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Industrial Production of Microalgae with Microplastic Contaminated Waters
Effects and biosolutions

MASTER DISSERTATION

Ivana Rita da Silva Mendonça
MASTER IN APPLIED BIOCHEMISTRY

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ORIENTATION
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INDUSTRIAL PRODUCTION OF MICROALGAE WITH MICROPLASTIC CONTAMINATED WATERS: EFFECTS AND BIOSOLUTIONS

Tese apresentada à Universidade da Madeira com vista à obtenção do grau de Mestre em Bioquímica Aplicada

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Sob a orientação de:
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LIST OF PUBLICATIONS

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Mendonça, I.; Cunha, C.; Kaufmann, M.; Faria, M.; & Cordeiro, N. (2023). Microplastics reduce microalgal biomass by decreasing single-cell weight: the barrier towards implementation at scale. *Environmental Science and Technology*. (submitted)

LIST OF CONFERENCES

Mendonça, I.; Sousa, J., Cunha, C., Faria, M., Ferreira, A., & Cordeiro, N. (2022). New strategy for microplastic bioremediation: cellulose biopolymer-based hydrogel. *MICRO 2022* (poster).

Mendonça, I., Sousa, J., Faria, M., Ferreira, A., & Cordeiro, N. (2022). Sustainable approach for microfibres removal: bacterial cellulose residues. *MICRO 2022* (poster).

Cunha, C.; Faria, M.; Mendonça, I.; & Cordeiro, N. (2022). Advancing bacterial cellulose biopolymers & hydrogels to remediate microplastic pollution. *MICRO 2022* (oral presentation).

Mendonça, I., Sousa, J., Cunha, C., Ferreira, A., & Cordeiro, N. (2022). Microplastics impacts on microalgae industrial production: growth vs biomass. *AlgaEurope 2022* (poster).

Faria, M.; Duarte, L.; Dias, M.; Mendonça, I.; & Cordeiro, C. (2022). Harvesting marine *Nannochloropsis gaditana* through extracellular bacterial bioflocculants. *AlgaEurope 2022* (poster).

ABSTRACT

Currently, microplastics (MPs) pose one of the most pressing environmental and urban issues, and aquatic systems are particularly vulnerable to these particles. Among the major sources of the problem are wastewater treatment plants. Biomass production relies heavily on water quality in microalgal-based industries. Therefore, industrially exploited microalgae, *Tetraselmis suecica*, *Scenedesmus armatus* and *Nannochloropsis gaditana*, were cultivated in PS- and PE-MPs-contaminated waters (5 and 10 mg/L). Simulating industrial procedure, production began 4 days before stationary phase, and chronic effects were assessed with 27-day long experiments. Both MPs induced species-specific algae density*: T. suecica* inhibited (up to 11%), *S. armatus* no significant changes and *N. gaditana* increased (up to 6%). However, all presented significant decrease in biomass yield (up to 24, 48 and 52%, respectively) - by a reduction in single cell weight (up to 14, 47, and 43%), and/or the production of smaller cells (e.g., *T. suecica*). Although chronic exposure showed adaptation signs in algae density, reduction in biomass production is still evident (up to 19, 49, and 35%). RSM revealed that microalgal biomass production using MPs contaminated waters is concentration-dependent. With growing interest in developing sustainable solutions, unused remnants of bacterial cellulose membranes were ground to form BC hydrogels as potential bioflocculants of MPs. BC hydrogel's viability in removing MPs from MPs-contaminated water was assessed for different environmental and operational conditions. BC hydrogel unveiled a very high flocculation rate (80.42%), most likely driven by the hydrogel's microporous nature. RSM showed BC hydrogel:MPs ratio and grinding times as the most critical variables modulating flocculation rates. Also, short exposure times (5 min) were sufficient to drive robust particle aggregation. These findings suggest that BC hydrogel could be an alternative to synthetic flocculants in wastewater remediation processes.

Keywords: Commercial microalgae; Industrial biomass production; Microplastic contaminated waters; Bacterial cellulose hydrogel; Flocculation; Bioremediation.

RESUMO

Atualmente, os microplásticos (MPs) representam uma ameaça ambiental e urbana emergente, e os sistemas aquáticos são particularmente vulneráveis a essas partículas. Uma das principais fontes deste problema são as estações de tratamento de águas residuais. A produção de biomassa depende muito da qualidade da água em indústrias baseadas em microalgas. Portanto, microalgas exploradas industrialmente, *Tetraselmis suecica*, *Scenedesmus armatus* e *Nannochloropsis gaditana*, foram cultivadas em águas contaminadas com PS e PE-MPs (5 e 10 mg/L). Simulando o procedimento industrial, produção iniciou 4 dias antes da fase estacionária, e os efeitos crónicos foram avaliados com experiências de 27 dias. Ambos os MPs induziram, na densidade celular respostas especificas para cada microalga: *T. suecica* foi inibida (até 11%), *S. armatus* sem alterações significativas e *N. gaditana* estimulada (até 6%). Porém, todas exibiram uma queda acentuada na produção de biomassa (até 18, 48 e 30%, respetivamente) – através de alterações bioquímicas nas microalgas, resultando na redução do peso por célula (até 14, 47 e 43%), e/ou na produção de células mais pequenas (ex., *T. suecica*). Embora a exposição crónica tenha mostrado sinais de adaptação na densidade algal, redução na produção de biomassa continua evidente (até 19, 49 e 35%). RSM revelou que a produção de biomassa microalgal usando águas contaminadas com MPs é dependente da concentração. Com o aumento do interesse no desenvolvimento de soluções sustentáveis, restos não utilizados de membranas de celulose bacteriana (BC) foram moídos para formar hidrogéis como potenciais biofloculantes de MPs. A viabilidade de hidrogel de BC na remoção de MPs de águas contaminadas com MPs foi avaliada para diferentes condições ambientais e operacionais. O hidrogel de BC revelou uma taxa de floculação muito alta (80,42%), provavelmente impulsionada pela natureza microporosa do hidrogel, muito superior a biofloculantes dispersivos comerciais como goma xantana e alginato. RSM mostrou a razão hidrogel de BC:MPs e os tempos de moagem como as variáveis mais críticas na modulação das taxas de floculação. Mais, tempos de exposição curtos (5 min) foram suficientes para conduzir uma agregação de partículas robusta. Estas descobertas sugerem que o hidrogel BC pode ser uma alternativa aos floculantes sintéticos em processos de remediação de efluentes.

Palavras-chave: Microalgas comerciais; Produção industrial de biomassa; Águas contaminadas com microplásticos; Hidrogel de celulose bacteriana; Floculação; Biorremediação.

TABLE OF CONTENTS

CHAPTER III. Solving urban water microplastics with bacterial cellulose hydrogels: leveraging predicative computational models

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

3D RGO	Three-dimensional-reduced graphene oxide
3D-EEM	Three-dimensional excitation-emission matrices
AFS	Atomic fluorescence spectrometry
AT	Austria
BC	Bacterial cellulose
BE	Belgium
BET-ASAP	Brunauer-Emmett-Teller surface area and porosity analyser
BG	Bulgaria
C	Concentration
CAM	Contact angle meter
CAS	Conventional activated sludge
CCD	Central composite design
CFS	Coagulation-flocculation-sedimentation
CH	Switzerland
CLSM	Confocal laser scanning, microscopy
CV	Coefficient of variation
CZ	Czech Republic
d.w.	Dry weight
DAF	Dissolved air flotation
DE	Germany
DK	Denmark
DLS	Dynamic light scattering
DOE	Design of the experiment
DOM	Dissolved organic matter
DWTP	Water treatment plants
ECD	Electron capture detector
EDS	Energy dispersive spectroscopy
EDX	Energy dispersive X-ray spectroscopy
EE	Estonia
EL	Greece

EPS	Exopolymer substances
ES	Spain
FCM	Flow cytometry
FESEM	Field emission scanning electron microscopy
FR	France
FSC	Forward scatter
FTIR	Fourier-transform infrared spectroscopy
FTIR-ATR	Fourier transform infrared spectroscopy – attenuated total reflectance
FW	Freshwater
GCHA	Guinea cornhusk ash
GT	Grinding time
HDPE	High-density polyethylene
HF	Hollow fibre
HPLC	High performance liquid chromatography
HU	Hungary
ICP-AES	Atomic emission spectroscopy with inductively coupled plasma
IS	Iceland
IT	Italy (chapter I)
IT	Immersion time (chapter III)
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LSCM	laser scanning confocal microscopy
LV	Latvia
MBC	Magnetic biochar
MBR	Membrane bioreactors
MPC MP	Concentrator
MPs	Microplastics
NL	Netherlands
NO	Norway
OD	Oxidation ditch (chapter I)
OD	Optical densities (chapter II and III)
PA	Polyamide
PALS	Phase analysis light scattering

PAM	Polymeric flocculant polyacrylamide (chapter I.1)
PAM	Pulse amplitude modulation (chapter I.2)
PAN	Polyacrylonitrile
PBS	Polybutylene succinate
PCR	Polymerase chain reaction
PDMS	Polydimethylsiloxane
PE	Polyethylene
PEA	Plant efficiency analyser
PEI	Polyethyleneimine
PET	Polyethylene terephthalate
PHB	Polyhydroxy butyrate
PMMA	Poly (methyl methacrylate)
PP	Polypropylene
PS	Polystyrene
PT	Portugal
PU	Polyurethane
PVC	Polyvinyl chloride
R	Ratio
R^2	Coefficient of determination
RSF	Rapid sand filtration
RSM	Response surface methodology
SD	Standard deviation
SE	Sweden
SEM	Scanning electron microscopy
SW	Seawater
T	Temperature (chapter III)
T	Time (chapter II)
TEM	Transmission electron microscopy
TLC	Thin-layer chromatography
UK	United Kingdom
UPLC-MS/MS	Ultra-performance liquid chromatography spectrome-tandem mass
w.w	Wet weight

WWTP	Wastewater treatment plant
XPS	X-ray photoelectron spectroscopy
XRD	X-ray diffractometer

AIM OF THE STUDY

The current Master's dissertation is organized into several chapters, each with the prospective to be submitted to scientific journals. Chapter II has already been submitted to Environmental Science and Technology journal, and Chapter III has been published in Chemosphere. The main goals of the present study are to determine the impact that microplastics (MPs) have on the biomass production of industrialy relevant microalgae, identifying the cellular impact driving those changes, and develop a biosustainable strategy to remove these particles from contaminated waters.

In detail, Chapter I gives an insight about the microalgal-based industries in Europe; on the interactions of microplastics and microalgae; and the current and emerging methods to remove MPs from aquatic systems. Chapter II aims to investigate the impact of microplastic (PS and PE) contaminated waters on commercial microalgae (*Tetraselmis suecica, Scenedesmus armatus,* and *Nannochloropsis gaditana*) in terms of biomass production. Following the discoveries made in Chapter II, Chapter III aims to find a solution for the removal of MPs from contaminated waters, evaluating the potential of BC hydrogels as bioflocculants and hetero-aggregators of MPs towards their prospective removal.

As supplementary work, a paper entitled *Bacterial cellulose biopolymers: The sustainable solution to water-polluting microplastics* has been published. Moreover, abstracts for poster presentations in the conferences *MICRO 2022* and *AlgaeEurope 2022* have been submitted. To MICRO posters entitled, *New strategy for microplastic bioremediation: cellulose biopolymer-based hydrogel,* and *Sustainable approach for microfibres removal: bacterial cellulose residues,* and oral presentation *Advancing bacterial cellulose biopolymers & hydrogels to remediate microplastic pollution*; and to AlgaeEurope, *Microplastics impacts on microalgae industrial production: growth vs biomass,* and *Harvesting marine Nannochloropsis gaditana through extracellular bacterial bioflocculants* were published.

CHAPTER I

Microplastic pollution impact and microalgal-based industries: an actual review

Microplastic pollution impact and microalgal-based industries: an actual review

1. Microalgal industry

Microalgae comprise a diverse array of photosynthetic microorganisms that grow in a variety of environments, including fresh-, sea- and brackish waters, soils, rocks, and, trees, (Borowitzka, 2018; Buono et al., 2014). Various bioactive compounds can be found in them, making them useful in several commercial applications. Production of microalgae on an industrial scale kicked off in Japan in the early 1960s, when *Chlorella* was introduced as a food additive (Araújo et al., 2021; Buono et al., 2014) -Fig. 1, *extraction* cluster in red. Between 1960 and 1990, there was a limited amount of development. However, the energy crisis at the start of the 21st century influenced the development of microalgae favourably toward the concept of a possible source of biofuels (de la Jara et al., 2016). With the high price of crude oil at the time, it was crucial to discover a sustainable and economically viable fuel alternative as soon as possible (D. Li et al., 2020) - *biomass* cluster in green. This crisis led major energy firms to become interested in microalgae biotechnology and invest a considerable sum in pursuing that goal. In spite of everything, the output from actual production systems fell considerably short of the expected values, and as a result, sufficient bioenergy production from microalgae is still not feasible. Nonetheless, this led to a breakthrough in technology and production capacity, which currently enables an increase in commercial microalgae use (Garrido-Cardenas et al., 2018). Nowadays, microalgae present with a wide range of emerging applications, including agriculture-related products (biofertilisers, biostimulants and biopesticides), wastewater treatment, bioplastics, and more (Fernández et al., 2021; Rumin et al., 2020) - *cultivation* cluster in blue.

According to the latest available data from EMODnet, there are currently 400 microalgae-producing industries based in Europe, spread between 20 countries (*Microalgae | EMODnet Human Activities*, 2022). France (FR), Italy (IT), Germany (DE), and Spain (ES) are the countries in Europe (Fig. 2A) with the largest number of microalgae industries (more than 20 producers each). However, France dominates the production landscape, with 50% of the production in Europe. Moreover, the majority of microalgae production occurs in inland areas.

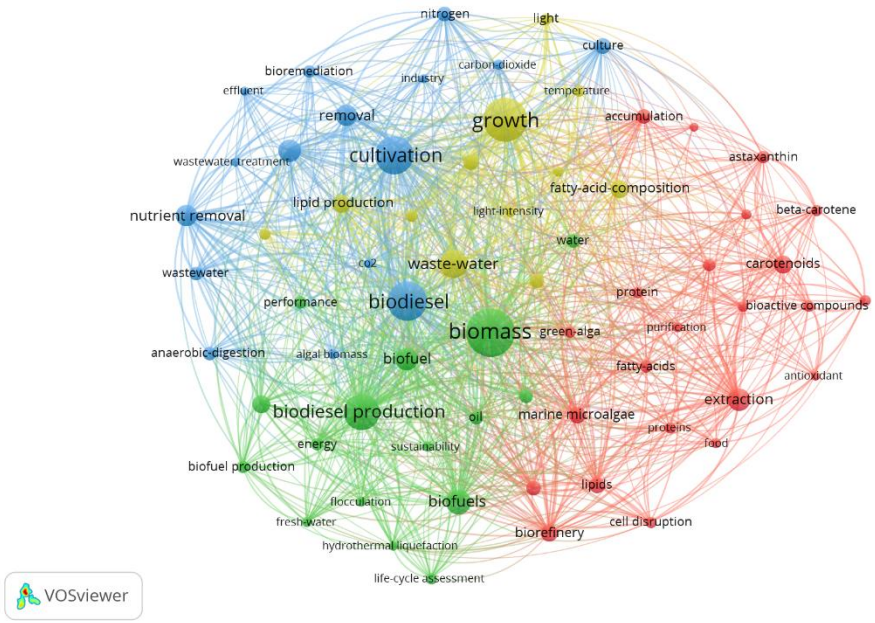


Fig. 1. Main concepts and concepts network, obtained from VOSviewer software **(*VOSviewer*, 2022)** and research of "*microalgae AND industry*" in the web of science database (Clarivate. Web of Science, 2022).

Despite being widely cultivated, only a small portion of the naturally occurring microalgae species (Fig. 2A) are used for commercial purposes (Fig. 1) - due to climatic, economic, and regulatory issues (Vigani et al., 2015). Along with *Arthrospira maximum* (Spirulina), microalgae *Chlorella* spp., *Nannochloropsis* spp., and *Haematococcus pluvialis* are the most extensively exploited, and over the last 20 years, have reportedly been among those employed the most, in biotechnology applications globally (Araújo et al., 2021; Fernandes & Cordeiro, 2021). Other microalgae species, *Tetraselmis*, *Isochrysis*, *Porphyridium*, and *Scenedesmus* genus are as well among the most cultivated microalgae in Europe (Araújo et al., 2021). Portugal (PT), Germany (DE) and Spain (ES) are the top three countries exploiting the greatest variety of species (Fig. 2A). European-wise, marine microalgae account for 64.6% of industrial production (Fig.2B). Moreover, inland countries tend to exploit more fresh- and brackish water microalgae.

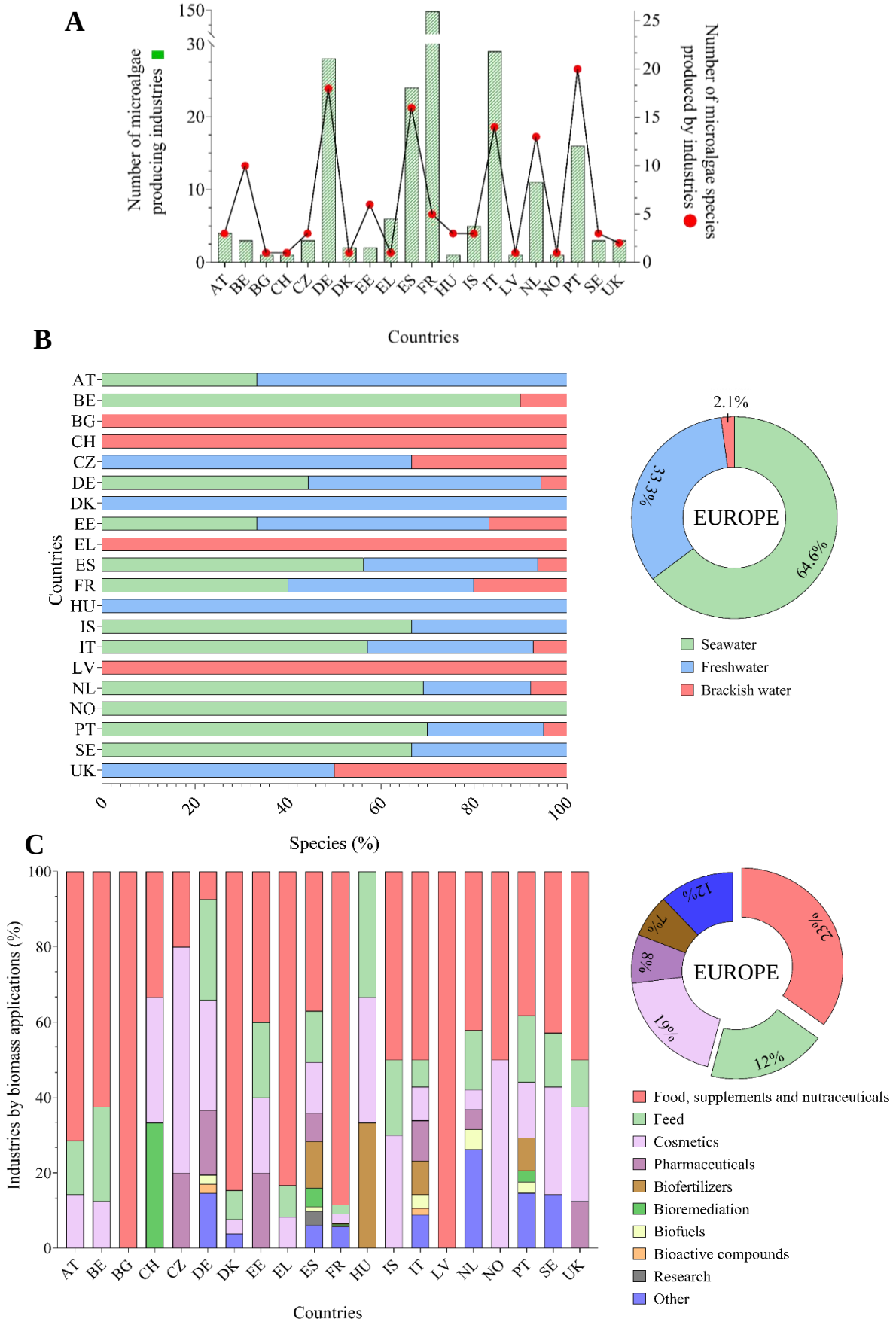

A
Number of microalgae producing industries
Number of microalgae species produced by industries
Countries
B
Countries
Species (%)
2.1%
33.3%
64.6%
EUROPE
Seawater
Freshwater
Brackish water
C
Industries by biomass applications (%)
Countries
23%
12%
19%
8%
7%
12%
EUROPE
Food, supplements and nutraceuticals
Feed
Cosmetics
Pharmaccuticals
Biofertilizers
Bioremediation
Biofuels
Bioactive compounds
Research
Other

Fig. 2. Microalgae production in Europe: (**A**) Number of industries producing microalgae biomass vs number of species exploited in Europe; Relative abundance charts of: (**B**) the different microalgae species in european countries and in Europe; (**C**) the different applications of biomass by industries in european countries and in Europe. AT - Austria; BE - Belgium; BG - Bulgaria; CH - Switzerland; CZ - Czech Republic; DE - Germany; DK - Denmark; EE - Estonia; EL - Greece; ES - Spain; FR - France; HU - Hungary; IS - Iceland; IT - Italy; LV - Latvia; NL - Netherlands; NO - Norway; PT - Portugal; SE - Sweden; UK - United Kingdom. Based on EMODnet and Joint Research Centre databases (***Microalgae | EMODnet Human Activities***, **2022;** ***The Bioeconomy in Different Countries | Knowledge for Policy***, **2022**).

The diverse bioactive compounds found in microalgae make them useful in a wide range of commercial applications. Over 54% of European microalgae industries primarily dedicate their biomass towards the feed and food markets (including human food, food supplements, nutraceuticals, and animal feed). Among these leading sectors, cosmetics, pharmaceuticals and fertilisers also account for prominent positions (Fig. 2C). Although microalgae produced in Europe today is allocated to these purposes, there are many additional applications that are steadily emerging in the market, in several countries. For instance, bioremediation is making notice in Switzerland (CH), and countries like Germany (DE) and Italy (IT) are investing in bioactive compounds.

Having stated that, despite microalgae biotechnology proving to be promising, microalgal biomass production requires specific environmental conditions to ensure quality. Enhancing biomass and/or biocompounds production will significantly influence the final costs of the marketable goods - higher biomass quality will lead to a more economically desirable end product. Therefore, the magnitude of these requirements is evident.

2. Microalgae vs microplastics

Given the significant role that microalgae play in the ecosystem and microalgae-based industries, there is a need to understand how these organisms respond to diverse types of contaminants. Considering that microplastics (MPs) represent a rising global worry, the mysteries regarding the potential effects of this pollutant on microalgae deserve further attention.

The National Oceanic and Atmospheric Administration defined MPs as plastic particles of all shapes and sizes, smaller than five millimetres (5 mm) in size (NOAA, 2020). These particles are enduring and pervasive contaminants that can either result from the fragmentation of larger polymers into smaller pieces due to environmental factors; or be produced intentionally as a result of industrial processes (Andrady, 2011a; Browne et al., 2011). MPs have been documented in practically every ecosystem, including urban networks (Dey et al., 2021), marine (from the equator to the polar regions) and freshwater (Bergmann et al., 2015; Novotna et al., 2019a) environments, as well as drinking, fresh and wastewater (Koelmans et al., 2019). Among the immense types of MPs found in these aquatic biospheres, some of the most frequent ones include polyethylene (PE), polypropylene (PP), polystyrene (PS), nylon, polyethylene terephthalate (PET), and polyvinyl chloride (PVC) (Erni-Cassola et al., 2019; Wan et al., 2019).

Numerous research on how MPs affect microalgae has been conducted (Table S1) (none done at an industrial scale), however, the information is still somewhat limited. The MPs that are most frequently employed in studies of this nature are those that are mostly prevalent in aquatic environments. The effects on microalgae from freshwater to seawater (mainly in the exponential growth phase) have been studied, using MPs from spherical to irregular particles, with concentrations ranging from 0.001 to 5000 mg/L, and diameters up to 1000 µm. Nevertheless, there are already some indications that MPs might have negative repercussions on these primary producers.

The impact on microalgae growth (microalgae density) is one of the most recurrent endpoints assessed in this kind of research (Fig. 3 and Table S1). Several studies have reported that these particles seem to induce growth inhibition in several microalgal species (Casado et al., 2013; Hazeem et al., 2020; S. Li et al., 2020; Lin et al., 2020; Mao et al., 2018). The most widely recognised and discussed theory to explain this finding is that MPs particles may contribute to this reduction in growth by encouraging shading effects, obstructing algal pores, inhibiting gas exchanges, negatively affecting photosynthesis and development (Bhattacharya et al., 2010). However, as observed by Canniff & Hoang (2018), Cunha et al. (2019), D. Wu et al. (2021), and Yokota et al. (2017), MPs exposure led to the opposite behaviour in certain species of microalgae. MPs has a stimulating effect on the microalgae (increase in microalgae density), suggesting that MPs may ultimately provide a favourable surface for their development. Furthermore, in other species, MPs might not even interfere in the growth process, as revealed by L. Sun et al. (2021), Tunali et al. (2020), and X. Zhu et al. (2020).

These variations in responses were influenced by the type, size, concentration, and length of exposure to the MPs, as well as the species of microalgae. Furthermore, given that different microalgae have different cell characteristics, it's possible that factors like the cell walls could influence MPs penetration or adsorption (Chae et al., 2019; Fu et al., 2019).

