

RESEARCH ARTICLE

## Effects of ABS microplastics on microalgae *Chlorella vulgaris* and *Raphidocelis subcapitata*

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**Abstract** – In recent years, there has been a growing interest in the impacts caused by the presence of microplastics (MP) in aquatic environments. The impacts of microalgae exposure to microplastics are still insufficiently investigated and further studies are needed to understand the possible outcomes. In addition, much of the literature has focused on the study of concentrations above those found naturally in the environment and in less toxic polymer matrices. Acrylonitrile-butadiene-styrene (ABS) plastics have a composition rich in additives and, so far, have been studied superficially. In the present study, two of the most commonly used green microalgae species in toxicity assays, *Chlorella vulgaris* and *Raphidocelis subcapitata*, were exposed to different concentrations of primary ABS-MP for a period of 6 days. Here, we observed physiological changes in cell growth and chlorophyll *a* content induced by the concentration and time of exposure to ABS-MP. The lowest concentration did not prove to be potentially toxic to cells, while the highest concentration was the most toxic. Primary consumers, such as microalgae, are essential for the proper functioning of entire ecosystems. Changes in these communities can lead to permanent damage to the communities of organisms at higher levels, so it is essential that their study be done carefully in the face of threats such as MP.

**Keywords:** Biological effects / ecosystem effects / plastic pollution / pollutants / toxicity

### 1 Introduction

The perception of the gravity of microplastics (MP) pollution is recent. Initially focused on marine environments, the studies have also investigated freshwater environments, such as lakes, rivers, and reservoirs (Buteler *et al.*, 2023; Cincinelli *et al.*, 2017; Luo *et al.*, 2019). Freshwater bodies close to human-occupied areas are more susceptible to MP pollution due to the release of domestic and industrial wastewater and inadequate disposal of large plastic materials (Xu *et al.*, 2020).

MP can remain in the water column and bottom sediment in the aquatic environment. Disponible in these compartments, plastic particles can interact and be ingested by organisms of different trophic levels (Ain Bhutto and You, 2022;

Garcia *et al.*, 2021; Krause *et al.*, 2021), such as microalgae (Chen *et al.*, 2020; Déniel *et al.*, 2020; Hazeem *et al.*, 2020), crustaceans (Canniff and Hoang, 2018; Portugal *et al.*, 2021), insects (Carrasco-Navarro *et al.*, 2021; Ribeiro-Brasil *et al.*, 2022), mollusks (Esterhuizen *et al.*, 2022; Hoellein *et al.*, 2021; Wang *et al.*, 2022), and fish (Abarghouei *et al.*, 2021; Batel *et al.*, 2020; Zhang *et al.*, 2021). However, algae represent a group superficially evaluated by studies that investigate the interaction of MPs with aquatic species.

Microalgae are organisms with extremely varied characteristics that can be found in almost all aquatic environments (Li *et al.*, 2022; Mutanda *et al.*, 2020). As primary producers, these organisms play a key ecological role in maintaining food chains. For this reason, these organisms have been widely used in toxicological tests. However, due to the great variability of algae characteristics, different responses can be observed according to species and pollutant (Song *et al.*, 2020).

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Many studies have reported the reduction in algae growth after exposure to different types of microplastics (MP), such as polystyrene (PS) (Casado *et al.*, 2013), polyvinyl chloride (PVC) (Fu *et al.*, 2019), polyacrylonitrile (PAN) (Lin *et al.*, 2020), polypropylene (PP) (Wu *et al.*, 2019), polyamide (PA) and polyethylene (PE) (Yang *et al.*, 2020). Other outcomes were also studied, such as oxidative stress, enzyme activity, photosynthetic activity and pigment production (Rani-Borges *et al.*, 2021). However, studies at environmentally relevant concentrations are still not common. As well as studies that consider more complex scenarios, such as the interaction with two or more pollutants, and more specific cellular impacts, such as the cellular internalization of particles, which according to Chen *et al.* (2020) can trigger negative effects, such as altering fertility and algal growth.

In the literature, significant effects have been observed in the different species of microalgae studied, although the results are not consistent in all studies. Many authors reported that no negative effects were found after the exposure period or even that population parameters benefited from the presence of MP in the medium, such as cell growth (Canniff and Hoang, 2018; Song *et al.*, 2020; Yokota *et al.*, 2017). Furthermore, according to the study by Song *et al.* (2020), although there were moderate changes in the chlorophyll *a* content, the differences were not statistically different from the control group. Overall, these mixed results suggest that the effects of microplastic exposure on microalgae are not yet well-established, and further research is needed to understand the underlying patterns of these interactions.

