



Marine vs freshwater microalgae exopolymers as biosolutions to microplastics pollution[☆]



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ABSTRACT

Microalgae can excrete exopolymer substances (EPS) with a potential to form hetero-aggregates with microplastic particles. In this work, two freshwater (*Microcystis panniformis* and *Scenedesmus* sp.) and two marine (*Tetraselmis* sp. and *Gloeocapsa* sp.) EPS producing microalgae were exposed to different microplastics. In this study, the influence of the microplastic particles type, size and density in the production of EPS and hetero-aggregates potential was studied. Most microalgae contaminated with microplastics displayed a cell abundance decrease (of up to 42%) in the cultures. The results showed that the formed aggregates were composed of microalgae and EPS (homo-aggregates) or a combination of microalgae, EPS and microplastics (hetero-aggregates). The hetero-aggregation was dependent on the size and yield production of EPS, which was species specific. *Microcystis panniformis* and *Scenedesmus* sp. exhibited small EPS, with a higher propensity to disaggregate, and consequently lower capabilities to aggregate microplastics. *Tetraselmis* sp. displayed a higher ability to aggregate both low and high-density microplastics, being partially limited by the size of the microplastics. *Gloeocapsa* sp. had an outstanding EPS production and presented excellent microplastic aggregation capabilities (adhered onto the surface and also incorporated into the EPS). The results highlight the potential of microalgae to produce EPS and flocculate microplastics, contributing to their vertical transport and consequent deposition. Thus, this work shows the potential of microalgae as biocompatible solutions to water microplastics treatment.

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1. Introduction

Microplastics (plastic particles smaller than 5 mm) pollution is a growing concern shared by scientists and many individuals across the world. Human consumerism gave rise to aquatic systems filled with microplastics that can enter into the food chain. Recent studies show that these debris are in aquatic and terrestrial ecosystems and have a worldwide geographic distribution, and can be found even in the most remote locations, such as the Antarctic (Waller et al., 2017) and Artic (Peeken et al., 2018) oceans. Microplastics may come from general consumer household and cosmetic products but can also derive from macroplastics that suffer from mechanical action and degradation, largely driven by UV-radiation-

induced-photooxidation, releasing increasingly smaller sized low-molecular-weight polymer fragments (Galloway et al., 2017). Two of the industrial most commonly employed plastics, that are found in aquatic environments, are poly(methyl methacrylate) (PMMA), known as acrylic (Browne et al., 2011) and polystyrene (PS) (Alimi et al., 2017). PMMA is a lightweight resistant synthetic fiber (Zeng et al., 2002), that is widely used in the building industry, as a substitute for glass due to its high light transmission and resistance (Harper, 2000). PS is also very versatile and widely used in packing, household and consumer goods (Maul et al., 2007), as this polymer is usually found in food containers (GESAMP, 2015). Microplastics have a strong influence in freshwater and marine ecosystems, as they are ingested and transferred through the planktonic web (Setälä et al., 2013). Furthermore, they affect higher trophic levels, (Farrel & Nelson, 2013; Cauwenbergh & Janssen, 2014; Besseling et al., 2015); modify genetic expression (Lagarde et al., 2016) and exhibit toxicity and mortality in marine plankton (Bergami et al., 2017). For these reasons, microplastics elimination/removal from

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the environment is important before they can enter the trophic chain.

Microalgae are unicellular species that have been recognized as a bioalternative of different stages and functions of wastewater treatment (Abdel-Raouf et al., 2012). Despite their actual usage in water treatment, there is still an immense potential of using microalgae for microplastics treatment. This is because microalgae have the potential to produce exopolymers substances (EPS), which are viscous gel-like structures that are species specific. EPS are long-chain polysaccharides, composed of repeating units of sugar derivatives, with a structural diversity arisen from a broad range of non-carbohydrate substituents and linkage types (Whitfield, 1988). EPS have been characterized, revealing prominent functional reactive groups, such as sulphate, hydroxyl or carboxylic (Shah et al., 2000; Castellane et al., 2015). In this regard, freshwater and marine microalgae can have the potential to affect microplastics bioavailability, via the secretion of EPS, with consequent formation of hetero-aggregates (composed by EPS, microalgae and microplastics) to microplastics removal from aquatic ecosystems. However, this potential is still untapped as very few studies have analysed the formation and composition of EPS and hetero-aggregates, as well as the interactions between EPS and microplastics.

This work aims to evaluate the potential of four phytoplanktonic species as compelling biocompatible solutions to marine, freshwater or urban water treatment, focusing on the hetero-aggregation potential in the presence of microplastics. Two freshwater microalgae, *Microcystis panniformis* and *Scenedesmus* sp., as well as two marine microalgae, *Tetraselmis* sp. and *Gloeocapsa* sp., all known to be EPS producers were used in the trials. Up until now, *Microcystis* sp. hepatotoxins (microcystins) quantitative analysis has been used as a reliable method for hazard control in water (Via-Ordonika et al., 2004) and *Scenedesmus obliquus* has proven to be an efficient microalga in coliform bacteria removal from domestic sewage (Sebastian & Nair, 1984). The marine *Tetraselmis suecica* also has the potential to inhibit pathogenic vibrios (Austin & Day, 1990) and bacterial fish pathogens (Austin et al., 1992). The removal of heavy metals such as Pb^{2+} has been performed with *Gloeocapsa* sp., with the results showing up to a 100% removal capacity (Raungsomboon et al., 2008).

The influence of microplastics type (PMMA and PS), size ($<106\text{ }\mu\text{m}$; $106\text{--}250\text{ }\mu\text{m}$), density (high and low) and concentration (12.5 mg L^{-1} and 125 mg L^{-1}) on the formation of hetero-aggregates and consequent deposition, was studied, in light of the different EPS yields of each species.

