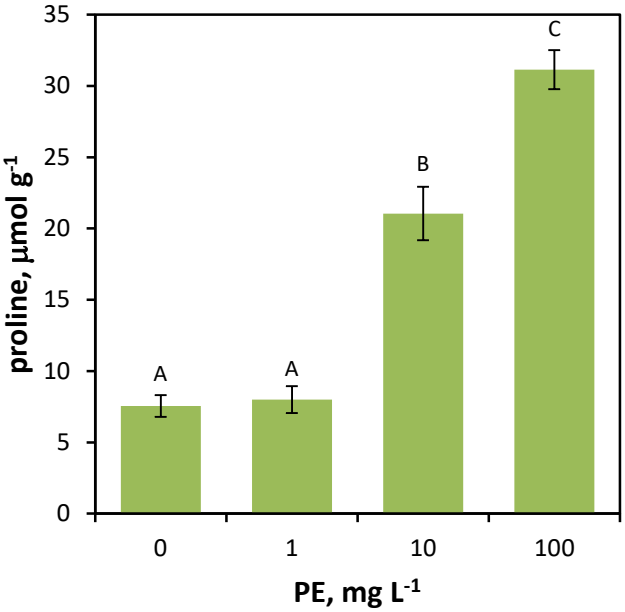
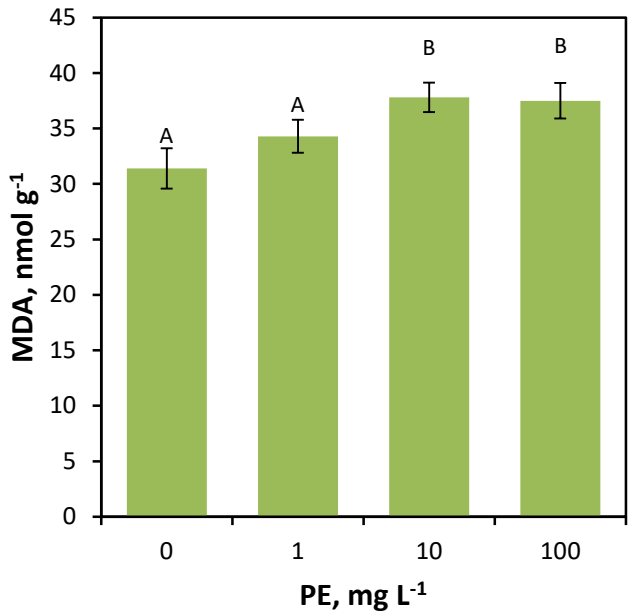


Figure 3: Concentrations of proline, normalised to dry weight of *A. platensis*, in the four treatments. Error bars represent one standard deviation about the mean (n = 4) and different letters indicate significant differences ($p < 0.05$).

Mean concentrations of MDA in *A. platensis* arising from the four treatments are shown in Figure 4. Here, differences are smaller between treatments than for proline concentrations and significant differences are only observed between the two highest exposures and the control and the lowest exposure.



200

201 Figure 4: Concentrations of MDA, normalised to dry weight of *A. platensis*, in the four treatments.
202 Error bars represent one standard deviation about the mean (n = 4) and different letters indicate
203 significant differences ($p < 0.05$).

204

205

206 Figures 5 and 6 show binocular and fluorescent microscopic images, respectively, of *A. platensis*
207 abstracted at the end of the experiment. Mature, helicoidal cells in the control reached lengths of
208 600 μm and with diameters of about 5 μm, but increasing concentration of PE microplastics was
209 accompanied by increasing fragmentation, with lengths often below 25 μm, swelling to about 8 μm,
210 and attachment of plastic. Also indicated in Figure 6 are microplastics attached or aggregated at
211 regions where fragmentation has occurred or is about to take place. The attachment of microplastics
212 to the algal surface more generally is evident when comparing the SEM images of the control and
213 exposure to 100 mg PE L^{-1} in Figure 7.