The study of less produced polymers, but with high toxic potential, has also been neglected. The use of copolymers by the industry has been increasingly frequent, since it is a way to obtain a final product with different characteristics and properties through the joining of two structurally distinct homopolymers (Tager, 1972). Acrylonitrile-butadiene-styrene (ABS) copolymer is a polymer widely used in building materials, electronics, automotive and transportation industries (Li *et al.*, 2017; Mao *et al.*, 2016; Scaffaro *et al.*, 2012). According to the risk assessment of polymers, ABS is highly toxic, mainly due to the presence of endocrine disruptors in the polymer matrix (Lithner *et al.*, 2011), which constitutes a major threat to aquatic biota. Furthermore, ABS microplastics (ABS-MP) have been detected in freshwater samples from different locations, indicating its wide environmental distribution. It has been found in drinking water treatment plants (Siegel *et al.* 2021), groundwater (Kim *et al.* 2023), surface water samples and sediment samples (Drabinski *et al.* 2023; Li *et al.* 2021), and internalized by species of insect larvae (Akindele *et al.* 2020; Ehlers *et al.* 2019).

Considering previous studies that show inconsistent results regarding the toxicity of MP to microalgae cells, the general objective of this study was to investigate the biological response of algae to a copolymer harmful to many species. The study of this interaction becomes a relevant source of ecological data, since algae play a fundamental role in the metabolism of the aquatic ecosystem. The species studied were *Chlorella vulgaris* and *Raphidocelis subcapitata*. Cultures were exposed for 6 days (chronic exposure) to three concentrations of ABS-MP and analyzed for growth inhibition and chlorophyll *a* content.

## 2 Material and methods

### 2.1 Microplastic characterization

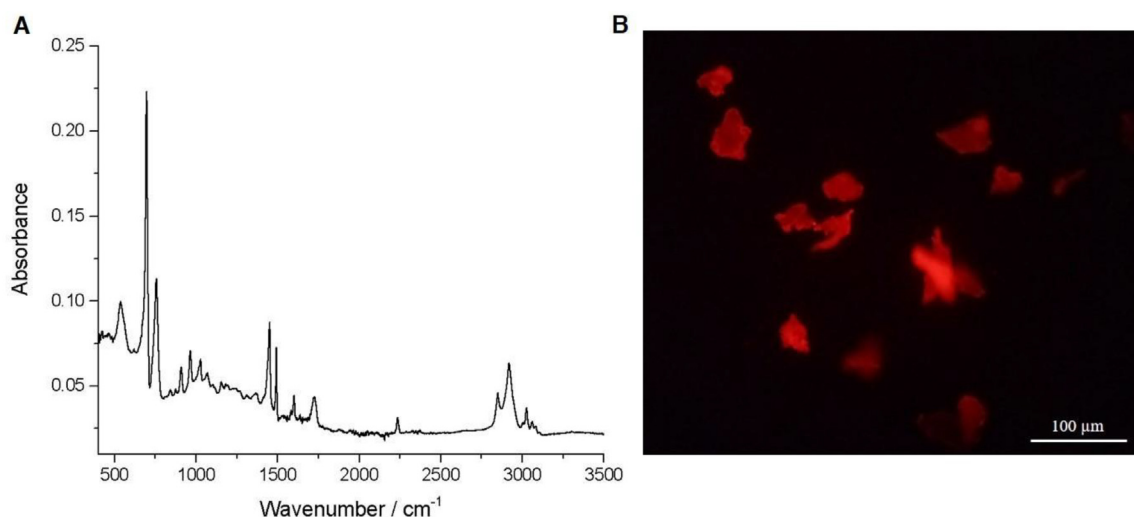
The MP used in the experiment was composed of primary ABS, with a size <100 µm, irregular shape and black color. The ABS sample was donated by a local industry. ABS was selected for this study due to its widespread use across various industrial sectors (Li *et al.*, 2017; Mao *et al.*, 2016; Scaffaro *et al.*, 2012), and it is a poorly studied polymer, despite its high toxicity, being considered one of the five most toxic polymers in the world (Lithner *et al.*, 2011). Therefore, it is important to carry out studies that can assess the responses of primary producers to this type of MP.