2. Materials and methods

2.1. Microalgae and culture conditions

In this study two freshwater species: *Microcystis panniformis* (Class Cyanobacteria; $3\text{--}5\text{ }\mu\text{m}$; Fig. 1a) and *Scenedesmus* sp. (Class Chlorophyceae; $5\text{--}13\text{ }\mu\text{m}$; Fig. 1b) were selected, as well as two marine microalgae: *Tetraselmis* sp. (Class Chlorodendrophyceae; $7\text{--}16\text{ }\mu\text{m}$; Fig. 1c) and *Gloeocapsa* sp. (Class Cyanobacteria; $38\text{--}42\text{ }\mu\text{m}$; Fig. 1d). *Gloeocapsa* sp., *Scenedesmus* sp. and *Microcystis panniformis* were obtained from the Spanish Algae Bank (BEA) and *Tetraselmis* sp. was obtained from a private collection of Mariculture Center of Calheta (Madeira).

The freshwater microalgae *Microcystis panniformis* (axenic) and *Scenedesmus* sp. (axenic) were grown in commercial BG-11 medium (Sigma-Aldrich), while the marine microalgae, *Gloeocapsa* sp. (axenic) and *Tetraselmis* sp. (non-axenic) were grown in commercial f/2 medium (Sigma-Aldrich).

The cultures used in the experiments (see 2.3.) were maintained

for 21 days at $20\pm1\text{ }^{\circ}\text{C}$, under the irradiance of 1738 lux (HOBO® Pendant® MX Temp MX2201) supplied by a cool white Osram L 18W 840 Lumilux lamp, with a 10/14 h (light/dark) photoperiod (Aralab CP500 growth chamber).

The initial cell concentration was $1.9\times10^6\text{ cells mL}^{-1}$ for *Microcystis panniformis*; $4.7\times10^5\text{ cells mL}^{-1}$ for *Scenedesmus* sp. and $3.3\times10^6\text{ cells mL}^{-1}$ for *Tetraselmis* sp.. *Gloeocapsa* sp. could not be counted due to its non-dissociable aggregates, as well as the impossibility to obtain homogeneous preparations. Microalgae cell growth was monitored using a Neubauer chamber (x10), sub-sampling every 2/4 days, for 3 weeks.

2.2. Microplastics

Two types of microplastics were used: fluorescent PMMA (green and purple) and fluorescent PS (yellow and blue). PMMA is a high-density plastic, whereas PS is a low-density plastic. The two types of irregular shaped microplastics were obtained by fragmentation using a milling machine (230 V ~ 50 Hz, 120 W). After this, all microplastics were mesh sieved (Analysensieb-Retsch), collected and separated according to their size. The green particles of PMMA and the yellow PS were separated into a $106\text{--}250\text{ }\mu\text{m}$ fraction and the purple particles of PMMA and blue PS were collected into a lower sized $<106\text{ }\mu\text{m}$ fraction. The microplastics were washed with dichloromethane, on a magnetic stirrer for 2 h, at room temperature. Then, the microplastics were filtered, oven-dried at $40\text{ }^{\circ}\text{C}$ overnight and kept in a dissector until analysis.

Two distinct stock solutions at two concentrations of microplastics were prepared for this study: high concentration was 250 mg L^{-1} and low concentration was 25 mg L^{-1} . In both concentrations, two types/densities and sizes of microplastics were used. As they are fluorescent with distinct colours, they are easily distinguishable. The solutions were prepared in f/2 and BG-11 medium, in glass flasks in order to minimize losses of microplastics due to the possible establishment of electrostatic bonds to the flask walls. The solutions were then kept at $4\text{ }^{\circ}\text{C}$ during the experimental period. And finally, Tween 20 (0.1%, v/v) was added to each solution to guarantee homogeneity.

2.3. Exposure conditions of microalgae to microplastics

Three experimental groups were used in triplicate: a control group, using the selected microalgae grown in their correspondent medium (Fig. 2a) and two other experimental groups in which the microalgae were subjected to the two distinct concentrations of microplastics (Fig. 2): 12.5 mg L^{-1} (low; Fig. 2b) and 125 mg L^{-1} (high; Fig. 2c). Each concentration contained purple and green PMMA (high density; $<106\text{ }\mu\text{m}$ and $106\text{--}250\text{ }\mu\text{m}$ size fractions), as well as blue and yellow PS (low density; $<106\text{ }\mu\text{m}$ and $106\text{--}250\text{ }\mu\text{m}$ size fractions).

The cultures were not renewed during the experimental period and were manually stirred 3 times a day. Nutrients were verified every other day to ensure that culture were nutrient sufficient (Nitrate and Phosphate test kits from Sigma Aldrich). Microalgal cell abundance was determined and used to distinguish the potential effects of the microplastics exposure to the countable species.

2.4. EPS determination

Modifications of Mota et al. (2013) method was used to determine the microalgae EPS production in the control treatments. EPS extraction was not performed in the culture with microplastics due to the fact that these cultures presented aggregates, that include EPS, that were further collected for analysis.