In addition to their impact on microalgal growth, researchers have found that MPs may also impact microalgal photosynthesis, as evidenced by the decreases in chlorophyll content and photosynthetic efficiency observed in response to the contaminant exposure (Tunali et al., 2020; Xiao et al., 2020; C. Zhang et al., 2017). Further, MPs may hinder photosynthesis by reduction in the expression of genes involved in the photosynthetic process (Lagarde et al., 2016), altering the electron donor sites, the photosynthesis centres and the electron transport chains, which can also result in electron build-up and generation of reactive oxygen species that contribute to oxidative stress (Bhattacharya et al., 2010; Mao et al., 2018). Moreover, the presence of MPs could lead to alterations in the morphology of microalgal cells, according to Mao et al. (2018) and Seoane et al. (2019) reported that cells exposed to MP may modify their energy metabolism to adapt to the stress conditions.

Nonetheless, plenty of these effects appear to be transient, with microalgae going through a susceptible phase, before recovering as a consequence of adaptive (Cunha, Lopes, et al., 2020; Yokota et al., 2017).

As previously stated, in the numerous studies on this matter, microalgal growth is one of the most frequently explored parameters (with almost 33%) (Fig. 3). This is followed by the analysis of the metabolites and photosynthetic activity. However, there are still fundamental gaps in the existing level of knowledge when it comes to biomass production. Only one recent study (Cunha, Lopes, et al., 2020b) assessed the impact of MPs on biomass yield. In light of this, it is crucial to determine and understand how this type of contaminant affects microalgae that are economical relevant to the industry. Furthermore, considering that in microalgae-based industries, water quality is an essential criterion and essential element when it comes to biomass production, it is imperial to find effective methods of mitigating the negative impacts brought on by the presence of MPs.

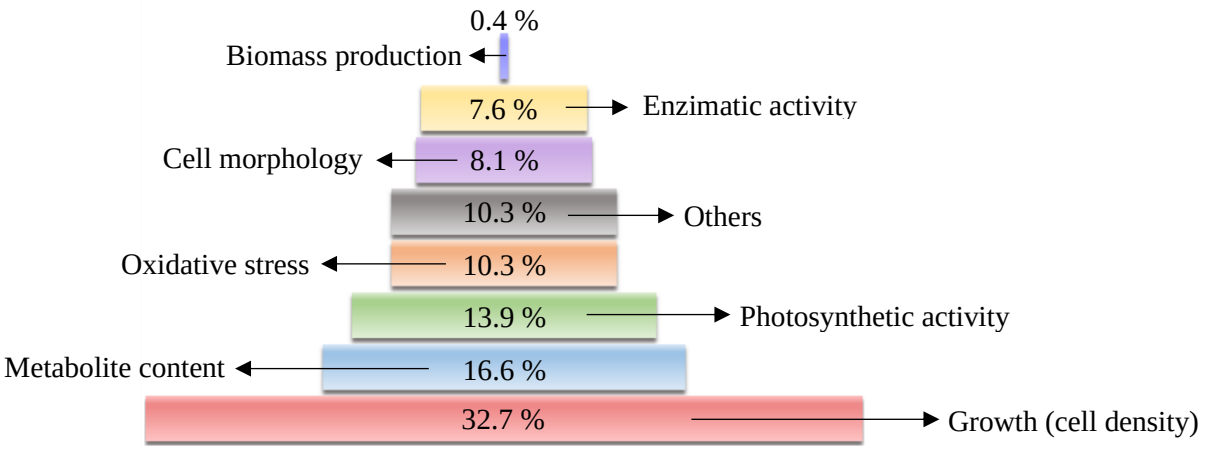


Fig. 3. Relative abundance chart of the different parameters explored in studies of the impact of MPs on microalgae.

3. Microplastic removal

Within the vast array of microalgal-based industries, each has its own distinctive manufacturing procedures and methods. As a result, the water used to grow the microalgae may very well be derived from a variety of sources. Potential sources of water for these industries include, wastewater treatment plants (WWTPs), drinking water treatment plants (DWTPs), or even from fresh and seawater environments. Bearing in mind that water quality is one of the most

vital parameters in the production of microalgae, it is imperative to ensure that it's free of contaminants that might have serious negative repercussions on the calibre of the end goods.

3.1 Wastewater treatment plants (WWTPs)

Among several other sources, the contribution of wastewater effluents to MPs pollution in aquatic environments has recently gotten a significant amount of attention (Carr et al., 2016; J. Sun et al., 2019).

Each treatment step in a WWTP (Fig. 4) has a particular aim that contributes to cleaning water flow in the overall process (Eerkes-Medrano et al., 2015; Z. Zhang & Chen, 2020). Note that, the amount and types of MPs vary across WWTP, according to the provincial and neighbouring industrial properties and influent periods (Cao et al., 2020; Zou et al., 2021).

While frequently exhibiting removal efficiencies exceeding 90%, wastewater treatment facilities (WWTPs), even with the recent development of techniques to remove MPs from wastewater (Poerio et al., 2019), lack the technological capabilities to extract and purify the waters of MPs (W. Liu et al., 2021; Novotna et al., 2019a), due to their smaller size and ongoing breakdown. As a result, despite the application of primary, secondary, and even tertiary procedures, the smallest particles frequently are still present in wastewater. MPs with sizes <100 μm tend to be particularly difficult to remove from urban waters (Azizi et al., 2022; Jiang et al., 2020; S. Sharma et al., 2021). However, the contribution of MPs released via wastewater effluent is unknown compared to other sources. Subsequently, these particles have the potential to disperse gradually and widely, entering fresh- and seawater environments, where they might have adverse effects (Bergmann et al., 2015; Thevenon et al., 2015). Hence WWTPs should develop some technologies in order to remove these particles (Talvitie et al., 2017).

Primary and secondary treatments are typically the two major methods (even though not the target) used to eliminate MPs. Primary treatment aims to remove suspended solids, mainly by sedimentation and cause them to settle at the bottom of the storage tank as sludge (Talvitie et al., 2017; M. Wu et al., 2021). Various studies have demonstrated that 50-98% of MPs may be eliminated during primary treatment. The primary treatment serves as the foundation for the secondary treatment, which involves further biochemical action treatment of the sewage (Michielssen et al., 2016), and most of the MPs >500 μm are removed (Conley et al., 2019; M.

Wu et al., 2021). The removal rate of MPs in the secondary treatment can be up to 99%. However, as mentioned previously most of the smaller particles tend to remain in the waters. Since most secondary effluents already meet the discharge criteria, tertiary treatment is optional (J. Sun et al., 2019). The seldom utilised tertiary treatment is capable of achieving a 99% removal efficiency.

The treatment method used in WWTPs significantly impacts the effectiveness of MPs elimination, with membrane-related technologies demonstrating the highest performance. Membrane bioreactors (MBR) are currently regarded as one of the most powerful methods for effective treatment of municipal and industrial wastewater worldwide. Studies have shown that MBR may effectively remove MPs around 99% (Fig. 4). Regardless of this, polymeric membranes (like MBR), can also contribute to MPs pollution, so efforts are increasingly being made to develop recyclable and biodegradable alternatives (Galiano et al., 2019). A recent study by Faria et al. (2022) shows bacterial cellulose (BC) biopolymers as a promising solution for replacing polymeric membranes. Results reveal that BC biopolymers have removal efficiencies up 99%. Conventional activated sludge (CAS), oxidation ditch (OD), rapid sand filtration (RSF), and dissolved air flotation (DAF) are methods that are also used, however present with a lower removal efficiency. An emerging process, the one of electrocoagulation, involves forming cations in the electrical field by metal electrodes. It generates coagulants using metal electrodes to trap and engulf suspended particles, such as MPs, and the removal efficiency has been shown to be 90-100% (Fig. 4).

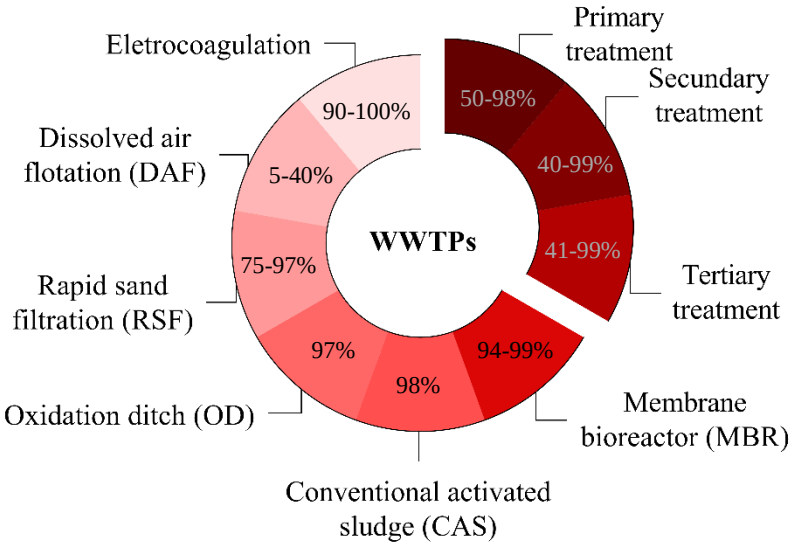


Fig. 4. MPs removal methods and effectiveness (%) in WWTPs. Based on: Alavian Petroody et al., 2020; Dris et al., 2015; Hongprasith et al., 2020; Michielssen et al., 2016; Murphy et al., 2016; Tagg et al., 2020; Bretas Alvim et al., 2021; Edo et al., 2020; H. Lee & Kim, 2018; Z. Zhang & Chen, 2020; Ziajahromi et al., 2021; Blair et al., 2019; Mintenig et al., 2019; Ziajahromi et al., 2017; Bayo et al., 2020; Lv et al., 2019; Lares et al., 2018; Hidayaturrahman & Lee, 2019; Bilgin et al., 2020; Talvitie et al., 2017; and Perren et al., 2018.

3.2 Drinking water treatment plants (DWTP)

To ensure the safety of drinking water, it has become increasingly significant to examine the MPs whereabouts in drinking water treatment plants (DWTP). So much so that MPs have already been tested for, in a few raw water samples from DWTPs, and their existence has been verified (Novotna et al., 2019a; Pivokonsky et al., 2018). Furthermore, these particles have also been found in tap, fresh and even bottled water (Eerkes-Medrano et al., 2019; Koelmans et al., 2019; Mintenig et al., 2019). Nevertheless, only a limited number of studies have focused on removing MPs from these facilities (Z. Wang et al., 2020). Among these, a majority were centred around coagulation-flocculation-sedimentation (CFS) procedures, since particle removal in modern DWTPs is typically achieved through coagulation-flocculation (often in conjunction with sedimentation) and granular media filtration or membrane filtration (Fig.5). Coagulation is mainly

induced by iron and aluminium-based salts; flocculants may be added to assist the process. Flocculation using alum or ferric and aluminium sulphate as coagulants, present a removal rate of up to 90% and 80%, respectively. With Fe-based coagulants, removal efficiency could be slightly improved by adding polymeric flocculant polyacrylamide (PAM). When associated with other procedures, sand filtration could also be used to improve the removal of MPs. However, this technology's biggest downside is that it tends to break down the MPs into smaller particles, making them even more difficult to remove, adding to MPs pollution (Phillips et al., 2016). Unfortunately, these methods are inefficient or are neither biodegradable nor ecologically friendly. Plus, they also pose several threats to human health and the environment (C. S. Lee et al., 2014). In addition to CFS, other removal methods, including reverse osmosis, ozonation/carbon filtering, and pulse cleansers, also demonstrate efficacy (Fig. 5), albeit not to the same extent. Even though DWTPs can prevent most MPs from contaminating tap water, MPs have been found in varying levels in the water supply, pipe scale, and distribution system (Chu et al., 2022). Because of this, MPs in pipe scales cannot be disregarded. In order to limit the amount of MPs entering the water supply system, it is essential to improve both the stability of pipe scales and the removal efficiency of MPs in DWTPs. With that said, considering that human, environmental and industrial wellbeing are undoubtedly affected by MPs in water, it is crucial to find a way to safely and effectively remove them from raw water.

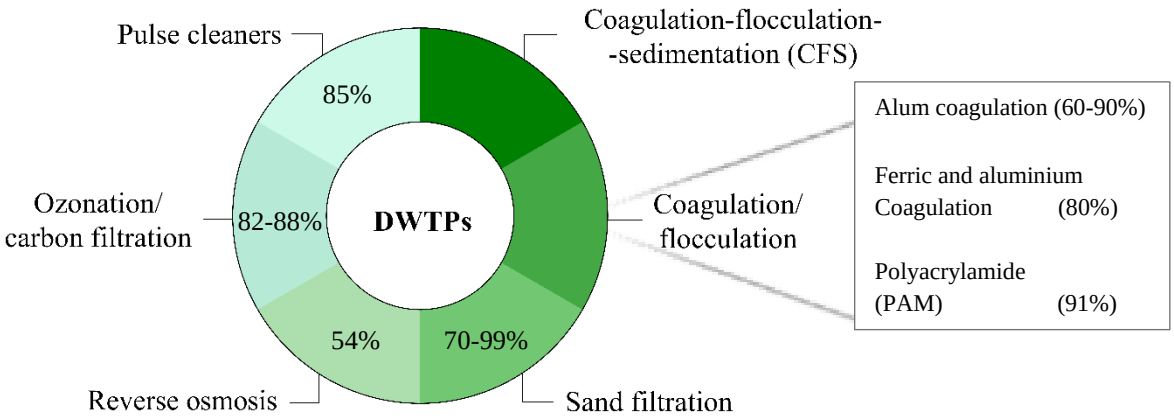


Fig. 5. MPs removal methods and effectiveness (%) in WWTPs. Based on: **Z. Zhang & Chen, 2020**; Novotna et al., 2019; Xue et al., 2021; Prokopova et al., 2021; Ma et al., 2019; Pivokonsky et al., 2018; **Z. Zhang & Chen, 2020**; Dalmau-Soler et al., 2021; Z. Wang et al., 2020; and Sarkar et al., 2021.

3.3 Sea- and freshwater environments

Besides WWTPs and DWTPs, it is also well known that MPs are present in all seas and freshwater environments, such as rivers and lakes, and are consumed by a wide variety of aquatic life (Kühn & van Franeker, 2020; J. Li et al., 2018a). However, it still remains unclear the quantity of microplastic that reaches and accumulates in the aquatic ecosystems (Khalid et al., 2021). The elimination of MPs from the sea- and freshwater environments is therefore essential, but there are still gaps in the available technology and a dearth of knowledge on doing so. Numerous research revealed encouraging prospects to eliminate MPs from the natural ecosystem, despite the lack of standardised procedures at this time.

3.4 Emerging solutions

In recent years, natural bio-based polymer materials derived from renewable resources have sparked interest due to their biodegradable, cost-effective, environmentally friendly, and efficient detoxifying properties with little impact on the environment (Vázquez-Núñez et al., 2020).

Biosorption (Fig. 6) has been proving to be a viable and innovative method to remove MPs from contaminated waters by using adsorbent materials. Research has shown that sponges (materials with 3D porous structured networks) can effectively remove numerous water contaminants (Z. Wang, Sun, et al., 2021). There have not been many papers on employing sponge material to remove MPs. However, the ones that have been published, demonstrated that sponges offer great reusability and biodegradability features, as well as removal efficiencies of 82%. A 3D (three-dimensional)-reduced graphene oxide (3D RGO) adsorbent has been recently developed, to separate PS-based MPs. According to the investigation, under ideal circumstances, the 3D RGO's maximum removal effectiveness was 67%. Biochar is another possible biosolution, that stands out due to its various advantages, including its abundant supply, low cost, easy synthesis, wide surface area, and developed pores. The biochar's capacity to adsorb can be significantly enhanced by adding ferromanganese oxide magnetic nanoparticles and creating magnetic biochar (MBC) – removal efficiency of up to 99%. Note that pH, temperature, adsorbent types, dissolved organic matter (DOM), and ions are just a few of the many variables that might impact the MPs

removal effectiveness (Z. Wang, Sun, et al., 2021). Due to its high MPs removal efficiency, low energy usage, and recyclable nature, adsorption is a desirable solution for bioremediation. However, the prospects of the MPs returning to the environment are possible since MPs must be separated from the adsorbent after usage.

In addition to adsorption, filtration (Fig. 6) also offers significant application potential for removing MPs in the aquatic environment. An affordable ceramic hollow fibre (HF) microporous membrane made of silica, developed using guinea cornhusk ash (GCHA), in the work of Yogarathinam et al., (2022) had a removal effectiveness of up to 97%. A new strategy for efficiently removing plastic particles in water, driven by solar energy, was presented by Wang et al. (2022). By using high-power density glass, sunlight was concentrated, causing convection and creating microbubbles at the interface. This way, plastic particles are convectively drawn into the microbubble, allowing them to be removed from water effectively over a long period, without requiring chemical, biological, or mechanical methods – maximum efficiency of 70%. Based on microfluidic technology, Chen et al. (2022) developed an MP concentrator (MPC) using polydimethylsiloxane (PDMS) as the material. MPC was able to concentrate and remove over 90% of MPs $\geq$ 19 μm, as well as obtain MP counts (per gram wet sediment). By combining anodisation and the liquid-phase deposition of lauric acid, Rius-Ayra and Llorca-Isern, (2021) were able to obtain a superwettable material with a superhydrophobic surface. This methodology exhibited a more than 99% efficiency and still prevented the MPs from degrading. Inspired by the natural mussel, Zhou et al. (2021) designed a biocompatible and cost-efficient method to remove MPs from aquatic environments. Adhesive PDA@Fe_3O_4 MagRobots were created by simply self-polymerising dopamine on the surface of commercial Fe_3O_4 nanoparticles. The magnetically powered adhesive microrobots can provide an ecologically safe, reliable, and stable platform for environmental remediation.

Jellyfish mucus has been exploited as a novel bioflocculant material and has been found to be capable of sequestrating polystyrene MPs in aquatic settings (Fig. 6). According to recent research, some microalgae can produce exopolymer substances (EPS) that have the right properties to aggregate (adhere and incorporate) MPs and create hetero-aggregates. Seawater microalgae *Tetraselmis* sp. and *Gloeocapsa* sp., as well as freshwater microalgae like *Cyanothece* sp., *Microcystis panniformis*, and *Scenedesmus* sp. presented with this ability and therefore, the potential to be employed in phytoremediation processes.

Microbial biopolymers, which are biodegradable polymers produced by living organisms, have been emerging as low-cost materials as another alternative to MPs removal. Among these, it has been shown that biofilms produced by bacteria *P. aeruginosa* revealed to be effective in accumulating MPs within the exopolymeric matrix. And incorporating a 'capture-release mechanism' into the biofilm's design improved the bioaccumulation of MPs while incorporating a release mechanism through dispersion, encouraging effective release and recovery of microplastics. Another promising biopolymer with high removal efficiencies is the one mentioned before, bacterial cellulose (BC) (study by Faria et al. (2022)) - removal efficiencies between 93 and 99%. BC in particular has already demonstrated its applicability and future promise in a wide range of industries including, food, biomedical, pharmaceutical, and cosmetic (Azeredo et al., 2019; Liu et al., 2020; Swingler et al., 2021). Further, has recently attracted interest regarding its potential use in the removal of contaminants such as dyes and heavy metals (Isik et al., 2018; Wanichapichart et al., 2002). This is possible due to its distinctive structural characteristics, high purity, biocompatibility, and biodegradability (Andriani et al., 2020), and therefore shows a lot of potential.

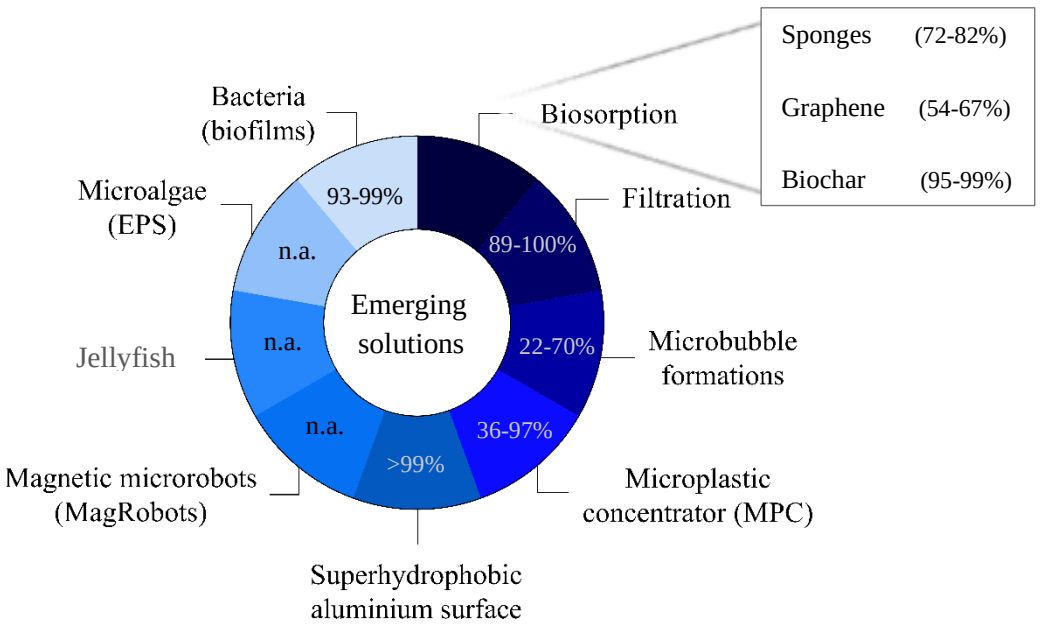


Fig. 6. MPs removal methods and effectiveness (%) in natural ecosystems. Based on: **Risch & Adlhart, 2021**; C. Sun et al., 2020; C. Sun et al., 2021; Z. Wang, Sun, et al., 2021; Yuan et al., 2020; Liang et al., 2019; Yogarathinam et al., 2022; Kuoppamäki et al.,2021 P. Wang et al., 2022; C. K. Chen et al., 2022; **Rius-Ayra**

& Llorca-Isern, 2021; Zhou et al., 2021; Cunha et al., 2019; Cunha, Silva, et al., 2020; Faria et al., 2022; S. Y. Liu et al., 2021; and Lengar et al., 2021.

In this respect, finding effective methods to extract, eliminate, or even minimise the MPs pollution is essential and crucial. Even with the various emerging options, more thorough research and technology advancements are required. Considering the need to implement biobased solutions across every economic sector, biopolymer-based remediation can offer an alternative in the future to help mitigate microplastic pollution. Besides showing potential to be used as a biofilter, bacterial cellulose, has also shown a true potential in removing contaminates such as heavy metals, proteins, dyes, and oil emulsions (Isik et al., 2018; Kurniawan and Yamamoto, 2013; Mohite and Patil, 2014; Wanichapichart et al., 2002). Therefore, the use of BC in the context of sustainable bioremediation systems for the circular bioeconomy is promising.

CHAPTER II

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

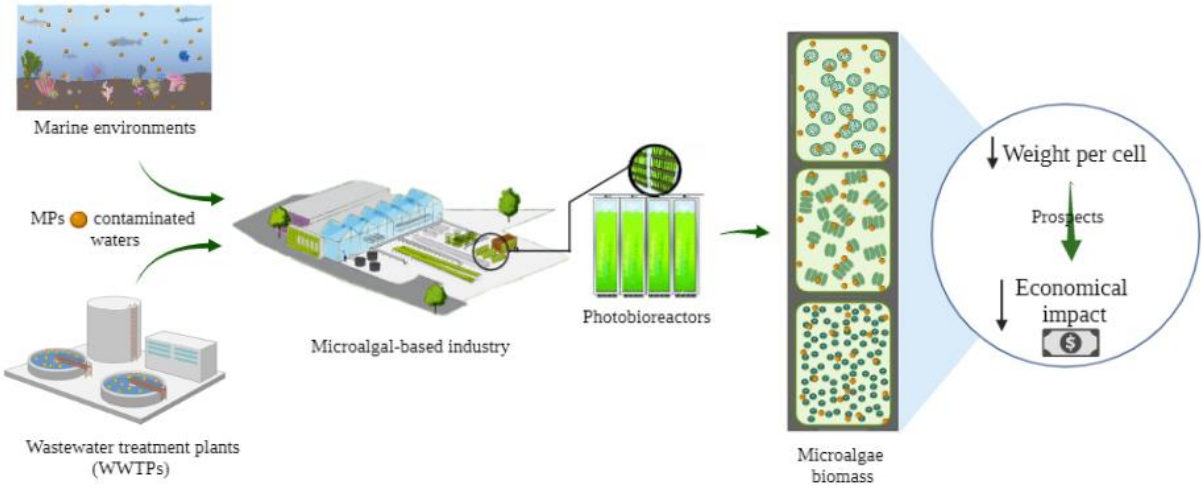
Microplastics reduce microalgal biomass by decreasing single-cell weight: the barrier towards implementation at scale

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 contaminated waters (5 and 10 mg/L). Following industrial empirical and ecotoxicological procedures, the production period was 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 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 production 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 some cell density adaptation signs. Despite cell density catching up, biomass production was still reduced compared to biomass production in clean water. Computational modelling demonstrated that MPs exposure had a concentration-dependent negative impact on microalgae biomass. The models allow 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.

Keywords: Microalgae; Biomass; Microplastics; Polystyrene; Polyethylene; Pollution

Graphical abstract



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., 2019a; Z. Wang et al., 2020). To address the issue 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 is wastewater treatment plants (WWTPs). These segregating infrastructures have been identified as focal points of MPs release into the fresh and marine environment (W. Liu et al., 2021; Novotna et al., 2019a; J. Sun et al., 2019). Despite recent years unveiling powerful microalgal- and bacterial-based biotechnological solutions, (Cunha et al., 2019; Cunha, Silva, et al., 2020;

Faria et al., 2022b) 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. Domestic wastewater has been shown to offer the ideal conditions for growing microalgae (Pacheco et al., 2015). Therefore, 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 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, including as a feedstock for biofuels, as well as in the food, feed, 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 a number of 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 dollars per kilogram for biofuel feedstocks to several hundred dollars per kilogram for human consumption products (Sathasivam et al., 2019). The demand for microalgal-based

bioproducts is expected to continue to grow 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 been done, that thoroughly investigated how MPs deplete biomass and most of its bioactive compounds (Cunha, Lopes, et al., 2020b). In that study, it was 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 in biomass yields of up to 82% 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 (freshwater), *Scenedesmus armatus* (seawater), and *Nannochloropsis gaditana* (seawater) present high industrial and economic potential. *Tetraselmisis*, 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 protein-rich microalgae 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.