The polymeric composition of ABS-MP was confirmed using a micro-Fourier transform infrared spectroscopy (micro-FTIR). The FTIR spectra were measured in a Bruker spectrometer, Alpha model, in the region of 400–4000 cm<sup>-1</sup>, with standard KBr beamsplitter and high sensitivity DLATGS detector. The spectra were recorded with the ATR (Attenuated Total Reflection) module: ATR Platinum, equipped with a diamond crystal as a reflective element. The spectra were obtained with 128 accumulations and with a resolution of 2 cm<sup>-1</sup> (Fig. 1A).

The MP was acquired in the form of powder and subsequently subjected to cryolamination. For a better visualization of the particles and to confirm the irregular shape of the particles, ABS-MP was stained with Nile Red and observed under an inverted fluorescence microscope (Leica DMi8) (Fig. 1B). The confirm size range of the particles and to determine the MP concentrations used in the toxicity tests, the Zeiss Discovery V12 stereomicroscope was used to count the particles present in 30 µL of the MP solution. The samples were previously vacuum filtered and the count was performed in four repetitions and the mean and standard deviation were calculated. For every 30 µL of the MP solution we found 196 ± 11 items. The selection of concentrations was based on studies conducted with environmental samples of freshwater. As demonstrated in the extensive review by Koelmans *et al.* (2019), reported MP concentrations vary significantly between different locations, spanning ten orders of magnitude (ranging from 1 × 10<sup>-2</sup> to 10<sup>8</sup>/m<sup>3</sup>). Taking this into account, we used a minimum concentration of 384 items L<sup>-1</sup>. For the medium and higher concentrations, we employed 3840 and 38400 items L<sup>-1</sup>, respectively, which are 10 and 100 times higher, aiming to induce more pronounced physiological responses.

### 2.2 Microalgae species

The species studied were *Chlorella vulgaris* and *Raphidocelis subcapitata*, both green microalgae commonly used in toxicity assays. The common use of *Chlorella* is mainly due to the fact that this genus is considered ideal for commercial and biotechnological purposes, due to its easy reproduction in the presence of sunlight, carbon dioxide, water and small amounts of nutrients, facilitating its cultivation (Masojidek and Torzillo, 2008). Furthermore, the ability to accumulate high concentrations of carotenoids makes *Chlorella* sp. even more interesting for its use in biotechnology. Regarding its shape,



**Fig. 1.** Fluorescence image of acrylonitrile-butadiene-styrene microplastic (ABS-MP) confirming the irregular shape of the particles.

*Chlorella* has a spherical shape, however, as the cells grow, its structural shape changes.

*R. subcapitata* belongs to the unicellular freshwater Chlorophyceae class, known to be more susceptible to chemical compounds than other species, so it is recommended for ecotoxicological assays (OECD, 2011). Its cultivation does not require many requirements, its growth rate is quite high and its genome has already been sequenced, such characteristics make this species ideal to be applied in several studies (Almeida *et al.*, 2019).

### 2.3 Experimental design and analysis

The green microalgae *C. vulgaris* and *R. subcapitata* were cultivated in NPK culture medium ( $10\text{--}5\text{--}10\text{ g L}^{-1}$ ) in an incubator with continuous lighting (1200–1900 lux), with a photoperiod of 12:12 hours (light:dark) and temperature of  $21 \pm 2^\circ\text{C}$ . The experiment was started when the cultures were in the exponential growth phase. The experiments were carried out in glass Erlenmeyer flasks containing 500 mL of culture of both species. The test was performed over a period of 6 days. Each treatment and negative control were performed in triplicate ( $n=3$ ,  $N=12$ ). Cultivation conditions were maintained during the experimental period, with the exception of aeration.

ABS-MP were added directly to the culture medium. The concentrations tested were Concentration 1:  $384\text{ items L}^{-1}$ ; Concentration 2:  $3840\text{ items L}^{-1}$  and Concentration 3:  $38,400\text{ items L}^{-1}$ .