After 21 experimental days, the control cultures were

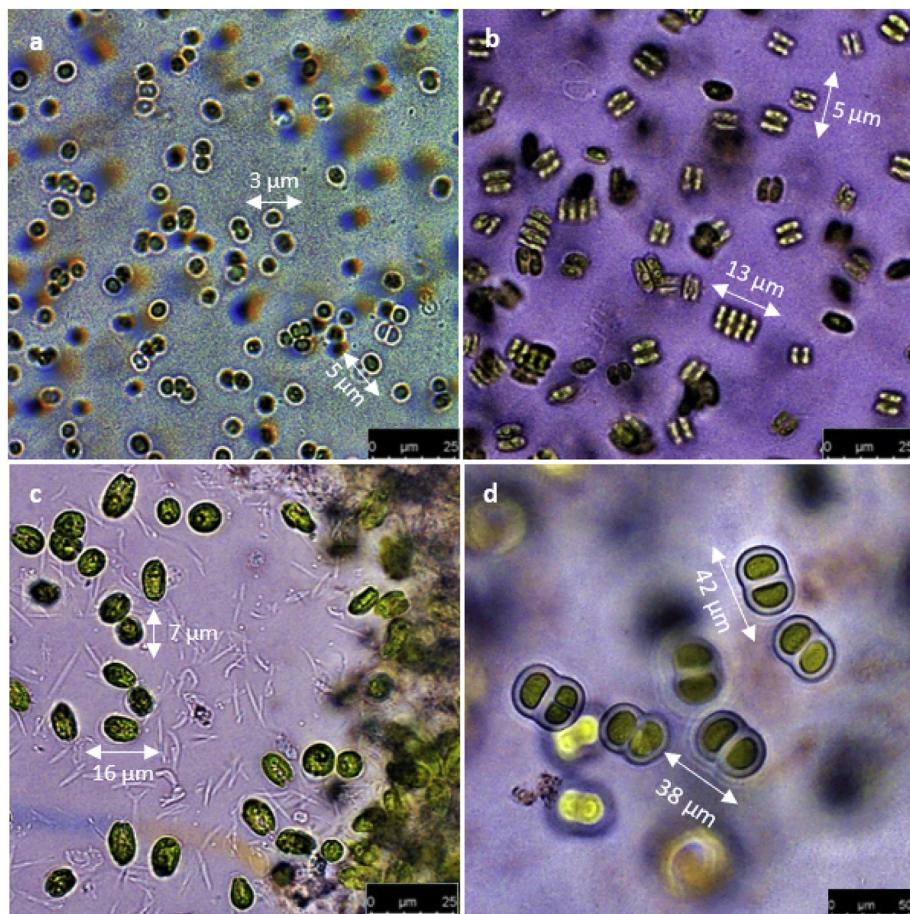


Fig. 1. Bright field micrographs of *Microcystis panniformis* (x20) (a), *Scenedesmus* sp. (x40) (b), *Tetraselmis* sp. (x40) (c) and *Gloeocapsa* sp. (x40) (d).

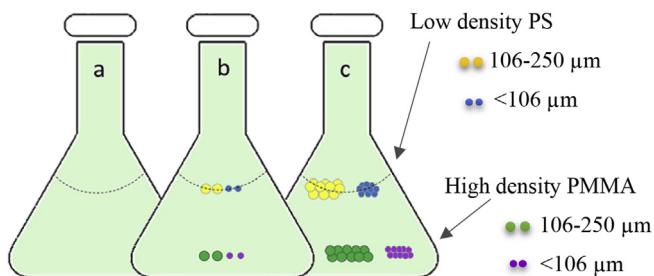


Fig. 2. Schematic of the experimental groups (exposure conditions to microplastics) applied to microalgae. (a) Control condition, without microplastics. (b) Low microplastics concentration culture (12.5 mg L⁻¹). (c) High microplastics concentration culture (125 mg L⁻¹). Each dot colour, size and density represent its corresponding microplastic. Low density microplastics: blue and yellow PS. High density microplastics: purple and green PMMA.

centrifuged at 6000 rpm for 30 min, at 25 °C to remove microalgae and other debris. The supernatant was concentrated on a magnetic stirrer at 60 °C for 12 h. For the precipitation of the EPS, methanol was gradually added to the concentrated supernatant, kept at 4 °C for 12 h and after centrifuged at 6000 rpm for 20 min. The precipitate was then washed with ethanol and re-dissolved in Milli-Q water. The dissolved EPS were dialysed against distilled water for 2 days to remove ions and salt. The dialysed EPS were frozen and freeze dried. The EPS yield was determined gravimetrically as EPS mg per mL of medium.

2.5. Characterization of hetero-aggregates

2.5.1. Fluorescent microscopy

Hetero-aggregates were collected from the bottom of the experimental flasks using a pipette. Each microplastic and different hetero-aggregates were individually analysed, under different microscopic filters. DAPI filter (excitation 340/80 nm, emission 425 nm) and I3 filter (excitation 450/90 nm, emission 515 nm). Observations were performed with a Leica DM2700P coupled with a CoolLED's pE-300^{lite} LED fluorescent illumination system.

2.5.2. Scanning electron microscope (SEM)

Before SEM analysis, the formed hetero-aggregates were washed three times with water and centrifuged at 2000 rpm, for 10 min, to remove any floating cells and culture medium compounds. Then, the hetero-aggregates were fixed using 2.5% (w/v) glutaraldehyde in 0.1 M sodium phosphate buffer, at pH 7.2, and stored overnight at 4 °C. After, the samples were washed two times with 0.1 M sodium phosphate buffer, at pH 7.2, and then serially dehydrated with ethanol (30%, 50%, 70%, 90% and dry 100%, v/v, ethanol/water) before freeze-drying. Samples were then preserved in a desiccator until analysis. Scanning electron micrographs of the surface samples were obtained by Scanning Electron Microscopy (SEM), with an HR-FESEM SU-70 Hitachi equipment operating at 4 kV, in the field emission mode. Samples were deposited on a steel plate and coated with carbon before analysis (EMITECH K950X Turbo Evaporator).

2.6. Statistics

Statistical analysis of microalgal growth rates were performed using IBM SPSS statistics software (V. 25). The differences in growth rates between different experimental conditions were assessed by one-way analyses of variance (ANOVA), with a level of statistical significance of p -value <0.05 .

