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## Polystyrene microbeads modulate the energy metabolism of the marine diatom *Chaetoceros neogracile*

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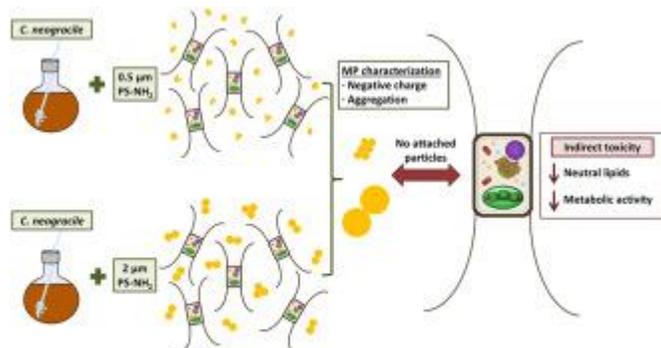
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### Abstract :

Due to the growing concern about the presence of microplastics (MP) in the environment, the number of studies evaluating the toxicity of these small persistent particles on different marine species has increased in recent years. Few studies have addressed their impact on marine phytoplankton, a subject of great concern since they are primary producers of the aquatic food web. The aim of this study is to unravel the cytotoxicity of 2.5 µg mL<sup>-1</sup> unlabelled amino-modified polystyrene beads of different sizes (0.5 and 2 µm) on the marine diatom *Chaetoceros neogracile*. In addition to traditional growth and photosynthesis endpoints, several physiological and biochemical parameters were monitored every 24 h in *C. neogracile* cells by flow cytometry during their exponential growth (72 h). Dynamic Light Scattering measurements revealed the strong aggregation and the negative charge of the beads assayed in the culture medium, which seemed to minimize particle interaction with cells and potentially associated impacts. Indeed, MP were not attached to the microalgal cell wall, as evidenced by scanning electron micrographs. Cell growth, morphology, photosynthesis, reactive oxygen species levels and membrane potential remained unaltered. However, exposure to MP significantly decreased the cellular esterase activity and the neutral lipid content. Microalgal oil bodies could serve as an energy source for maintaining a healthy cellular status. Thus, MP-exposed cells modulate their energy metabolism to properly acclimate to the stress conditions.

## Graphical abstract



Graphical abstract

## Highlights

► Effects of 0.5 and 2 µm PS-NH<sub>2</sub> microplastics (MP) were evaluated on *C. neogracile*. ► MP showed negative charge, were aggregated and were not attached to the cell wall. ► Exposure to MP decreased the cellular metabolic activity and neutral lipid content. ► Cells modulate their energy metabolism to properly acclimate to the stress conditions. ► Microalgal oil bodies serve as an energy source for maintaining a healthy status.

**Keywords** : microalgae, microplastic, flow cytometry, cytotoxicity, scanning electron microscopy.

## 44 1. Introduction

45 Plastic wastes constitute a major portion of marine litter (Ruiz-Orejón, 2016). Synthetic  
46 polymers have excellent properties for many packaging applications and manufacturing  
47 processes that have led to their increasing use throughout the last decades, reaching an  
48 annual production of 335 million tons in 2016 (Plastics Europe, 2017). However, the  
49 characteristics that make plastics useful materials (low cost, high durability, low density) also  
50 make them a menace to the environment (Ryan, 2015). Plastics may persist in the  
51 environment for many years and can be easily dispersed by oceanic currents even to remote  
52 areas of the world, far away from the source of contamination (Barnes et al., 2009; Peeken et  
53 al., 2018; Pham et al., 2014; Ryan et al., 2009).

54 The concern of the scientific community about plastic pollution and its impacts on marine  
55 organisms has increased in recent years especially in regards with the high proportion of small  
56 persistent particles commonly called microplastics (MP) (Andrady, 2015). MP are defined as  
57 plastic particles with a size smaller than 5 mm (NOAA, 2008). Lately, a new category of plastic  
58 debris, named nanoplastics (NP), was described as particles <100 nm (Galloway et al., 2017) or  
59 <1  $\mu\text{m}$  (Gigault et al., 2018). In this study, we will keep the NP definition as particles <100 nm  
60 and therefore, considering MP as particles from 100 nm to 5 mm in size. Both MP and NP may  
61 originate from the fragmentation of larger plastic debris (*e.g.*, bags, bottles and fishing nets) in  
62 the marine environment caused by a combination of physical and chemical processes such as  
63 mechanical abrasion, photochemical and thermo-oxidation, hydrolysis or even biological  
64 degradation (Dawson et al., 2018; Gigault et al., 2016; Lambert and Wagner; 2016). In addition,  
65 MP could also reach the aquatic environment directly since many products used plastics at the  
66 micrometric size (*e.g.*, personal care and household cleaning products or microfibers from  
67 synthetic clothing) (Andrady, 2015), while the increased use of NP in diverse industries  
68 (cosmetics, drugs, lubricants) may lead to their release in the environment (Hernandez et al.,  
69 2017; Lusher et al., 2017). MP have been found to be ubiquitously present in all environmental  
70 compartments of the aquatic environment, from surface waters and water column to deep-sea  
71 sediments (Reviewed in Paul-Pont et al., 2018). In reference to object counts, MP constitute  
72 more than 92 % of floating plastics in the oceans (Cole et al., 2011; Eriksen et al., 2014).  
73 Regarding reported environmental concentrations, some of the highest have been detected in  
74 the southern North Sea reaching 1,700,000 items  $\text{m}^{-3}$  ( $\sim 8.5 \text{ mg L}^{-1}$ ) for plastic particles > 80  
75  $\mu\text{m}$  (Dubai and Liebezeit, 2013). However, due to the unavailability of methods, there are no  
76 field determinations of MP as small as those used in most experimental studies (< 20  $\mu\text{m}$ ;  
77 Filella, 2015). For nanoplastics, no quantitative data exists *in situ* and only indirect evidence of  
78 their presence has been debated recently (Ter Halle et al., 2017).