The toxicity of MP exposure was measured by analyzing cell growth and chlorophyll *a* concentration (Strickland and Parsons, 1972). Analyzes of growth and pigment content were performed on the initial (day 0) and final (day 6) days of exposure. The growth of the algal culture was calculated by counting cells in a Neubauer chamber under light microscopy with the aid of a manual counter. For pigment analysis, 50 mL of the culture was vacuum filtered through glass fiber filters (Millipore 47 mm with a pore size of  $0.7\text{ }\mu\text{m}$ ). The filters were placed in 15 mL tubes and frozen at  $-20^\circ\text{C}$  protected from light until processing. 24 hours before the procedure, 5 mL of

DMSO + 90% acetone solution was added to each tube and stored at  $4^\circ\text{C}$ . After this period, the samples were centrifuged (3500 rpm,  $4^\circ\text{C}$  for 10 min) and the supernatant was transferred to a 5 mL cuvette for reading in a spectrophotometer (Femto, Cirrus 80) at 433, 480, 510, 647, 667 and 750 wave length.

The results of algae growth and chlorophyll *a* content were displayed as a percentage (%) calculated from the results obtained from the results of control group at each sampling time (days 0 and 6).

### 2.4 Statistical analysis

To determine whether there were significant differences between treatments (different concentrations of MP) and the control group, and between treatments according to exposure time, Dunnett's and Fisher's exact statistical tests were used, respectively, in addition to descriptive analyzes (mean and relative standard deviation). All analyzes were performed in triplicate and statistical significance was accepted at  $p < 0.05$  level. The software used was Minitab v.14.

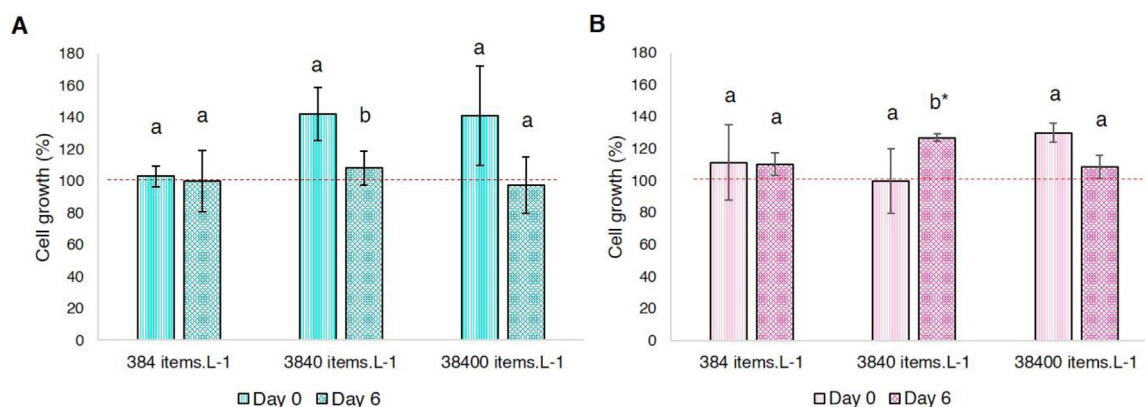
## 3 Results and discussion

### 3.1 Growth inhibition

After 6 days of exposure to ABS-MP, samples of *C. vulgaris* and *R. subcapitata* were analyzed to assess the effects of this pollutant on cell growth.

The growth of *C. vulgaris* (Fig. 2A) remained stable at all concentrations tested, when compared to the control group (Dunnett's test,  $p < 0.05$ ). At Concentration 2 ( $3840\text{ items L}^{-1}$ ), growth was inhibited at the end of the 6-day exposure period (Fisher test,  $p < 0.05$ ). In treatments with *R. subcapitata* (Fig. 2B), cell growth was observed in relation to the control group only at Concentration 2 ( $3840\text{ items L}^{-1}$ ) after 6 days of exposure (Dunnett test,  $p < 0.05$ ), which also differed in relation to the first timepoint (Day 0) (Fisher test,  $p < 0.05$ ).

The stability in cell number observed for both species in the treatment under Concentration 1, shows that ABS-MP in



**Fig. 2.** Cell growth (%), normalized to the negative control group (100%, represented by the red dashed line), and relative standard deviation of days 0 (initial) and 6 (final) of exposure to acrylonitrile-butadiene-styrene microplastics (ABS-MP) at three concentrations: 384, 3840 and 38,400 items L<sup>-1</sup>. (A) *Chlorella vulgaris* (in green). (B) *Raphidocelis subcapitata* (in pink). Equal letters indicate no significant difference and different letters indicate a significant difference between the groups at different exposure times (days 0 and 6) (Fisher's exact test). The asterisk symbol (\*) represents a statistically significant difference between the treatments and the control group (Dunnett's test). *p* value considered significant *p* < 0.05.