3. Results

3.1. Microalgae growth

The microalgae growth for control conditions are presented in Fig. 3. Results show that after 21 days of growth, without renewal, *Tetraselmis* sp. was at stationary phase, while *Microcystis panniformis* and *Scenedesmus* sp. were still at exponential phase. For *Gloeocapsa* sp., the initial dilution performed ensured the exponential phase at the end of the experimental period.

At the end of the experimental period, cell abundance of each microalga, in both high and low concentrations of microplastics was also determined and compared with the control groups (Table 1). Both freshwater microalgae species displayed a cell abundance decrease in the microplastics condition cultures when compared with the control groups (Table 1). *Microcystis panniformis* exhibited a considerable and significant decrease ($p < 0.05$) in both microplastic concentrations; in the high microplastics concentration there was a decrease of 31% in cell abundance, but a more accentuated decrease (42%) was observed in the low microplastics concentration. In *Scenedesmus* sp. cultures, though the decrease in cell abundance was higher for the high microplastics concentration condition (21%), no significant differences were found between microplastic concentrations treatments. The marine microalgae, *Tetraselmis* sp. did not present a significant difference in cell abundance between both microplastic exposure conditions and control treatment ($p > 0.05$). For *Gloeocapsa* sp., no information regarding microplastics effect could be inferred for the number of cells per mL (see 2.1.).

3.2. Formation of EPS and hetero-aggregates

Table 2 presents the results of EPS and hetero-aggregates formation. After 21 experimental days, EPS production was higher for the marine *Gloeocapsa* sp. (2.146 ± 0.093 mg mL $^{-1}$), followed by *Tetraselmis* sp. (1.287 ± 0.090 mg mL $^{-1}$), *Microcystis panniformis* (0.047 ± 0.019 mg mL $^{-1}$) and *Scenedesmus* sp. (0.012 ± 0.002 mg mL $^{-1}$).

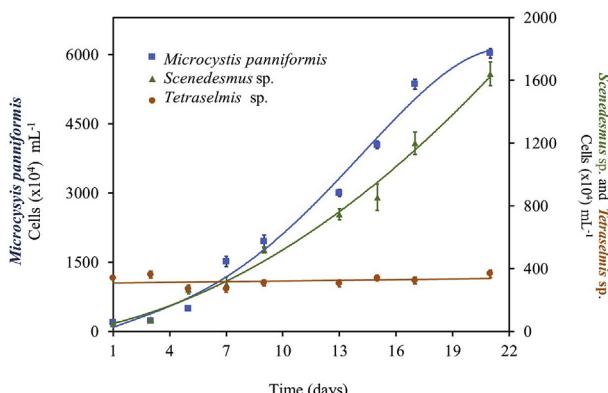


Fig. 3. Growth curves for control conditions of each countable microalga. Initial concentration was 1.9×10^6 cells mL $^{-1}$ for *Microcystis panniformis*, 4.7×10^5 cells mL $^{-1}$ for *Scenedesmus* sp. and 3.3×10^6 cells mL $^{-1}$ for *Tetraselmis* sp.

Fig. 4 presents macroscopic photographs of the aggregates formed at the bottom of the culture flasks, after 21 days of culture. The freshwater microalga *Microcystis panniformis* presented no distinguishable hetero-aggregates (Fig. 4a), unlike *Scenedesmus* sp. (Fig. 4b), that presented a clear and visible formation of hetero-aggregates. For *Tetraselmis* sp. (Fig. 4c) there were no visible hetero-aggregates, whilst *Gloeocapsa* sp. (Fig. 4d) flasks displayed identifiable hetero-aggregates.

Macroscopically, it was also possible to observe that at the bottom of the flasks these hetero-aggregates incorporated the microplastics. The low density fluorescent yellow PS microplastics were the most identifiable ones at the bottom of the flasks in these aggregation conditions, as pointed by the arrows in Fig. 4, due to its more intense and vivid colour. The other microplastics exhibit a paler colour that does not make them so easily distinguishable. In the case of *Gloeocapsa* sp. cultures, the microplastics were trapped within its viscous EPS. As observed in Fig. 4b and d, the low-density yellow PS sunk to the bottom of the flask, confirming its aggregation and the microplastics vertical transport. Since not all the different microplastics could be identified at the bottom of the flasks, to confirm if the aggregates were composed of microalgae/EPS alone (homo-aggregates) or microalgae/EPS and microplastics (hetero-aggregates), they were analysed by fluorescence microscopy.

3.2.1. Detection of hetero-aggregates by fluorescence microscopy

The micrographs revealed aggregation across all microalgae. *Microcystis panniformis* (Fig. 5a and b) exhibited a bright field observation of the EPS, as depicted by the arrows, confirming the aggregation of the microplastics (formation of hetero-aggregates). The EPS produced by this smaller sized *Microcystis panniformis* were abundant, but small (between 10 and 110 μ m) and easily disaggregated (shaking of the culture flasks).

In the case of *Scenedesmus* sp., the micrographs taken under I3 filters (Fig. 5c) revealed a considerable amount of EPS, ranging between 40 and 200 μ m in size. The EPS produced by this microalga is comparable to the one produced by *Microcystis panniformis*, in the perspective of its overall size and easiness to disaggregate. The aggregation of high density purple PMMA (1) was observed in Fig. 5c. A second micrograph taken under DAPI filters (Fig. 5c1) exhibited considerable low-density blue (2) PS aggregation and minor low-density yellow PS (3) aggregation. The results showed that *Scenedesmus* sp. exhibited a higher amount of aggregates when compared to *Microcystis panniformis*.

