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Microplastics reduce microalgal biomass by decreasing single-cell weight: The barrier towards implementation at scale

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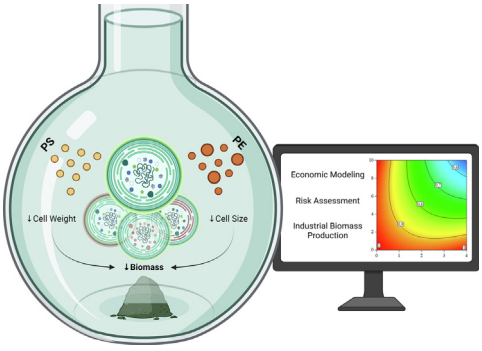
HIGHLIGHTS

- Research on industrial microalgae exposure to microplastics is scarce.
- Microplastics affect significatively cell density and biomass production.
- Computational modelling evaluates systematic risks of MPs' exposure to microalgae.
- Biological factors were identified for those changes and their economic implications.

GRAPHICAL ABSTRACT



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ABSTRACT

Microplastics (MPs) are a widespread environmental threat, especially to aquatic and urban systems. Water quality is vital for biomass production in microalgal-based industries. Here, industrially relevant microalgae *Tetraselmis suecica*, *Scenedesmus armatus*, and *Nannochloropsis gaditana* were exposed to PS- and PE-MPs (polystyrene and polyethylene, respectively – 10-20 μm) contaminated waters (5 and 10 mg/L). Following industrial empirical and ecotoxicological procedures, the production period was established as four days (exponential growth phase). 27-long day experiments were conducted to determine the chronic effects of MPs contamination in microalgal biomass yields. MPs induced different responses in cell density: *T. suecica* decreased (up to 11 %); *S. armatus* showed no changes; and *N. gaditana* increased (up to 6 %). However, all three microalgae exhibited significant decreases in biomass production (up to 24, 48, and 52 %, respectively). *S. armatus* exposed to PS-MPs and *N. gaditana* exposed to PE-MPs were the most impacted regarding biomass production. The decrease in biomass yield was due to the reduction in single-cell weight (up to 14, 47, and 43 %), and/or the production of smaller-sized cells (*T. suecica*). In response to chronic exposure, microalgae showed signs of cell density adaptation. Despite cell density normalizing, biomass production was still reduced compared to biomass production in clean water. Computational modelling highlighted that MPs exposure had a concentration-dependent negative impact on microalgae biomass. The models allow the evaluation of the systematic risks that MPs impose in microalgal-based industries and stimulate actions towards implementing systems to contain/eliminate MPs contamination in the waters used in microalgae production.

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1. Introduction

Microplastics (MPs) are tiny particles of plastic that are smaller than 5 mm in size (NOAA, 2020). They can be generated from a variety of sources, including consumer products and the breakdown of larger plastic items. MPs are a growing concern because of their reported harmful effects on both terrestrial and aquatic organisms (Silva et al., 2018). MPs can be classified into two main categories: primary and secondary. Primary MPs are those that are intentionally produced and used in products, such as microbeads in personal care products. Secondary MPs are those that are generated through the breakdown of larger plastic items, such as plastic bottles or bags (Galloway et al., 2017).

Several studies have found the presence of MPs in waste-, fresh-, drinking- (Koelmans et al., 2019), bottled- (Oßmann et al., 2018) and tap-water (Pivokonsky et al., 2018). Regrettably, there is still no legal framework for the number of MPs that can be found in drinking water and no specific technology to remove them (Novotna et al., 2019; Wang et al., 2020). To tackle the problem of MPs, it is important to reduce their production and use, as well as implement effective waste management and clean-up strategies. One prime example are wastewater treatment plants (WWTPs). These segregating infrastructures have been identified as focal points of MPs release into freshwater and marine environments (Liu et al., 2021; Novotna et al., 2019; Sun et al., 2019). Despite recent years unveiling powerful microalgal- and bacterial-based biotechnological solutions (Cunha et al., 2020b, 2019; Faria et al., 2022) these approaches require extensive scaling and awareness to make the transition from the bench to the industry. Hence, new questions arise as MPs become ubiquitous across multiple natural and urban domains.

Globally, a lot of work has been developed in characterizing and understanding the extent and nuances of MPs pollution, from environmental occurrence and persistence to their effects on living organisms. However, few have focused on understanding the industrial disruption and economic impact of such widespread pollution. Particularly, the microalgal biomass industry has held a lot of promise over the last decade, but these multifaceted biochemical factories have not been able to break the economic viability barrier.

Microalgae are a diverse group of microorganisms that can be found in a wide range of environments, including freshwater, marine, and terrestrial ecosystems. They are an important part of the global ecosystem, as they play a vital role in the carbon and nitrogen cycles and are a primary producer of oxygen on Earth (Rizwan et al., 2018). Microalgal biomass represents a natural and net-positive renewable source of a wide range of high-value bioactive products, such as carbohydrates (polymers, flocculants), antioxidant photosynthetic pigments (chlorophylls, carotenes, phycobiliproteins), vitamins (A, B1, B2, B6, B12, C, E), minerals (calcium, magnesium, potassium, iron, iodine), and proteins (Koyande et al., 2019; Sathasivam et al., 2019). One of the main advantages of microalgae as a source of bioactive compounds is that they can be grown in a variety of environments, including in open ponds or closed photobioreactors, using non-arable land and saltwater (Rizwan et al., 2018). This makes them an attractive alternative to traditional crops, which can be more resource-intensive to grow.

The production of microalgae biomass has gained increasing attention in recent years due to the potential of microalgae as a sustainable and renewable source for use in a wide range of applications. These include feedstock for biofuels, as well as in the food, aquaculture, wastewater treatment, pharmaceutical, nutraceutical, cosmetics, and personal care industries (Camacho et al., 2019; Tang et al., 2020). On the commercial side, the market price of algal biomass can vary significantly depending on several factors, including the microalgae species, the specific bioactive compounds it contains, the intended application, the formulation, and the associated production costs (Alam et al., 2020; Vieira, 2016). Prices can range from a few US dollars per kilogram for biofuel feedstocks to several hundred US dollars per kilogram for human consumption products (Sathasivam et al., 2019). The demand for microalgal-based bioproducts is expected to continue to growing in the coming years, driven by the increasing demand for sustainable and renewable alternatives to traditional products.

From a research perspective, several studies have focused on understanding the effects of MPs in fundamental biological and biochemical aspects such as microalgal growth, photosynthetic activity, metabolite content, and cell morphology (Larue et al., 2021). However, few have focused on assessing how MPs affect microalgae biomass. To our knowledge, only one study has thoroughly investigated how MPs deplete biomass and most of its bioactive compounds (Cunha et al., 2020a). That study reported that exposing *Phaeodactylum tricornutum* to concentrations as low as 0.5 mg/L of polystyrene (PS) and polymethyl methacrylate (PMMA) led to exorbitant reductions of up to 82 % in biomass yields while cell density remained constant throughout. Therefore, there is an interest in evaluating these effects in microalgae relevant to the biomass industry and understanding the biological mechanisms underlying the severe reduction in biomass yields, considering that the seemingly intuitive linear relationship between cell density and biomass yield was not observed.

Tetraselmis suecica (marine), *Scenedesmus armatus* (freshwater), and *Nannochloropsis gaditana* (marine; currently regarded as a synonym of *Microchloropsis gaditana* (Guiry, 2015)) present high industrial and economic potential. *Tetraselmis*, is rich in vitamin E, carotenoids, chlorophyll, and tocopherols (Sansone et al., 2017) and is well known to be sourced in pharmaceutical/nutritional industries, as well as for feeds in aquaculture (Sathasivam et al., 2019). *Scenedesmus* is a protein-rich microalga and a source of mono-unsaturated, polyunsaturated, and saturated fatty acids in animal and fish feed (Yukesh Kannah et al., 2021). *Nannochloropsis* can synthesise high-quality pigments and accumulate high concentrations of saturated fatty acids (Yukesh Kannah et al., 2021) which is also often used in aquaculture and pharmaceutical/nutritional industries (Cuellar-Bermudez et al., 2015; García et al., 2017; Rocha et al., 2003).