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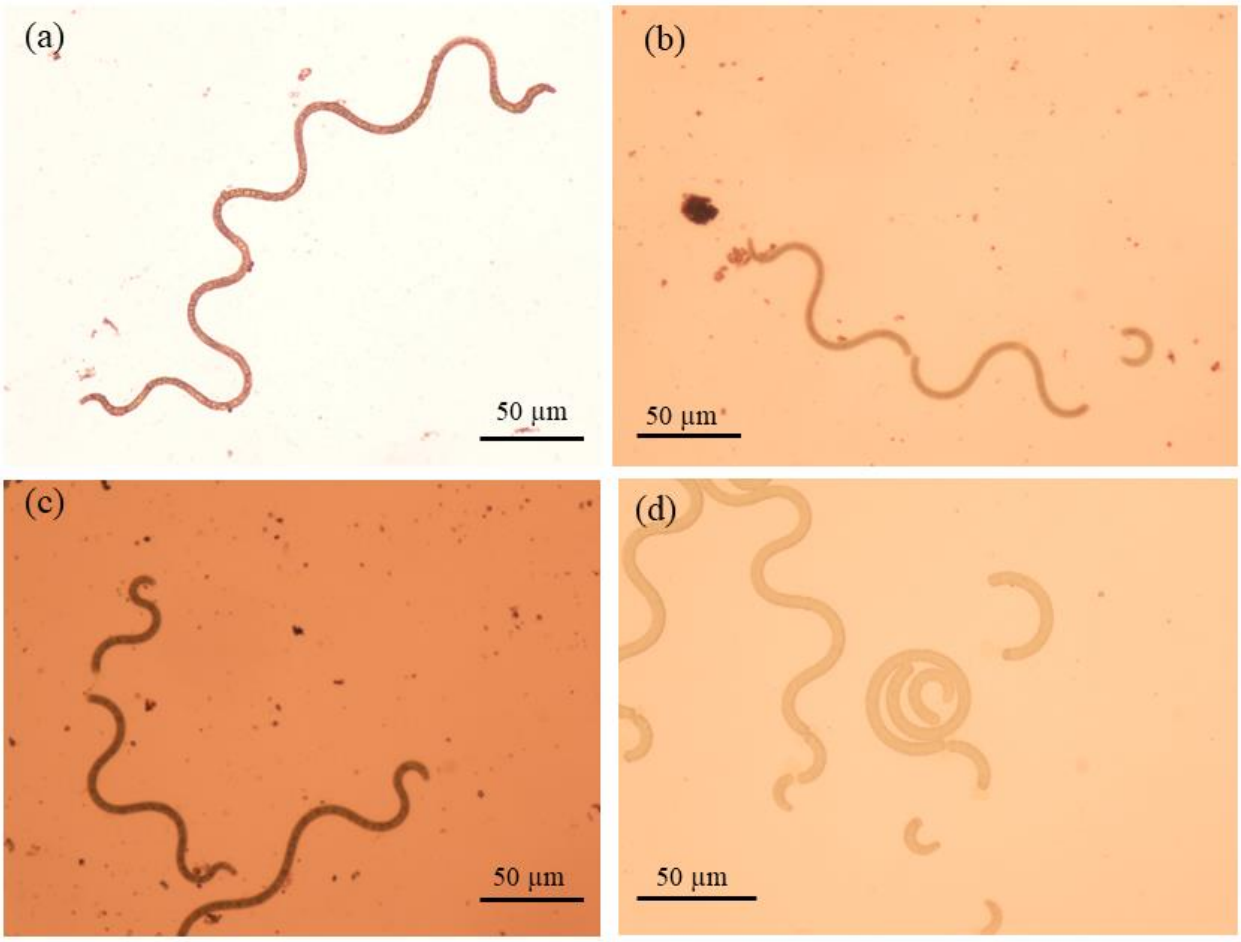

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217 Figure 5: *A. platensis* under the binocular microscope in (a) the control, (b) exposed to 1 mg L^{-1} PE,

218 (c) 10 mg L^{-1} PE, (d) 100 mg L^{-1} PE.