The micrographs of *Tetraselmis* sp. showed a high amount of EPS (Fig. 5d), ranging from 50 to 300 μ m in size. When observed under DAPI filters (Fig. 5e), these aggregates revealed a high amount of low density yellow (1) and blue (2) PS microplastic aggregation. A second micrograph (Fig. 5e1) taken under I3 filters, showed aggregation of high density green (3) and purple (4) PMMA, although in much lower quantities. *Tetraselmis* sp. also displayed the ability to colonize and aggregate microplastic much larger than its size (Fig. 5f).

Gloeocapsa sp. was the most distinct microalga, due to its abundant, dense, thick and viscous EPS mesh. The micrographs taken under DAPI filters (Fig. 5g and g1) showed abundant aggregation for every type of microplastic studied. From the same photograph (Fig. 5g), it is possible to infer that for this microalga the two types of microplastic did not only adhere to the surface of the exopolymer but were also incorporated.

3.2.2. Scanning electron microscope images

The SEM images (Fig. 6a) confirmed the smaller size of the EPS produced by *Microcystis panniformis*. These micrographs also showed that the size and type of exopolymer produced by

Table 1

Cell abundance (cells mL^{-1}) for each microalga studied, at the end of the experimental period. Cell abundance variation was calculated for the microplastic conditions against the control, for each microalga.

Microalgae	Microplastic concentration	Cell abundance ($\times 10^4$ cells mL^{-1})	Cell abundance variation
<i>Microcystis panniformis</i>	control	$6030 \pm 102^{\text{a}}$	—
	high	$4135 \pm 182^{\text{b}}$	-31%
	low	$3497 \pm 200^{\text{c}}$	-42%
<i>Scenedesmus</i> sp.	control	$1641 \pm 72^{\text{a}}$	—
	high	$1298 \pm 123^{\text{b}}$	-21%
	low	$1430 \pm 84^{\text{b}}$	-13%
<i>Tetraselmis</i> sp.	control	$368 \pm 24^{\text{a}}$	—
	high	$375 \pm 7^{\text{a}}$	+1%
	low	$365 \pm 44^{\text{a}}$	-1%

The values are mean of three replicates \pm SD; for each microalga species. Different letters indicate significant differences in cell concentration/abundance ($p < 0.05$).

Table 2

Summary table of the observations, including the EPS yield, size and stability and the main microplastics aggregated as well as the main type of aggregation.

Microalgae	EPS			Main Aggregation	
	Yield (mg mL^{-1})	Size (μm)	Stability	Microplastics	Type
<i>Microcystis panniformis</i>	0.047 ± 0.019	10–110	disaggregated	<106 μm PMMA	colonized
<i>Scenedesmus</i> sp.	0.012 ± 0.002	40–200	disaggregated	<106 μm PMMA and PS	adhered
<i>Tetraselmis</i> sp.	1.287 ± 0.090	50–300	stable	<106 μm and 106–250 μm PS	colonized and incorporated
<i>Gloeocapsa</i> sp.	2.146 ± 0.093	—	stable	all	adhered, incorporated and colonized

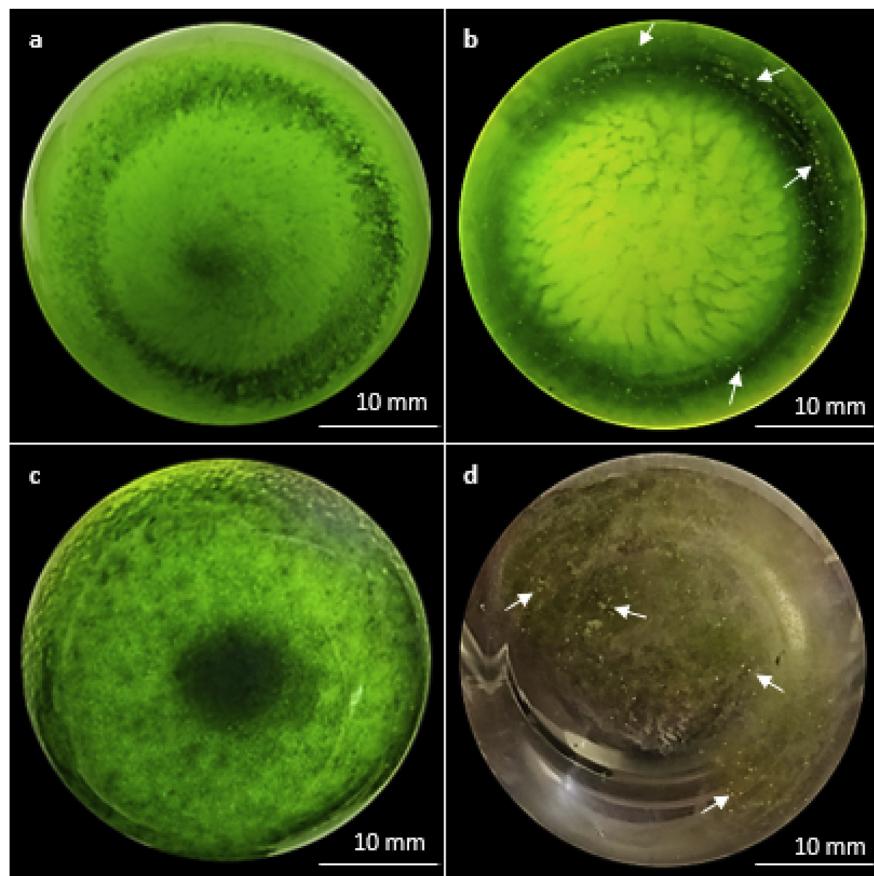


Fig. 4. Photographs of each microalga at high microplastics concentration condition culture (the high concentration was used for the photographs for better clarity), as observed from below the culture flasks. Each culture was photographed at the end of the experiment, using a digital camera. (a) *Microcystis panniformis* (b) *Scenedesmus* sp. (c) *Tetraselmis* sp. (d) *Gloeocapsa* sp.