79 Exposure laboratory experiments do not reflect the complexity of the marine environment;  
80 however, they may contribute to disclose and aware of the effect of plastics debris on marine  
81 organisms (Paul-Pont et al., 2018; Phuong et al., 2016). Several studies have investigated how  
82 MP can be ingested by aquatic animals leading to negative consequences for them and for the  
83 aquatic food chain (Yokota et al., 2017). Nevertheless, there is still a scarcity of knowledge  
84 about the impacts of MP on phytoplankton, which constitutes the basis of the aquatic food  
85 webs. In particular, diatoms are considered one of the most diverse and ecologically important  
86 phytoplanktonic groups and are responsible for approximately 20 % of the overall primary  
87 production on Earth, playing a central role in the biogeochemical cycling of important nutrients  
88 (Malviya et al., 2016; Rosenwasser et al., 2014).

89 The aim of this study was to assess the potential effects of two different sizes (0.5  $\mu\text{m}$  and 2  
90  $\mu\text{m}$ ) of unlabelled polystyrene (PS) beads on the marine diatom *Chaetoceros neogracile*, trying  
91 to disclose differential impacts depending on particle size, as smaller particles are expected to  
92 be more toxic (Sjollema et al., 2016). To perform the experiments, PS beads with amino ( $-\text{NH}_2$ )  
93 surface modifications were used at 2.5  $\mu\text{g mL}^{-1}$ , concentration set to compare with the 72 h  
94  $\text{EC}_{50}$  value of 50 nm PS- $\text{NH}_2$  NP (data not shown). PS is one of the most used plastics worldwide  
95 and it is one of the plastic polymers type most frequently detected as micro-debris in marine  
96 environments (Andrady, 2011). PS- $\text{NH}_2$  beads have been amply used as model particles in  
97 ecotoxicology and have been shown to cause severe damages on different organisms of the  
98 aquatic trophic chain (Bergami et al., 2017; Bhattacharya et al., 2010; Canesi et al., 2017;  
99 Manfra et al., 2017; Marques-Santos et al., 2018; Pinsino et al., 2017; Tallec et al., 2018).  
100 Particle behaviour was measured in Milli-Q water, seawater and in the culture medium using  
101 Dynamic Light Scattering (DLS) to assess potential particle aggregation or changes in the  
102 particle surface charge. To assess PS- $\text{NH}_2$  MP effects on *C. neogracile* several physiological and  
103 biochemical parameters such as cell morphology, autofluorescence, esterase activity, reactive  
104 oxygen species (ROS) levels, cytoplasmic membrane potential and neutral lipid content were  
105 monitored by flow cytometry (FCM) in addition to traditional growth and photosynthesis  
106 endpoints. Furthermore, potential structural damages and disposition or adsorption of  
107 unlabelled PS- $\text{NH}_2$  beads on microalgal surface were determined using scanning electron  
108 microscopy (SEM).

## 109 **2. Materials and methods**

### 110 **2.1. Microalgal cultures**

111 *Chaetoceros neogracile* (*Bacillariophyceae*) was obtained from the Culture Collection of Algae  
112 and Protozoa of the Scottish Marine Institute (strain CCAP 1010-3). *C. neogracile* is a non-  
113 motile centric marine diatom (width = 4  $\mu\text{m}$ , length = 7  $\mu\text{m}$ ) encased in lightly siliceous valves

114 (frustules), covered with an organic coating (Hecky et al., 1973). This species was selected  
115 based on its predominance in marine phytoplankton communities (Malviya et al., 2016). Stock  
116 microalgal cultures were maintained in filtered (pore size: 0.2  $\mu\text{m}$ ) and autoclaved natural  
117 seawater (FSW) supplied with 1 mL L<sup>-1</sup> of Conway medium (Walne, 1966) and enriched with  
118 silica ( $\text{Na}_2\text{SiO}_3 \cdot 9\text{H}_2\text{O}$ ) ( $1.07 \cdot 10^{-4}$  M). Flasks were kept at 20 °C, with continuous aeration and  
119 light at 100  $\mu\text{mol photon m}^{-2}\text{s}^{-1}$ . CO<sub>2</sub> was supplied to keep the pH between 7.5 and 7.9.