this amount of particles (384 items L<sup>-1</sup>), did not show toxicity at the population level. Lagarde *et al.* (2016) reported in their 78-day study that the culture of *Chlamydomonas reinhardtii* did not undergo any change in its growth pattern before 63 days of exposure to polyethylene and to polypropylene MP. Despite this, the concentration used in this study was much higher than that used in the present work, therefore, it is not possible to infer whether in a long exposure the results would be different. Casado *et al.* (2013) used *R. subcapitata* with realistic concentrations and observed a reduction in growth, however, the physical characteristics of the MP used were quite different from the ABS-MP used here, being ~11 µm. Smaller particles are known to generally have greater toxicity. All studies found in the literature with *C. vulgaris* used concentrations considered high (>10 mg L<sup>-1</sup>), making comparison with our results impossible (Fu *et al.*, 2019; Hazeem *et al.*, 2020; Sjollema *et al.*, 2016; Song *et al.*, 2020).

In treatment with *C. vulgaris* under Concentration 2, cells were significantly inhibited when compared with day 0 (*p* < 0.05). This result can be attributed to a combination of factors that induce different responses in cells, such as the shading effect, increased turbidity of the medium, cell internalization process and adhesion of MP to the cell wall, which inhibit cell growth (Lagarde *et al.*, 2016). After these processes, cells can undergo a period of adaptation and self-regulation, returning to the state of exponential growth (Rani-Borges *et al.*, 2021). To observe this event, studies with longer exposure times should be performed. Furthermore, the toxic potential of ABS comes mainly from the release of additives present in its polymer matrix (Lithner *et al.*, 2011). Therefore, it is to be expected that the exposure time is a determining factor for the increase in the toxicity of this material.

On the other hand, *R. subcapitata* showed growth at Concentration 2, both compared with Day 0 and control treatment (*p* < 0.05). This phenomenon was also observed by Canniff and Hoang (2018) with the same species exposed to polyethylene (PE) MP with a size range within the one used here (63–75 µm). It is suggested that this event occurs due to the surface of MP that can serve as a substrate for the adhesion

and growth of microorganisms, which favors the increase of algae concentration (Canniff and Hoang, 2018; Song *et al.*, 2020; Yokota *et al.*, 2017).

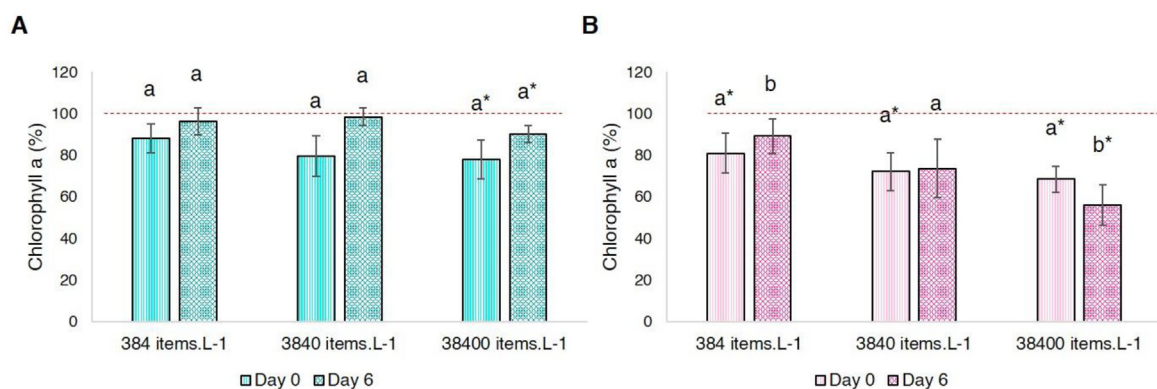
It is noteworthy that the size of MP particles can be an important factor of toxicity. The microalgae *Skeletonema costatum*, when exposed to PVC particles of 1 µm, in concentrations between 20 and 200 mg L<sup>-1</sup>, had its cell growth significantly inhibited. The same does not occur when exposed to 1 mm PVC particles (Zhang *et al.*, 2017). The growth of the microalgae *Dunaliella tertiolecta* also seems to be affected by particle size. MP-PE caused a more significant inhibitory effect with smaller particle sizes (Sjollema *et al.*, 2016). The fact that smaller particles can be more toxic is very worrying, especially if we consider that, as the plastic material remains in the environment, smaller and smaller particles are formed and, consequently, can increase the polluting potential of these contaminants.