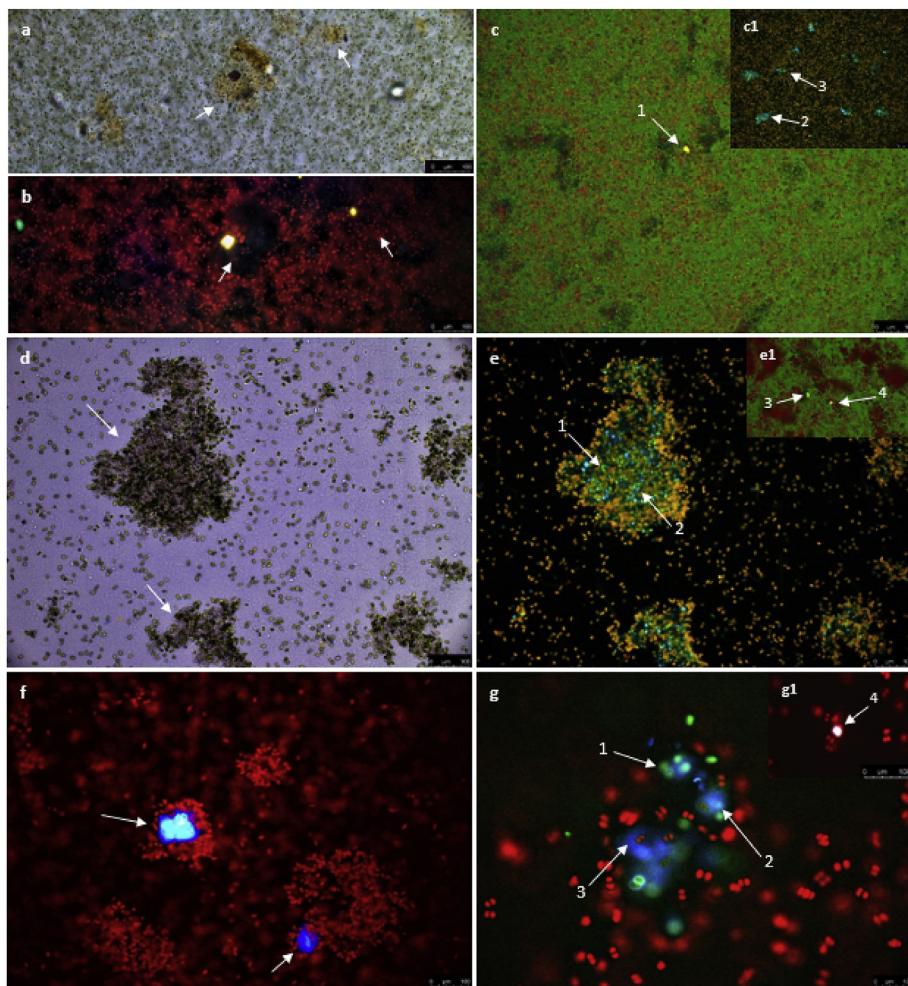


Fig. 5. (a) Bright field micrograph of *Microcystis panniformis* (high microplastics concentration) hetero-aggregates (x40). (b) Same micrograph seen in (a), but under DAPI filters (Excitation 340/80 nm, Emission 425 nm), being visible in the incorporation of the fluorescent microplastics in the hetero-aggregates. (c) Micrograph of *Scenedesmus* sp. (high microplastics concentration), under I3 filters (Excitation 450/90 nm, Emission 515 nm), showing the abundance of EPS and the microplastics aggregation (x10). (c1) Micrograph of *Scenedesmus* sp. (low microplastics concentration) under DAPI filters, exhibiting aggregation of different microplastics. Each arrow represents a different type of microplastic: the low density yellow (1) and blue (2) PS and the high-density green (3) and purple (4) PMMA. (d) Bright field micrograph of *Tetraselmis* sp. (low microplastics concentration) considerable sized hetero-aggregates (x10). (e) Same micrograph seen in (d), but under DAPI filters, showing hetero-aggregates composed of microalgae (orange) and microplastics (blue/green) and EPS (x10). (e1) Micrograph of *Tetraselmis* sp. hetero-aggregates (high microplastics concentration), under I3 filters, also revealing aggregation. (f) Micrograph of *Tetraselmis* sp. (high microplastics concentration) showing colonization of microplastics by the microalga, under DAPI filters. (g/g1) Micrographs of microplastics trapped in *Gloeocapsa* sp. (low microplastics concentration) thick EPS, under DAPI filters (x20). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Microcystis panniformis does not allow for a proper aggregation of microplastics larger than 25 μm but revealed its microplastics colonization potential.

The SEM images of *Scenedesmus* sp. showed that the EPS tend to form a network, benefiting the captation, aggregation and adhesion of microplastics to the *Scenedesmus* sp. EPS (Fig. 6b). *Tetraselmis* sp. The SEM images (Fig. 6c) showed the microalga and EPS agglomerates (homo-aggregates), with EPS creating a viscous mesh. As previously indicated by fluorescence microscopy, *Tetraselmis* sp. tends to colonize and coat the microplastics, with no microplastics being visibly aggregated on the surface.

In the case of *Gloeocapsa* sp., the SEM images (Fig. 6d) exhibited a fibrous, dense and viscous nature of its EPS. The SEM micrographs also showed that this microalga has the potential to colonize, aggregate and adhere microplastics to its surface, independent of its fraction size. In detail, Fig. 6d1 reveals the colonization potential of *Gloeocapsa* sp., as well as the ability to form hetero-aggregates, using microplastics as support.