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. Growth culture media were described in Supplementary Information 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) supplied by a cool white Osram L 18W 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). 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:

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

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

$CD = 70.216 \times OD_{750} - 3.7285$ ($R^2 = 0.9989$) *Nannochloropsis gaditana* **Eq. 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. (Y. Chen et al., 2020; Mao et al., 2018; L. Sun et al., 2021; Tunali et al., 2020; C. Zhang et al., 2017; Zhao et al., 2019; X. 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

The effects of PS- and PE-MPs were studied on the cell growth and biomass production of the three commercial microalgae species mentioned. The experimental design is shown in Table S2. To determine the acute and chronic effects in microalgae, these were grown in PS- and PE-MPs contaminated waters, ensuring exposition for the entirety of, respectively, 4- and 27-day-long experiments. To simulate industrial procedure, the production period started 4 days preceding 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 *xg*, 10 min) (HERLMEZ 360 Centrifuge) and gravimetrically determined after freeze-drying (Savant RVT400).

2.4. Flow cytometry

Flow cytometry analyses were performed using CytoFLEX, 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: flow cytometry did not discriminate between algal and microplastic particles; however, given the concentrations, the added microplastics' contribution to the responses is expected to be negligible compared to the microalgae.

2.5. Response surface methodology (RSM)

RSM modelling (Supplementary S2) was performed with Design Expert software (version 13) 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 (Supplementar Information S2; Table S2-S4). (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.

$$\text{Error } (\%) = \frac{\text{experimental - predicted}}{\text{experimental}} \times 100 \qquad \textbf{Eq. 4}$$

2.6. Statistics

Data representation and statistics of the data outside of the Design Expert software were performed using GraphPad Prism 9. 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 clear water and MPs-exposed conditions are shown in Fig. 7, 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, *T. suecica* and *N. gaditana* both achieved a steady-state cell density that was somewhat higher (36% and 20%, respectively) than that of Ulloa et al. (2012) and Fernandes (2015), 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), (10.14 ± 0.59) ×10^{6} cells/mL for *S. armatus* (day 12), and (46.73 ± 0.73) ×10^{6} cells/mL for *N. gaditana* (day 12).

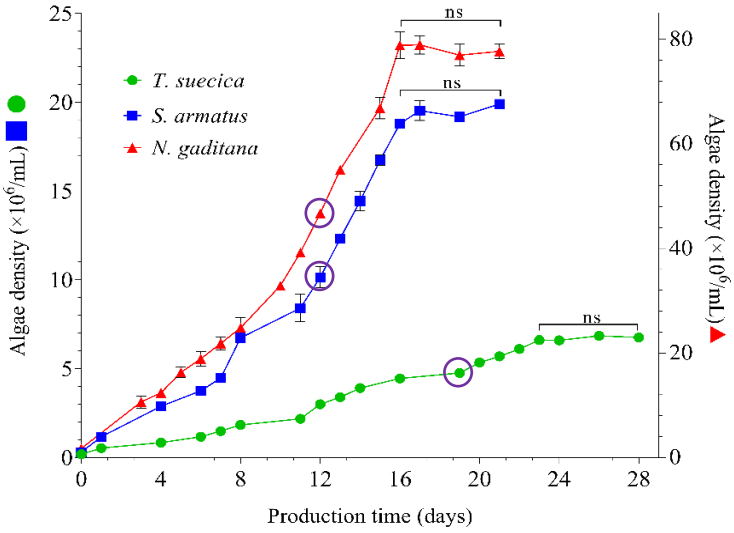


Fig. 7. Growth curves for *T. suecica*, *S. armatus*, and *N. gaditana*. *ns* - represents no significant difference among days (*p-value* ≥ 0.05) – stationary phase. Highlighted in a circle are the densities corresponding to four days before reaching the stationary phase, used in the acute and chronic experiments.

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 S2). 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. 8. 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. 8 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. 8A). 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. 8A, Table S2). 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 MPs particles might promote shading effects, block membrane pores 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, a decrease of up to 40%, effect on the diatom growth of *Skeletonema costatum* after 4 days of exposure.

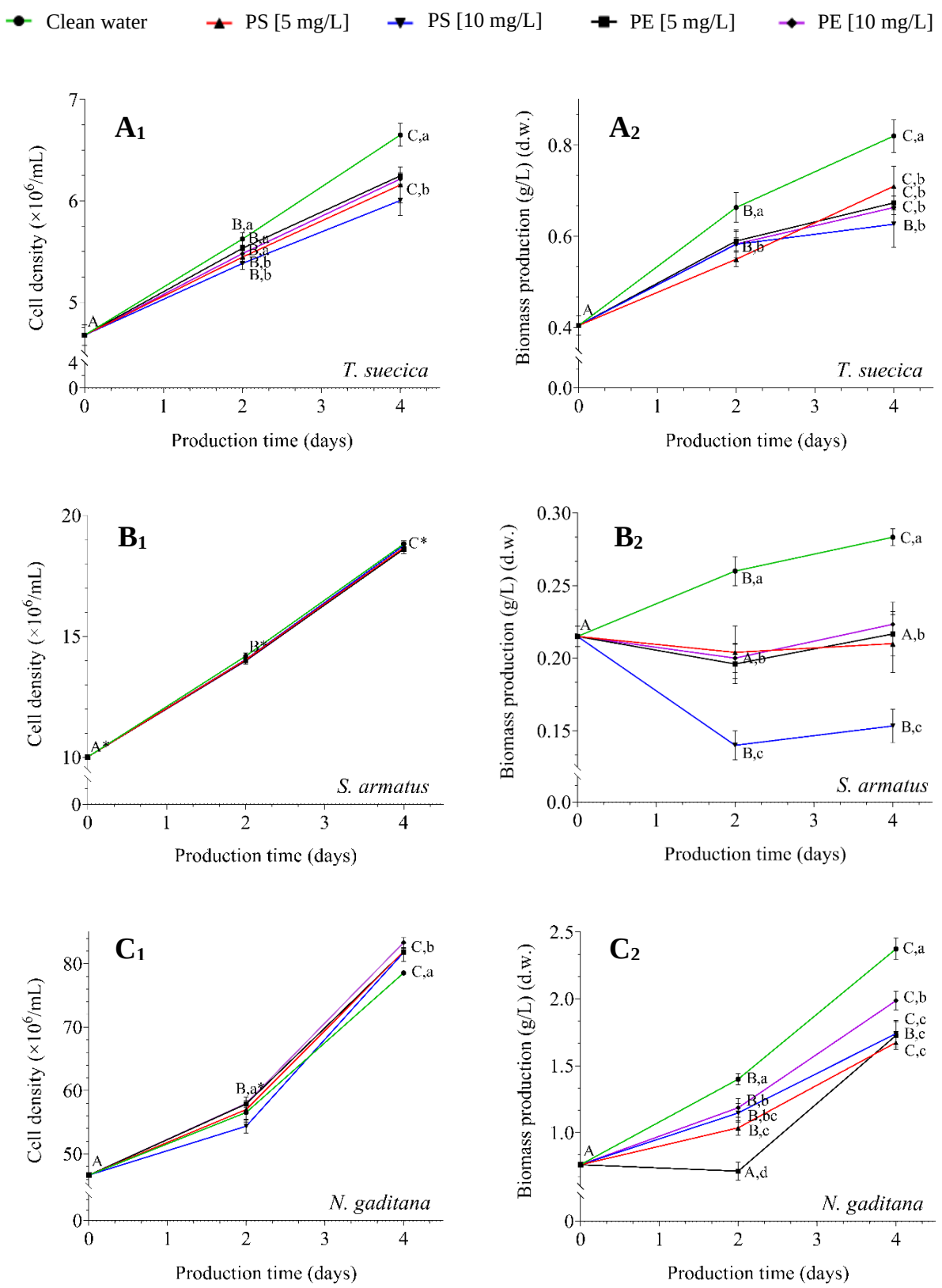


Fig. 8. Algae density (**A1, B1, C1**) and biomass production (**A2, B2, C2**) for each microalga cultivated in uncontaminated water and PS-MPs contaminated water (5 and 10 mg/L) throughout a 4-day production period of *T. suecica* (**A**), *S. armatus* (**B**), and *N. gaditana* (**C**). Different letters 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).

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. $9A_1$ 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. 10A), 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. $8A_2$). 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 S3). 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.

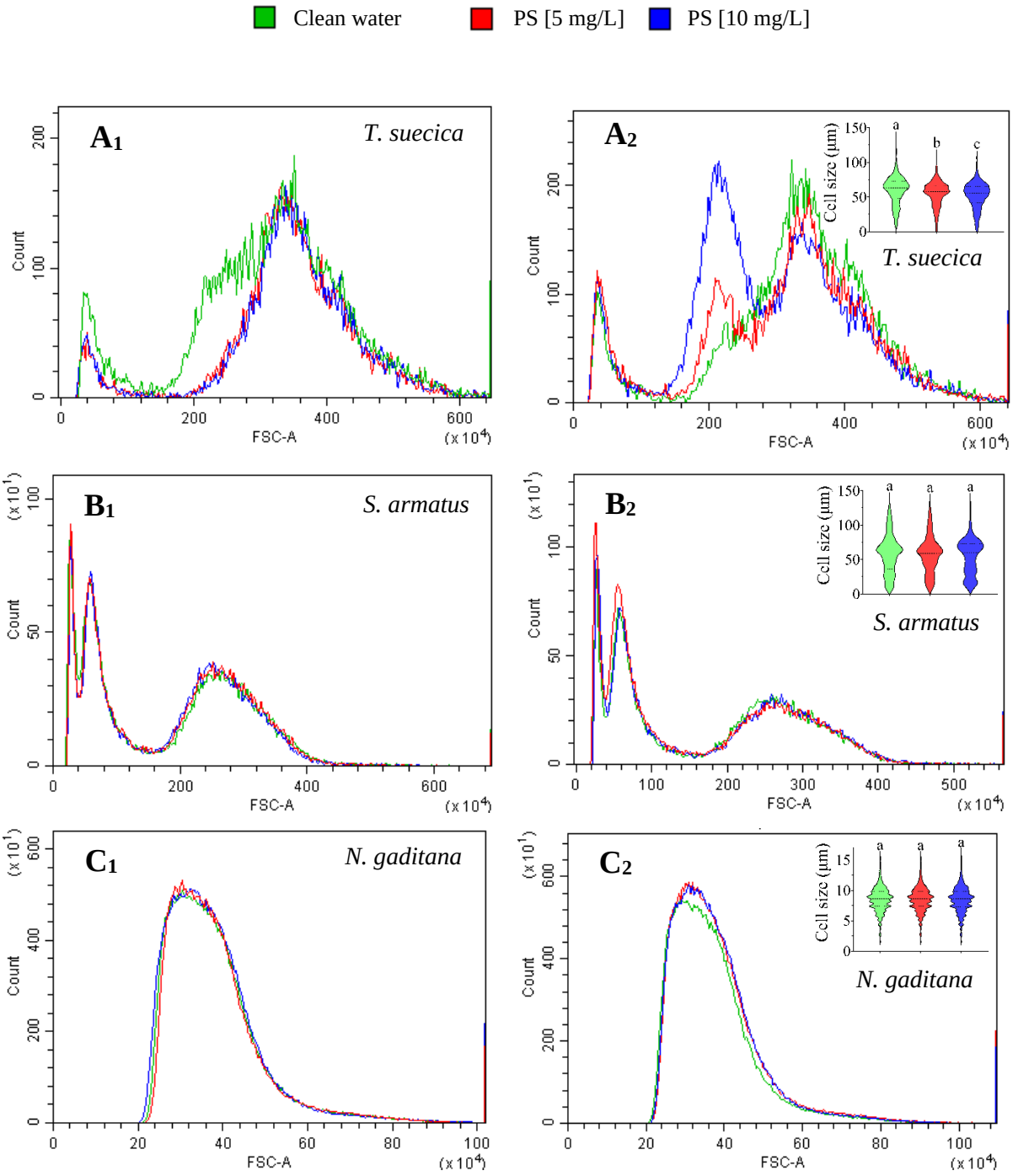


Fig. 9. Representative Representative forward (size) scattering histograms, and insert in histograms are the cell size graphics (obtained through fluorescence microscopy), of the clean water and for PS-MPs-

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.

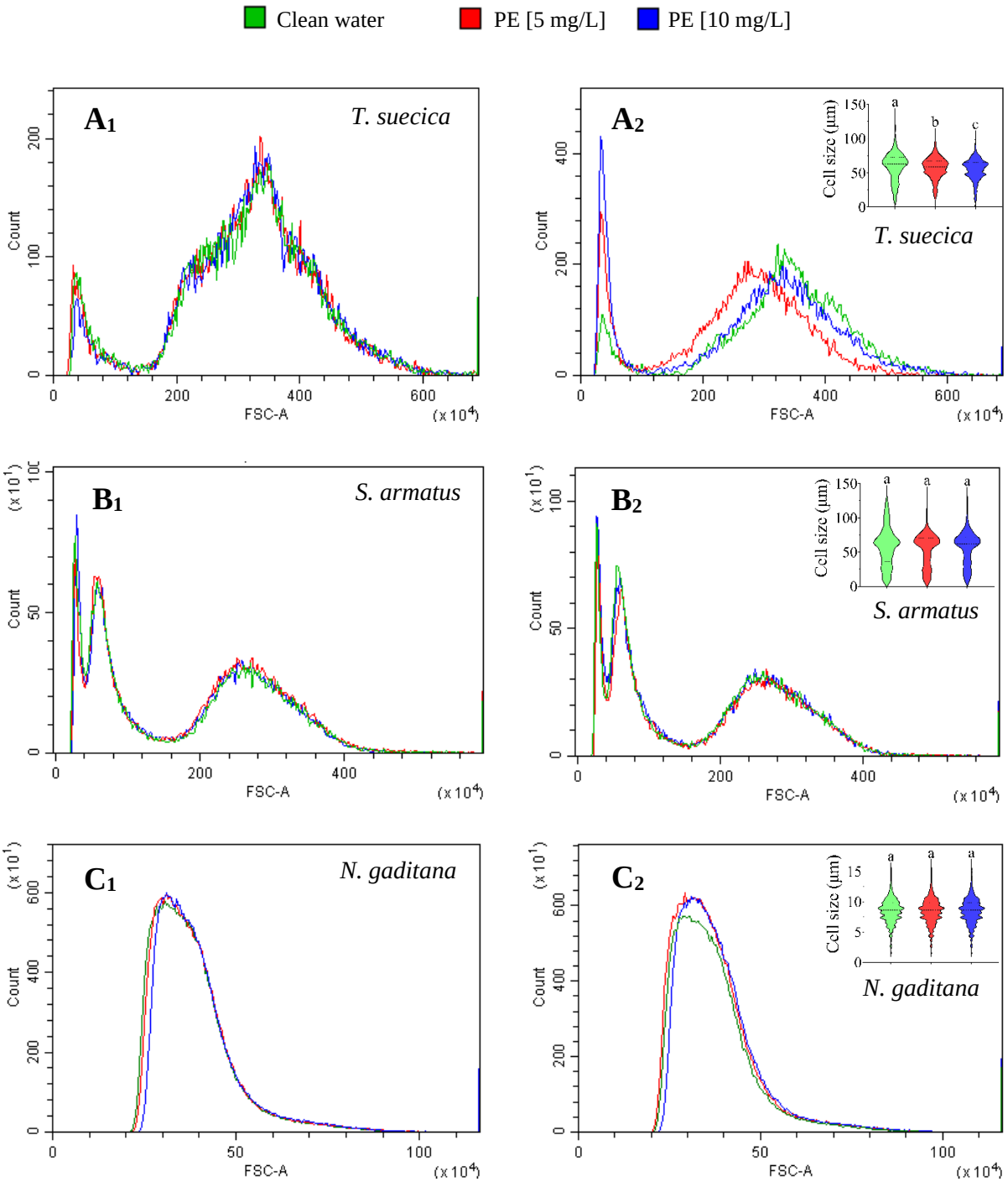


Fig. 10. Representative forward (size) scattering histograms, and insert in histograms are the cell size graphics (obtained through fluorescence microscopy), of the clean water and for PS-MPs-contaminated water conditions (5 and 10 mg/L), *T. suecica* (**A**), *S. armatus* (**B**), and *N. gaditana* (**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.

S. armatus demonstrated to counteract the presence of PS- and PE-MPs differently than *T. suecica*. Different microalgae possess distinct microalgal properties which dictate how MPs affect microalgae (Chae et al., 2019; Fu et al., 2019). *S. armatus* (Fig. $8B_1$) showed no significant differences in cell density throughout the 4-day production period for any MPs. Other research works 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), 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 cytometric histograms, compared to the clean water (Figs. 9B 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. $8B_2$ and Table S3), 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, it is hypothesised that this decrease in biomass productivity 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 16-44%), 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. 8C. In contrast to *T. suecica*, both MPs exhibited a significant stimulating effect of up to 6% in cell density on day 4 (Table S2). 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 & 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, 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 cytometric histograms (Figs. $9C_2$ and $10C_2$) in the same size, which might explain the increase in cell abundance presented in Fig. 8. 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. $8C_2$; Table S3), 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 exposition, *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. $8C_2$; Table S3). 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 when it comes to 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 the most vulnerability. On the other hand, lower concentrations of PE-MPs yielded the greatest impact on the 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 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. (2020), in which 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 *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. Further, 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 unknowingly shadowing the economic viability of microalgal-based industries.

Table 1. Variation of the weight per cell (normalised values) 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}	92.3 ± 1.14^{b,AB}	92.80 ± 1.99^{b,A}
	4	87.20 ± 0.38^{b,B}	87.44 ± 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). * Weight per cell (normalised values = microalgal cell weight in contaminated water / microalgal cell weight in clean water x 100; of the same day)

3.2.2. Chronic exposure

To the best of our knowledge, only one research 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-based industries (Cunha, Lopes, et al., 2020b). 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 at the same densities. Overall, this concentration impacted microalgae more and therefore was selected. After 27 days of production (Fig. 11A):

- ***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 registered 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. 11B), 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.

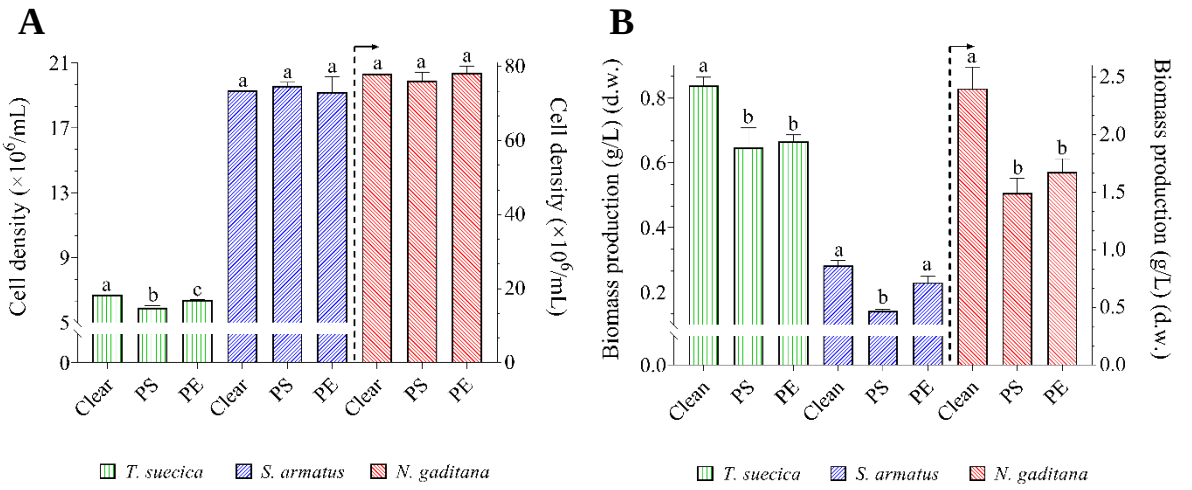


Fig. 11. Cell density (**A**) and biomass production (**B**) for the clean water and PS- and PE-MPs contaminated water conditions (10 mg/L) for a 27-day production period of *T. suecica*, *S. armatus*, and *N. gaditana*. Different letters represent significantly different means of the correspondent day (*p*-value < 0.05).

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. 14 and 15, respectively. Cell densities and biomass productions with lower rates are depicted in blue while red represents higher values. The data revealed:

- to ***T. suecica*** the data revealed that increasing the exposure time and the concentration of PS- and PE-MPs, respectively, caused a decline in the cell density. Biomass production (Fig. 13A) showed the same trend, in concordance with the previously discussed results;

- to ***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. 13B reveal that biomass production is expected to decrease by increasing the concentration of MPs;

- to ***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 intensified. In contrast, considering the variables examined (Fig. 13C_1), biomass production is at its lowest when the microalgae are exposed to high concentrations of PS-MPs for longer. The contour plot for biomass production in PE-MPs contaminated waters in Fig. 13C_2, illustrates that lower concentrations of PE-MPs lead to more severe decreases in biomass production, aligning with the previous results.

Equations 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 it knows the concentration of MPs in the water used and the production time, 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% to *T. suecica* (0.84 normalised values) and 26% to *S. armatus* (0.74 normalised values). Table S5 presents the regression equations that can be used to predict cell density.

The biomass production/cell density ratio (Table S4) was determined to establish the direct relationship between cell growth and biomass production and give indirect intel on the economic implications of using waters contaminated with MPs in the microalgal industry. Fig. S2 illustrates how exposure to high concentrations of PS- and PE-MPs during longer culture times generally have a detrimental impact on biomass output. Based on these response surface plots, this model enables the determination of the conditions that would most highly affect biomass. Table S6 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: *i*) no significant lack-of-fit (*p*-value > 0.05) was found for any of the models (*e.g.* Fig. S3); *ii*) value obtained experimentally R^2 and the model predicted values R^2 showed no significant differences (*e.g.* Fig. S4); *iii*) *p*-value less than 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.

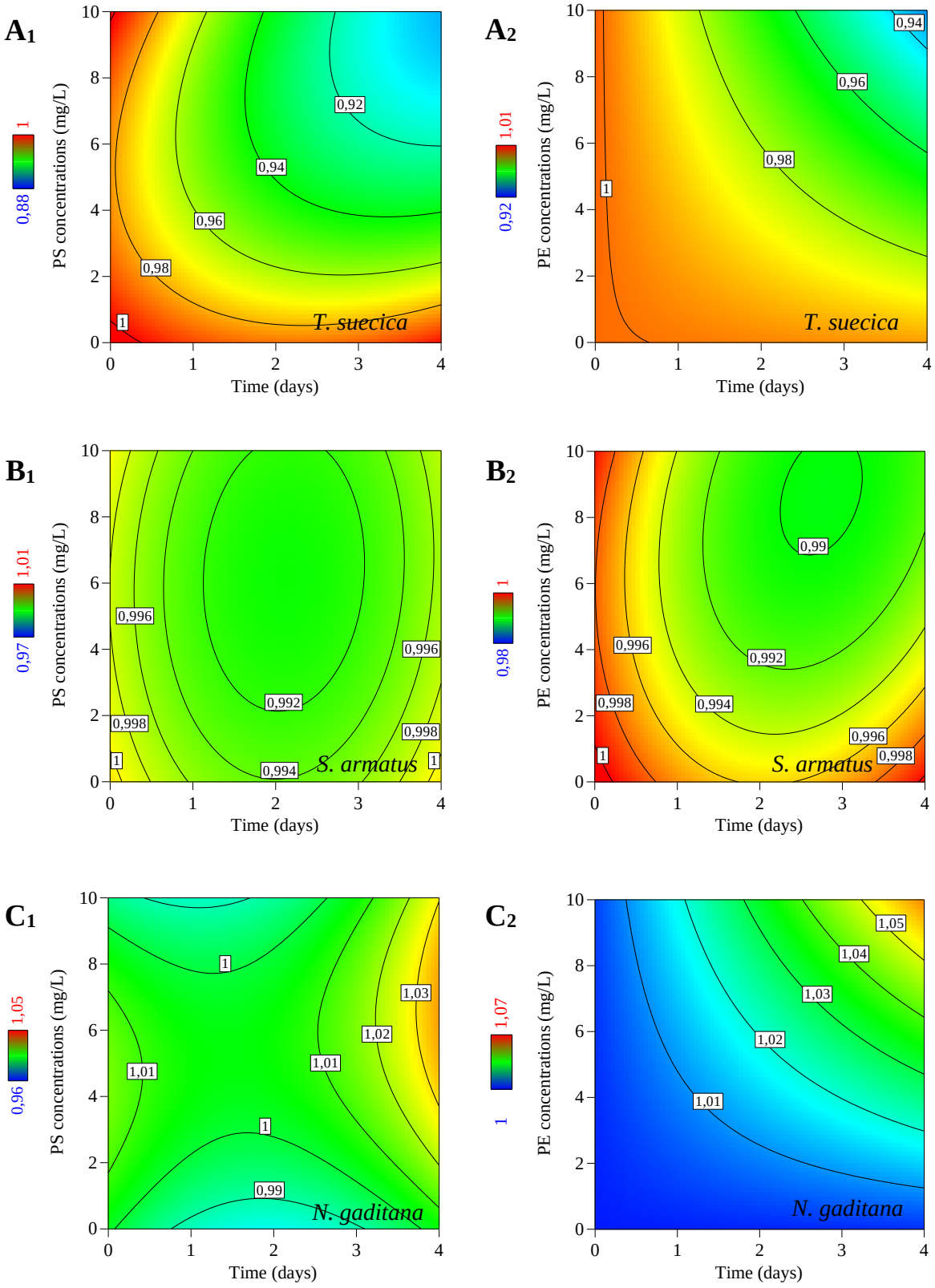


Fig. 12. Contour plots of cell density as a function of MPs concentration: PS (**1** in subscript: **A_1, B_1, C_1**), and PE (**2** in subscript: **A_2, B_2, C_2**); and the production time of T. *suecica* (**A**), *S. armatus* (**B**), and *N. gaditana* (**C**).