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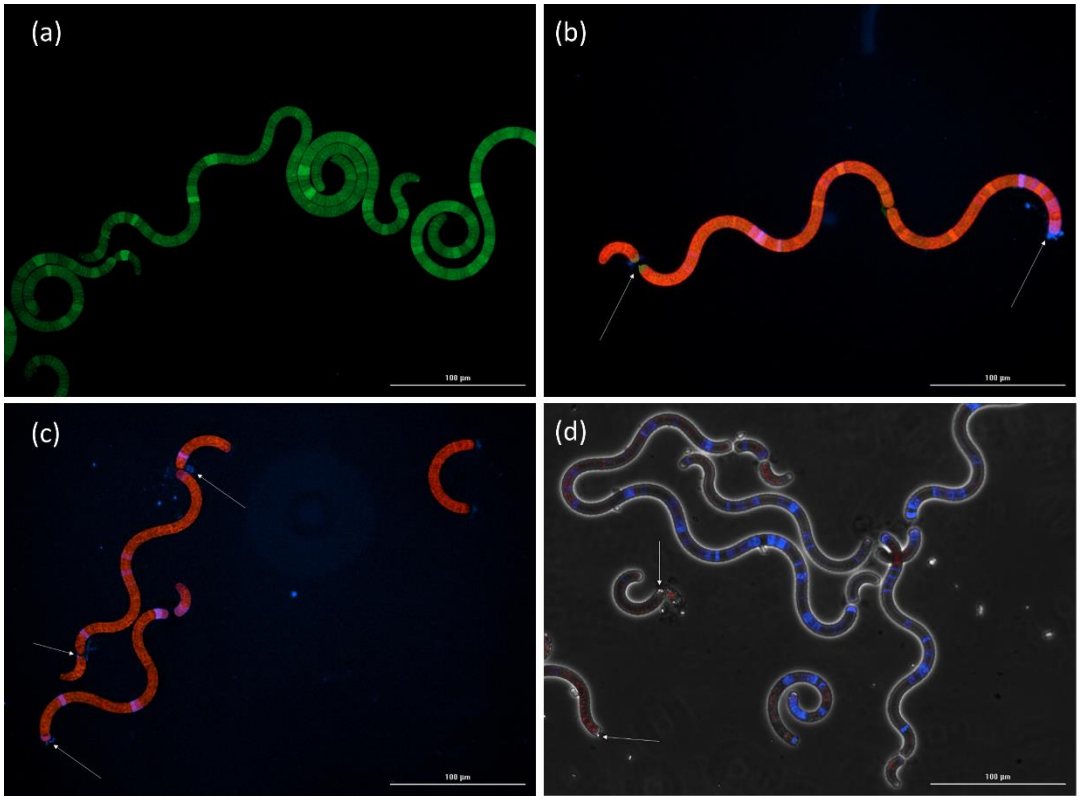

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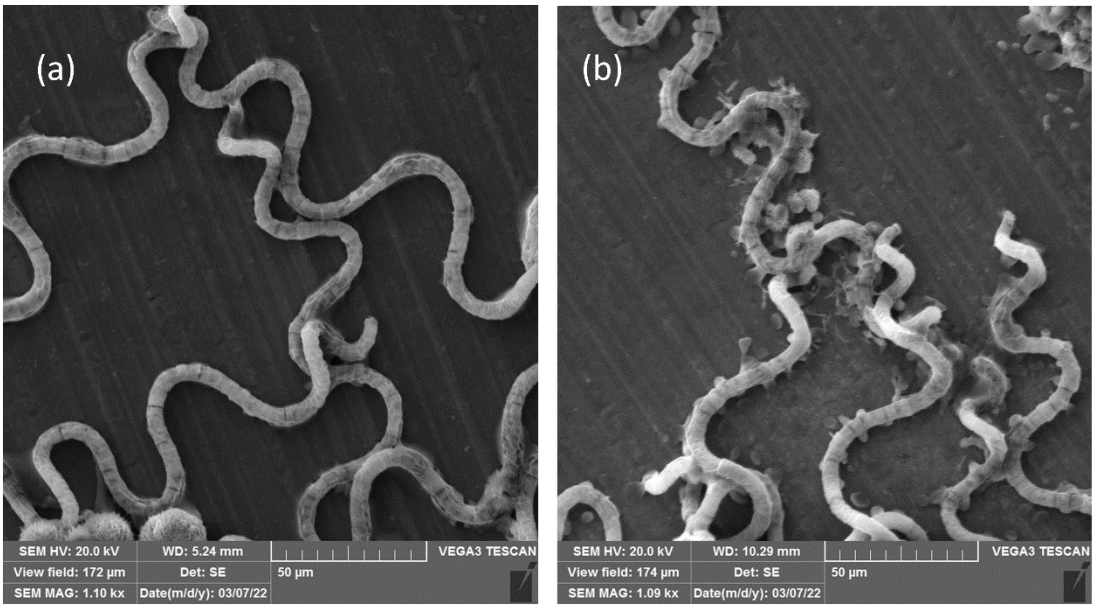
221 Figure 6: *A. platensis* under the fluorescent microscope in (a) the control, (b) exposed to 1 mg L^{-1} PE,
222 (c) 10 mg L^{-1} PE, (d) 100 mg L^{-1} PE. Arrows show fragmented or fragmenting regions where
223 microplastics appear to be attached or aggregated.



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225 Figure 7: SEM images of *A. platensis* in (a) the control and (b) exposed to 100 mg L^{-1} PE.