4. Discussion

Following on the hypothesis that hetero-aggregation could be linked to cell physiology and EPS production (Lagarde et al., 2016), this study characterized and differentiated the interactions between different freshwater/marine microalgae and two types of microplastics. It aimed to assess the potential of microalgae to be used in water treatment plants based on their capability to produce EPS with their sticky properties. The hetero-aggregation observed in the present study between microplastics, microalgae and EPS is in line with recent field observations that suggest that hetero-aggregation is species specific (Long et al., 2017) and dependent on the polymer type (Lagarde et al., 2016).

4.1. Microplastics effect on microalgal growth

The results of this study showed that under our experimental conditions (12.5 mg L⁻¹ for the lower concentration and 125 mg L⁻¹

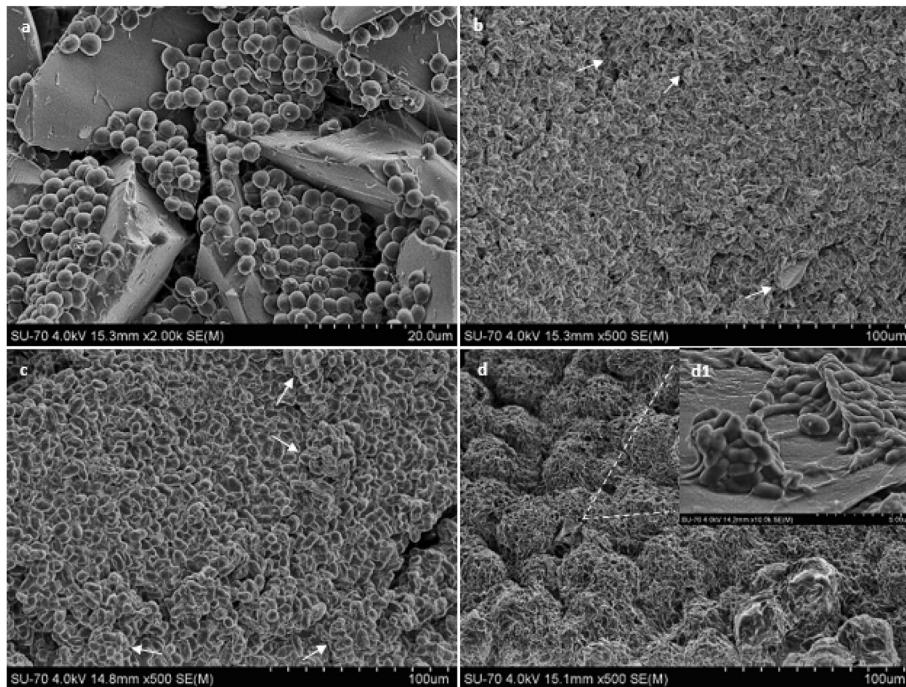


Fig. 6. Scanning electron microscopy (SEM) images of microalgae hetero-aggregates. (a) SEM image of microplastics colonized by *Microcystis panniformis* cells (x2000). (b) SEM image of *Scenedesmus* sp. cells, EPS and microplastics trapped in hetero-aggregates (arrows) (x500). (c) SEM image of *Tetraselmis* sp. cells forming homo-aggregates (arrows) (x500). (d) SEM image of *Gloeocapsa* sp. EPS fibrous mesh (x500). (d1) Amplified SEM image of a microplastic trapped on the surface of the EPS, in image (d), showing entanglement and colonization of the microplastics (x10000).

for the higher concentration), both *Microcystis panniformis* and *Scenedesmus* sp. cultures with microplastics included in culture medium, presented lower cell abundance when compared with the controls. These results could be explained by (i) the shading effect of the microplastics (reducing the access of microalgae to light), affecting the photosynthesis negatively (Sjollema et al., 2016), and/or (ii) the adsorption effect of the microplastics to the microalgae cells, which cause mobility reduction and consequently the decrease in microalgae growth (Davarpanah & Guilhermino, 2015). However, for *Microcystis panniformis* the higher depression in cell abundance was observed in the cultures with low microplastic concentration. The formation of micro-scale microplastic aggregates is an important factor when determining if the interactions and growth inhibition takes place (Bergami et al., 2017). Long et al. (2017) showed that microplastics tend to form micro-aggregates, which are larger than the microplastic alone. In this present work, the original size of the microplastics tested were 0–250 µm, but it was observed by microscopic analysis that larger particles were formed due to micro-aggregation, with smaller particles being more likely to interact and affect microalgae mechanisms. And therefore, these particles were more unlikely to adsorb onto smaller microalgae cells. Considering that microplastics in low concentrations tend to aggregate less, the adsorption to microalgae cells will be greater, reducing the mobility and consequently the microalgae growth. In the case of *Scenedesmus* sp., this would not affect its processes, since it is a non-moving microalga. For the marine *Tetraselmis* sp., no significant effect was observed for both the microplastics concentration condition. This resistance may be partially explained by the fact that *Tetraselmis* sp. presents a thick, rigid and complex cell wall (Domozych et al., 1981), that acts as an important barrier to microplastic interactions. Moreover, *Tetraselmis* sp. is a resistant species that exhibits an ease to grow even in unfavourable conditions (Fabregas et al., 1984; Michels et al., 2014).

Considering the obtained results, it can be inferred that the

interactions between microplastics and microalgae varies with microalga size and species characteristics, namely mobility and cell wall composition.

4.2. EPS influence and hetero-aggregates formation

All genus of microalgae used in this study are known to be EPS producers (Zhu et al., 2014; Li et al., 2015; Passow, 2002; Sharma et al., 2008). The freshwater microalgae *Microcystis panniformis* and *Scenedesmus* sp. produced a smaller quantity of EPS compared to the marine microalgae *Tetraselmis* sp. and *Gloeocapsa* sp.