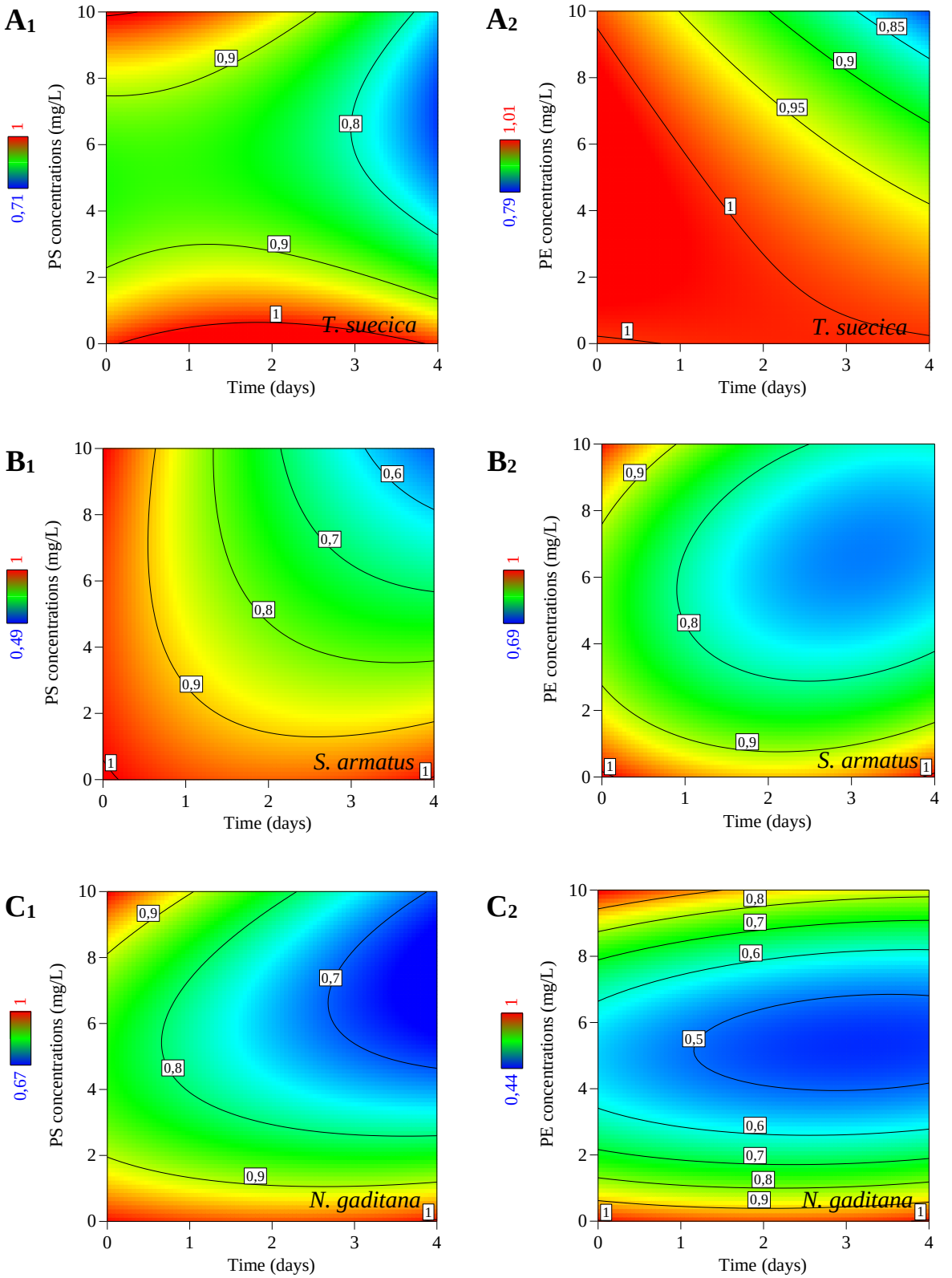


Fig. 13. Contour plots of biomass production as a function of MPs concentration: PS (**1** in subscript: **A_1, B_1, C_1**), and PE (**2** in subscript: **A_2, B_2, C_2**); and the production time of T. *suecica* (**A**), *S. armatus* (**B**), and *N. gaditana* (**C**).

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). *normalised values = contaminated water / clean water; of the same day. Biomass production decrease % = 100 x (1 – biomass normalised values)

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 present for the different microalgae the higher impactful conditions that give rise to the highest impact on biomass production. *N. gaditana* shows the greatest reduction in biomass production of 51% only with 52 hours of production using water contaminated with 5.40 mg/L of PE-MPs.

To confirm the applicability of this RSM function, up-scale production was carried out under the higher impactful conditions. 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 to verify the accuracy of the model. It can be concluded that the RSM has high prognostic ability and accuracy.

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*)		
		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

*normalised values = contaminated water / clean water; of the same conditions. Biomass production decrease % = 100 x (1 – biomass normalised values)

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 of up to 82% (Cunha, Lopes, et al., 2020b) 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 of 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 works are being done towards achieving these goals and urge others to do the same. Despite this, it hasn't 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.

CHAPTER III

Solving urban water microplastics with bacterial cellulose hydrogels: leveraging predictive computational models

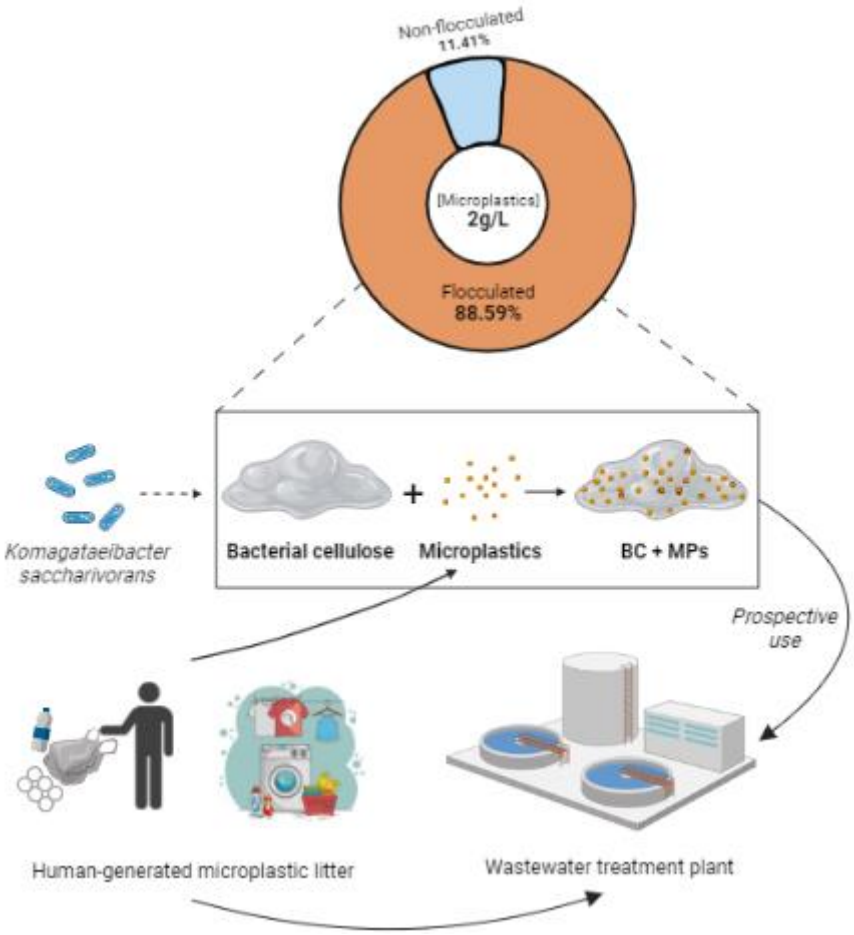
Solving urban water microplastics with bacterial cellulose hydrogels: leveraging predictive computational models

Abstract

The prevalence of microplastics (MPs) in both urban and aquatic ecosystems is concerning, with wastewater treatment plants being considered one of the major sources of the issue. As the focus on developing sustainable solutions increases, unused remnants from bacterial cellulose (BC) membranes were ground to form BC hydrogels as potential bioflocculants of MPs. The influence of operational parameters such as BC:MPs ratio, hydrogel grinding, immersion and mixing time, temperature, pH, ionic strength, and metal cations on MPs flocculation and dispersion were evaluated. A response surface methodology based on experimental data sets was computed to understand how these parameters influence the flocculation process. Further, both the BC hydrogel and the hetero-aggregation of MPs were characterised by UV–Vis, ATRFTIR, IGC, water uptake assays, fluorescence, and scanning electron microscopy. These highlights that the BC hydrogel would be fully effective at hetero-aggregating MPs in naturally-occurring concentrations, thereby not constituting a limiting performance factor for MPs' optimal flocculation and aggregation. Even considering exceptionally high concentrations of MPs (2 g/L) that far exceed naturally-occurring concentrations, the BC hydrogel was shown to have elevated MPs flocculation activity (reaching 88.6%: 1.77 g/L). The computation of bioflocculation activity showed high reliability in predicting flocculation performance, unveiling that the BC:MPs ratio and grinding times were the most critical variables modulating flocculation rates. Also, short exposure times (5 min) were sufficient to drive robust particle aggregation. The microporous nature of the hydrogel revealed by electron microscopy is the likely driver of strong MPs bioflocculant activity, far outperforming dispersive commercial bioflocculants like xanthan gum and alginate. This pilot study provides convincing evidence that even BC remainings can be used to produce highly potent and circular bioflocculators of MPs, with prospective application in the wastewater treatment industry.

Keywords: Bacterial cellulose; Biopolymer; Microplastics; Flocculation; Bioremediation.

Graphical abstract



1. Introduction

A growing widespread concern about deeply entrenched environmental changes caused by small plastic particles has arisen (Andrady, 2011b). These micropolymeric fragments, denominated microplastics (MPs), represent a global threat to several ecosystems: aquatic marine (Cole et al., 2011) and freshwater biospheres (Novotna et al., 2019); remote antarctic (Waller et al., 2017) and artic polar waters (Peeken et al., 2018); and urban networks (Sun et al., 2019).

MPs are defined as plastic particles with a diameter inferior to 5 mm (Arthur et al., 2009). Despite constituting the majority of marine debris, it is still not fully clear how MPs transversely affect aquatic biodiversity. Nonetheless, several studies have thoroughly reported extensive toxicity in marine vertebrates, microbiome, and benthic communities (Khalid et al., 2021; Ugwu et al., 2021), as well as in freshwater ecosystems (J. Li et al., 2018b). Moreover, MPs can also

induce toxic effects on fundamental layers of the aquatic biome through the release of several adsorbed persistent organic pollutants (Cunha et al., 2019; Khalid et al., 2021; Wang et al., 2020).

Less studied but not of lesser importance are urban water ecosystems. Given how critically important wastewater treatment plants (WWTPs) are to urban water distribution, these infrastructures have recently undergone extensive scrutiny. Currently, WWTPs do not possess the technological expertise to remove MPs, thus becoming a focal source of MPs' release into the environment. (Carr et al., 2016; Dey et al., 2021; Liu et al., 2021; Sarkar et al., 2021; Sun et al., 2019). Even with the recent development of techniques to remove MPs from waters (Poerio et al., 2019), these are still early-stage technologies with unsatisfactory environmental setbacks. Despite sustainability concerns, membrane systems such as membrane bioreactors (MBRs) show considerable promise. However, its membranes still represent a critical point of apprehension given their fossil-based/non-biodegradable nature and environmental unsustainable fouling removal mechanisms (Poerio et al., 2019; Wang et al., 2014). Therefore, it is imperative to advance the R&D of biomembranes to tackle this issue.

Accordingly, bacterial cellulose (BC), first reported by Dr. Adrian Brown in the 19th century (Brown, 1886), is a natural extracellular polymer produced as an output of bacterial biosynthetic processes (Blanco Parte et al., 2020; Rangaswamy et al., 2015). BC production is often restricted to bacterial species belonging to the *Komagataeibacter* genus, also known as *Acetobacter* and *Gluconacetobacter* (Lin et al., 2020; Yamada et al., 2012). Aside from its biosustainable nature, structurally-stable BC can be produced using cost-effective raw and cheap alternative carbon sources, such as agricultural, industrial and food wastes (Islam et al., 2017; Vazquez et al., 2013).

The BC biofilm exhibits unique structural features, such as a hydrated ultrafine three-dimensional nanofibril structure and high porosity, resulting in the formation of a hydrogel. This hydrogel displays a strong water absorption and retention capacity, chemical stability, large specific surface area, excellent mechanical strength/degree of polymerisation, and exceptional biocompatibility (Andriani et al., 2020; Blanco Parte et al., 2020; Choi and Shin, 2020; Klemm et al., 2005; Shah et al., 2013; Skvortsova et al., 2019). These distinctive properties are highlighted as BC has shown applicability and further promise in a wide range of fields, such as biomedical and tissue engineering (Liu et al., 2020; Moniri et al., 2017; Pang et al., 2020), food science (Azeredo et al., 2019; Lin et al., 2020; Paximada et al., 2016), cosmetics/pharmaceutical industries (Swingler et al., 2021; Ullah et al., 2016), and paper/textile manufacturing (Reis et al., 2019).

Despite some early environmental-related work focusing on BC and cellulose-based composites (Hussain et al., 2019; Ul-Islam et al., 2016), the most exciting advances regard the bioremediation potential of BC hydrogels in removing pollutants such as heavy metals, proteins, dyes and oil emulsions (Isik et al., 2018; Kurniawan and Yamamoto, 2013; Mohite and Patil, 2014; Wanichapichart et al., 2002). This highlights that BC might act as a highly multipurposed and sustainable alternative for complex bioremediation processes such as water treatment in WWTPs.

The fact that WWTPs still use inorganic flocculants presenting low flocculation efficiencies, no biodegradability, and several high health risk hazards/environmental sludge generation is daunting (Lee et al., 2014). Remarkably, several environmental-friendly alternatives such as microbial and plant-derived bioflocculants have gained recognition as truly unrealised potential sources of biomaterials with high flocculating power (Das et al., 2021; Li et al., 2020; Rebah et al., 2018). Still, and despite research having begun showing that microorganismal-derived biopolymers are highly effective in removing MPs from polluted water (Cunha et al., 2019; Cunha et al., 2020; Faria et al., 2022), there is still a complex road ahead in aligning their environmental benefits with industrial and economic demands.

Following the urgent need to develop top-performing sustainable alternatives, this laboratory-scale work aims to evaluate the potential of BC hydrogels as bioflocculants and hetero-aggregators of MPs towards their prospective removal from contaminated WWTP waters. Besides, the production of BC membranes yields unused and leftover BC remnants. To fully embrace the circularity aspect of the process, only BC remnants were used to create the hydrogel. A response surface methodology was employed to predict and deepen the optimisation of parameters such as grinding times, BC:MPs ratio, temperature, and immersion time. The influence of different mixing times, pH, salinity, and presence of metal ions was investigated. To understand the BC/MPs hetero-aggregates durability, the retention capacity of the MPs in the BC hydrogel was also assessed.

2. Materials and methods

2.1. BC hydrogel production and characterization

Komagataeibacter saccharivorans was used to produce the BC hydrogel (Supplementary ES1). The highest statistically significant BC production was achieved at 7 days (Fig. S2), after which the biofilm was isolated, treated with 0.5 M NaOH (80 ºC for 45 min), and washed with distilled water until neutral pH, and kept at 4°C pending usage (Faria, 2015; Faria et al., 2022). A moisture balance (Gibertini, Eurotherm, Novate Milanese, Italy) was used to determine the dry weight (106 ºC during 120 min). The BC was characterised by infrared spectroscopy (ATR-FTIR; Perkin Elmer, Llantrisant, United Kingdom), inverse gas chromatography (IGC; Surface Measurements Systems, London, United Kingdom), and water uptake capacity (Supplementary ES2-ES4). Visually, the hydrogel was characterised by fluorescence (Leica Microsistemas, Barcelona, Spain) and scanning electron microscopy (Hitachi, Montignyle-Bretonneux, France). To obtain the hydrogel, BC remnants (scraps) were ground from 1 to 20 min (Kunft, Worten, Portugal).

2.2. Microplastics

Commercial polystyrene (PS) (UV-Granulate, Magic Pyramid Bruecher & Partner KG, Frechen, Germany) was used in this study (Cunha et al., 2020a). This particular polymer was chosen given its vast occurrence in wastewater treatment plants (Sun et al., 2019). A milling machine (Kunft, Worten, Portugal) was used to fragment the plastics, which were then sieved (Analysensieb–Retsch, Scansci, Vila Nova de Gaia, Portugal) to obtain a fraction of MPs<100 µm (particle fraction difficult to remove from urban waters and known to be translocated into human tissues) (Cox et al., 2019; Sharma et al., 2021). A solution of polystyrene microplastics (PS-MPs) was prepared at a concentration of 2 g/L.

2.3. Flocculation activity

The flocculation activity of the BC hydrogel was measured using a standard clay-based methodology, as described by Kurane et al., 1986. Briefly, 1% of Ca^{2+} was added to the bentonite

clay suspension (Fonte da Areia, Porto Santo, Cordeiro et al., 2010) (4 g/L). The final solution had a pH of 7, to which BC was mixed into in a ratio of 25:1 (BC hydrogel wet weight (w.w)/MPs or clay dry weight (d.w.)). At 20 ± 2°C, the mixture was stirred at 300 rpm (Agimatic-E JP Selecta, V.Reis, Lisboa, Portugal) for 5 min and left resting for another 5 min. Subsequently, the solution was filtered (mesh size of < 112 µm; Krefeld, V.Reis, Lisboa, Portugal) and the optical densities (OD) of the unfiltered (without BC; OD_C) and filtered (OD_S) solutions were measured at 750 nm. The OD of the flocculant dispersion (OD_F) was subtracted to the OD_S, and the flocculating rate was calculated according to **Eq. (11)** (adaptation from Ndikubwimana et al., 2016):

$$\text{Flocculation rate (\%)} = \frac{OD_C-(OD_S- OD_F)}{OD_C} \times 100 \qquad \textbf{Eq. (11)}$$

The same experimental process was performed to assess the flocculation activity of the BC hydrogel relative to the particles being aggregated. All the experiments were conducted in triplicate.

2.4. MPs removal efficiency with BC hydrogel

2.4.1. Removal process

The MPs removal process was performed using a solution of PS-MPs with an exceedingly high concentration of 2 g/L. The BC hydrogel was added to the MPs-contaminated water, and different parameters were tested (in triplicate) to determine the hydrogel's behaviour in relation to several operational conditions (Supplementary Table S5). The mixture was adjusted relative to grinding times, BC:MPs ratio, temperature, pH, salinity and metal cations. The mixture was stirred at 300 rpm (Agimatic-E JP Selecta, V.Reis, Lisboa, Portugal) to potentiate hetero-aggregation formation (mixing times) and left resting for aggregate settling (immersion time) (Fig. S3) (based in Ma et al., 2019). The BC/MPs hetero-aggregate's height was measured to determine the dispersion (**Eq. 12**), after which the solution was filtered (mesh size of < 112 µm; Krefeld; V.Reis, Lisboa, Portugal). The absorbance of the MPs-contaminated water (solution of MPs or bioflocculant-free control) and the filtered solution were measured by the turbidity at 750 nm (UV-6300PC Spectrophotometer; V.Reis, Lisboa, Portugal). Moreover, the OD of the bioflocculant was subtracted to the OD obtained for each sample. The flocculation rate was

calculated according to **Eq. (11)** and the dispersion was determined using **Eq. (12)**, where *m* is the mass of BC (g), and *v* is the volume of the BC/MPs hetero-aggregate formed after the immersion time:

$$\text{Dispersion } (cm^3/g) = \frac{v}{m} \qquad \textbf{Eq. (12)}$$

2.4.2. Flocculation: computation

A response surface methodology (RSM) was used in the present work, which is a combination of mathematical and statistical tools that may be applied in multi-variable processes and to optimise the parameters of an experimental design. Within this method, the design of the experiment (DOE) model is employed to establish the correlation between the variables (in this case, grinding time, BC:MPs ratio, temperature, and immersion time) that are influencing a specific variable (in this case, the flocculation rate, and the dispersion) and the output/response of that process. Despite the empirical nature of the resulting quantitative connection, there is a good chance that a viable model will be created that can be used to separate ineffective factors from effective ones and optimise operations (Breig & Luti, 2021). Several statistical analyses are taken into account to evaluate the model's validity (Domagalski et al., 2015), namely, the examination of the residuals (difference between the data point and the regression line), lack of fit (difference between the model prediction values and the experimental data), and analysis of the *p*-value and F-value (ANOVA analysis). The Minitab software was used to design the experiments considering the following parameters: grinding time (1– 10 min), BC:MPs ratio (1.25:1–37.5:1 w. w./d.w.), temperature (15–30 ± 2 °C) and immersion time (0–120 min) – Supplementary Table S6.

2.4.3. Flocculation: experimental

All the flocculation tests (Supplementary Table S5) were conducted using the above mentioned method. Different ratios of BC (wet weight) and MPs solution (2 g/L) were explored to determine its influence on the flocculating activity (1.25:1 – 37.5:1, w.w./d.w.). The impact of the grinding times of the hydrogel (1-20 min), the effects of different immersion times (0 – 360 min), mixing times (5-30 min), and temperatures (4-30°C) were tested. The influence of the pH (3-8) was also considered: MPs solutions were adjusted (HI96100, Hanna Instruments Póvoa de Varzim, Portugal) using HCl (0.1M) and NaOH (0.5M). In addition, various salinity

concentrations (Hand Held Refractometer, Labbox, Spain) of 0, 10, 15 and 37‰, as well as the effects of metal ions (4.5 mM) such as Fe^{3+}, Ca^{2+}, Mg^{2+}, and K^{+} were measured. Supplementing this parameter, Fe^{3+} was chosen to evaluate the effects of several cation concentrations (4.5, 9 and 18 mM). All the experiments were conducted in triplicate.

2.4.4. Retention capacity

The capacity of the BC hydrogel to retain MPs was evaluated. After collecting the BC/MPs hetero-aggregates formed with the ratios BC:MPs of 25:1 and 50:1, the hetero-aggregates were kept under agitation (300 rpm) for 24h in distilled water to clear the hydrogel of MPs. This will ensure the release of all removable microparticles. The BC/MPs hetero-aggregates were formed with the following conditions: 2 min of grinding time, 5 min of mixing time, and 60 min of immersion time at 20°C. One wash cycle for each of the ratios was performed in triplicate. The OD of the solutions was measured at 750 nm and the MPs retention rate was calculated according to **Eq. (13)**, where the OD_S is the sample before the wash cycle, OD_{MPs} is the MPs-contaminated water, and OD_{SW} is the sample after the wash cycle, respectively:

$$\text{Retention rate } (\%) = \frac{OD_{SW}}{OD_{MPs}-OD_S} \times 100 \qquad \textbf{Eq. (13)}$$

2.5. Fluorescence microscopy

Fluorescence microscopy was used to document the retention of MPs in the BC hydrogel, using a Leica DM2700 device attached to a Leica DFC450 C digital camera and a CoolLED pE-300 lite lighting system (Leica Microsistemas, Barcelona, Spain). The MPs' fluorescence was observed with a 450-490 nm/515-565 nm excitation/emission filter I3.

2.6. Scanning electron microscopy

The BC hydrogel and BC/MPs hetero-aggregates were analysed using scanning electron microscopy (SEM; Montigny-le-Bretonneux, France). Samples were lyophilised, coated with a thin layer of carbon (EMITECH K950X Turbo Evaporator) and deposited on a steel plate. An HR-FESEM SU-70 Hitachi Scanning Electron Microscopy equipment (5 kV beam; 15.6 mm

working distance; field emission mode) was used to acquire the SEM micrographs. Images were collected at magnifications of 100x and 400x.

2.7. MPs removal with commercial bioflocculants

The BC hydrogel flocculation capacity was compared to commercial bioflocculants, xanthan gum (11472781/MP Biomedicals) and alginate (A3249/PanReac). The flocculating tests were conducted using the method described previously: after an immersion time of 1h, 1 mL was removed (*ca.* 3.5 cm from the top) and its OD absorbance was measured at 750 nm.

2.8. Data and statistical analysis

The results were presented as the mean values ± standard deviation (SD) of three replicates. Data representation and statistics were performed using GraphPad Prism 8. The D'Agostino-Pearson omnibus and Kolmogorov-Smirnov normality tests were used to assess the Gaussian distribution of data. 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. BC physicochemical characterisation

The ATR-FTIR spectra (Fig. S4) of the bacterial cellulose (BC) exhibited the characteristic absorption bands of cellulose at around: 3347 cm^{-1} (O-H stretching vibrations); 2899, 1434, 1373 and 1312 cm^{-1} (C-H and C-H_2 stretching vibrations); 1632 (C-H or O-H bending vibration), and 1064 cm^{-1} (C-O stretching vibration) (Faria et al., 2019; Pecoraro et al., 2007; Pretsch et al., 2009).