Overall, the rate of algal growth inhibition is one of the most used parameters in laboratory ecotoxicological studies using algal cultures. These experiments allow us to suggest the risks of a xenobiotic on an algae population and, consequently, to estimate the impact of this contaminant on the entire ecosystem, since algae constitute a group of extreme importance for the maintenance of balance in aquatic environments.

### 3.2 Chlorophyll a

Chlorophyll *a* is a photosynthetic pigment located in chloroplast membranes that absorbs light energy to be used in photosynthesis, a fundamental process for the biological activities of microalgae. Chlorophyll *a* content was measured at the beginning of the exposure period (day 0) and at the end (day 6) in order to evaluate the effects of ABS-MP on the production of this pigment.

The species *C. vulgaris* did not show significant differences (Fisher test, *p* < 0.05) in any of the concentrations tested between days 0 and 6 of exposure. However, the culture



**Fig. 3.** Chlorophyll *a* content (%), normalized with the negative control group (100%, represented by the red dashed line), and relative standard deviation of days 0 and 6 of exposure to acrylonitrile-butadiene-styrene microplastics (ABS-MP) at three concentrations: 384, 3840 and 38400 items. L<sup>-1</sup>. (A) *Chlorella vulgaris* (in green) (B) *Raphidocelis subcapitata* (in pink). Equal letters indicate no significant difference and different letters indicate a significant difference between the groups at different exposure times (days 0 and 6) (Fisher's exact test). The asterisk symbol (\*) represents a statistically significant difference between the treatments and the control group (Dunnett's test). *p* value considered significant *p* < 0.05.

exposed to Concentration 3 was statistically different from the control group (Dunnett's test, *p* < 0.05) at both evaluated times (Fig. 3A). On the other hand, *R. subcapitata* showed an increase and decrease in chlorophyll *a* content under Concentrations 1 and 3, respectively (Fisher test, *p* < 0.05), after 6 days of exposure (Fig. 3B). When compared to the control group, *R. subcapitata* showed a significant reduction at the beginning of the experiment (day 0) at all concentrations tested, and at day 6 at the highest concentration (38,400 items L<sup>-1</sup>) (Dunnett test, *p* < 0.05).

In the experiments with *C. vulgaris* there was no significant difference over the 6-day exposure period (Fisher test, *p* < 0.05). With the exception of Concentration 3, which had production inhibited, the groups exposed to Concentrations 1 and 2 did not differ from the control (Dunnett test, *p* < 0.05). Song *et al.* (2020) reported a similar result in a study with *Chlorella* sp., after tests under different experimental conditions (concentration of 200 mg L<sup>-1</sup> for 96 h) and MP consisting of other polymers (PE, PET, PP and PVC; with a size of 74 μm). On the contrary, Tunali *et al.* (2020) demonstrated a reduction in chlorophyll production by *C. vulgaris* after exposure to polystyrene MP (0.5 μm) and that the reduction was accentuated as the concentration increased. Thus, with these results it is possible to suggest that the physiological responses of pigment production will be intrinsic to the physical and chemical characteristics of the particles to which the alga will be exposed as well as the species itself and the particle size. All these parameters may be a fundamental factor for the understanding of the toxic effects of MP on algae.

Concerning *R. subcapitata*, curiously, under Concentration 1 shows a significant increase in the production of chlorophyll *a* (Fig. 3B), even though there was no cell growth in this same treatment (Fig. 2B). At the same time, under Concentration 2, no increase in chlorophyll *a* production was observed, even though there was an increase in cell growth (Fig. 2B). This suggests that the lower concentration may not have caused damage at the physiological level, while under 3840 items L<sup>-1</sup> the photosynthetic apparatus suffered some damage, even with the increase in population. These conclusions should be taken

into account with caution, as there was a reduction in chlorophyll *a* content at all concentrations tested on day 0 compared to the control group (equivalent to 100% chlorophyll content).

The greatest vulnerability of *R. subcapitata* to the presence of ABS-MP occurred in the exposure of ABS-MP to 38,400 items L<sup>-1</sup>, with a reduction of pigment production of ~12% in 6 days of experiment. Although lower concentrations than those normally found in the literature were used, the results demonstrate the same pattern observed in other studies, where the rate of chlorophyll inhibition was dose-dependent (Li *et al.*, 2020; Tunali *et al.*, 2020; Wang *et al.*, 2020; Wu *et al.*, 2019). However, in the present study, this effect was observed only for *R. subcapitata*.