As microalgae are a critical part of the global ecosystem, understanding the impacts of microplastics on their growth and productivity is important for understanding the potential cascading effects on the entire ecosystem. Additionally, as microalgae are a valuable source of bioactive compounds and are used in a wide range of industries, understanding the impacts of microplastics on their growth and biomass production is essential for the sustainability and viability of these industries. The present research aims to investigate the impact on the biomass production of *Tetraselmis suecica*, *Scenedesmus armatus*, and *Nannochloropsis gaditana,* identifying the cellular impact driving those changes. Given the timeframes for biomass generation at scale, the acute (4-day experiment) and chronic (27-day experiment) effects of exposure to both polystyrene (PS)- and polyethene (PE)-MPs (5 and 10 mg/L) were determined. To simulate empirical industrial practices and ecotoxicological procedures, the production period started in the exponential growth phase (four days preceding the stationary phase). For all three microalgae, growth, biomass production, and single-cell weight paths were evaluated in parallel. Further, a laboratory scale-up was performed to ensure the reproducibility of the data. The results of this study are important for understanding the impacts of MPs on commercial microalgae and the potential implications for microalgal-based industries' economies of scale.

2. Materials and methods

2.1. Microalgae selection and culture conditions

For this study, one freshwater species *Scenedesmus armatus* (Class Chlorophyceae), and two marine microalgae *Tetraselmis suecica* (Class Chlorodendrophyceae) and *Nannochloropsis gaditana* (Class Eustigmatophyceae) were selected and obtained from the Spanish Algae Bank (BEA) collection (BEA 1402B, REC 0033B, REC 0099B, respectively). Growth culture media (Waris-H for *S. armatus*, Asp-12 for *T. suecica,* and *N. gaditana*) are described in Supplementary data S1.

The cultures used in the experiments were maintained for the production period at a temperature of 20 ± 1 °C, under the irradiance of

8.262 μmol/m^2/s (HOBO® Pendant® MX Temp MX2201; this sensor measures light in lux, therefore a conversion factor of 0.0135 was applied to obtain photon flux) supplied by a cool white Osram L 18 W 840 Lumilux lamp, with a 14/10 h (light/dark) photoperiod (Aralab CP500 growth chamber). During the production period, the growth was monitored using a spectrophotometer at 750 nm (UV-6300PC Double BeamSpectrophotometer). The samples were placed in 1.5 mL plastic cuvettes (1 mL of sample was added) and measured (with the culture medium as the control). For this, the cell number in the medium was measured using a Neubauer improved chamber. A calibration curve plotting cell density (CD – cell number/mL) against absorbance was used to determine the cell density of each microalga (Fernandes et al., 2020):

$$CD = 19.464 \times OD_{750} - 2.876. \quad (R^2 = 0.9909)\ \textit{Tetraselmis suecica} \quad (1)$$

$$CD = 27.432 \times OD_{750} - 1.4364. \quad (R^2 = 0.9826)\ \textit{Scenedesmus armatus} \quad (2)$$

$$CD = 70.216 \times OD_{750} - 3.7285. \quad (R^2 = 0.9989)\ \textit{Nannochloropsis gaditana} \quad (3)$$

2.2. Microplastics

Spherical polystyrene microplastics (PS-MPs) with a diameter of 10 μm were purchased from Thermo Scientific™ (G1000) as a 1 % (*w/v*) suspension with excitation and emission wavelengths of 468 and 508 nm, respectively. Spherical polyethylene microplastics (PE-MPs) with a diameter of 10–20 μm were purchased from Cospheric LLC™ (UVMS-BG-1.025) as a dry powder form, with excitation and emission wavelengths of 414 and 515 nm, respectively. A suspension of 1 % (w/v) was prepared. This study used concentrations of 5 and 10 mg/L of MPs, as they are two of the most common concentrations used in these types of studies, based on the concentrations found in the environment (Chen et al., 2020; Mao et al., 2018; Sun et al., 2021; Tunali et al., 2020; Zhang et al., 2017; Zhao et al., 2019; Zhu et al., 2020). Both solutions were prepared in glass flasks to reduce the establishment of electrostatic interactions with their walls and stored at 4 °C until further use.

2.3. Exposure conditions

To determine the acute and chronic effects in microalgae, these were grown in PS- and PE-MPs contaminated waters, ensuring exposition for the entire duration of the 4- and 27-day-long experiments. To mimic industrial procedures and the Organization for Economic Cooperation and Development (OECD) ecotoxicological guidelines (OECD, 2011) as accurately as possible, the production period started in the exponential growth phase (four days preceding the stationary phase). By day 27, the microalgae naturally had entered the stationary phase. The cultures were not renewed and allowed to grow with aeration to ensure complete contact between the MPs and the microalgal cells.

At the end of the production period, the biomass was recovered by centrifugation (4430 ×*g*, 10 min) (HERLMEZ 360 Centrifuge) and gravimetrically determined (as dry weight - d.w.) after freeze-drying (Savant RVT400).

2.4. Flow cytometry

Flow cytometry analyses were performed using a CytoFLEX instrument (Beckman Coulter) with a blue laser (excitation radiation of 488 nm). The samples were characterised according to “forward scatter” (FSC) – curve area. The CytExpert software (Beckman Coulter) was used to analyse the output data. Note that flow cytometry was not discriminative of algal and microplastic particles. However, given the low concentrations of the added MPs, their contribution to the responses was negligible against the high microalgal cell density. The parameters selected for the measurements were a FSC of 226, a volume of 20 μL, and a medium flow rate of 30 μL/min (all the measurements were done in triplicate).

2.5. Response surface methodology (RSM)

RSM modelling (Supplementary data S2) was performed with Design Expert software version 13 (Stat-Ease, Inc.) and generated a three-level factorial design with two variables (Tables S1-S3). The range of variables included the concentration of MPs (0–10 mg/L) and time of exposure (0–4 days). The biomass and cell density were measured as the responses. Both responses and the ratio biomass production/cell density, with posterior normalisations (contaminated water / clean water; of the same day), were implemented into the model. This ratio allowed to determine the direct relationship between growth and biomass. For each, the Design Expert provided the equations that can be applied to determine the respective response in the function of the variables. The fitting of RSM mathematical models towards the responses (*measured/experimental* cell density and biomass production and *calculated/predicted* biomass production/cell density ratio) were investigated using Design Expert software (Supplementary data S2; Table S1-S3) (Mendonça et al., 2022). The validity of the model was assessed through the analysis of variance (ANOVA), the comparison (R^2), and by calculating the error percentage (Eq. 4) between the experimental and predicted values.

$$\text{Error}\ (\%) = \frac{\text{experimental} - \text{predicted}}{\text{experimental}} \times 100 \quad (4)$$

2.6. Statistics

Data representation and statistics of the data outside the Design Expert software, already described, were performed using GraphPad Prism 9 (GraphPad Software). The D'Agostino-Pearson omnibus and Kolmogorov-Smirnov normality tests were used to assess the Gaussian data distribution. Parametric unpaired *t*-tests (or one-way ANOVA) were applied for normally distributed data, while non-parametric unpaired Mann-Whitney (or Kruskal-Wallis) tests were applied for non-Gaussian distributed data (statistical significance: p-value <0.05). Statistical analysis was performed in at least three independent experiments.

3. Results and discussion

3.1. Growth curves

The growth curve for each microalga (*T. suecica*, *S. armatus*, and *N. gaditana*) under clean water and MPs-exposed conditions are shown in Fig. 1, until all species reached the stationary phase. Throughout each growth phase, microalgae cells undergo several changes, which vary according to the strain, leading to different growth rates (Aziz et al., 2020; Machado et al., 2016). The growth rate of each microalga is, therefore, species-specific (Krzemińska et al., 2014; Vello et al., 2018). In the current study, both *T. suecica* and *N. gaditana* both achieved a steady-state cell density that was somewhat higher (36 % and 20 %, respectively) than that found by Ulloa et al. (2012) and Fernandes et al., 2020, respectively. The different conditions to which the cultures were exposed may account for these differences. Lastly, *S. armatus* presented an algal density comparable (with a difference of 1 %) to that of the previously obtained by Czarny et al. (2019).