Further, in terms of industrial applicability and economic scalability, BC drying greatly increases logistics and implementation practicality. Thus, the BC remnants were ground and oven-dried. Firstly, the water content of the hydrogel was determined to be 98.70 ± 0.08%. Also, considering that the BC is fully immersed in water during the MPs removal process, it is crucial

to understand how the water uptake capacity (swelling behaviour) of the BC evolves with time. The re-hydration potential, which plays a significant role in allowing for particle aggregation (Hamidi et al., 2008), is central in allowing the BC hydrogel to recover its full structural integrity. Accordingly, the oven-dried (40 ± 2°C) BC hydrogel exhibited a solid swelling behaviour of 300% after 2 hours and saturated at 580% its dried weight after 8 hours (Fig. S5). This confirms that the BC hydrogel can be easily dried and re-hydrated to regain its original properties.

3.2. Flocculation activity

Clay is typically used as a standard in flocculation assays (Cunha et al., 2020b). A flocculation activity assay using clay showed that the BC hydrogel (500 mg d.w./L) flocculated 27.12 ± 0.63% of particles present in the water. Despite the BC hydrogel not flocculating clay particles as efficiently, this biopolymer still showed a solid theoretical basis for fulfilling its objective: the flocculation and hetero-aggregation of MPs. Thus, the flocculation of MPs was studied under the same conditions used for the clay flocculation, and a MPs flocculation rate of 74.54 ± 3.00% was obtained **(**Fig. S6**)**. The differences in the flocculation rate of BC with clay and PS-MPs are due to their acid/base properties. Inverse Gas Chromatography (Supplementary ES3) reveals that BC has an approximately amphoteric character (K_b/K_a=0.80) and that clay has a basic character (K_b/K_a =5.50) much more basic than PS-MPs (K_b/K_a = 1.94). Thus, interactions between the acidic and basic groups of BC hydrogel are more significant with the PS-MPs.

So, the first aim of applying the BC hydrogel was to predict how the flocculation rate of the BC hydrogel would vary under several potential operational conditions found in wastewater treatment plants. Thus, response surface methodology (RSM) was employed (Supplementary Table S6) to determine the degree to which parameters such as grinding time, ratio, temperature, and immersion time might influence the flocculation rate of the BC hydrogel (Fig. 14). Based on registered experimental values and computational modulation, the parameter exhibiting the most statistically significant impact on the flocculation of MPs was the ratio (BC:MPs). The grinding time, immersion time and temperature ranked as the following most impactful parameters to flocculation activity. Therefore, these parameters were sequentially explored to determine the optimal conditions for flocculation of MPs using the BC hydrogel.

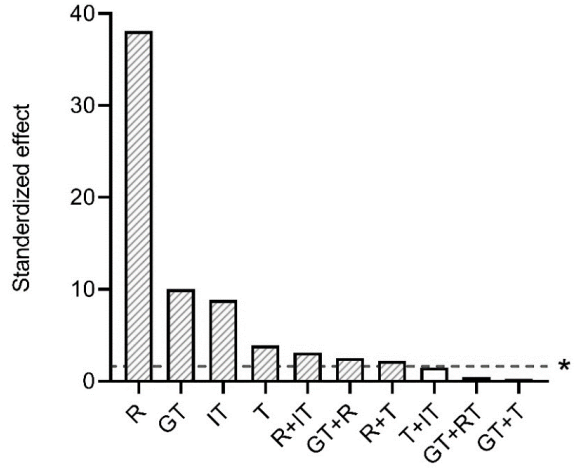


Fig. 14. Pareto chart on the degree of influence of four parameters (grinding times, ratio, temperature, and immersion times) in determining flocculation activity. * The horizontal line represents the threshold of statistical significance. **R**: Ratio; **GT**: Grinding time; **IT**: Immersion time; **T**: Temperature.

Under favourable conditions, the best biosorption performance is usually reached at the optimal bioflocculant dosage (Salehizadeh and Yan, 2014; Zeng et al., 2019). Despite a greater porous surface area available theoretically leading to an increase in particle flocculation, there is a saturation point at which the bioflocculant concentration no longer improves flocculation performance (Maksoud et al., 2020). Based on the model in Fig. 14, the effect of the BC:MPs ratio was analysed to determine the most cost-effective dosage for the MPs flocculation process. As shown in Fig. 15A, the percentage of MPs removal reached a maximum of 80.42 ± 2.48% with a BC:MPs ratio of 25:1. At this ratio, the BC hydrogel is no longer the limiting factor of optimal flocculation and aggregation of MPs. Further, it is shown that the flocculation activity is at its highest when the dispersion is at its lowest. It is noteworthy that the dispersion is calculated after flocculation.

To understand how different grinding times affect the efficiency of the hydrogel as a bioflocculator in the optimal ratio (25:1 BC:MPs), the BC biopolymer was ground from 1-20 min (Fig. 15B). Unsurprisingly, it becomes apparent that the hydrogel's structural integrity is critical in modulating particle aggregation. The highest flocculation rates (83.67 ± 0.79% and 80.42 ± 2.48%) were obtained for grinding times of 1 and 2 min, with no significance found between. A consistent decrease in flocculation rate is observed with increased grinding times, reaching a plateau at 10 min. Dispersion does not show to move inversely to flocculation rates.

According to the RSM model in Fig. 14, the third parameter affecting flocculation the most is the hydrogel's immersion time. Using the optimal parameters of 25:1 BC:MPs ratio and 2 min of grinding time, the effect of progressively increased immersion times is shown in Fig. 155C. Over time, the flocculation rate increased in contrast to decreased dispersion. It is documented that a maximum flocculation rate is reached at an immersion time of 60 min. Longer immersion times are expected to yield better flocculation results, given that time is a crucial variable in potentiating particle adsorption, providing MPs a longer frame to access the biopolymer's active sites (Maksoud et al., 2020) and reach an equilibrium (Sargin et al., 2019).

Lastly, the temperature has been documented as a modulationg factor during the flocculation process (Maksoud et al., 2020; Vajihinejad et al., 2019). Fig. 15D shows the relationship between temperature and flocculation rate at optimal conditions (25:1 BC:MPs ratio, 2 min of grinding and 60 min of immersion time). The BC hydrogel showed a lower flocculation rate at lower temperatures (4 and 15°C). Room temperatures (20-25 °C) facilitate the flocculation process, highlighting the lack of necessity for specific operating conditions using BC hydrogels. The relation between lower flocculation activity and lower temperature has been hypothesised to be driven by the attenuation in the binding force between the bioflocculant and the particles (Maksoud et al., 2020). Considering that the dispersion is not inversely affected here, temperature might play a role in particle binding but not particle release.

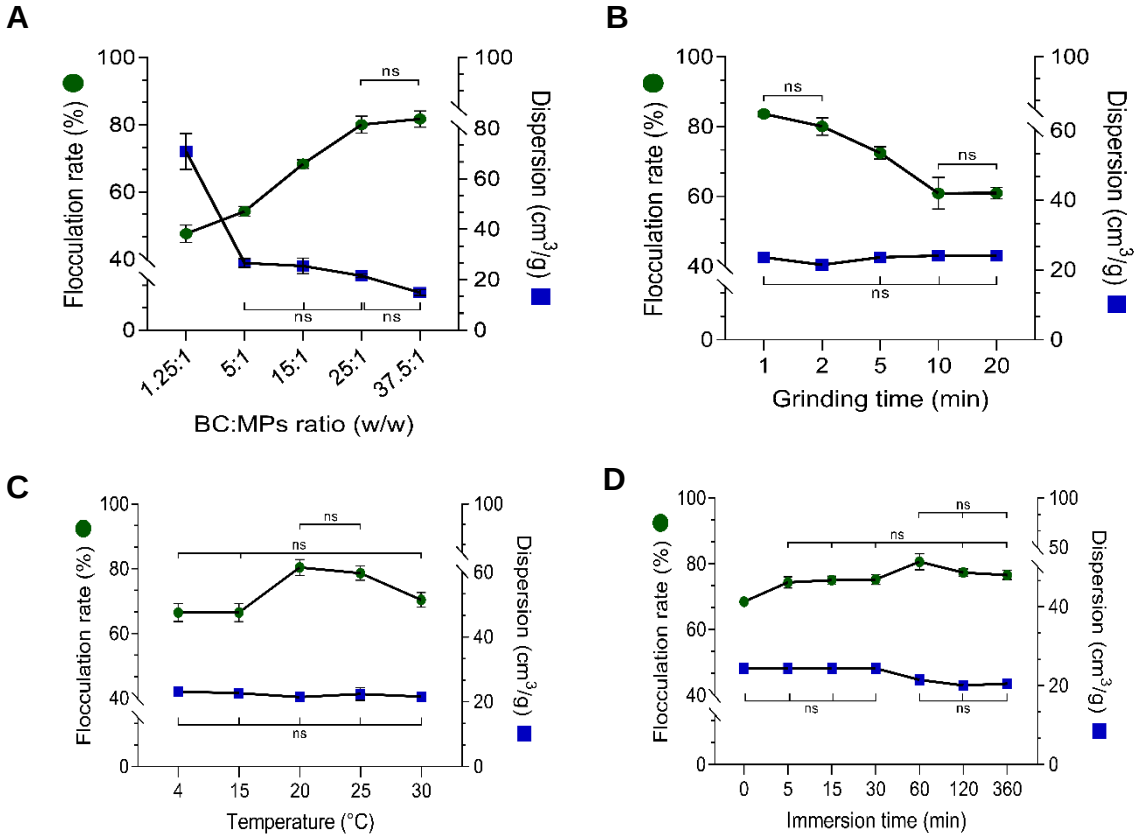


Fig. 15. Effect of different conditions on the flocculation and dispersion activities of the BC hydrogel regarding MPs. (**A**) BC hydrogel:MPs ratio; (**B**) grinding time; (**C**) immersion time; (**D**) temperature. *ns*: non significantly different ($p \geq 0.05$).

3.3. Microscopy: BC hydrogel and MPs hetero-aggregation

Fluorescence and scanning electron microscopy were employed to visualise the retention of MPs in the BC hydrogel network. The micrographs unveiled that the MP particles are retained in the cellulosic network of the bioflocculant (Fig. 16B), with a substantial reduction in the number of particles outside the hydrogel (Fig. 16A, C).

Scanning electron microscopy confirmed the expected three-dimensional (16D) fibrillar network and porous gel-like microstructure of the BC hydrogel. Considerable structural differences between the BC hydrogel ground for 1 min (Fig. 16D) and 20 min (Fig. 16E) are observed. Moreover, both the adsorption and incorporation of MPs in the hydrogel are displayed in Fig. 16F. The porous nature of the hydrogel is likely the primary factor driving the flocculation

and aggregation of MPs. As a consequence of excessive grinding, the breakage in the 3D structural integrity and consequent pore collapse likely causes the decreased flocculation rates observed in Fig. 16B.

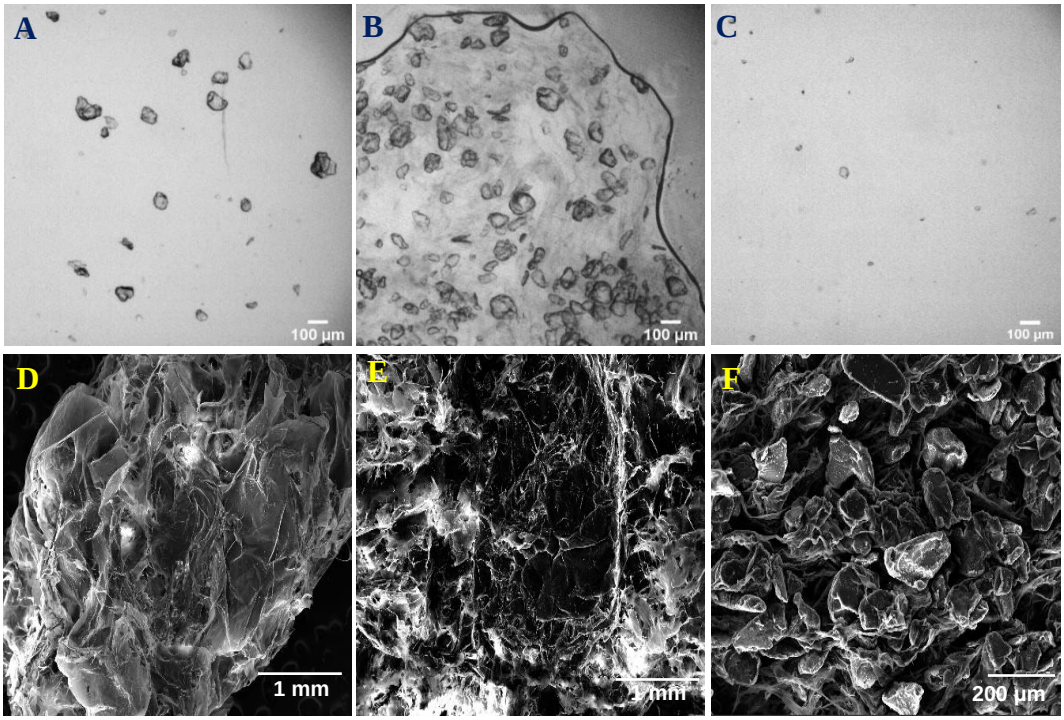


Fig. 16. Fluorescence micrographs: (**A**) MPs solution before flocculation; (**B**) BC/MPs hetero-aggregate; (**C**) MPs remaining after flocculation. Scanning electron micrographs: (**D**) BC hydrogel after 1 min of grinding; (**E**) BC hydrogel after 20 min of grinding; (**F**) MPs adsorbed and embedded in the BC hydrogel.

3.4. Experimental meets computational flocculation

In order to understand how flocculation would vary in a vast array of experimental conditions, a contour plot was computed. Based on the experimental design parameters expressed in Supplementary Table S6, contour plots of the variables under study were generated to display the region of optimal factor setting for both flocculation (Fig. 17A) and dispersion (Fig. 17B). With the model assuming a hold temperature of 22.5 °C and an immersion time of 60 min, it is clear that decreasing grinding times while increasing the ratio of BC:MPs are expected to yield higher flocculation rates (Fig. 17A). On the other hand, assuming a grinding time of 5.5 min and

a temperature of 22.5 ºC is expected to yield higher dispersion at lower ratios of BC:MPs independently of the immersion time.

To experimentally confirm the reliability and predictability of the computed model, several previously untested values were trialled. As shown in Fig. 17A, a 30:1 BC:MPs ratio with a grinding time of 3 min (a*) yielded the expected >80% flocculation rate (87.2%). Further, a 20:1 BC:MPs ratio with a grinding time of 5 min (b*) yielded the expected 64-72% flocculation rate (71.48%). A 1.25:1 BC:MPs ratio with a grinding time of 9 min (c*) yielded a flocculation rate of 52.57%. Despite this latter not matching the exact predicted region of values, the experimental value still falls in the lower flocculation rate region (blue). This highlights that the model can reliably predict and distinguish high from low-performance MPs bioflocculant conditions for the BC hydrogel, widely facilitating potential industrial optimisations. The same line of thought was applied for the computation of dispersion (Fig. 17B). A 30:1 BC:MPs ratio with an immersion time of 20 min (a*) yielded a dispersion of 8.06 cm^3/g. The same was observed for the 20:1 BC:MPs ratio with an immersion time of 60 min (b*), yielding the expected 15-20 cm^3/g dispersion (17.62 cm^3/g). Predicted dispersive values of 3.75:1 BC:MPs ratio and an immersion time of 40 min (c*) yielded a dispersion of 158.12 cm^3/g, highlighting not how exactly predictive the model is but how it can separate optimal from poor bioflocculant performance. Both predicted and confirmed experimental values corroborate the data presented in Fig. 14, showing that the ratio and grinding times are the most important variables modulating the flocculation rate.

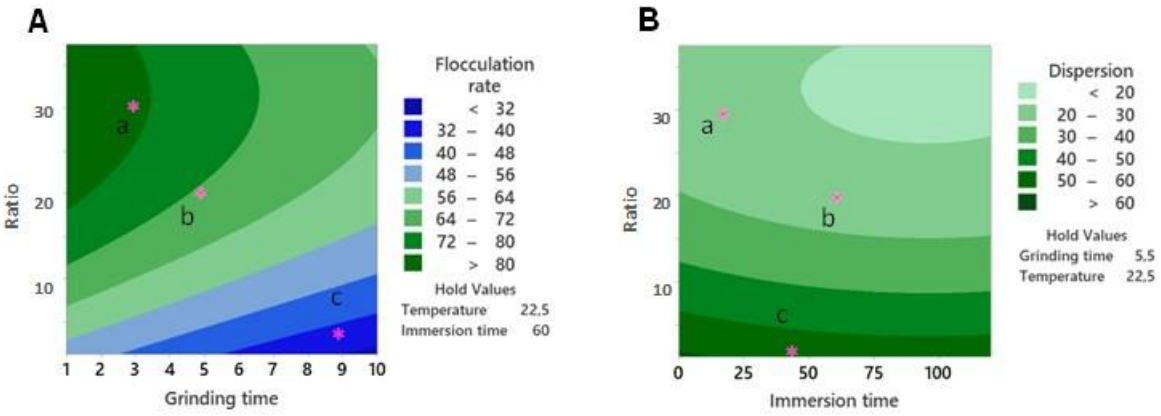


Fig. 17. Contour plots: (**A**) flocculation rate as a function of the ratio and grinding times, with constant temperature and immersion times; (**B**) dispersion as a function of the BC hydrogel:MPs ratio (w.w/d.w.) and immersion times. *experimentally-confirmed values.

Fig. 18 shows a simplified factorial plot exhibiting the flocculation and dispersion in response to each variable. Individual plots exhibit the value in which the flocculation/dispersion is highest and lowest. The optimised paraments are presented as a grinding time of 1 min, a BC:MPs ratio of 31.65:1 (BC hydrogel:MPs ratio), temperature of 22.9°C and an immersion time of 76 min. The optimal values were used experimentally and confirmed to yield the highest flocculation rate (88.59 %) and the lowest dispersion (17.62 cm^3/g). This confirms that lower grinding times, higher BC:MPs ratios, room temperatures, and increased immersion times are associated with increased flocculation rates and decreased dispersion.

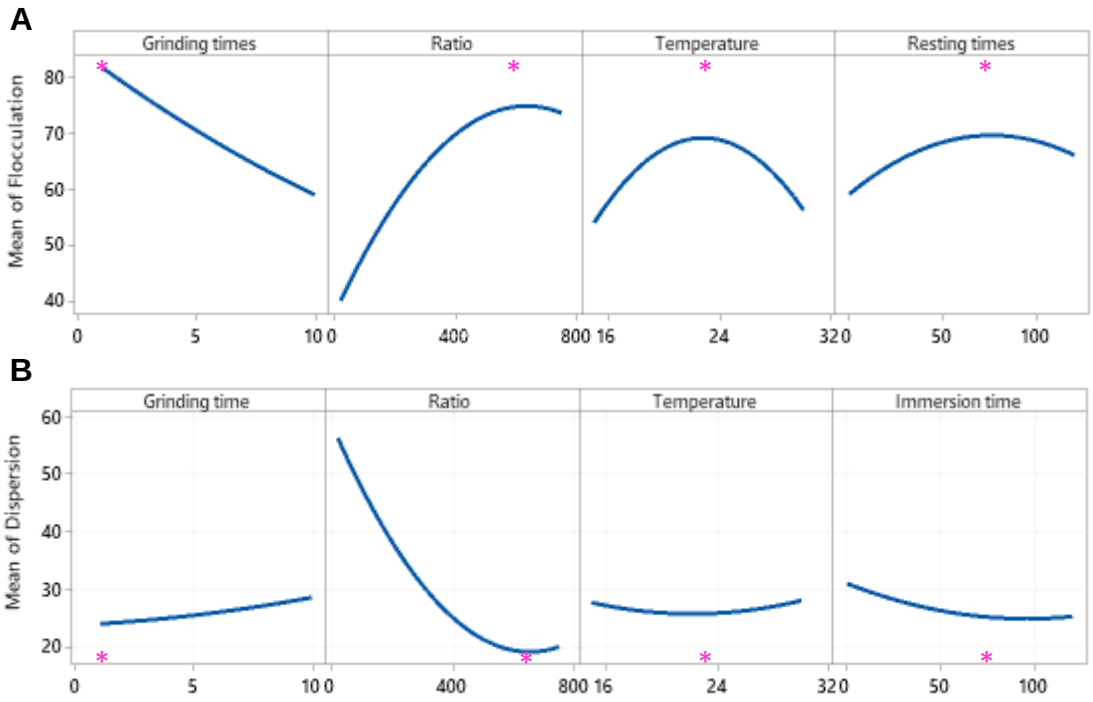


Fig. 18. Factorial plot of (**A**) flocculation rate and (**B**) dispersion, in function of grinding times, BC:MPs ratio, temperature, and immersion times. *experimentally-confirmed values.

The regression equations defining the experimental correlations are displayed in **Eq. (14)** and **Eq. (15)**, where *GT* is the grinding time, *R* is the BC:MPs ratio, *T* is the temperature, and *IT* is the immersion time. These equations can be applied to predict the response of flocculation or dispersion when adjusting the levels of each variable.

$$\text{Flocculation rate (\%)} = -86.2 - 3.12GT + 0.118R + 11.30T + 0.275IT + 0.0566GT^2 - 0.000093R^2 - 0.2478T^2 - 0.001814IT^2 \quad \textbf{Eq. (14)}$$

$$\text{Dispersion (cm}^3\text{/g)} = 81.0 + 0.21GT - 0.1224R - 1.67T - 0.127IT + 0.027GT^2 + 0.000094R^2 + 0.0376T^2 + 0.00063IT^2 \quad \textbf{Eq. (15)}$$

The applicability of the models was evaluated using residuals, lack of fit, and ANOVA analysis. The residual vs. predicted values shows a uniform distribution, and the lack of outliers suggests no overtclear indicators of normality (Figs. S6A and S6C). None of the models had a significant lack-of-fit (*p*-value > 0.05), and each model had a coefficient of determination over 0.96 (Figs. S6B and S6D), indicating the suitability of the models. The values of the adjusted R^2 and the predicted R^2 did not differ significantly (< 0.2). Additionally, since the coefficient of variations was low, the values are considered to be good indicators of the reproducibility of the responses studies. The *p*-value less than 0.05 and the *F*-value of 152.5 for the flocculation rate and 112.04 for the dispersion, also led to the conclusion that the model is applicable.

Taking into account the knowledge of the optimal ratio (31.65:1) and considering a MPs-contaminated water with 2 g/L, the BC hydrogel was able to hetero-aggregate roughly 1.77 g/L of polystyrene MPs, which is far beyond any urban concentration reported to date. Also, the optimal amount of BC to use for different levels of MPs contamination can be determined. For example, for a MPs-contaminated water with 50 mg/L, a mass of 2.06 g of BC hydrogel or 18.2 mg of dry BC remnants per litre should be used (Fig. S7).

3.5. Mixing time, pH, salinity and cations

Beyond the two parameters linked to the bacterial cellulose (BC) hydrogel: BC:MPs ratio and grinding time and the two parameters associated with operational conditions: temperature and immersion time, variables such as mixing time, pH, salinity, and the presence of metal cations would be anticipated to considerably influence the flocculating activity of the biopolymer.

Mixing during the flocculation process is expected to facilitate particle adsorption to the biopolymer, leading to the formation of hetero-aggregates formed by BC and microplastics

(BC:MPs) (Al-Shamrani et al., 2002; Aljuboori et al., 2015). Fig. 19A demonstrates that a shorter mixing time of 5 min displays a higher flocculation rate (80.42 ± 2.48%) compared to longer mixing times of 10 or 30 min. These findings denounce that short exposure times are enough to drive strong particle aggregation, highlighting that MPs absorb and hetero-aggregate in the BC hydrogel fairly quickly. Importantly, increased mixing times can lead to the breakage of BC/MPs hetero-aggregates and consequent decrease in flocculation rates (Gregory, 1991). However, dispersion does not change as mixing times increase, dismissing the possibility of meaningful polymer destruction and strengthening the hypothesis that particle aggregation happens rapidly. The fact that a mixing time of 5 and 30 min yielded statistical similar flocculation rates suggest that over a long enough period of time, BC/MPs hetero-aggregates are able to re-arrange and re-form.

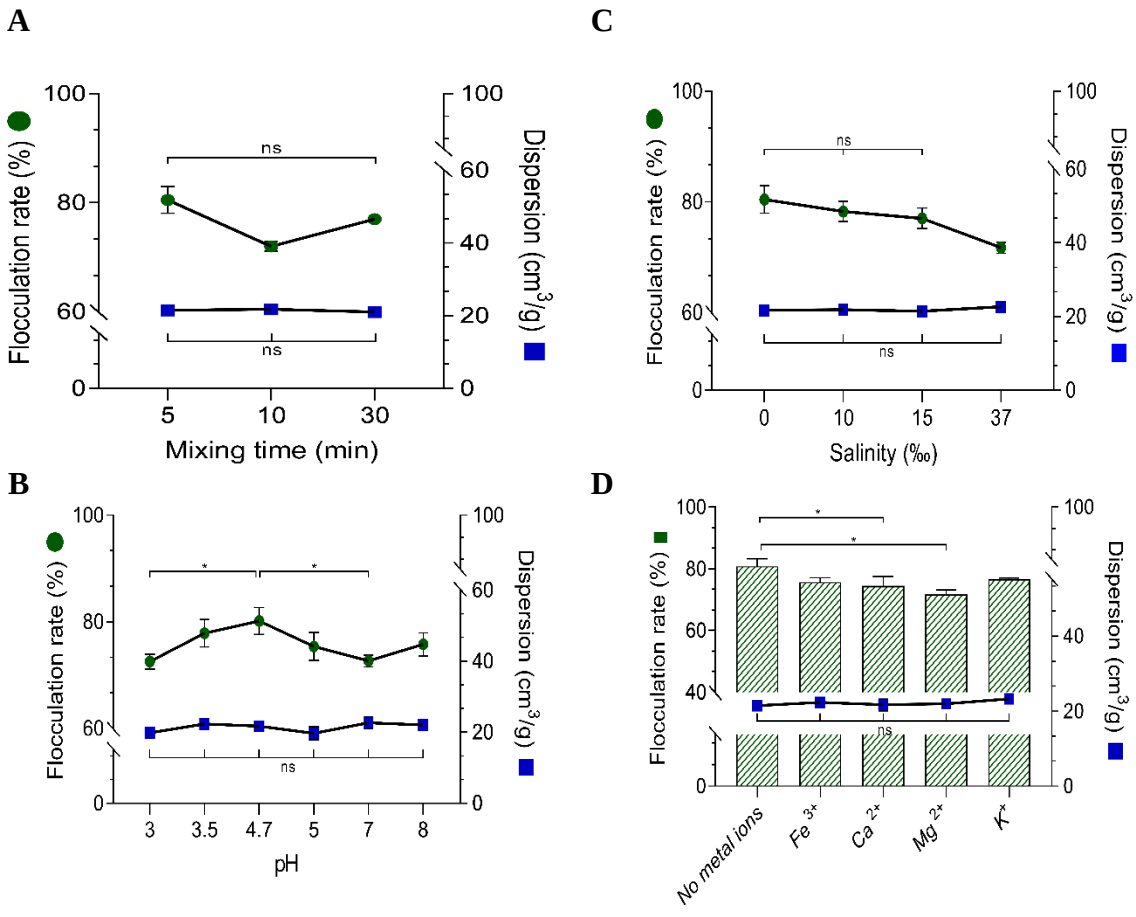


Fig. 19. Effect of different conditions on the flocculation rate of the BC hydrogel towards MPs-contaminated water and dispersion after flocculation. (**A**) mixing time; (**B**) pH; (**C**) salinity; (**D**) metal ions presence (4.5 mMol). * significantly different ($p < 0.05$). *ns*: non significantly different ($p \geq 0.05$).