Raman spectra between wave numbers of 900 and 1200 cm^{-1} are shown for *A. platensis* abstracted at the end of the experiment and for the PE microplastics in Figure 8. The peaks centred at 1153 cm^{-1} are related to carotenoid substances, and intensities are similar for the control and lowest concentrations of microplastics but at the highest concentration of particles, and (at least qualitatively) consistent with the observations in Figure 2, the peak is supressed. The peak at about at 1067 cm^{-1} is related to polyethylene (C-C stretching) and is clearest for the microplastics. However, this peak is also evident in *A. platensis* exposed to microplastics and with an intensity that decreases with microplastic concentration, confirming the algal-microplastic associations observed microscopically above.

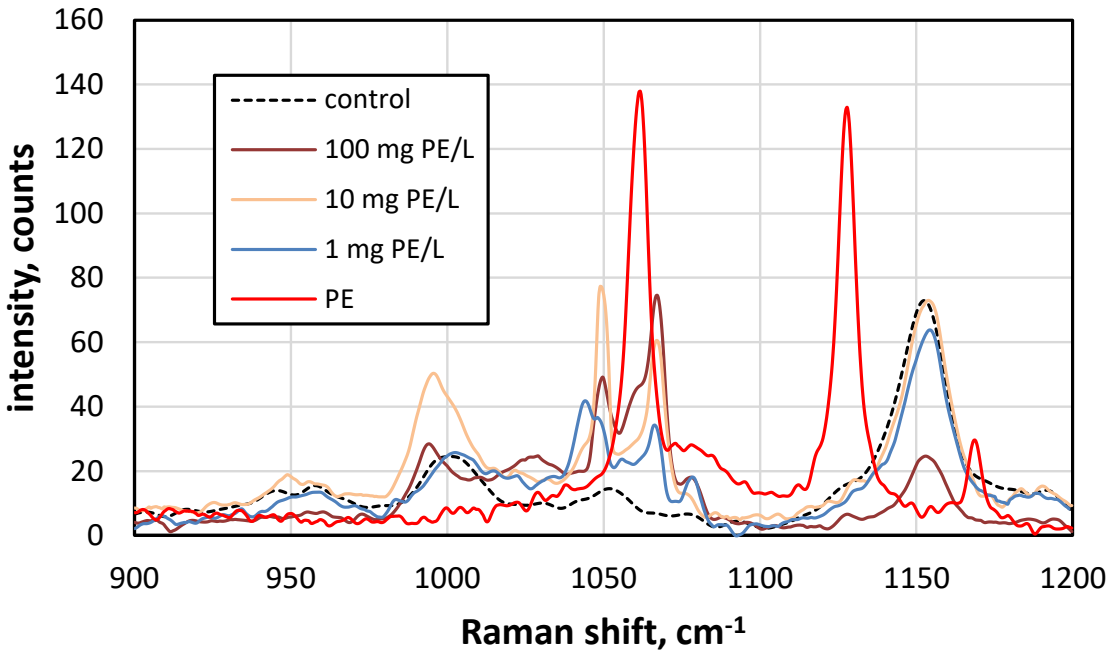

Figure 8: Raman spectra for *A. platensis* sampled at the end of the experiment and for the PE microplastics.

4. Discussion

The results of this study show that PE microplastics do not have lethal effects on the cyanobacteria, *A. platensis*, but are responsible for physiological and biochemical changes. Specifically, PE microplastics inhibit the growth and reduce photosynthesis in *A. platensis* over the range of

concentrations studied (1 to 100 mg L^{-1}), with an EC-50 for growth on a dry mass basis of about 100 mg L^{-1}. Concentrations of 10 and 100 mg L^{-1} also cause oxidative stress leading to lipid peroxidation, and result in the intracellular production of proline.

In a recent review, Prato et al. (2019) highlighted the limited number of studies on the effects of microplastics and nanoplastics on microalgae, and the lack of consensus among these studies. With respect to growth, for example, the presence of microplastics have revealed inhibition (e.g. Besseling et al., 2014; Mao et al., 2018), promotion (e.g. Lagarde et al., 2016; Canniff and Hoang, 2018) and no effects (e.g. Davarpanah and Guilhermino, 2015; Yokota et al., 2017).