To follow the empirical industrial procedures and the OECD ecotoxicological guidelines (OECD, 2011), the start of microalgal growth for biomass harvest was performed in the exponential phase of growth (four days before reaching the stationary phase). Thus, based on the growth curves, this cell density was determined and posteriorly used in acute and chronic experiments. The 4th day of growth before the stationary phase corresponded to a cell density of (4.76 ± 0.07) × 10^6 cells/mL for *T. suecica* (day 19),

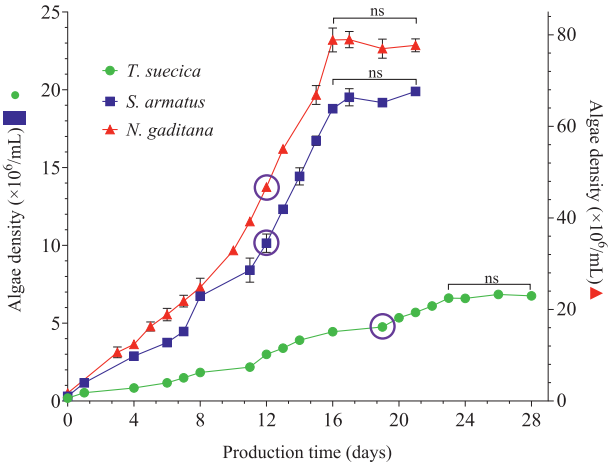

Fig. 1. Growth curves for *T. suecica*, *S. armatus*, and *N. gaditana*. *ns* - represents no significant difference among days (*p*-value >0.05) – indicator of the stationary phase. Highlighted in a circle are the densities corresponding to four days before reaching the stationary phase, used in the acute and chronic experiments.

$(10.14 \pm 0.59) \times 10^6$ cells/mL for *S. armatus* (day 12), and $(46.73 \pm 0.73) \times 10^6$ cells/mL for *N. gaditana* (day 12).

3.2. Microalgal density and biomass production

3.2.1. Acute effects of MPs exposure

To evaluate the acute effects of microalgal exposure to MPs, *T. suecica*, *S. armatus,* and *N. gaditana* were exposed to waters contaminated with PS- and PE-MPs for four days (Table S1). The cell density and biomass production of each microalga exposed to different conditions (clean water and contaminated water with 5 and 10 mg/L PS- and PE-MPs) are shown in Fig. 2 (and Supplementary data Tables S6 and S7). The exposure to MPs revealed a species-specific outcome regarding cell abundance and biomass productivity. Independently of inter-microalgae differences in the cell abundance to biomass productivity ratio, the results in Fig. 2 show that biomass production was significantly lower (12–52 %) across all microalgae (*T. suecica*, *S. armatus*, and *N. gaditana*), all MPs (PE and PS), and concentrations (5 and 10 mg/L) tested compared to the microalgae grown in clean water.

T. suecica grew rapidly when exposed to different concentrations of PS- and PE-MPs, in a consistent upward trend (Fig. 2A). However, the cultures exposed to MPs-contaminated waters grew at a slower rate than the microalgae grown in clean water. After 2 days, a significant growth suppression of around 4 % was observed when *T. suecica* was exposed to both concentrations of PS-MPs (compared to the clean water). This suppression continued until day 4 (decrease of 8–10 %), still independent of the concentration of PS-MPs (Fig. 2A, Table S1). When grown with PE-MPs, cell density was only inhibited by day 4 compared to clean water. Overall, the decrease in cell growth might be explained by hetero-aggregation between the MPs particles and microalgae that may cause damage to the cell walls or membranes.

This could also be because MP particles might promote shading effects, block membrane pores by external adhesion, or prevent gas exchanges, negatively interfering with photosynthesis and, therefore, growth. The results of this study are in accordance with Chen et al. (2020), in which 10 mg/L of 1–2 μm sized PS-MPs had significant inhibitory effects on *Platymonas helgolandica* var. *tsingtaoensis* and *Scenedesmus quadricauda* growth, after 4 days. Yan et al. (2021) demonstrated that an exposure of *Chlamydomonas reinhardtii* to 500 mg/L PS 100 μm led to a decrease of 23 % in growth after 4 days. Zhao et al. (2019) reported that *Karenia mikimotoi*'s cell density inhibition peaked at 46 % when exposed to 100 mg/L of 1 μm PVC-MPs after 1 day. Zhang et al. (2017) observed that 50 mg/L PVC-MPs (1 μm) had an evident negative effect, a decrease of up to 40 %, on the diatom growth of *Skeletonema costatum* after 4 days of exposure.

In response to the presence of MPs, microalgal cells may undergo a stress response, resulting in alterations in microalgal cell size, so flow cytometry was employed. The representative histograms in Fig. 3A_1 exhibit a clear shift in the curve area within the forward (size) scatter profile of *T. suecica*, comparing the cultures grown in clean water against the cultures grown in water contaminated with PS-MPs on day 2. This shift indicated a decrease in the number of cells which confirms the decrease in cell density previously observed. On day 4, exposure to both concentrations of PS-MPs led to a split of the curve, compared to the clean waters, indicating a decrease in cell size. This effect was more noticeable with 10 mg/L of PS-MPs, showing that higher concentrations of MPs have a more severe impact on the size of *T. suecica* cells and possibly in their biochemistry, molecular and cell biology. When it comes to PE-MPs exposure (Fig. 4A), the effects are less noticeable. However, highlighting day 4, the cultures exposed to 5 mg/L of PE-MPs exhibited a shift in the profile, with an increase in the number of cells with reduced size. Exposure to 10 mg/L of PE-MPs also yielded an increase in the number of smaller sized cells. These results confirm the decreased cell abundance previously observed for exposure to both MPs.

Furthermore, the presence of both PS-/PE-MPs not only showed negative effects on the cell density but also on the biomass production of *T. suecica* (Fig. 2A_2). Although biomass production kept increasing throughout the 4-day period, a decrease in yield was observed when compared to clean water. The observed reduction was up to 24 % for PS-MPs (more significant for the concentration of 10 mg/L) and 19 % for PE-MPs (independently of the concentration) (Table S2). This decrease in biomass is thought to be caused by the MPs triggering a stress response that might result in cell metabolism adaptions to produce less dense molecules or discharge stock molecules. Accordingly, *T. suecica* showed a decline in single-cell weight (compared to the production in clean water), which supports the biomass productivity results. Single-cell weight was calculated through the quotient between microalgal cell weight in contaminated water and microalgal cell weight in clean water (%), on the same day.

S. armatus demonstrated to counteract the presence of PS- and PE-MPs differently than *T. suecica*. Different microalgae possess distinct cellular properties which dictate how MPs affect their biology (Chae et al., 2019; Fu et al., 2019). *S. armatus* (Fig. 2B_1) showed no significant differences in cell density throughout the 4-day production period for any MPs. Other studies have also described the lack of microalgal growth inhibition in the presence of this type of contaminant. Zhu et al. (2020) reported that 1 μm PVC-MPs with concentrations lower than 6 mg/L had no adverse effect on the growth of the diatom microalgae *Skeletonema costatum* after 4 days of exposure. Sjollema et al. (2016) observed that exposure of *Dunaliella tertiolecta* to a concentration of 25 mg/L of 0.5 μm PS-MPs had no significant difference between treatments and clean water in terms of microalgal growth after 3 days. No significant repressive effect on cell growth was detected by Sun et al. (2021) when exposing *Euglena gracilis* to 1 μm PS-MPs (concentration up to 25 mg/L) after 4 days. Furthermore, Tunali et al. (2020) determined that 1 and 5 mg/L of 0.5 μm PS-MPs had no impact on the growth of *Chlorella vulgaris* during the first 4 days.