The pH is another important factor that is known to affect the activity of bioflocculants (Nwodo and Okoh, 2013) by influencing the stability of the microparticles and the hetero-aggregate formation (Ugbenyen and Okoh, 2014). Thus, a wide variation of pH ranging from 3–8 was tested to understand its influence on flocculation and dispersion (Fig. 19B).

Results show that the highest flocculating activity was achieved at a pH of 4.7. Despite both lower and higher pH ranges showing slightly decreased flocculation but not dispersion, these findings show that the bioflocculant would be suitable for prospective application in neutral, acid, and alkaline wastewaters. The differences observed here are likely due to a pH-dependent shift in zeta potentials (Qiu et al., 2020) and bridging/charge neutralisation mechanisms likely to influence particle aggregation (Yu and Somasundaran, 1996).

Ionic strength, or salinity, is known to influence biopolymers' ability to flocculate target particles (Maksoud et al., 2020). Results in Fig. 19C show that only in high salinity is the flocculation rate of the BC hydrogel affected. Despite constituting a slight decrease, this effect may be due to electrolytic interference with the active sites of the BC, as they may obstruct the adsorption of the MPs (Abdel Maksoud et al., 2020). The dispersion was not affected by increased salinity conditions, highlighting that the BC hydrogel flocculates MPs stably under severely distinct ionic strength environments.

Lastly, metal ions are frequently added to enhance flocculation activity (Elkady et al., 2011; Gong et al., 2008; Ugbenyen et al., 2012) and are likely to be ubiquitous during wastewater treatment. In general terms, cations are known to promote flocculation by neutralising/stabilising negative charges of suspended particles and bridging them together, thus increasing particle adsorption onto the bioflocculant (Wu and Ye, 2007; Yim et al., 2007). Therefore, metal cations (Fe^{3+}, Ca^{2+}, Mg^{2+}, and K^{+}) were added to understand their influence on flocculation activity. The flocculation rate of the BC hydrogel remained statistically unchanged, except for when exposed to Mg^{2+} (Fig. 19D). Despite a small decrease observed there and the fact that the dispersions were unchanged, highlights that the bioflocculant capacity of the BC hydrogel is vastly resistant to chemical changes in operational parameters. Moreover, given that Fe^{3+} is one of the most widespread toxic components in wastewater effluents (Chai et al., 2021) and that different cation concentrations can also influence flocculation rates (Lee et al., 2012), different concentrations were tested (Fig. S8). Despite high Fe^{3+} concentrations, the flocculation rate of the BC hydrogel

was barely affected. The dispersion was also not affected, further strengthening the potential for this biopolymer to perform even under harsh operational conditions.

3.6. Retention capacity

Importantly for understanding the BC/MPs hetero-aggregates durability, the ability of the BC hydrogel to irreversibly retain the MPs was evaluated. After flocculation, the BC/MPs hetero-aggregates were subjected to a wash cycle of 24 hours to evaluate if the hydrogel releases previously retained MPs. With a 25:1 (w.w./d.w.) BC:MPs ratio, only 13.01±0.94% of the MPs were removed after a wash cycle of 24 hours (Fig. 20). The BC hydrogel's capacity to retain aggregated MPs highlights its structural fortitude that pairs strong particle aggregation with high particle retention. Aside from its microporous nature, electrostatic interactions might help to explain the strength of particle aggregation observed here (Cunha et al., 2020a; Salehizadeh and Shojaosadati, 2001).

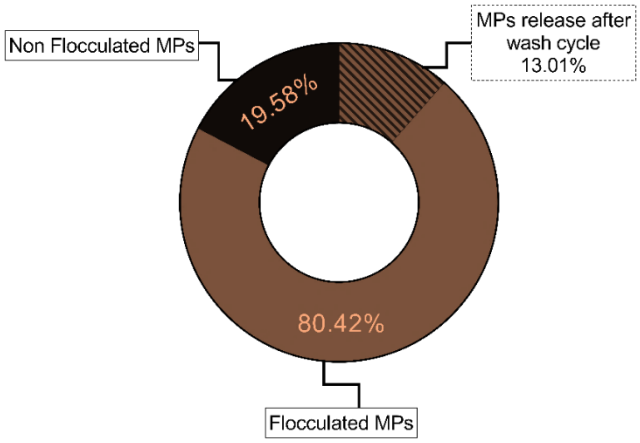


Fig. 20. Retention capacity MPs by the BC hydrogel after a 24 hours wash cycle.

Increasing the used ratio of BC:MPs to 50:1 (similar to less polluted wastewater) significantly decreased the release of MPs to 1.54 ± 0.03%. Notably, the concentration used here (2 g/L) far exceeds any naturally occurring abundance of MPs, highlighting that the BC hydrogel would never act as the limiting factor to proper bioremediation in real wastewater treatment scenarios.

3.7. MPs removal with commercial bioflocculants

To benchmark the performance of the BC hydrogel, its flocculation capacity was compared to two most common commercially available flocculants: *(i)* xanthan gum, a natural exopolysaccharide produced by *Xanthomonas campestris* with several industrial applications (García-Ochoa et al., 2000; Lee and Song, 2015); *(ii)* alginate, a natural organic polymer obtained from brown algae (*Phaeophyceae*) that is also widely used industrially (Augst et al., 2006; Lee and Mooney, 2012). Both xanthan gum and alginate are unsatisfactory flocculants (-95.18 ± 0.10% and -87.75 ± 4.55%, respectively; Fig. S9), compared to the BC hydrogel. Not only do these commercial bioflocculants not flocculate the microplastic particles but end up effectively dispersing them. Given that xanthan gum is a negatively charged polyelectrolyte (Mrokowska and Krztoń-Maziopa, 2019), the presence of negatively charged polystyrene MPs (Zeta potential of -31.3; Cunha et al., 2020a) might result in electrostatic repulsion. The same is expected with alginate (Kundu et al., 2020), which again promotes MPs' dispersion, not flocculation. In the case of BC, it is a positively charged biopolymer (Zeta potential of 14.09), which explains the higher flocculation rate.

3.8. Call for research

At this point, it is expected that exponentially available data will reinforce the apprehension and clear away any remaining skepticism and indifference regarding MPs pollution. Environmental contamination by MPs is arguably the most substantial ramification of the whole plastic pollution issue. The urban outgrowth and by-products are extremely worrisome. As the demand for biosustainable alternatives grows across every economic sector, a bacterial-based alternative for urban water remediation is presented here. This follows reports from our laboratory of similarly effective microalgal-based extracellular polymeric substances in aggregating and removing MPs (Cunha et al., 2020a; 2020b). Also, a recent work from our laboratory (Faria, 2015) showed the potential of BC membranes as biosustainable and ecologically inert alternatives to conventional hazardous fossil-based polymeric membranes used in wastewater treatment plants WWTPs for the removal MPs. Given how particle concentration might affect flocculation capacity, this study follows our previous data demonstrating that these biopolymers perform just as well in the presence of low concentrations of MPs (Cunha et al., 2020a; 2020b; Faria et al.

2022). Thus, our focus was to understand how these biopolymers and hydrogels perform longitudinally even in the upmost extreme pollutive conditions. It is now critical to understand how to optimise culture conditions at an industrial scale to produce and create BC membranes at cost-efficiency. Also, it is still technically unfeasible to quantitatively analyse the removal of emerging micropollutants such as MPs from real wastewater samples. Accordingly, future studies that focus on modulating and re-creating quantitative(-able) complex matrixes that closely models real WWTPs water is currently in development. From a financial point of view, it is important to calculate the CAPEX (capital expenditure) required to achieve economic breakeven and potential margin expansions, compared to the current input costs of inorganic and synthetic polymeric membranes. Still, it is critical to realise that the economic outlook is a vital constant of the equation but cannot outweigh the main concern: to develop a biodegradable, biosustainable and truly circular alternative to current applications. The fact that BC membranes and its scraps/remnants/remainings can be easily manipulated further strengthens its application narrative. Taking everything into consideration, the pollution is caused by MPs not just another issue. It is one of the greatest generational issues of our time and should be addressed as such.

4. Conclusions

This pilot research aimed to assess how several operational parameters would affect the flocculation performance of microplastics (MPs) using a bacterial cellulose (BC) hydrogel assembled with BC remnants. Also, the main priority remains to develop sustainable, circular, and ecologically inert solutions to bioremediation processes in wastewater treatment plants. Results show that the BC hydrogel can achieve a MPs flocculation rate of 88.59% under optimal conditions of 31.65:1 BC hydrogel:MPs ratio (or 3.2:10 BC dry weight:MPs ratio), grinding time of 1 min, immersion time of 76 min and temperature of 22.9 ºC. It was confirmed that the BC:MPs ratio and the BC grinding time equate the most crucial variables potentiating the flocculation process. Further, the microporous cellulosic network of the BC hydrogel, revealed by scanning electron microscopy, and its acid character and positively charged nature are the likely main contributors to the strong hetero-aggregation of MPs observed using fluorescence microscopy.

The computational and experimental confirmation of the flocculation model provides largely facilitating conditions for potential industrial optimisations. Interestingly, the BC hydrogel was shown to be vastly resistant to a wide range of chemical variations in operational parameters,

such as ionic strength and metal cations concentration. Short exposure times were shown to be sufficient in driving a strong aggregation of MPs, highlighting the speed at which the hetero-aggregation process occurs. Lastly, the BC hydrogel was shown to be substantially more effective in flocculating MPs, unlike commercial alternatives such as xanthan gum and alginate, which dispersed the micropolymeric particles instead. The data presented here is compelling in showing that a BC hydrogel forged with unused scraps from BC membranes carries immense promise as a truly sustainable and circular solution to bioremediation processes.

FINAL CONCLUSIONS AND FUTURE PERSPECTIVES

The present work aimed to assess the influence that MPs-contaminated waters have on industrially exploited and economically relevant microalgae *Tetraselmis suecica, Scenedesmus armatus* and *Nannochloropsis gaditana*, with emphasis on microalgal biomass; and to evaluate the viability of BC hydrogels (assembled with BC remnants) as a potential sustainable, circular, and ecologically inert solution to bioremediation processes:

- Even though MPs (PS- and PE-MPs) affected growth (cell density) in a species-dependent manner, biomass production of industrially exploited microalgae was severely affected.
- The mathematical models provided by RSM, makes it feasible to create settings that are significantly conductive to future industrial optimisations. By adjusting the levels of each variable, it is possible to predict the response biomass production, allowing industrialists to define the economic risk they're willing to assume.
- Biomass production was negatively affected in a way that led to reduction in single cell weight and/or a superior production of cells with a smaller size.
- As a result, the industrial biomass production of these microalgae can have detrimental economic impacts and critically endanger its economic scalability - containing/eliminating MPs' contamination is crucial.
- The computational and experimental confirmation of the flocculation model provided by RSM, provides largely facilitating conditions for potential industrial applications - The optimised paraments to achieve a MPs flocculation rate of 88.59% under optimal conditions of 31.65:1 BC hydrogel:MPs ratio, grinding time of 1 min, immersion time of 76 min and temperature of 22.9 ºC.
- The microporous cellulosic network of the BC hydrogel, and its acid character and positively charged nature are the likely major contributors to the strong hetero-aggregation of MPs.
- BC hydrogel was shown to be vastly resistant to a wide range of chemical variations in operational parameters, and highly efficient with short exposure times.
- BC hydrogel is substantially more effective in flocculating MPs, unlike commercial alternatives such as xanthan gum and alginate.

- BC hydrogel made with unused scraps from BC membranes carries immense promise as a truly sustainable and circular solution to bioremediation processes.

As future perspectives, since MPs altered the microalgae's biomass production, the molecular mechanisms underlying this phenomenon must be unveiled. Future studies should be done focusing on understanding the molecular mechanisms driving the reduction of single cell weight and assess the scale of these effects in industrial settings. Further, the impact of these and other MPs should be evaluated in other species of microalgae. Regarding, BC hydrogels, studies should be done to determine the removal efficiency of not only other types of MPs, but also microfibres. Microfibres are the predominant microplastic type in domestic wastewater effluents, and to date there are no published works in regard of their removal from the aquatic environments.

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APPENDIX

Appendix I

1. Experimental section (ES)

ES1. Microalgae growth conditions

The freshwater microalgae *Scenedesmus armatus* were grown in Waris-H medium [0.1 g/L KNO_3; 0.02 g/L $MgSO_4.7H_2O$; 0.02 g/L $(NH_4)_2HPO_4$; 0.1 g/L $Ca(NO_3)_2.4H_2O$; 0.24 g/L HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid); PII Metals (0.003 g/L Titriplex III; 0.001 g/L H_3BO_3; 0.00014 g/L $MnCl_2.4H_2O$; 0.00021 g/L $ZnSO_4.7H_2O$; 0.000004 g/L; Fe-EDTA (0.0052 g/L Tritiplex II; 0.0049 g/L $FeSO_4.7H_2O$; 0.054 mL/L KOH; Vitamins (0.0002 mg/L Vitamin B_{12}; 0.001 mg/L Biotin; 0.1 mg/L Thiamine-HCl; 0.0001 mg/L Niacinamide); 10 mL/L Soil Extract], while the marine microalgae, *Tetraselmis suecica* and *Nannochloropsis gaditana* were grown in ASP-12 medium [0.714 g/L HEPES; 28 g/L NaCl; 0.696 g/L KCl; 7 g/L $MgSO_4.7H_2O$; 4 g/L $MgCl_2.6H_2O$; 1.48 g/L $CaCl_2.2H_2O$; 0.1 g/L $NaNO_3$; 0.013 g/L $K_3PO_4.3H_2O$; 0.0069 g/L Na_2-Glycerophosphate; 0.151 g/L $Na_2SiO_3.9H_2O$; 0.1 g/L Tritiplex I; Vitamins (0.0002 mg/L Vitamin B_{12}; 0.001 mg/L Biotin; 0.1 mg/L Thiamine-HCl; 0.0001 mg/L Niacinamide); Trace Metals (4.36 g/L $Na_2EDTA.2H_2O$; 3.15 g/L $FeCl_3.6H_2O$; 0.0019 g/L K_2CrO_4; 0.0119 g/L $CoCl_2.6H_2O$; 0.0025 g/L $CuSO_4.5H_2O$; 0.178 g/L $MnCl_2.4H_2O$; 0.0199 g/L $Na_2MoO_4.2H_2O$; 0.00263 g/L $NiSO_4.6H_2O$; 0.00129 g/L H_2SeO_3; 0.00184 g/L Na_3VO_4; 0.023 g/L $ZnSO_4.7H_2O$)].

ES1. Microalgae growth conditions

The RSM is a collection of mathematical and statistical methods that can be employed in processes with multiple variables, in which the desired response (output) is impacted by the input variables (Nazarpour et al., 2022; P. Sharma & Sahoo, 2022). One of the standard methods under RSM is the quadratic model central composite design (CCD). CCD is helpful when optimising multiple process variables by expediting the protocol design based on designing the most efficient variable to number of experimental conditions/trials (Roy et al., 2022). Multiple

regression analysis is calculated using a quadratic polynomial equation (Banerjee et al., 2022; Mehra & Jutur, 2022):

$$y = \beta_0 + \sum_{i=1}^{n} \beta_i A_i + \sum_{i=1}^{n} \beta_{ii} A_i^2 + \sum_{i<j}^{N} \beta_{ij} A_i A_j \quad \textbf{Eq. S1}$$

where y is the predicted response, β_0 is the intercept term, βi is the linear effect, βii is the squared effect, βij is the interaction effect, and A_i and A_j are input variables that influence the response variable y (Banerjee et al., 2022).

Several statistical analyses can be used to evaluate the model's validity [6,7] namely the: *i)* analysis of the lack-of-fit (difference between the model prediction values and the experimental data). If there are no significant differences, a linear equation with a high coefficient of determination (R^2) can be drawn; *ii*) residual analysis (difference between the data point and the regression line). If a satisfactory coefficient of determination is obtained, it indicates that the models were well-adjusted to the data.; and *iii*) analysis of the *F*-value and *p*-value, provided by the ANOVA test. If the found values are lower than critical values, the model is valid.

ES1. BC production

Komagataeibacter saccharivorans was used to produce BC hydrogel. This bacterium was cultured in static conditions of Hestrin and Schramm (HS) liquid medium at a temperature of 30°C, and an initial OD_{600} of 0.4 (UV-6300 PC Double Beam). HS medium – 2% (w/v) glucose (G7021/Sigma-Aldrich), 0.5% (w/v) peptone (84616.0500/VWR Chemicals), 0.5% (w/v) yeast extract (84601.0500/VWR Chemicals), 0.27% (w/v) disodium hydrogen phosphate (02494C/VWR Chemicals), 0.115% (w/v) citric acid (0529-500G/VWR Chemicals), 0.4% (w/v) ethanol, pH 3.25. Sterilisation of the medium was done at 121°C, for 15 min.

ES2. Infrared Spectroscopy (ATR-FTIR)

Attenuated total reflection Fourier transform infrared spectroscopy (ATR–FTIR) was employed to obtain the infrared spectra of BC hydrogel. Before sample analysis, BC was dried at 40 ◦C to remove moisture. FTIR spectra were recorded by a Perkin Elmer FTIR System

Spectrum Two coupled with a Diamond ATR accessory (DurasamplIR II, Smiths Detection, UK), with a resolution of 4 cm^{-1} and an accumulation of 32 scans.

ES3. Inverse Gas Chromatography (IGC)

Inverse gas chromatography (IGC) measurements were carried out on a commercial inverse gas chromatograph (IGC, Surface in diameter and 30 cm in length Measurements Systems, London, UK) equipped with both flame ionization (FID) and thermal conductivity (TCD) detectors. The system is automatized with SMS IGC Controller v1.8 control software and data were analysed using IGC Standard v1.3 and Advanced Analysis Software v1.25. IGC measurements were done following the study done by Cordeiro et al. (2011). The samples were placed in standard glass silanised (dimethyldichlorosilane; Repelcote BDH, UK) columns by vertical tapping for 2 hours. The columns were acclimatized at 40 ºC for 8 hours, after which pulse injections were carried out with a 0.25 mL gas loop.

ES4. Water uptake capacity (Swelling behavior)

To determine the water uptake capacity of BC, the biofilm was previously dried (at 40 °C). Posteriorly, the swelling was determined by immersing the samples (1 x 1 cm^2) in distilled water at room temperature (20 ± 2 °C), in triplicate (minimum). For a period of 10 hours, the weight increase was periodically measured, for which the samples were taken out of the water, dried off with filter paper, weighed, and then re-immersed. The water uptake/swelling (SW) was calculated according to **Eq. S1**, where W_w and W_d are the weights of BC biofilm after and before (dry) immersion in water, respectively.

$$\text{SW } (\%) = \frac{W_w - W_d}{W_d} \text{ x } 100 \qquad \textbf{Eq. S1}$$

2. Results section

Table S1. Parameters used in bioassays: exposure of microalgae to microplastics.

Microalgae	Reason for microalgae choice	Polymer	Shape	Size (μm)	Tested concentration (mg/L)	Exposure duration (h)	Growth cycle	Effect criteria/ Endpoints	Reference
Acutodesmus obliquus (FW)	• Not mentioned	HDPE PP PVC	Powder	0.1	5, 10, 15, 25, 100, 150, 200, and 250	504	Exponential (log) growth phase	• Growth • Photosynthetic activity • Carbohydrate content • Lipid content • Protein content	(Ansari et al., 2021)
Tisochrysis lutea (SW)	• Common marine ecotoxicological model	PE PVC PA PHB	Fragments	<250 and <20	10	96	Not mentioned	• Growth	(Beiras et al., 2021)
Raphidocelis subcapitata (FW)	• Natural prey for Daphnia magna (used in this study)	PE	Microbeads	63-75	130	120	Exponential (log) growth phase	• Growth	(Canniff & Hoang, 2018)
Pseudokirchneriella subcapitata (FW)	• Known to be especially sensitive to a wide range of pollutants	PS-PEI (+)	Amorphous Silica Beads	0.11	0.1-0.8	72	Exponential (log) growth phase	• Growth	(Casado et al., 2013)
Dunaliella salina (SW)	• Used as a base material in food colouring, cosmetics, and health food	PE	Microspheres	180-212	50, 100, 150, 200, 250, 300 and 350	144	Exponential (log) growth phase	• Growth • Photosynthetic activity • Cell morphology	(Chae et al., 2019)

Scenedesmus obliquus (FW)	• Widely recognized as a food resource for Daphnia magna (used in this study)	PS	Round-shaped microbead	5	6, 6000, 12000, 24000, 60000, 120000, 240000, 600000 and 900000 (particle mL^{-1})	72 120	Exponential (log) growth phase	• Growth • Oxidative stress	(Q. Chen et al., 2020)
Scenedesmus quadricauda (FW)	• Model phytoplanktonic freshwater organism	PS	Microspheres	1, 2, 3, 4 and 5	10	96	Exponential (log) growth phase	• Growth • Photosynthetic activity	(Y. Chen et al., 2020)
Phaeodactylum tricornutum (SW)	• Often used as an experimental object in toxic researches • Plays an important role in aquaculture, bioenergy, medical treatment, and environmental protection	PS	Particles	5	10, 20, 50, and 100	168	Exponential (log) growth phase	• Growth • Photosynthetic activity	(Z. Chen et al., 2022)
Microcystis panniformis (FW) *Scenedesmus sp.* (FW) *Tetraselmis sp.* (SW) *Gloeocapsa sp.* (SW)	• All known to be EPS producers	PS PMMA	Irregular Fragments	106-250 and <106	25 and 250	504	Exponential (log) growth phase	• Growth • EPS	(Cunha et al., 2019)
Phaeodactylum tricornutum (SW)	• Numerous applications in biotechnology	PS	Irregular Fragments	70-80	0.5 and 50	648	Exponential (log) growth phase	• Growth • Biomass • Photosynthetic pigments	(Cunha, Lopes, et al., 2020b)

	• Explored for biodiesel production	PMMA		40-60				• Extracellular carbohydrates content • Extracellular proteins content	
Tetraselmis chuii (SW)	• Model phytoplanktonic organism in ecotoxicological studies	PE	Microspheres	1-5	0.046, 0.092, 0.184, 0.368, 0.736 and 1.472	96	Exponential (log) growth phase	• Growth	(Davarpanah & Guilhermino, 2015)
Tetraselmis chuii (SW)	• Model phytoplanktonic organism in ecotoxicological studies	Not Mentioned	Microspheres	1-5	0.3, 0.9 and 4	96	Exponential (log) growth phase	• Growth	(Davarpanah & Guilhermino, 2019)
Chlamydomonas reinhardtii (FW)	• Model phytoplanktonic organism in ecotoxicological studies	PS	Particles	0.1	2	192	Exponential (log) growth phase	• Growth	(Déniel et al., 2020)
Chlamydomonas reinhardtii (FW)	• A model algae species • exists widely in the aquatic environment • has a strong tolerance of trivalent arsenic (used in this study)	PS	Particles	0.1, and 5	10, 25, 50, and 100	180	Exponential (log) and stationary phases	• Growth • Oxidative stress • Enzymatic activity • Chlorophyll content • Photosynthetic and respiration rates	(Y. Dong et al., 2021)
Phaeodactylum tricornutum (SW)	• most used algal species in marine bioassays due to its easy cultivation and its sensitivity to metals and organic compounds	PMMA	Particles	1	10, 25, 50, and 100	240	Exponential (log) growth phase	• Growth • Photosynthetic activity • Chlorophyll a content • Protein content • Enzymatic activity	(J. Dong et al., 2022)