The growth inhibition ratio, IR, for *A. platensis* exposed to PE microplastics was calculated from optical densities at 20 h in each exposure (E20) and control (C20) (Ansari et al., 2021):

$$IR\ (\%) = (1 - E_{20}/C_{20}) \times 100\%$$

We note that ratios of 3.6%, 5.6% and 9.0% for PE concentrations of 1, 10 and 100 mg L^{-1}, respectively, are lower than the typical range reported in the literature for different genera of microalgae exposed to various polymers at concentrations between 50 and 250 mg L^{-1} (about 20 to 50%, and where growth inhibition was observed; Besseling et al., 2014; Li et al., 2020; Ansari et al., 2021). However, our results are, quantitatively, more consistent with growth rate inhibitions reported for *Spirula* sp. exposed to microplastic concentrations above 25 mg L^{-1} (Abed et al., 2021; Hadiyanto et al., 2021; Hadiyanto et al., 2022). Presumably, discrepancies in the literature relate to differences in species morphology and cell characteristics, as well as plastic particle size and surface charge, the concentration and type of any additives present, and the exposure period employed (Nava and Leoni, 2021).

Moderate growth rate suppression by microplastics in the present study was accompanied by swelling of cells and fragmentation of filaments (Figures 5 and 6). The propagation of *A. platensis* takes place by fragmentation of trichomes that are subsequently elongated by binary fission until maturity (Jung et al., 2021). Increasing fragmentation in the presence of increasing quantities of MPs may reflect a progressive delay of the propagation phase, or some response of mature microalga to plastic. In a study of *A. platensis* exposed to cadmium, Rangsayatorn et al. (2001) attributed fragmentation to a defence mechanism. Specifically, low concentrations (< 8 mg L^{-1}) of Cd resulted in a small number of disordered cells, with higher concentrations resulting in severe injury. Nevertheless, and despite cellular damage, growth and division continued. In the present study, the attachment or aggregation of microplastics at the surface of the microalgae, evident in microscopic imagery, may contribute to the weakening of filaments or trigger some defence. This may explain

why microplastic accumulation is concentrated at the cell terminal (Figure 6). Alternatively, it is possible that MPs are attracted to the cytoplasm content that is released in this region during cell fragmentation (Jung et al., 2021). The precise mechanisms behind this process and the potential for small micro- or nanoplastics to be internalised during fragmentation are not known and warrant further study.

As well as inhibiting the growth of *A. platensis*, exposure to PE microplastics affects the photosynthetic activity of the microalga. Moreover, a reduction in the concentration of pigments essential for photosynthesis was accompanied by increases in MDA content, as an indication of cell membrane lipid peroxidation by reactive oxygen species (ROS), and proline, as an ROS scavenger and antioxidant. Thus, ROS are formed when electrons are diverted to O_2 and not CO_2 when photosynthetic activity decreases (Bhattacharya et al., 2010). Recent studies have also demonstrated a reduction in pigment content of freshwater microalgae following exposure to microplastics of different polymeric compositions at concentrations on the order of 100 mg L^{-1} (Li et al., 2020; Tunali et al., 2020). Potential causes of photosynthetic inhibition by microplastics include shading from incident light (Zhang et al., 2017) and adherence to the algal surface via extracellular polymeric substances, thereby reducing nutrient and gas exchange and trapping harmful metabolites (Mao et al., 2018). It is not clear whether the reduction of photosynthetic activity is related to or independent of growth inhibition and fragmentation in the presence of plastic but we note that accumulation of ROS by *A. platensis* can result in spiral breakage by oxidizing lipids of the sheath or cell membrane (Ma and Gao, 2010).

Although the effects observed here are broadly consistent with those reported elsewhere in the literature, their ecological relevance is likely to be low because unrealistically high concentrations of well-defined microplastics are typically considered in the presence of a single microalgal species. Nevertheless, relatively small disruptions at the population level, including subtle changes to morphology and palatability, could have more serious implications for ecosystem services and functioning (Prata et al., 2019; Nava and Leoni, 2021). More specifically, and given its importance as a human source of human protein (Habib et al., 2008), population-level alterations to *A. platensis* could have significant impacts on food security. Conversely, it has been suggested that *A. platensis* could be exploited to assist in the degradation of microplastics (Hadiyanto et al., 2021) or act as a bioremediator through hetero-aggregation (Abed et al., 2021).

5. Conclusions

The present study has shown that exposure of the cyanobacterium, *A. platensis*, to different concentrations of PE microplastics over a 20-d period results in various adverse effects, including a reduction in growth rate and photosynthesis, oxidative stress, and the fragmentation and swelling of trichomes. Although these impacts were observed for environmentally unrealistic concentrations of well-defined, customised microplastics, small disruptions arising from lower concentrations could have adverse effects at the population level. Because of the potential implications for ecosystem services, further research into the impacts and interactions of lower concentrations of more representative and heterogenous microplastics (in terms of size, polymer composition, surface charge and degree of weathering, for example) is warranted.

Acknowledgements

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