When exposed to either concentration of both MPs, *S. armatus* showed similar forward scatter profiles in the flow cytometer histograms, compared to the clean water (Figs. 3B and 4B), which indicates that MPs did not cause significant changes with the relative size/number. On the other hand, both MPs led to a reduction in the biomass production of *S. armatus*. The sharpest decrease, of 46–48 % (compared to the clean water; Fig. 2B_2 and Table S2), was observed when this species was exposed to a concentration of 10 mg/L of PS-MPs. The other concentrations led to reductions of 21–26 %. As a result of MPs exposure, microalgal cells have been proven to undergo morphological changes, which are associated with damage to pyrenoids, thylakoids, plasma, and the cell wall (Mao et al., 2018; Yokota et al., 2017), which might result in a reduction of cell size. As there were no changes in cell density, this decrease in biomass productivity

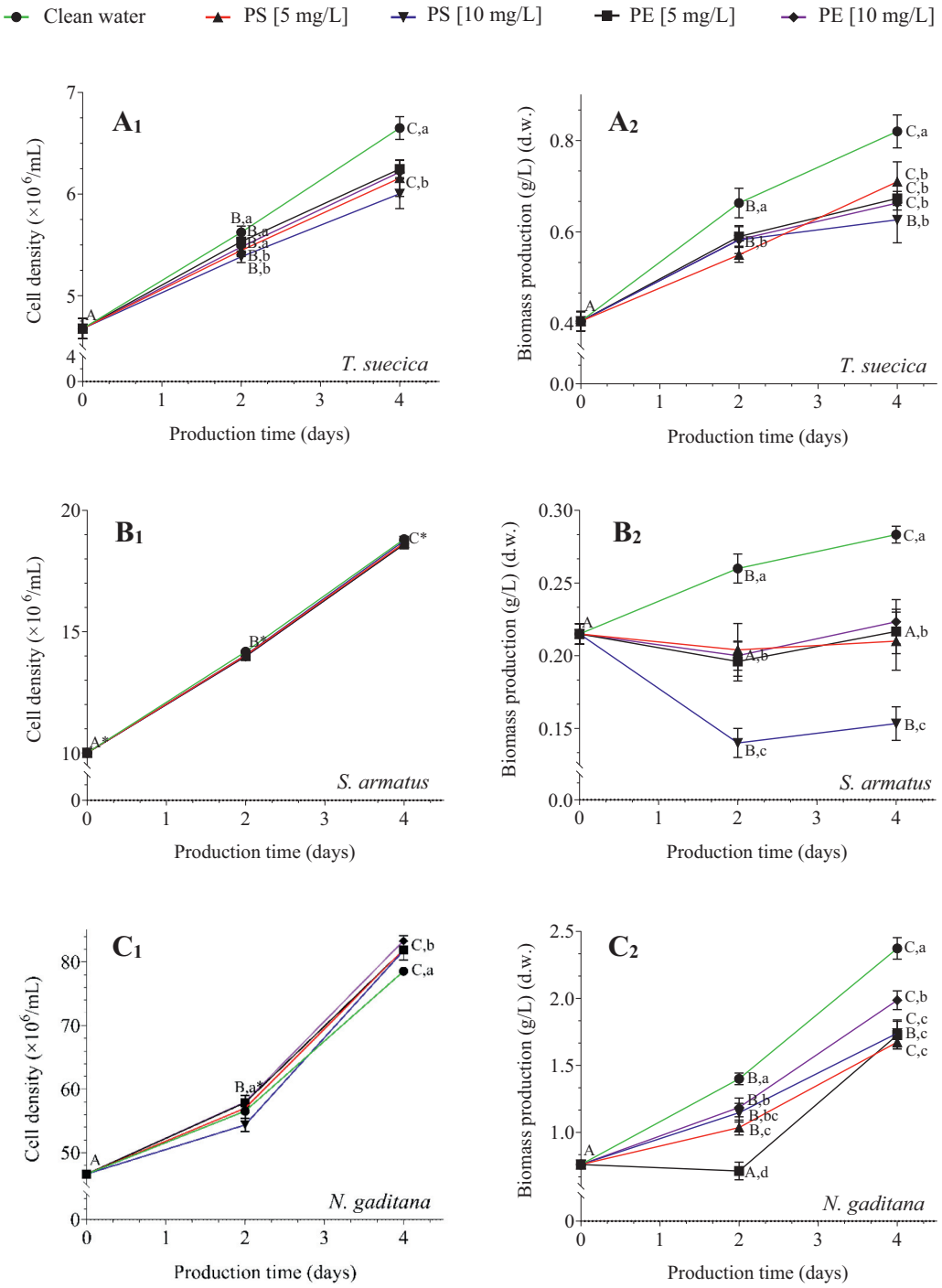

Fig. 2. Cell density (1 in subscript: A_1, B_1, C_1) and biomass production (2 in subscript: A_2, B_2, C_2) for each microalga cultivated in clean water and PS-/PE-MPs contaminated water (5 and 10 mg/L) throughout a 4-day production period of *T. suecica* (A), *S. armatus* (B), and *N. gaditana* (C). Different letters represent significantly different means of the correspondent day (small letters) and different water contamination (capital letters) (p-value ≤ 0.05); * represents no significant differences between all conditions (p-value > 0.05).

implies that exposure to MPs should be impacting either microalgal cell-size or cell-weight, which were both tested. The results in Table 1 show that the weight per cell of *S. armatus* seems to be heavily affected by day 2 (a weight loss between 16 and 44 %), without any variation throughout the rest of the 4-day production period in neither of the MPs nor concentrations.

N. gaditana's growth curves under the addition of different concentrations of PS- and PE-MPs are shown in Fig. 2C. In contrast to *T. suecica*, both MPs exhibited a significant stimulating effect up to 6 % in cell density on day 4 (Table S1). This increase is likely linked to the usage of MPs, by some microalgae species, as a substrate to enhance growth and colonise said MPs (Canniff and Hoang, 2018; Cunha et al., 2019; Yokota et al., 2017). Canniff and Hoang (2018) showed that exposure to 63–75 μm PE-MPs at a concentration of 130 mg/L after 5 days enhanced the growth of freshwater microalgae *Raphidocelis subcapitata*, where PS-MPs act as substrates for algal growth. Chae et al. (2019) determined that exposing *Dunaliella salina* to PE-MPs of 200 μm in concentrations of 200, 250, 300, and 350 mg/L stimulated cell growth (125–140 %) after 6 days. Moreover,