Microcystis aeruginosa (FW)	• common freshwater microalga is found worldwide in rivers and lakes • produces numerous cyanotoxins harmful to the health of other aquatic organisms and that of human	PS	Microbeads	0.1, 1, 10, and 100	1, 10, and 100	96	Exponential (log) growth phase	• Growth • Photosynthetic activity • Oxidative stress • Microcystin production	(Fan et al., 2022)
Chlorella vulgaris (FW)	• Model phytoplanktonic organism	PVC	Particles	93.8-197	10, 100 and 1000	240	Exponential (log) growth phase	• Growth • Enzymatic activity	(Fu et al., 2019)
Phaeodactylum tricornutum (SW)	• A standard species used in toxicology tests	PE	Irregular Particles	1-500	0.01-25	72	Exponential (log) growth phase	• Growth	(Gambardella et al., 2019)
Isochrysis galbana (SW)	• A biological model for the assessment of MP toxicity	PE	Mixture Of Different Shapes (Powder)	1.4-42	0.5, 1, 10 and 25	72	Exponential (log) growth phase	• Growth	(Garrido et al., 2019)
Akashiwo sanguínea (SW) *Heterosigma akashiwo* (SW) *Karenia mikimotoi* (SW)	• Flagellated microalgae with different cell sizes • Microalagae with the cell wall:P. donghaiense, P. micans, P. minimum, and A. tamarense • Microalagae without the cell wall: A. sanguinea, H.	PS	Microspheres	1	10	192	Exponential (log) growth phase	• Growth • Pigment content • Photochemical efficiency • Antioxidant mechanisms • Cell morphology	(Ge et al., 2022)

Prorocentrum donghaiense (SW) *Prorocentrum micans* (SW) *Prorocentrum minimum* (SW) *Alexandrium tamarense* (SW)	akashiwo, and K. mikimotoi								
Phaeodactylum tricornutum (SW)	• A standard species used in toxicology tests	PE PVC	Powders	150 250	50, 1000 and 5000	96 and 216	Exponential (log) and stationary phases	• Growth • Lipid content	(Guo et al., 2020)
Chlorella sorokiniana (FW)	• Extensively used in controlled laboratory experiments as a food source for consumers	PS	Irregular, Fragmented and Mostly Angular	<70	60	672	Exponential (log) growth phase	• Lipid content • Fatty acids content • Chlorophyll content	(Guschina et al., 2020)
Spirulina sp. (FW)	• The protein in Spirulina sp., phycocyanin is mostly used for its antioxidant, anti-cancer, and anti-inflammation properties, in food and feed applications	PE PP	flake-like shape	0.5–1 (mm^2)	1000	720	Exponential (log) growth phase	• Growth • Protein content (phycocyanin)	(Hadiyanto et al., 2021)
Spirulina sp. (FW)	• Used as food sources (containing hydrocarbon, lipids and protein and other	PET PP	Particles	1-2	300, 500 and 550	2688	Long term toxicity test	• Growth	(Hamdan et al., 2019)

	high added value compounds)						Exponential (log) growth phase		
Chlorella vulgaris (FW)	• Model phytoplanktonic organism	PS PS-COOH	Particles	0.5	250	912	Long term toxicity test Exponential (log) growth phase	• Growth • Chlorophyll a content • Oxidative stress • Enzymatic activity • Cell morphology	(Hazeem et al., 2020)
Microcystis aeruginosa (FW)	• Known as an extracellular polymeric substance (EPS) producing algae	PS	Particles	0.1, and 1	1, 10, and 100	408	Lag phase to exponential (log) growth phases	• Growth • Photosynthetic activity • Oxidative stress • EPS	(Jiao et al., 2022)
Chlorella vulgaris (FW)	• Model phytoplanktonic organism • Highly sensitive to environmental pollution	PS–NH_2	Spheres	0.2, and 0.3	25, 50, 100, and 200	72	Exponential (log) growth phase	• Growth • Pigment content • Cell morphology	(Khoshnamvand et al., 2021)
Chlamydomonas reinhardtii (FW)	• Model freshwater algae, commonly used in eco-toxicological studies	HDPE PP	Fragments	400-1000	400	1872	Exponential (log) growth phase	• Growth • Hetero aggregation • Stress Response / Photosynthesis/ Apoptosis/ Sugar biosynthesis genes	(Lagarde et al., 2016)
Phaeodactylum tricornutum (SW)	• Not mentioned	PS	Not mentioned	0.1	25, 50, 100, and 200	1, 24, 48, 72, and 96	Exponential (log) growth phase	• Growth • Chlorophyll a content • Photosynthetic activity • Reactive oxygen species (ROS)	(Lang et al., 2022)

Scenedesmus quadricauda (FW)	• Not mentioned	PS PU HDPE PP	Particles	18.9 76.7 220 171.3	3	504	Not mentioned	• Growth • Photosynthetic activity • Oxidative stress	(Lazareva et al., 2021)
Chlamydomonas reinhardtii (FW)	• Model freshwater algae, commonly used in eco-toxicological studies	PS	Powder	0.3-0.6	5, 25, 50 and 100	240	Exponential (log) growth phase	• Growth • Chlorophyll a fluorescence • Photosynthetic activity • Oxidative stress • EPS • Soluble proteins cointent • Cell morphology	(S. Li et al., 2020)
Euglena gracilis (FW)	• Model freshwater microalgae to assess the toxicity of pollutants • Sensitive to environmental stress	PS	Spheres	1	1, and 5	96	Not mentioned	• Growth • Photosynthetic activity • Pigment content • Protein content • Oxidative stress	(X. Li et al., 2022)
Chlorella pyrenoidosa (FW)	• Used as feed in aquaculture • Commonly used in eco-toxicological studies	PAN	Not mentioned	0.1-0.8	50, 150, 250, and 500	144	Exponential (log) growth phase	• Growth • Photosynthetic pigments • Chlorophyll a fluorescence • Protein content • Enzyme activity	(Lin et al., 2020)

Scenedesmus obliquus (FW)	• Model phytoplanktonic organism	PS PS-NH_2 PS-COOH	Spheres	0.1	1	96	Exponential (log) growth phase	• Growth • Oxidative stress • Enzyme activity	(Y. Liu et al., 2019)
Scenedesmus obliquus (FW)	• Model phytoplanktonic organism	PS PS-NH_2	Beads	0.1, 0.5, 1 and 2 0.1	75	336	Exponential (log) growth phase	• Growth • Chlorophyll fluorescence • Cell morphology	(G. Liu et al., 2020)
Alexandrium pacificum (SW)	• Produces paralytic shellfish toxins (PST) • PST can be transfered to higher trophic level organisms along food chains	PS	Spheres	0.1 and 1	5, 25 and 100	504	Exponential (log) growth phase	• Growth • Chlorophyll a content • Photosynthetic activity • PST production	(C. Liu et al., 2021)
Chaetoceros neogracile (SW) *Tisochrysis lutea* (SW) *Heterocapsa triquetra* (SW)	• Predominance in marine phytoplankton communities • Common inclusion in bivalve diets	PS	Beads	2	0.00396 and 0.0396	840	Entire culture cycle, from seeding to stationary growth phase	• Growth • Hetero aggregation • Chlorophyll fluorescence	(Long et al., 2017)
Rhodomonas baltica (SW)	• Widely used as food source for different invertebrate species cultivated under laboratory conditions • Natural prey for Oxyrrhis marina (used in this study)	PS	Microbeads	10	75 and 7500 (particle L^{-1})	264	Lag, exponential (log) and stationery growth phases	• Growth • Chlorophyll content	(Lyakurwa, 2017)

Chlorella sp. (FW)	• High growth rate and nutrient removal efficiency	PVC	Spherical	0.2	50, 100, 150, and 200	240	Exponential (log) growth phase	• Growth • pH • Nutrients removal • Enzymatic activity	(Manzi et al., 2022)
Chlorella pyrenoidosa (FW)	• Widely distributed in freshwater systems all over the world • Commonly used in eco-toxicological studies	PS	Not Mentioned	0.1 1	10, 50 and 100	720	From the lag to the earlier exponential (log) growth phases	• Growth • Photosynthetic activity • Cell morphology	(Mao et al., 2018)
Chlorella sp. (FW)	• commonly used in ecotoxicological studies • sensitive to a broad group of toxic substances	PE PP PS PVC PET	Irregular	100-300, 300-500, and 500-700	10, 250, 500, 750, and 1000	72	Exponential (log) growth phase	• Growth	(Miloloža et al., 2021)
Scenedesmus sp. (FW)	• easy to cultivate, has a short life cycle, its sensitivity to various contaminants has already been confirmed, and the cost of its toxicity tests was low	PE PP PS PVC PET	Fragments	200, 400, and 600	50, 250, 500, 750, and 1000	72	Not mentioned	• Growth	(Miloloža et al., 2022)
Phaeodactylum tricornutum (SW)	• Not mentioned	PE	Microbeads	10-20, 50-63, and 90-106	1.25×10^2–1.25×10^7 (particles/L)	72	Exponential (log) growth phase	• Growth	(Niu et al., 2021)
Pseudokirchneriella subcapitata (FW)	• Known to be especially sensitive to a wide range of pollutants	PS-COOH	Particles	0.11	10	72	Exponential (log) growth phase	• Growth	(Nolte et al., 2017)

Tetraselmis chuii (SW)	• Model phytoplanktonic organism in ecotoxicological studies	PE	Microspheres	1-5	0.75, 1.5, 3, 6, 12, 24 and 48	96	Exponential (log) growth phase	• Growth • Chlorophyll content	(Prata et al., 2018)
Chlorella pyrenoidosa (FW)	• Model phytoplanktonic organism	PS	Not Mentioned	0.7	20	96	Exponential (log) growth phase	• Growth • Oxidative stress • Apoptosis	(Qu et al., 2020)
Tetraselmis suecica (SW) *Amphora subtropica* (SW)	• food resources for Pseudodiaptomus annandalei (used in this study)	PS-NH_2	Beads	0.1 2	250, 500, and 750	240	Exponential (log) growth phase	• Growth • Chlorophyll content • Lipid content	(Raju et al., 2022)
Scenedesmus armatus (FW) *Microcystis aeruginosa* (FW)	• Model phytoplanktonic organisms	PE	Microspheres	250−300 and 212−250	0.25, 0.5 and 1	672	Exponential (log) growth phase	• Growth • Photosynthetic activity • Microcystins content	(Sánchez-Fortún et al., 2021)
Phaeodactylum tricornutum (SW)	• A standard species used in toxicology tests	PS	Particles	0.1, 1, 5,10, 20 and 50	0.1	72	Exponential (log) growth phase	• Growth • Oxidative stress • Photosynthetic stress • Cytoplasmic and mitochondrial membrane potential • Viability/ membrane cell damage • Genotoxicity • Protein content	(Sendra et al., 2019)

Chaetoceros neogracile (SW)	• Predominance in marine phytoplankton communities	PS-NH_2	Microbeads	0.5 and 2	2.5	72	Exponential (log) growth phase	• Growth • Photosynthetic activity • Cell morphology • Esterase activity • Reactive oxygen species (ROS) • Cytoplasmic membrane potential • Neutral lipid content	(Seoane et al., 2019)
Thalassiosira pseudonana (SW) *Skeletonema grethae* (SW) *Phaeodactylum tricornutum* (SW) *Dunaliella tertiolecta* (SW)	• These species were chosen based on our previous published studies examining toxicology of oil related pollutants on marine phytoplankton and POPs	PS	Microspheres	1 and 6	0.0001, 0.001, 0.01, 0.1, 1, 10, 50 and 250	48	Exponential (log) growth phase	• Chlorophyll content • EPS	(Shiu et al., 2020)
Dunaliella tertiolecta (SW) *Dunaliella tertiolecta* (SW)	• Thalassiosira pseudonana has a silicate cell wall • Dunaliella tertiolecta doesn't have a cell wall • Chorella vulgaris, has a polysaccharidic cell wall • Model phytoplanktonic organisms	PS PS-COOH	Microspheres	0.5 and 6 0.5	25 and 250	72	Exponential (log) growth phase	• Growth • Photosynthetic activity	(Sjollema et al., 2016)

Chlorella vulgaris (FW) *Thalassiosira pseudonana* (SW)									
Chlorella sp. (FW) *Phaeodactylum tricornutum* (SW)	• Model phytoplanktonic organisms	PP PE PET PVC	Particles	74	200	96	Whole growth cycle	• Growth • Chlorophyll and carotenoid content • Enzymatic activity • EPS	(Song et al., 2020)
Chlorella vulgaris (FW)	• Model phytoplanktonic organism	PE PA PLA PBS	Not mentioned	77.75 59.88 57.41 53.33	10, 100, and 1000	264	Exponential (log) growth phase	• Growth • Photosynthetic activity • Enzymatic activity • EPS content	(Su et al., 2022)
Euglena gracilis (FW)	• sensitive to environmental stress due to lack of cell wall • a model organism to evaluate the ecotoxicity of various chemicals	PS	Sphere	0.1	1, 5, and 25	192	Not mentioned	• Photosynthetic activity • Motility	(L. Sun et al., 2021)
Chlorella sp. (FW)	• Model phytoplanktonic organism	PS PS-NH_2 PS-COOH	Particles	6	1000	72	Exponential (log) growth phase	• Growth • Oxidative stress • Lipid peroxidation	(Thiagarajan et al., 2019)

Chlorella sp. (FW)	• A food resource for marine microcrustacean Artemia salina (used in this study)	PS PS-NH_2 PS-COOH	Spheres	6	1	48	Exponential (log) growth phase	• Growth • Oxidative stress	(Thiagarajan et al., 2021)
Chlorella vulgaris (FW)	• Model phytoplanktonic organism	PS	Microspheres	0.5	1, 5, 10, 50, 100 and 1000	480	Growth reached the stationary phase	• Growth • Chlorophyll a content	(Tunali et al., 2020)
Chlorella sp. (FW) *Pseudokirchneriella subcapitata*	• Chlorella sp. is an indigenous tropical microalga • P. subcapitata is a recommended model for toxicity testing	PS	Not mentioned	10	47.7, 57.3, 68.8, 82.5, and 99	384	Exponential (log) growth phase	• Chlorophyll-a content • Cell morphology • Oxidative stress • Lipid peroxidation	(Wan et al., 2021)
Phaeodactylum tricornutum (SW) *Chaetoceros gracilis* (SW) *Thalassiosira sp.* (SW)	• Standard Marine diatoms	PVC	Microspheres	1	25, 50, 100 and 200	96	Exponential (log) growth phase	• Growth • Pigments content • Cell morphology	(S. Wang et al., 2020)
Chlamydomonas reinhardtii (FW)	• Model freshwater algae, commonly used in eco-toxicological studies	PVC	Beads	50-100	10, 20, 50, 100 and 200	96	Exponential (log) growth phase	• Growth • Chlorophyll content • Enzymatic activity	(Q. Wang et al., 2020)
Chlorella vulgaris (FW)	• Model phytoplanktonic organism	PS PVC	Particles	250 150	10, 100 and 1000	168	Exponential (log) growth phase	• Growth • Enzymetic activity	(Z. Wang, Fu, et al., 2021)

Chlorella pyrenoidosa (FW) *Microcystis flos-aquae* (FW)	• Most widely distributed and common types of algae in freshwater environments • Commonly used in eco-toxicological studies	PVC PP	Particles	111, 157 and 216 64, 172 and 236	5, 10, 50, 100, 250 and 500	264	Exponential (log) growth phase	• Chlorophyll a content • Photosynthetic activity	(Y. Wu et al., 2019)
Microcystis aeruginosa (FW)	• a common species in blooms • produces hepatotoxins (microcystins (MCs)) that are toxic to organisms and harmful to water supplies	PS	Particles	0.1, and 1	5	96	Exponential (log) growth phase	• Growth • Photosynthetic activity • Oxidative stress • Hepatotoxins (microcystins) content	(D. Wu et al., 2021)
Euglena gracilis (FW)	• Model freshwater microalgae to assess the toxicity of pollutants • Sensitive to environmental stress	PS	Not Mentioned	0.1 and 5	0.5, 1, 10, and 50	96	Exponential (log) growth phase	• Growth • Pigments content • Oxidative stress • Gene expression	(Xiao et al., 2020)
Chlamydomonas reinhardtii (FW)	• the most widely distributed algal species in freshwater environments • commonly used in ecotoxicological studies	PS	Microbeads	0.1 and 100	50, 250, and 500	96	Exponential (log) growth phase	• Growth • Chlorophyll content • Oxidative stress • Cell morphology • EPS content • Enzymetic activity	(Yan et al., 2021)
Chlorella pyrenoidosa (FW)	• Widely distributed in freshwater systems all over the world • Commonly used in eco-toxicological studies	PE PA PS	Not Mentioned	13 and 150	10, 30, 50, 70 and 100	96	Exponential (log) growth phase	• Growth • Chlorophyll fluorescence • Enzymatic activity	(Yang et al., 2020)

Chlorella pyrenoidosa (FW)	• Widely distributed green algal species • Model organism commonly used in eco-toxicological studies	PS	Particles	0.55 and 5	0.05, 0.5 and 5	96	Exponential (log) growth phase	• Growth • Cell morphology	(Yi et al., 2019)
Dolichospermum flos-aquae (FW) *Microcystis aeruginosa* (FW)	• Model phytoplanktonic organisms	PS	Microbeads	20-350	66.7	504	Exponential (log) growth phase	• Growth • Cell morphology • Hetero aggregation	(Yokota et al., 2017)
Skeletonema costatum (SW)	• Sensitive to toxic substances	PVC	Particles Bulk debris	1 1000	1, 5, 10 and 50 50, 500, 1000 and 2000	96	Exponential (log) growth phase	• Growth • Chlorophyll content • Photosynthetic activity	(C. Zhang et al., 2017)
Chlorella pyrenoidosa (FW)	• Model organism commonly used in eco-toxicological studies • A food resource for Daphnia magna (used in this study)	PS-NH2 PS-COOH	Microspheres	1	0.09, 0.19, 0.45 and 0.47	72	Exponential (log) growth phase	• Growth • Oxidative stress • Enzyme activity	(F. Zhang et al., 2020)
Platymonas subcordiformis (SW) *Dicrateria zhanjiangensis* (SW)	• Primary producers of the marine ecosystem • Major feed species for the aquaculture of shellfish and finfish	PS	Spherical particles	0.49	0.29	24	Exponential (log) growth phase	• Photosynthetic efficiency • Energy supply • Oxidative stress	(W. Zhang et al., 2021)
Prorocentrum donghaiense (SW)	• dominant red tide species in the East China Sea	PS	Microspheres	1.12	0, 1, 5, and 10	168	Exponential (log) growth phase	• Growth • Pigment contents,	(J. Zhang et al., 2022)

								• Relative electron transfer rate • Oxidative stress • Enzymatic activity	
Karenia mikimotoi (SW)	• Harmful microalgae species widely distributed in offshore waters of China • Has occurred red tide	PVC	Beads	1	5, 25, 50, 100	96	Exponential (log) growth phase	• Growth • Chlorophyll content • Photosynthetic activity	(Zhao et al., 2019)
Karenia mikimotoi (SW)	• Widely distributed in offshore waters of China	PS	Beads	0.1 and 1	1 and 10	72	Exponential (log) growth phase - short term toxicity test	• Growth • Enzyme activities • total protein	(Zhao et al., 2020)
						312	Whole growth cycle - long term toxicity test		
Microcystis aeruginosa (FW)	• a common microalga in freshwater • often used as a model aquatic organism for toxicity experiments	Nylon	Irregular	1-3	25, 50, and 100	720	Exponential (log) growth phase	• Growth • Chlorophyll content • Photosynthetic activity • Oxidative stress • Morphologic properties • RNA and transcriptional response	(Zheng et al., 2022)

Skeletonema costatum (SW)	• Sensitive substances	to	toxic	PVC	Spheres	1	3, 6, 10, 15, 20 and 30	96	Exponential (log) growth phase	• Growth • Cell morphology	(X. Zhu et al., 2020)
Skeletonema costatum (SW)	• Sensitive substances	to	toxic	PE PS PVC PVC	Not Mentioned	74 1	10, 20, 50 and 100	144	Exponential (log) growth phase	• Growth • Oxidative stress • Enzymatic activity	(Z. lin Zhu et al., 2019)

FW: Freshwater; SW: Seawater; HDPE: High-density polyethylene, PP: Polypropylene, PVC: Polyvinyl chloride, PE: Polyethylene, PA: Polyamide, PHB: Polyhydroxy butyrate, PS: Polystyrene, PEI: Polyethyleneimine, PMMA: Poly (methyl methacrylate), PET: Polyethylene terephthalate, PU: Polyurethane, PAN: Polyacrylonitrile, PBS: polybutylene succinate; PAM: Pulse amplitude modulation, FTIR: Fourier-transform infrared spectroscopy, DLS: Dynamic light scattering, FCM: Flow cytometry, CLSM: Confocal laser scanning, microscopy, XRD: X-ray diffractometer, SEM: Scanning electron microscopy, TEM: Transmission electron microscopy, AFS: Atomic fluorescence spectrometry, UPLC-MS/MS: Ultra-performance liquid chromatography spectrome-tandem mass try, TLC: Thin-layer chromatography, HPLC: High performance liquid chromatography, EDX: Energy dispersive x-ray spectroscopy, 3D-EEM: Three-dimensional excitation-emission matrices, FESEM: Field emission scanning electron microscopy, PCR: Polymerase Chain Reaction, CAM: contact angle meter, ECD: Electron capture detector, ICP-AES: atomic emission spectroscopy with inductively coupled plasma, PEA: Plant efficiency analyser, XPS: X-ray photoelectron spectroscopy, LC-MS/MS: Liquid chromatography tandem mass spectrometry, FTIR-ATR: Fourier transform infrared spectroscopy – attenuated total reflectance, LSCM: laser scanning confocal microscopy, EDS: Energy dispersive spectroscopy, BET-ASAP: Brunauer-Emmett-Teller surface area and porosity analyser, PALS: phase analysis light scattering

Table S2. Central composite design matrix with two variables and one response – cell density - for *T. suecica*, *S. armatus*, and *N. gaditana*.

Run No.	Variables		Response: Cell density (normalised values)*					
	Production time (days)	MPs concentration (mg/L)	*T. suecica*		*S. armatus*		*N. gaditana*	
			PS	PE	PS	PE	PS	PE
1	0	0	---	---	---	---	---	---
2	0	5	---	---	---	---	---	---
3	0	10	---	---	---	---	---	---
4	2	0	---	---	---	---	---	---
5	2	5	$0.94 \pm 0.02^{A,a}$	$0.98 \pm 0.02^{A,b}$	$0.99 \pm 0.01^{A,a}$	$0.99 \pm 0.01^{A,a}$	$1.01 \pm 0.04^{A,a}$	$1.02 \pm 0.01^{A,a}$
6	2	10	$0.93 \pm 0.03^{A,a}$	$0.94 \pm 0.01^{A,b}$	$1.00 \pm 0.01^{A,a}$	$0.99 \pm 0.01^{A,a}$	$1.04 \pm 0.01^{A,a}$	$1.03 \pm 0.01^{A,a}$
7	4	0	---	---	---	---	---	---
8	4	5	$0.94 \pm 0.01^{B,a}$	$0.97 \pm 0.02^{B,a}$	$0.99 \pm 0.01^{B,a}$	$0.99 \pm 0.01^{B,a}$	$0.96 \pm 0.02^{B,a}$	$1.05 \pm 0.01^{B,a}$
9	4	10	$\mathbf{0.90 \pm 0.02}^{B,a}$	$0.93 \pm 0.01^{B,a}$	$1.00 \pm 0.01^{B,a}$	$0.99 \pm 0.01^{B,a}$	$1.03 \pm 0.02^{B,a}$	$1.05 \pm 0.01^{B,a}$

All the samples are represented as average of at least three replicates ± S.D.; *normalised values = contaminated water / uncontaminated water; of the same day

Table S3. Central composite design matrix with two variables and one response – biomass production - for *T. suecica*, *S. armatus*, and *N. gaditana*.

Run No.	**Variables**		Response: Cell density (normalised values)*					
	Production time (days)	MPs concentration (mg/L)	*T. suecica*		*S. armatus*		*N. gaditana*	
			PS	PE	PS	PE	PS	PE
1	0	0	---	---	---	---	---	---
2	0	5	---	---	---	---	---	---
3	0	10	---	---	---	---	---	---
4	2	0	---	---	---	---	---	---
5	2	5	$0.94 \pm 0.02^{A,a}$	$0.98 \pm 0.02^{A,b}$	$0.99 \pm 0.01^{A,a}$	$0.99 \pm 0.01^{A,a}$	$1.01 \pm 0.04^{A,a}$	$1.02 \pm 0.01^{A,a}$
6	2	10	$0.93 \pm 0.03^{A,a}$	$0.94 \pm 0.01^{A,b}$	$1.00 \pm 0.01^{A,a}$	$0.99 \pm 0.01^{A,a}$	$1.04 \pm 0.01^{A,a}$	$1.03 \pm 0.01^{A,a}$
7	4	0	---	---	---	---	---	---
8	4	5	$0.94 \pm 0.01^{B,a}$	$0.97 \pm 0.02^{B,a}$	$0.99 \pm 0.01^{B,a}$	$0.99 \pm 0.01^{B,a}$	$0.96 \pm 0.02^{B,a}$	$1.05 \pm 0.01^{B,a}$
9	4	10	$\mathbf{0.90 \pm 0.02}^{B,a}$	$0.93 \pm 0.01^{B,a}$	$1.00 \pm 0.01^{B,a}$	$0.99 \pm 0.01^{B,a}$	$1.03 \pm 0.02^{B,a}$	$1.05 \pm 0.01^{B,a}$

All the samples are represented as an average of at least three replicates ± S.D.; *normalised values = contaminated water / clean water; of the same day.