Although for *Microcystis panniformis* there was a considerable production of EPS (0.047 ± 0.019 mg mL⁻¹), EPS were small, easily disaggregable and the abundance of hetero-aggregates was low. The smaller microplastics sized fraction (<106 µm) and the high density purple PMMA were the most commonly found, agreeing with the small size of the EPS observed. The apparent low EPS viscosity observed while handling *Microcystis panniformis* cultures may play a crucial role in explaining not only the low rate of microplastics aggregation but also why this microalga could not aggregate the low density small sized blue PS. SEM images of *Microcystis panniformis* (Fig. 6a) support the fact that this microalga is too small to aggregate microplastics much bigger than 25 µm, but still retains the ability to heavily colonize the microplastics, for this a reason microplastics could not be observed in Fig. 4a. Colonization of microplastics by microalgae leads to an increased plastic density (Lagarde et al., 2016). *Scenedesmus* sp. showed a good amount of EPS production (0.012 ± 0.002 mg mL⁻¹), with satisfactory characteristics for aggregation of smaller sized microplastics. Although the production of EPS from *Scenedesmus* sp. was lower than *Microcystis panniformis* EPS production, *Scenedesmus* sp. exhibited a greater abundance of overall aggregates. The fact that only the smaller sized fractions (<106 µm) of both types of microplastics were aggregated reinforces the above referred, about the

EPS produced by *Scenedesmus* sp., not being able to aggregate microplastics bigger than the size of the exopolymer produced. For this microalga, the type of microplastic is not a determining factor for aggregation, but rather for its size. SEM images of *Scenedesmus* sp. (Fig. 6b) confirmed the aggregation and adhesion of microplastics (arrows). This corroborates the macroscopic observations made in Fig. 4b, with visible hetero-aggregates at the bottom of the flasks. And therefore, indicates the higher stability of the EPS produced by *Scenedesmus* sp. in comparison with the EPS produced by *Microcystis panniformis*, evidenced by the abundance and size of its EPS. This aspect benefits the captation and consequent microplastics aggregation.

In the case of *Tetraselmis* sp., the production of EPS was higher than any of the freshwater microalgae (1.287 ± 0.090 mg mL $^{-1}$). *Tetraselmis* sp. exhibited a different type of EPS, with more favourable characteristics for the aggregation of smaller sized microplastics.

Consequently, this greater production embodies a higher microplastics aggregation, when compared with *Microcystis panniformis* and *Scenedesmus* sp. The way the microplastics are aggregated reveal a tendency for *Tetraselmis* sp. to aggregate, colonize and completely coat the microplastics that it cannot fully aggregate, contributing to its increase in density and consequent sinking, as shown in Figs. 5f and 6e. These observations also justify those made in Fig. 4c, with the lack of visible hetero-aggregates in the bottom of the flask.

Gloeocapsa sp. is a microalga different from the others under observation, due to its non-dissociable, abundant, dense, thick and viscous EPS. Of all microalgae studied, *Gloeocapsa* sp. proved to be the best microalga for both EPS production (2.146 ± 0.093 mg mL $^{-1}$) and microplastics aggregation. The results provided by the fluorescence (Fig. 5g and g1) and SEM (Fig. 6d and d1) micrographs reinforce the observations made in the culture flasks (Fig. 4d), that *Gloeocapsa* sp. has the capability to aggregate both high and low concentration and density microplastics. Furthermore, both the size fractions, contribute to an observable and pronounced vertical deposition of every type and size of microplastics that were studied.

According to the aggregation observed in the present study between microplastics and microalgae, we may advocate that this hetero-aggregation is species specific, depending on the size, strength, viscosity of the microalgal EPS produced. Since the EPS structural and chemical properties were not measured in this work, further studies are required to test the hypothesis that EPS structure and chemical properties (namely viscosity) can be correlated with hetero-aggregation. Results of the present study also showed that the concentrations of microplastics used did not influence the amount of hetero-aggregates formed. However, depending on the microalga species, microplastics concentration may affect growth as shown previously by other authors (see also Sjollema et al., 2016; Long et al., 2017; Lagarde et al., 2016), alongside the EPS production. Furthermore, the results highlight the potential for microalgae to be explored as biosolutions for the removal of microplastics in marine, freshwater or urban waters, due to its aggregation capabilities.

5. Conclusions

The aim of this study was to evaluate the relation between EPS and hetero-aggregation, and also characterize and differentiate the interactions between marine/freshwater microalgae and microplastics, with distinct sizes, shapes, densities and concentrations. And to explore them as possible biosolutions for the elimination/removal of microplastics from aquatic systems. The obtained results showed that:

- the effect of microplastics in microalgae growth varies with the microplastic size and microalga specie characteristics (cell wall and mobility);
- freshwater microalgae *Microcystis panniformis* and *Scenedesmus* sp., and marine microalgae *Tetraselmis* sp. and *Gloeocapsa* sp. have the potential to colonize microplastics and produce EPS capable of aggregating (adhering and incorporating) microplastics;
- there is a species-specific correlation between EPS yield, size and stability and the type/size of the microplastic when defining aggregation;
- in all microalgae studied, *Gloeocapsa* sp. proves to be the most suitable microalga regarding EPS production and consequent microplastics (of different types, densities and size fractions) aggregation;
- the microplastics aggregation may contribute to the vertical transport from the water surface to the sediment of microplastics with different characteristics.

The results of this work anticipate the application of EPS, produced by microalgae, in microplastic aggregation to water treatment.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.envpol.2019.03.046>.

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