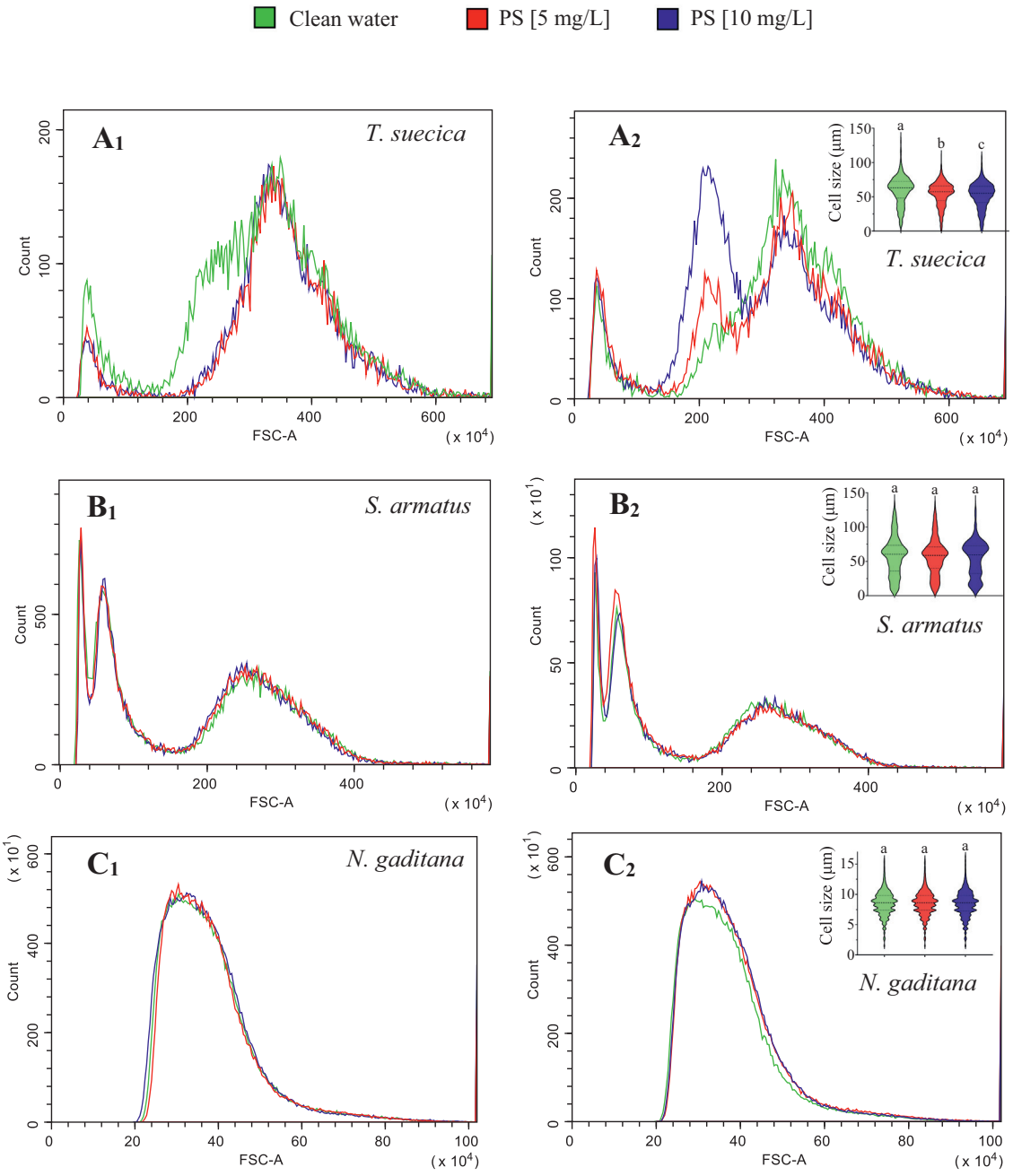

Fig. 3. Representative forward (size) scattering histograms, and insert in histograms are the cell size graphics (obtained through fluorescence microscopy), of the clean water and for PS-MPs-contaminated water conditions (5 and 10 mg/L), *T. suecica* (A), *S. armatus* (B), and *N. gaditana* (C), on day 2 (1 in subscript: A_1, B_1, C_1), and day 4 (2 in subscript: A_2, B_2, C_2) of culture.

within 4 days culture cycle, the presence of PE, PET, and PVC (74 µm), at a concentration of 200 mg/L, also led to a growth increase of *Chlorella* sp. *L38* (Song et al., 2020), suggesting the presence of a strong adaptive capacity to microplastics. Furthermore, a 1 µm PS exposure (5 mg/L) after 4 days affected *Microcystis aeruginosa* by stimulating algal growth (Wu et al., 2021).

In the present work, the *N. gaditana* cultures exposed to both concentrations of PS- and PE-MPs, on day 4, revealed slightly increased shifts in the curve area of the representative flow cytometer histograms (Figs. $3C_2$ and $4C_2$) in the same size, which might explain the increase in cell abundance presented in Fig. 2. Despite the *N. gaditana* cultures in MPs-contaminated waters growing more rapidly, compared to the ones in clear water, a decrease in biomass production was observed across all cultures exposed to MPs. The most substantial reduction in biomass production of *N. gaditana*, when exposed to PS-MPs contaminated waters was on day 4 of around 27–29 % (Fig. $2C_2$; Table S2), with either concentration. When exposed to PE-MPs, biomass production significantly decreased by 52 % by day 2 with a concentration of 5 mg/L. However, despite the drastic decline after 2 days of MPs exposure, *N. gaditana* displayed an adaptive response by increasing biomass production on day 4. Still, the biomass yield was around 29 % lower than cultures grown in clear water (Fig. $2C_2$; Table S2). The same tendency is shown when it comes to weight per cell (Table 1). Considering these results, it is likely that, even though cell growth increased, gene expression, cell morphology, and colony size are influenced by the presence of MPs, leading to a possible decrease in cell weight.

In conclusion, these results show that out of all three microalgae, *T. suecica* appears to be the most affected regarding cell density when exposed to either PS-MPs or PE-MPs. In terms of biomass production in a higher concentration of PS-MPs contaminated waters, *S. armatus* showed

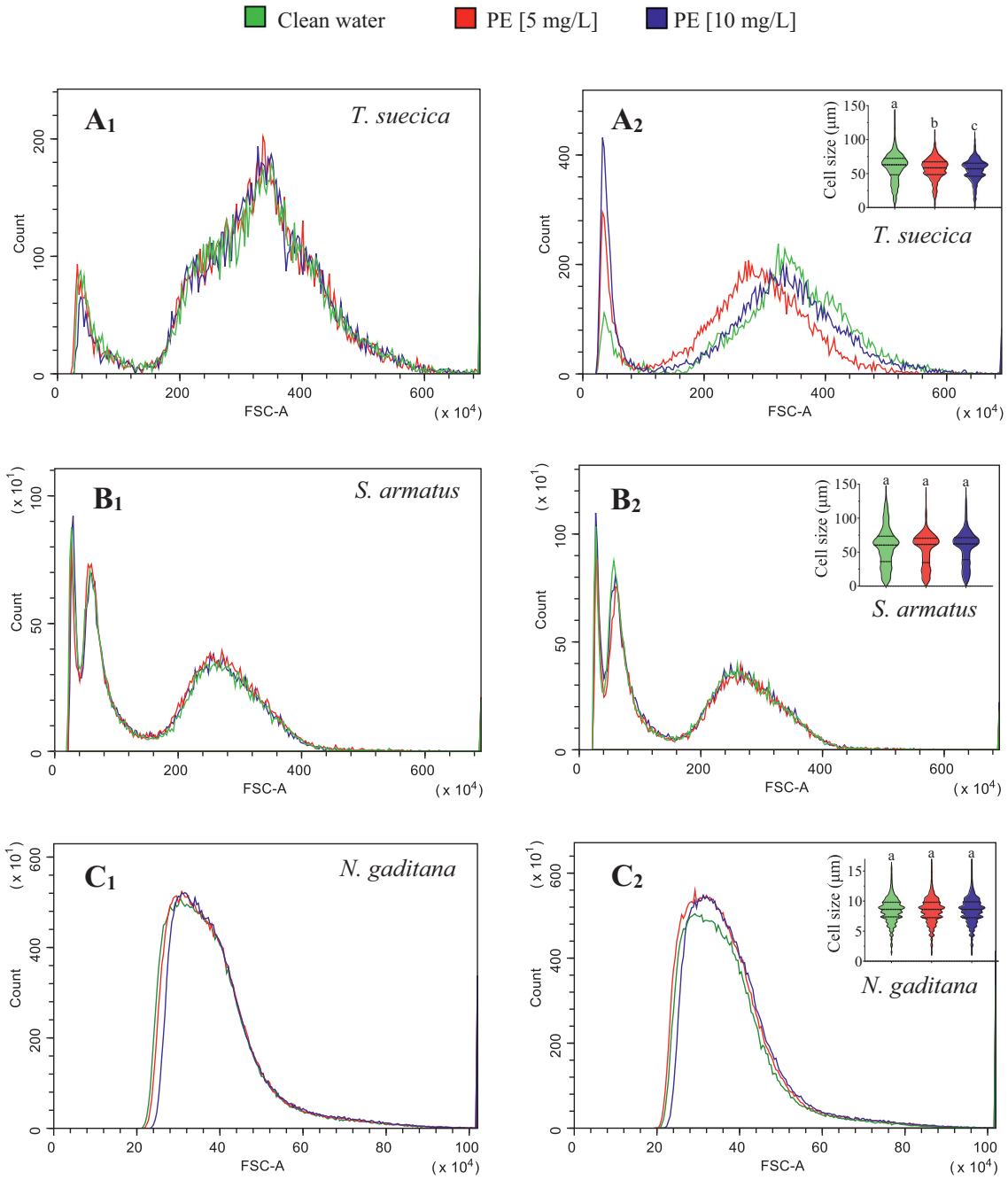

Fig. 4. Representative forward (size) scattering histograms, and insert in histograms are the cell size graphics (obtained through fluorescence microscopy), of the clean water and for PE-MPs-contaminated water conditions (5 and 10 mg/L), *T. suecica* (A), *S. armatus* (B), and *N. gaditana* (C), on day 2 (1 in subscript: A_1, B_1, C_1), and day 4 (2 in subscript: A_2, B_2, C_2) of culture.

the greatest vulnerability. On the other hand, lower concentrations of PE-MPs yielded the greatest impact on biomass production in *N. gaditana*. Thus, it should be emphasised that the undesirable effects of MPs on microalgae appear to be species-specific and can be influenced by the concentration and exposure time. This study also shows that under the same concentrations and size, the effect of PS- and PE-MPs on the cell density of all three microalgae varied significantly. Biomass production was affected negatively regardless of the type of MPs and independent of microalgal cell density. This was also shown by Cunha et al. (2020b), where there was a significant decrease in the biomass yield of *Phaeodactylum tricornutum* in a cell-density independent manner, regardless of the type of MPs used in the experiment (PS or PMMA). These results show that *T. suecica* appears to be affected in both cell size and cell weight. In contrast, the decrease in biomass production for both *S. armatus* and *N. gaditana* are not due to changes in cell size but instead, a reduction in cell weight (highlighted in Table 1). Thus, future research should focus on understanding the mechanisms responsible for these changes. Furthermore, considering the evident loss in biomass productivity shown here (up to 50 %), the economic impact of using waters contaminated with even low concentrations of MPs might be one of the factors that unwittingly overshadow the economic viability of microalgal-based industries.