Table S4. Central composite design matrix with two variables and one response - biomass production/cell density ratio - for *T. suecica*, *S. armatus*, and *N. gaditana.*

Run No.	**Variables**		Response: Biomass production/cell density ratio (normalised values)*					
	Production time (days)	MPs concentration (mg/L)	*T. suecica*		*S. armatus*		*N. gaditana*	
			PS	PE	PS	PE	PS	PE
1	0	0	---	---	---	---	---	---
2	0	5	---	---	---	---	---	---
3	0	10	---	---	---	---	---	---
4	2	0	---	---	---	---	---	---
5	2	5	$0.91 \pm 0.04^{A,a}$	$1.02 \pm 0.02^{A,a}$	$0.79 \pm 0.07^{A,a}$	$0.73 \pm 0.02^{A,a}$	$0.75 \pm 0.01^{A,ab}$	$0.46 \pm 0.03^{B,a}$
6	2	10	$1.04 \pm 0.05^{A,a}$	$0.90 \pm 0.04^{A,a}$	$0.54 \pm 0.04^{B,b}$	$0.78 \pm 0.04^{A,a}$	$0.85 \pm 0.04^{A,a}$	$0.81 \pm 0.04^{A,b}$
7	4	0	---	---	---	---	---	---
8	4	5	$1.07 \pm 0.07^{A,a}$	$0.93 \pm 0.07^{A,a}$	$0.74 \pm 0.07^{A,a}$	$0.77 \pm 0.05^{A,a}$	$1.15 \pm 0.03^{C,b}$	$1.19 \pm 0.06^{C,b}$
9	4	10	$0.85 \pm 0.05^{B,a}$	$0.87 \pm 0.01^{A,a}$	$\mathbf{0.54 \pm 0.04}^{B,b}$	$0.79 \pm 0.05^{A,a}$	$1.20 \pm 0.05^{B,b}$	$1.35 \pm 0.03^{C,a}$

All the samples are represented as an average of at least three replicates ± S.D.; *normalised values = contaminated water / clean water; of the same day.

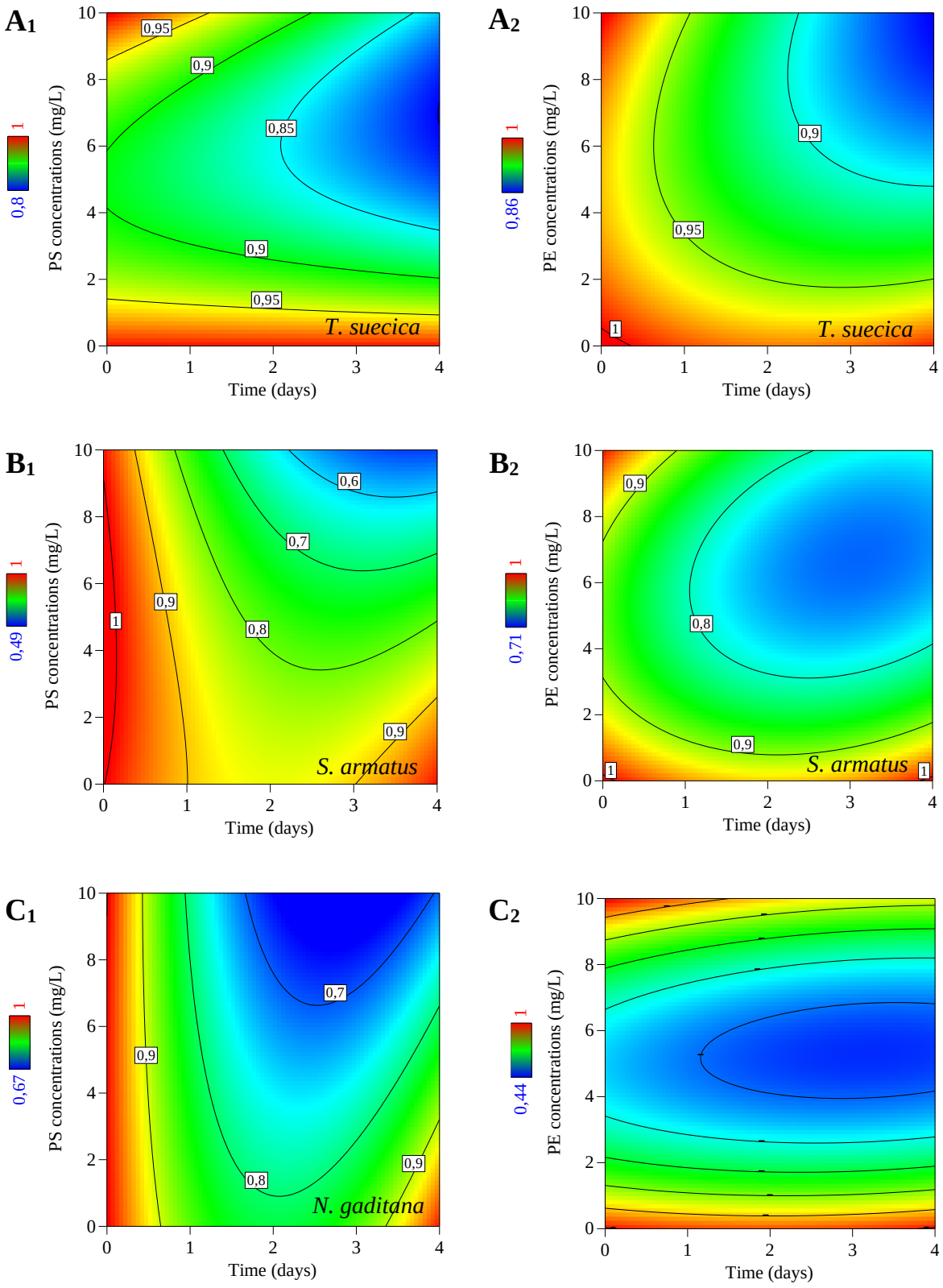

Fig. S1. Contour plots of biomass production/cell density ratio as a function of MPs concentration: PS (**1** in subscript: **A_1, B_1, C_1**), and PE (**2** in subscript: **A_2, B_2, C_2**); and the production time of *T. suecica* (**A**), *S. armatus* (**B**), and *N. gaditana* (**C**).

Table S5. Model equations for the predicted cell density (normalised values*) of *T. suecica*, *S. armatus*, and *N. gaditana*

Species	MPs	Model equations	**Eq.**
T. suecica	PS	$1.00578 - 0.01694T - 0.00925C - 0.00290TC + 0.00390T^2 + 0.00089C^2$	**(S2)**
	PE	$1.00066 - 0.00101T + 0.00010C - 0.00162TC$	**(S3)**
S. armatus	PS	$1.00094 - 0.00687T - 0.00107C - 0.00006TC + 0.00172T^2 + 0.00009C^2$	**(S4)**
	PE	$1.00102 - 0.00498T - 0.00102C - 0.00019TC + 0.00120T^2 + 0.00009C^2$	**(S5)**
N. gaditana	PS	$1.00141 - 0.01870T + 0.00627C + 0.00083TC + 0.00484T^2 - 0.00071C^2$	**(S6)**
	PE	$1.00168 + 0.00027T + 0.00031C + 0.00137TC$	**(S7)**

T is the time (days), and *C* is the concentration of MPs (mg/L). *normalised values = contaminated water / clean water; of the same day. Cell density decrease % = 100 x (1 – cell density normalised values)

Table S6 Equations for the predicted biomass production/cell density ratio of *T. suecica*, *S. armatus*, and *N. gaditana*.

Species	MPs	Equations	**Eq.**
T. suecica	PS	$1.0001 - 0.00005T - 0.04122C - 0.004TC + 0.0001T^2 + 0.00412C^2$	**(S8)**
	PE	$1.00672 - 0.02009T - 0.01313C - 0.00337TC + 0.00450T^2 + 0.00126C^2$	**(S9)**
S. armatus	PS	$1.00214 - 0.13501T + 0.01141C - 0.01144TC + 0.03348T^2 - 0.00128C^2$	**(S10)**
	PE	$1.00901 - 0.06445T - 0.04990C - 0.00515TC + 0.01613T^2 + 0.00481C^2$	**(S11)**
N. gaditana	PS	$1.00011 - 0.18445T - 0.00259C - 0.00736TC + 0.04610T^2 + 0.00028C^2$	**(S12)**
	PE	$1.00672 - 0.35186T - 0.04652C - 0.00490TC + 0.08712T^2 + 0.00487C^2$	**(S13)**

T is the time (days), and *C* is the concentration of MPs (mg/L)

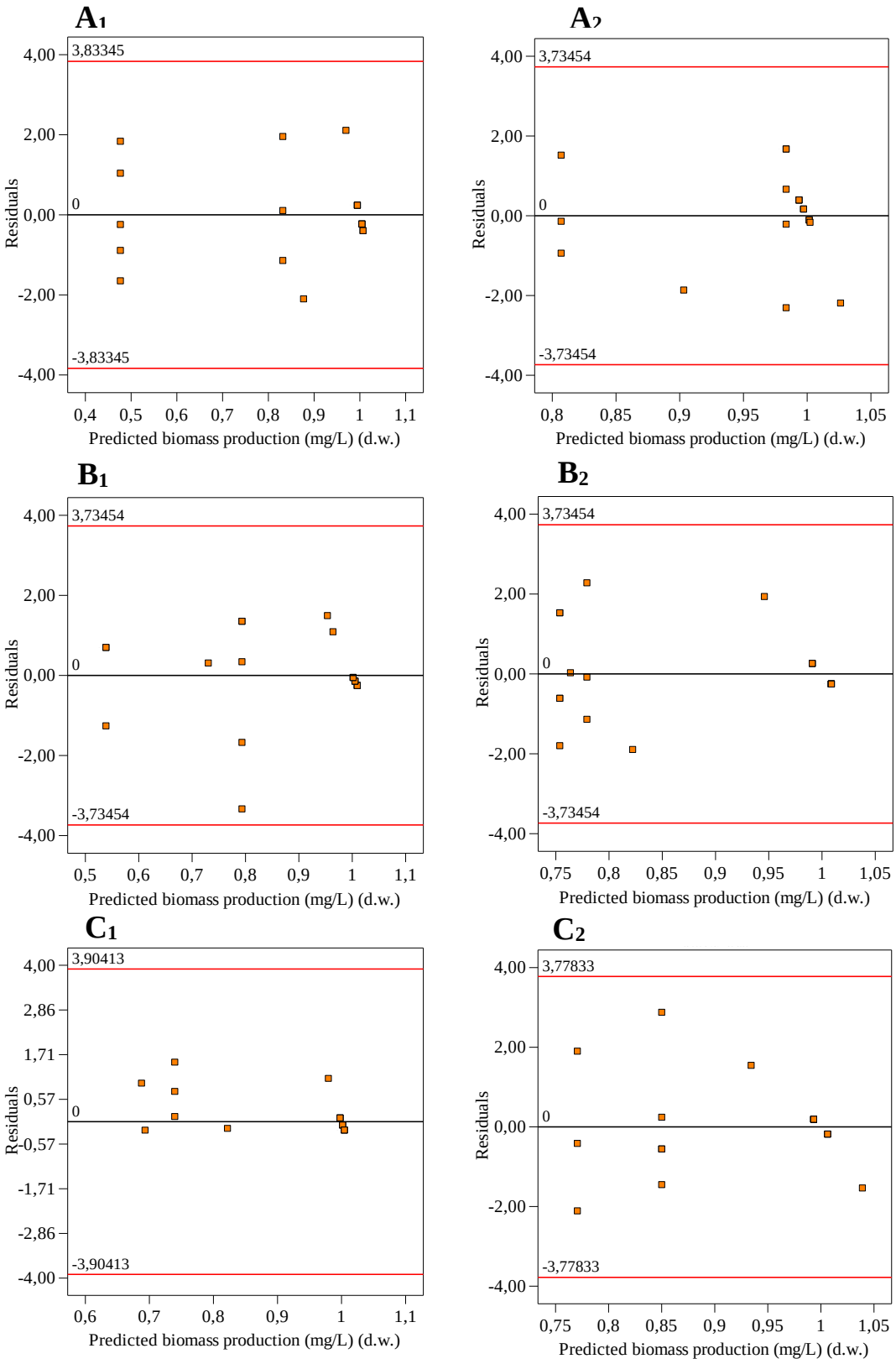

Fig. S2. Residual plot for predicted of biomass production for *T. suecica* (**A**), *S. armatus* (**B**), and *N. gaditana* (**C**) when cultivated in PS (**1** in subscript: **A_1, B_1, C_1**) and PE-MPs **(2** in subscript: **A_2, B_2, C_2**) contaminated waters.

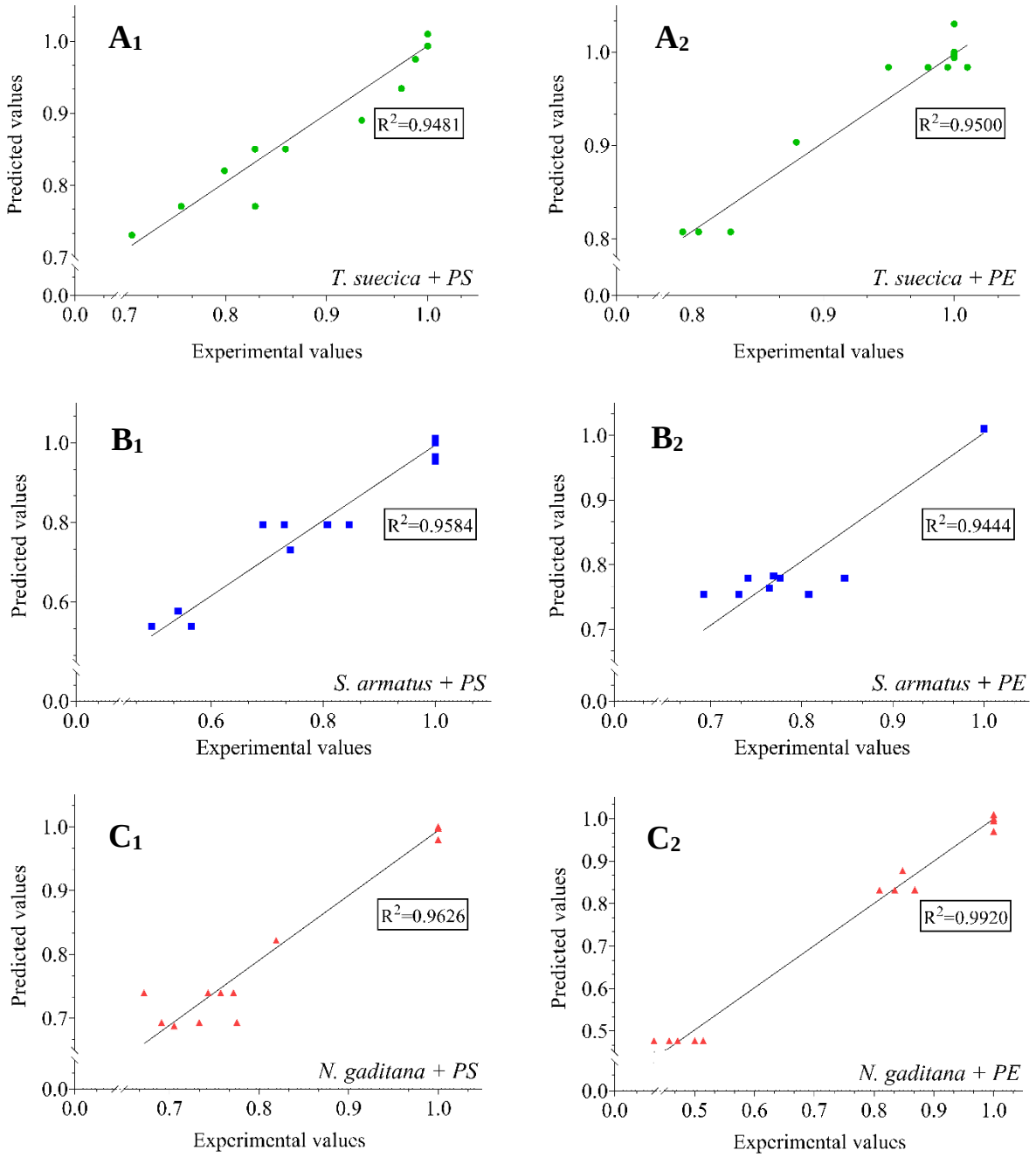

Fig. S3. Relation between the value obtained experimentally and predicted values to biomass production for *T. suecica* (**A**), *S. armatus* (**B**), and *N. gaditana* (**C**) when cultivated in PS (**1** in subscript: **A_1, B_1, C_1**) and PE-MPs (**2** in subscript: **A_2, B_2, C_2**) contaminated waters.

Table S7. Operational parameters used to the MPs removal efficiency studies.

Grinding time (min)	BC:MPs ratio (w.w./d.w.)	BC:MPs ratio (d.w./d.w.)	BC concentration (g d.w./L)	Temperature (°C)	Mixing time (min)	Immersion time (min)	Salinity (‰)	pH	Metal cations	Fe^{3+} (mMol)
	1.25:1	1.6:100	0.032			0		3		
1				4						
	5:1	3.4:100	0.13			5	0	3.5	Ca^{2+}	0
2				15	5					
	15:1	1.5:10	0.39			15	10	4.7	Fe^{3+}	4.5
5				20	10					
	20:1	2.0:10	0.52			30	15	5	Mg^{2+}	9
10				25	30					
	25:1	2.5:10	0.65			60	37	7	K^{+}	18
20				30						
	37.5:1	3.75:10	0.97			120		8		

w.w.: wet weight; d.w.: dry weight.

Table S8. Three level and four factor experimental design and associated response - flocculation rate (%).

Run	Grinding time (min)	BC:MPs ratio (wet weight/dry weight)	Temperature (°C)	Mixing time (min)	Flocculation rate **(%)**
1	1	1.25:1	15	0	29.50 ± 2.33
2	10	1.25:1	15	0	24.50 ± 0.42
3	1	37.5:1	15	0	74.71 ± 1.87
4	10	37.5:1	15	0	63.77 ± 1.58
5	5	20:1	20	60	71.48 ± 2.50
6	1	1.25:1	15	120	30.17 ± 2.51
7	10	1.25:1	15	120	23.78 ± 0.15
8	1	37.5:1	15	120	75.71 ± 0.73
9	10	37.5:1	15	120	70.88 ± 1.27
10	1	1.25:1	30	0	32.97 ± 2.15
11	10	1.25:1	30	0	34.40 ± 0.68
12	1	37.5:1	30	0	84.71 ± 2.80
13	10	37.5:1	30	0	73.21 ± 1.80
14	1	1.25:1	30	120	47.85 ± 2.72
15	10	1.25:1	30	120	43.88 ± 3.14
16	1	37.5:1	30	120	83.79 ± 1.35
17	10	37.5:1	30	120	70.91 ± 2.79

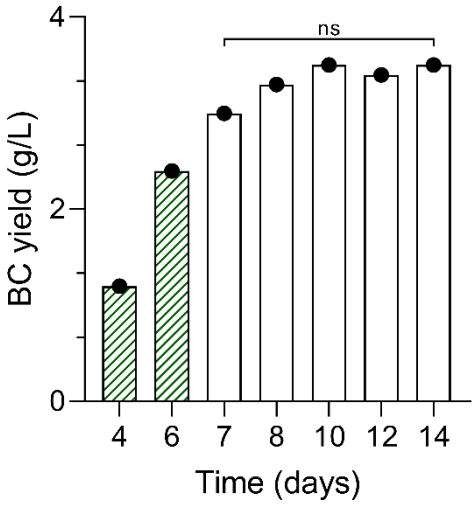

Fig. S4. Bacterial cellulose (BC) production by *Komagataeibacter saccharivorans* in function of time. *ns*: non significantly different ($p \geq 0.05$).

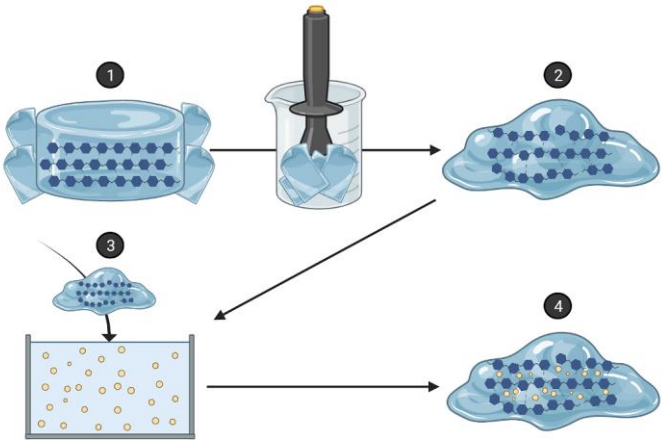

Fig. S5. Schematic representation of the microplastics (MPs) removal process. Bacterial cellulose (BC) remnants hydrogel before (**1**) and after grinding (**2**); (**3**) BC hydrogel immersion in MPs-contaminated water; (**4**) BC/MPs hetero-aggregate formed. Simplified illustration of the MPs bound within the chemical structure of the BC membrane.

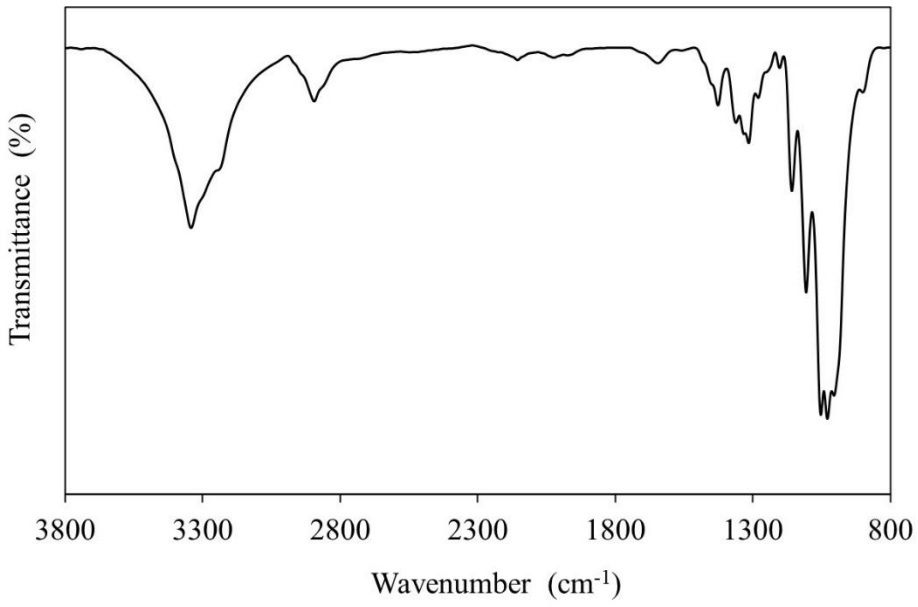

Fig. S6. ATR-FTIR spectrum of oven-dried bacterial cellulose.

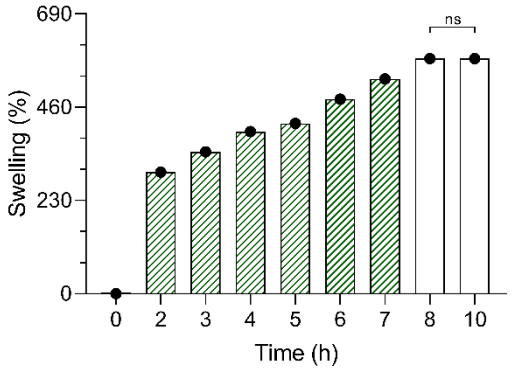

Fig. S7. Swelling behaviour of oven-dried bacterial cellulose (BC) remains, in function of time. *ns*: non significantly different ($p \geq 0.05$).

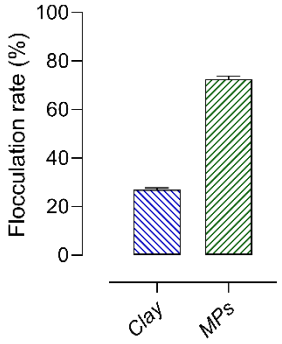

Fig. S8. Effect of bacterial cellulose (BC) on the flocculation rate towards clay and microplastics particles (MPs).

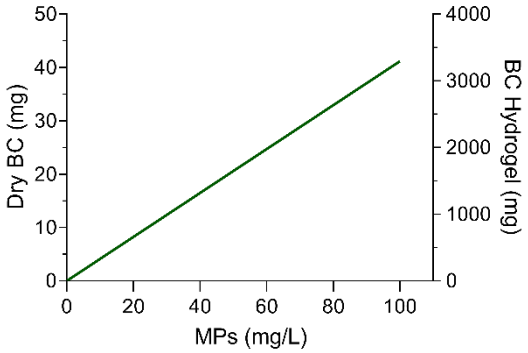

Fig. S9. Amount of dry BC or BC hydrogel to use for different levels of MPs-contaminated wastewater, to 23 °C, immersion time of 76 min, and a BC grinding time of 1 min.

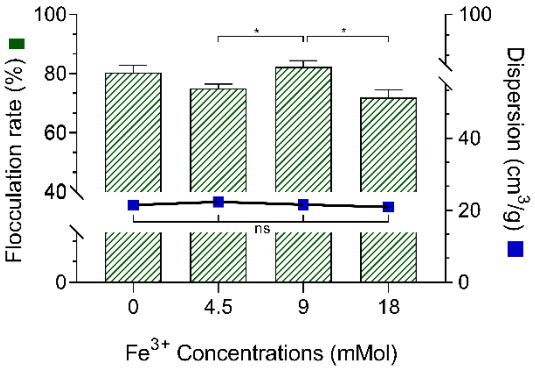

Fig. S10. Effect of different concentrations of Fe^{3+} on the flocculation rate and dispersion of the BC hydrogel towards MPs from contaminated water. *Significantly different ($p < 0.05$). *ns*: non significantly different ($p \geq 0.05$).

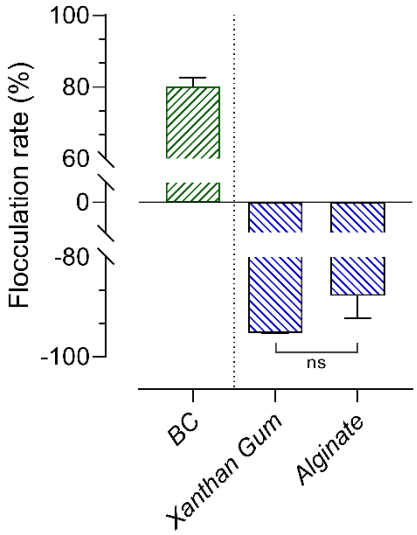

Fig. S11. Performance of different bioflocculants in flocculating MPs. *ns*: non significantly different ($p < 0.05$).

3. References

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