### 3.3 Environmental implications of the interaction between MP and microalgae

The effects observed through experiments with *C. vulgaris* and *R. subcapitata* could potentially have ecosystem implications. However, it is difficult to predict how these effects might manifest under real conditions in freshwater environments due to the complexity of these ecosystems. Microalgae represent a key group for maintaining ecological balance in aquatic environments (Wu *et al.*, 2017). The changes caused by increasing levels of microplastic pollution can not only affect this community, but also negatively impact the ecosystem as a whole (Nava and Leoni, 2021). For example, the association of microalgae and MP can change its palatability, detectability and make it difficult for herbivores to manage (Yokota *et al.*, 2017). On the other hand, the colonization of plastic particles by microalgae may make them more attractive to herbivores, resulting in higher rates of ingestion by these organisms. Thus, it is expected that zooplanktonic species may be affected by this association. The concern is not only with the direct impacts caused by MP ingestion, as already reported in the literature (Cole *et al.*, 2013; Jemec *et al.*, 2016; Jeong *et al.*, 2016), but also with the ability of these particles to adsorb other contaminants on its

surface (Caruso, 2019; Wang *et al.*, 2021). In this case, herbivores could be more susceptible to the effects caused by these aquatic pollutants and the combined effects of this association.

Considering the importance of the phytoplankton community in aquatic ecosystems and considering that any negative effect on the algal community can disturb the balance of an entire ecosystem, interactions between MP and microalgae can jeopardize the quality of aquatic environments. As is known, phytoplankton can be impacted by these particles, which can cause several adverse effects as a result of exposure, ranging from growth inhibition, induction of oxidative stress and even decreased photosynthetic efficiency (Wan *et al.*, 2018). The effect of MP on microalgae is not limited to their populations. According to Wang *et al.* (2019), exposure to plastic particles can induce a variety of adverse effects on aquatic biota, from primary producers to higher levels of the food chain. Therefore, the phytoplankton community is an important model for the assessment of the aquatic environment, whether marine or continental, mainly because these organisms respond directly to environmental changes, whether seasonal or punctual. Thus, the assessment of this community in aquatic ecosystems is important for environmental monitoring, aiming at the maintenance of ecological processes, aquatic communities, economic and public health conditions (Käse and Geuer, 2018).

## 4 Conclusions

As they are organisms that form the basis of the food chain of these ecosystems, the impacts of MP on algae can reflect on the flow of energy in aquatic food webs and on the dynamics of an entire aquatic ecosystem. Microalgae have ecological and economic relevance; therefore, it is necessary to understand the processes and mechanisms involved after periods of exposure to MP and how these particles can affect them.

This preliminary study presents evidence that ABS-MP can negatively affect microalgae growth and pigment content. The results showed that there are differences between the biological responses depending on the species and the concentrations to which they are exposed. While *C. vulgaris* seems not to be affected by the presence of ABS-MP, with regard to chlorophyll production, the same cannot be said about *R. subcapitata*, as it was observed that the higher the concentration of these particles, the more physiological damage to the cells of these algae can be observed. No other study with this class of organisms and this type of MP polymer was found in the literature, making this an unprecedented study with preliminary results on this interaction.

The use of MP composed of polymers rich in additives and with high toxicity in laboratory tests, as in the case of ABS used in the present study, is fundamental to evaluate the environmental responses to these pollutants. Here, we highlight a large knowledge gap in relation to ABS studies, not only in microalgae, but in the entire MP study area.

## Authors contributions

Bárbara Rani-Borges: Conceptualization, Methodology, Formal analysis, Data curation, Investigation, Visualization,

Writing – original draft, Writing – review and editing, Funding acquisition. Lucas G. Queiroz: Conceptualization, Methodology, Formal analysis, Data curation, Investigation, Visualization, Writing – original draft, Writing – review and editing. Karen S. Ferreira: Writing – original draft, Writing – review and editing. Thaís F.G. Martins: Writing – original draft, Writing – review and editing. Rômulo A. Ando: Formal analysis, funding acquisition. Marcelo Pompêo: Supervision, Writing – review and editing, Funding acquisition.

## Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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