3.2.2. Chronic exposure

To the best of our knowledge, only one study has conducted a systematic experiment on trying to understand the effects of long-term exposure to MPs on microalgal populations and the potential effects on microalgal-

Table 1
Variation of the weight per cell (normalised to clean water in %) for PS- and PE-MPs contaminated water conditions (5 and 10 mg/L) throughout a 4-day production period of *T. suecica*, *S. armatus*, and *N. gaditana*.*

Species	Time (days)	[5 mg/L]		[10 mg/L]	
		PS	PE	PS	PE
T. suecica	2	89.61 ± 1.86^{b,B}	89.28 ± 1.54^{b,B}	92.30 ± 1.14^{b,AB}	92.80 ± 1.99^{b,A}
	4	87.20 ± 0.38^{b,B}	87.4 ± 1.98^{b,B}	86.48 ± 3.82^{b,B}	86.53 ± 2.19^{b,A}
S. armatus	2	84.21 ± 2.24^{b,B}	77.93 ± 4.50^{b,B}	56.31 ± 2.75^{c,B}	79.94 ± 2.76^{b,B}
	4	77.69 ± 4.99^{b,B}	74.75 ± 5.03^{b,B}	**53.07 ± 5.00^{c,B}**	81.79 ± 5.03^{b,B}
N. gaditana	2	75.10 ± 1.16^{b,B}	57.04 ± 2.55^{d,B}	82.45 ± 3.15^{c,B}	78.32 ± 2.89^{bc,B}
	4	69.16 ± 1.15^{b,C}	57.99 ± 1.73^{d,B}	72.99 ± 2.88^{bc,C}	77.89 ± 1.69^{c,B}

Different letters represent significantly different means of the correspondent day (small letters) and different water contaminations (capital letters) (p-value ≤0.05). PS= Polystyrene; PE= Polyethlyene.

* Weight per cell (normalised values = microalgal cell weight in contaminated water / microalgal cell weight in clean water × 100; of the same day).

based industries (Cunha et al., 2020a). Thus, to follow up on those results, the chronic effects of MPs exposure at 10 mg/L over 27 days were studied for the same three species starting with similar cell densities.

Overall, this concentration impacted microalgae more and therefore was selected. After 27 days of production (Fig. 5A):

- *T. suecica* presented a significant reduction in cell density when produced in both PS- and PE-MPs contaminated waters. Nevertheless, these cultures had already reached the stationary phase by day 4 (no significant differences between day 4 and 27), just like the ones produced in clean water. No significant differences were recorded between acute and chronic exposure to MPs, suggesting a capacity of the microalgae to adapt to their presence;
- *S. armatus*, showed that despite the long-term exposure to either MPs, this species could strongly resist the adverse environment, indicating its capacity to adapt in terms of growth. Similarly, to *T. suecica*, acute and chronic exposure did not differ statistically;
- *N. gaditana* revealed a reduction in cell density from day 4 to 27 in both MPs tested. A comparison with clean water revealed no significant differences in terms of the chronic effect. Essentially, the longer exposure allowed the microalga to regain its cell density as a result of an alternative type of adaptation.

These results show that short-lived and adaptive responses accompanied by the initial impairment or enhancement can lead to cell density recovery.

Despite this capacity to resist or adapt to the depression of cell density in the presence of MPs, the biomass production still decreased considerably across all three microalgal species (Fig. 5B), in both short- and long-term exposure to PS- and PE-MPs. This reveals that PS- and PE-MPs induced irreversible effects on these microalgae, which is corroborated by cell weight changes (Table 1). *T. suecica* showed a cell-weight loss of approximately 16 %, *S. armatus* of 50 %, and *N. gaditana* of 33 % when cultivated in PS-contaminated waters. The losses were 22, 21 and 28 %, for *T. suecica*, *S. armatus,* and *N. gaditana*, respectively, when cultivated in PE-contaminated waters. Note that neither of these values is statistically different from day 4 onwards, highlighting the irreversible and lasting damage the presence of these particles exerts on microalgae.

3.3. Response surface methodology (RSM)

A response surface methodology (RSM) is an ensemble of tools that can be employed to modulate the parameters of an experimental design. This method was applied to model and predict how the cell density and biomass production of each microalga would be affected when exposed to PS- and PE-MPs varying concentrations and time. The implemented experimental design parameters for cell density and biomass production generated the contour plots presented in Figs. 6 and 7, respectively. Cell densities and biomass productions with lower rates are depicted in blue while red represents higher values. The data revealed:

- for *T. suecica*, increasing the exposure time and the concentration of PS- and PE-MPs, respectively, caused a decline in the cell density. Biomass production (Fig. 7A) showed the same trend, in concordance with the previously discussed results;
- for *S. armatus* neither the presence, absence, or exposure time to MPs, had any significant effect on the cell density. However, the contour plots of Fig. 7B reveal that biomass production is expected to decrease by increasing the concentration of MPs;
- for *N. gaditana*, as stated previously, both MPs have a stimulating effect causing noticeable increases in cell density. If the concentration of MPs and culture time is increased to the highest values, the response is

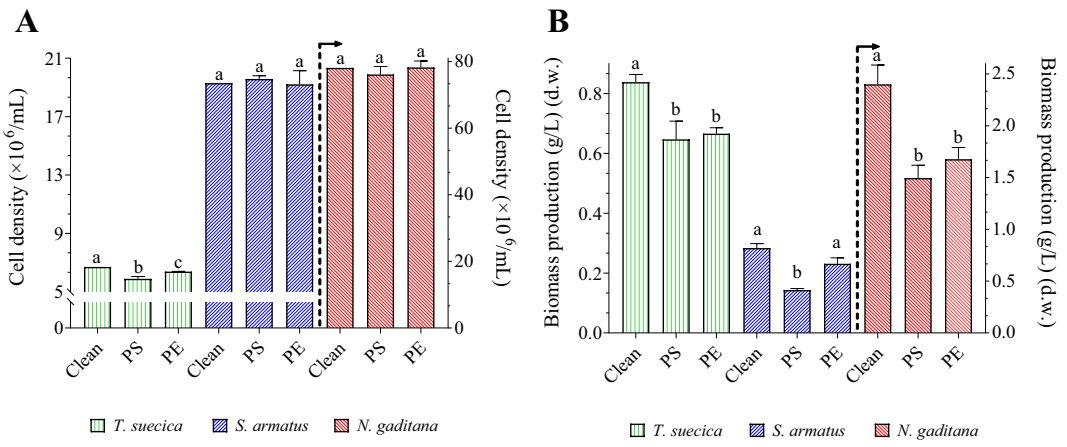

Fig. 5. Cell density (A) and biomass production (B) for the clean water and PS- and PE-MPs contaminated water conditions (10 mg/L) for a 27-day production period of *T. suecica*, *S. armatus*, and *N. gaditana*. Different letters represent significantly different means of the correspondent day (*p*-value ≤0.05).

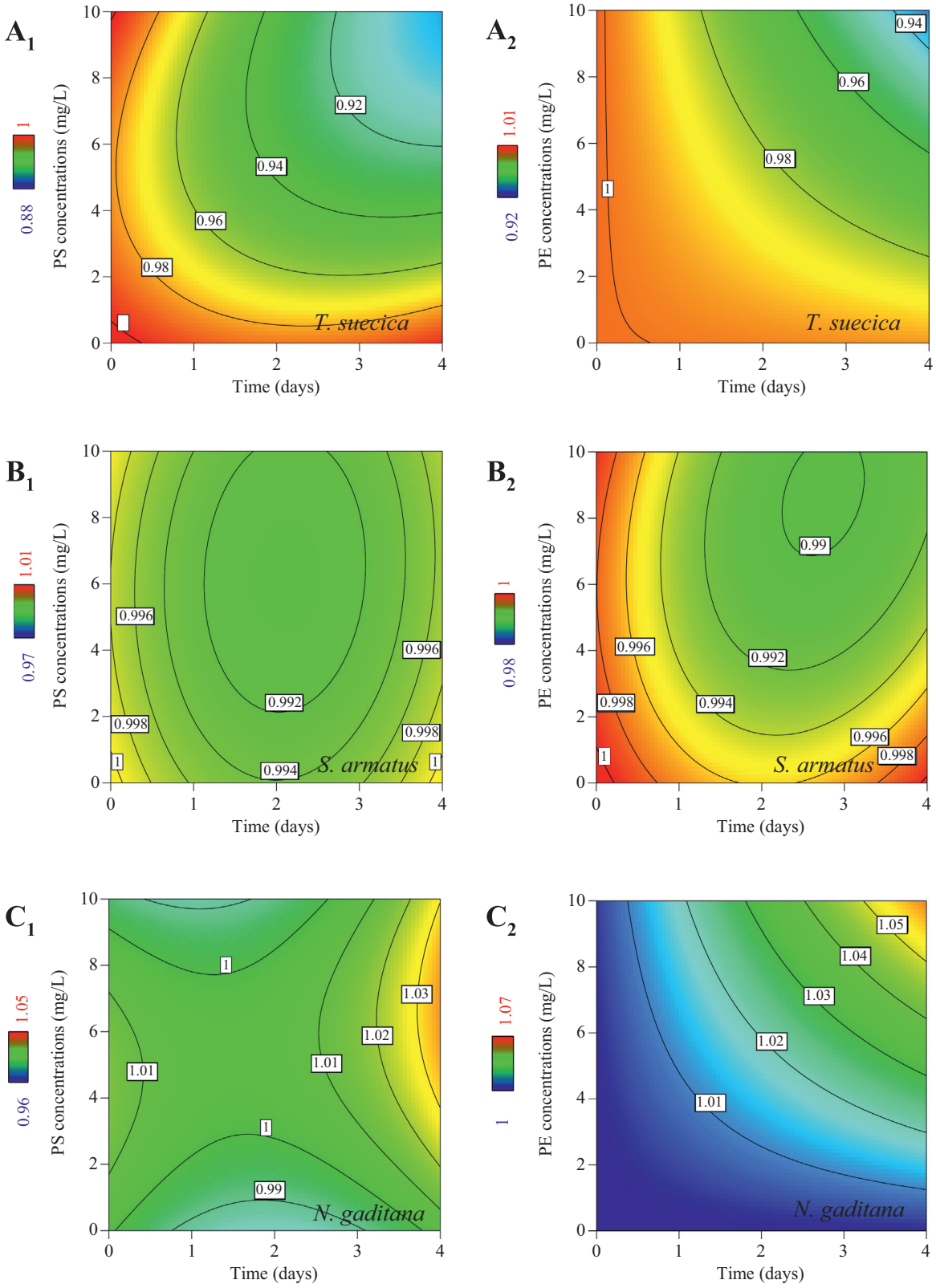

Fig. 6. Contour plots of cell density as a function of MPs concentration: PS (1 in subscript: A_1, B_1, C_1), and PE (2 in subscript: A_2, B_2, C_2); and the production time of *T. suecica* (A), *S. armatus* (B), and *N. gaditana* (C).

intensified. In contrast, considering the variables examined (Fig. 7C_1), biomass production is at its lowest when the microalgae are exposed to high concentrations of PS-MPs for longer periods of time. The contour plot for biomass production in PE-MPs contaminated waters in Fig. 7C_2, illustrates that lower concentrations of PE-MPs lead to more severe decreases in biomass production, aligning with the previous results.

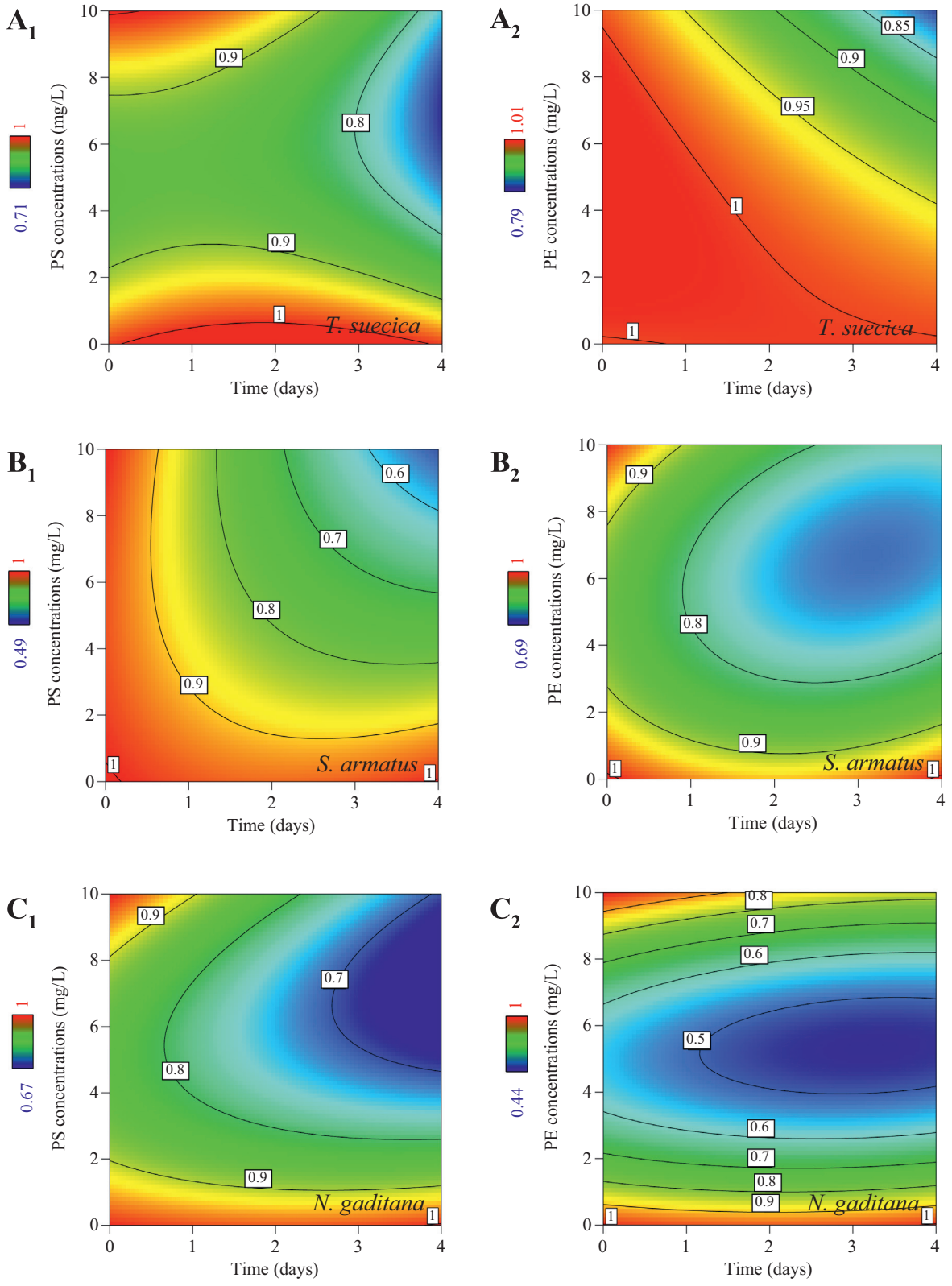

Fig. 7. Contour plots of biomass as a function of MPs concentration: PS (1 in subscript: A_1, B_1, C_1), and PE (2 in subscript: A_2, B_2, C_2); and the production time of *T. suecica* (A), *S. armatus* (B), and *N. gaditana* (C).

Eqs. 5 to 10 presented in Table 2 were the model equations that define the experimental correlations with the concentration of MPs and culture time as variables. If the concentration of MPs and the production time are known, these equations can be used to predict biomass production. For example, a concentration of PS-MPs in the water of 6.5 mg/L and for 2 days of production it's expected a biomass reduction of 15 %

Table 2
Model equations for the predicted biomass production (normalised values*) of *T. suecica*, *S. armatus*, and *N. gaditana*.

Species	MPs	Model equations	Eq.
T. suecica	PS	$0.99345 + 0.04589T - 0.05336C - 0.00589TC - 0.01147T^2 + 0.00547C^2$	(5)
	PE	$0.99739 + 0.00395T + 0.01195C - 0.00478TC - 0.00071T^2 - 0.00123C^2$	(6)
S. armatus	PS	$1.00978 - 0.05478T - 0.01740C - 0.01147TC + 0.01343T^2 + 0.00166C^2$	(7)
	PE	$1.00892 - 0.06283T - 0.05389C - 0.00529TC + 0.01572T^2 + 0.00521C^2$	(8)
N. gaditana	PS	$1.00195 - 0.02284T - 0.06482C - 0.00768TC + 0.00589T^2 + 0.00644C^2$	(9)
	PE	$1.00498 - 0.03546T - 0.17966C - 0.00414TC + 0.00902T^2 + 0.01787C^2$	(10)

T is the time (days), and *C* is the concentration of MPs (mg/L). Biomass production decrease % = 100 × (1 – biomass normalised values).
* Normalised values = contaminated water / clean water; of the same day.

to *T. suecica* (0.84 normalised values) and 26 % to *S. armatus* (0.74 normalised values). Table S4 presents the regression equations that can be used to predict cell density.

The biomass production/cell density ratio (Table S3) was determined to establish the direct relationship between cell growth and biomass production and to provide indirect intel on the economic implications of using waters contaminated with MPs in the microalgal industry. Fig. S1 illustrates how exposure to high concentrations of PS- and PE-MPs during longer culturing periods generally have a detrimental impact on biomass output. Based on these response surface plots, this model allows for the identification of conditions that would most strongly affect biomass yields. Table S5 presents the model equations that can be used to predict biomass production/cell density ratio.

The applicability and validity of the model equations were assessed through the analysis of variance (ANOVA), and by the comparison (R^2) between the experimental and predicted values. Based on the ANOVA: *i*) no significant lack-of-fit (*p*-value >0.05) was found for any of the models (e.g. Fig. S2); *ii*) value obtained experimentally R^2 and the model predicted values R^2 showed no significant differences (e.g. Fig. S3); *iii*) *p*-value <0.0001 and the *F*-value above 19.06 for all models; led to the conclusion that all the model equations are valid and confirm the reliability and predictability of all the computed models obtained.

The refinement of an intended outcome is among one of the most relevant steps in RSM. This step aims to determine the most selective levels for each input variable to achieve the desired outcome. Thus, to uncover the parameters that would affect biomass production the most and potentially affect the microalgae biochemistry and cell morphology, the levels for each factor were determined. Considering the economic point of view for microalgal-based industries, the minimum number of production days and PS- or PE-MPs concentration necessary to reach the lowest amount of biomass production was selected for the study. Table 3 shows the conditions with the highest impact on biomass production for the different microalgae. *N. gaditana* shows the greatest reduction in biomass production of 51 % only with 52 h of production using water contaminated with 5.40 mg/L of PE-MPs.

Table 3
Conditions of the highest impact of MPs (PS and PE) exposure on biomass production with experimental and predicted values (and model validation error %; Eq. 4) of *T. suecica*, *S. armatus*, and *N. gaditana*.

Species	MP	High impact conditions		Biomass production (normalised values[a])		
		Production time (days)	MPs concentration (mg/L)	Predicted values	Experimental values	% Error
T. suecica	PS	4.00	7.04	0.72	0.74 ± 0.04	2.79
	PE	4.00	10.00	0.82	0.78 ± 0.03	4.31
S. armatus	PS	4.00	10.00	0.54	0.52 ± 0.04	4.07
	PE	3.14	6.76	0.73	0.77 ± 0.06	5.70
N. gaditana	PS	3.94	6.77	0.65	0.70 ± 0.06	6.36
	PE	2.18	5.40	0.47	**0.49 ± 0.02**	3.68

[a] Normalised values = contaminated water / clean water; of the same conditions. Biomass production decrease % = 100 × (1 – biomass normalised values).

In order to confirm the applicability of this RSM function, up-scale production was carried out under conditions with a higher level of impact. The experimental values obtained (Table 3) were very close to the predicted values, indicating that the conditions succeeded in reaching the target stated above. The corresponding low error (2.79–6.36 %) between the experimental and predicted model values of the response also allows verifying the accuracy of the model. It can be concluded that the RSM has high prognostic ability and accuracy.

3.4. Implications and recommendations

Microalgal biomass is a rich source of various bioactive compounds and can be leveraged in a wide range of critically important industrial applications. However, it is increasingly clear that maximizing biomass generation is a more complex operational affair than previously thought. The discovery that a seemingly harmless but highly ubiquitous pollution source such as MPs decreased biomass yields by up to 82 % (Cunha et al., 2020a), raised alarming bells to variables that were previously unknown to be detrimental towards the generation of highly valuable biomass. Here, these results follow up on those findings to report that these effects are reproducible across microalgae and types of MPs, while also uncovering some of the mechanisms driving the loss of biomass. Since decreases in biomass yields were up to 50 %, MPs might single-headedly endanger the economic scalability of microalgal biomass. Hence, containing/eliminating MPs' contamination is crucial. Future work needs *(i)* to scale this research to industrial setups to confirm these findings in more adequate and relevant translational setups and *(ii)* to understand the molecular mechanisms driving the reduction of single-cell weight and assess the scale of these effects in industrial settings. It should start by assessing pollution sources and performing rigorous water quality checks for the presence of MPs. Current research is being developed towards achieving these goals and we urge others to do the same. Despite this, it has not been possible to achieve economies of scale, and environmental pollutants might be one of the central pieces of the puzzle. Thus, tackling one of the most ubiquitous pollutants out there might be the barrier separating scalability and implementation from failure and disappointment. Enough evidence has been gathered to alert for the severely negative economic impacts that MPs might cause on extremely valuable biomass productions that play a critical part in the sustainable future we all envision.

4. Conclusions

The present research was performed by exposing industrially- and economically-relevant microalgae *Tetraselmis suecica*, *Scenedesmus armatus,* and *Nannochloropsis gaditana* to waters contaminated with PS- and PE-MPs, in order to mimic water contamination in industrial environments and understand the potential effects that MPs exert of microalgal biomass production. Growth-wise, these microalgae were affected in a species-dependent manner. However, biomass yields were severely affected across all species, independently of the MP-type, and concentration. It was shown that the presence of MPs affected biomass production by decreasing single-cell weight and/or cell-size. MPs may alter the metabolic pathways of microalgae, by favoring the production of certain

metabolites. The biomass production model obtained makes it possible to determine the direct relationship between biomass production and the level of MPs contamination at a defined time. This tool allows microalgal industries to extrapolate information about the economic implications of MPs-contamination in terms of biomass output. As a result of the ubiquitous presence of MPs in waters, biomass production at the industrial level might be severely affected and have detrimental economic impacts. Since decreases in biomass yields up to ~50 % were observed, MPs might singlehandedly endanger the economic scalability of microalgal biomass. Hence, containing/eliminating MPs' contamination is crucial. Future studies should focus on understanding the molecular mechanisms driving the reduction of single-cell weight and assess the scale of these effects in industrial settings.

CRediT authorship contribution statement

IM, MF, and NC contributed to the conception and design of the study. IM and CC executed the experiment. IM, CC, MF, and NC analysed, interpreted the data, and wrote the manuscript. NC and MK made possible the execution of the experiment by providing administrative and financial support, supervising the experiment, and making critical revisions regarding important intellectual content of the manuscript. All authors read and approved the final manuscript.

Data availability

Data will be made available on request.

Declaration of competing 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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Appendix A. Supplementary data

Supplementary data includes: *(i)* Additional information about the materials and methods; *(ii)* Tables relatively the RSM factorial design and mathematical models equations; *(iii)* Contour plots of biomass production/cell density ratio *(iv)* Figures related to RSM validation. Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2023.162950.

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