## 120 **2.2. Particle characterization**

121 Unlabelled 0.5  $\mu\text{m}$  and 2  $\mu\text{m}$  PS-NH<sub>2</sub> particles were purchased from Micromod (Micromod  
122 Partikeltechnologie GmbH). Both particles were characterized by Dynamic Light Scattering  
123 (DLS) using Zetasizer Nano Series ZS (Malvern instruments) as conducted by Tallec et al.  
124 (2018). Size (Z-average in nm), charge ( $\zeta$ -potential in mV) and aggregation state (Polydispersity  
125 Index, Pdl in arbitrary units (a.u.)) were measured in Milli-Q water, FSW and also in filtered (0.2  
126  $\mu\text{m}$ ) microalgal culture medium. When Pdl exceeds 0.2 a.u. particles were considered to be  
127 aggregated. Measurements were carried out in triplicate, each containing 13 runs of 10 s for Z-  
128 average and 20 runs, 3 s delay for  $\zeta$ -potential following the protocol described in Della Torre et  
129 al. (2014). Data were analyzed using Zetasizer Nano Series software, version 6.20.

## 130 **2.3. Microalgal exposure to microplastics**

131 Microalgae cells were exposed to both particles in different batch cultures for 72 h, during the  
132 exponential growth phase, as recommended in most standardized growth inhibition tests with  
133 microalgae (OECD 201, 2011). It has also been shown that during exponential growth phase  
134 MP impaired more drastically the major cellular and physiological parameters (Mao et al.,  
135 2018). Exposures were performed in triplicates in 500 mL glassware balloon flasks filled with  
136 300 mL of microalgal culture under the same environmental conditions as stock cultures.  
137 Microalgal cells in early exponential growth phase were used as inoculum and initial cell  
138 density was adjusted to  $4 \times 10^5$  cells mL<sup>-1</sup>. The MP concentration used ( $2.5 \mu\text{g mL}^{-1}$ )  
139 corresponds to the 72 h EC<sub>50</sub> value of 50 nm PS-NH<sub>2</sub> NP (data not shown) and was established  
140 to compare results with other laboratory studies (Long et al., 2017; Mao et al., 2018; Zhang et  
141 al., 2017), but not as high as in most published works, so that we could get closer to worst  
142 environmental scenarios (Paul-Pont et al., 2018). This same mass concentration for the two  
143 beads tested results in differences in the nominal concentration in number of particles per mL,  
144 corresponding to  $3.7 \times 10^7$  particles mL<sup>-1</sup> for the 0.5  $\mu\text{m}$  MP and to  $5.7 \times 10^5$  particles mL<sup>-1</sup> for  
145 the 2  $\mu\text{m}$  MP. Before the experiment, MP stock solutions were diluted in Milli-Q water. The  
146 volume of the diluted MP solutions added to microalgal cultures to reach the final MP  
147 concentration used for the assay ( $2.5 \mu\text{g mL}^{-1}$ ) never exceeded 0.3 % of the final culture  
148 volume. Therefore, in the whole process an approximate dilution of 1/20000 of the

149 commercial MP stock was made. Analyses were performed in fresh samples every 24 h during  
150 the 72 h of the test. Additionally, after 48 h of exposure, halfway in the experiment, control  
151 and exposed samples were fixed for microscopy observations.

#### 152 **2.4. Scanning electron microscopy**

153 Samples of control and MP exposed cultures were diluted to  $10^5$  cells mL<sup>-1</sup> and fixed in 6%  
154 glutaraldehyde in 0.1 M sodium cacodylate buffer (1.75% w/v of NaCl, pH 7.2). Suspensions  
155 were incubated for 2 hours at 4 °C before being filtered through polycarbonate filters  
156 (Nucleopore PC) with a 2 µm pore size. Then, samples were prepared for scanning electron  
157 microscopy (SEM) observation following the method previously described by Foulon et al.  
158 (2016). Finally, samples were observed with a Hitachi S-3200N microscope.

#### 159 **2.5. Flow cytometric analyses**

160 Flow cytometric (FCM) analyses were performed using an Easy-Cyte Plus 6HT flow cytometer  
161 (Guava Merck Millipore®) equipped with a 488 nm argon excitation laser, detectors of forward  
162 (FSC) and side (SSC) light scatter and three fluorescence detectors: green (525 nm ± 15), yellow  
163 (583 nm ± 13) and red (680 nm ± 15). All fluorescence measurements were obtained in a  
164 logarithmic scale and data were computed as the mean fluorescence value of the cell  
165 population in arbitrary units (a.u.). Collected data from the FCM were analyzed with the  
166 software InCyte (Millipore).

##### 167 **2.5.1. Cellular density and growth rate**

168 Direct absolute cell counts were carried out daily by FCM to determine the cellular density of  
169 control and treated cultures. Growth rates ( $\mu$ ) were also calculated as described in Seoane et  
170 al. (2017a).

##### 171 **2.5.2. Morphological parameters and chlorophyll *a* fluorescence**

172 Forward light scatter (FSC) and side light scatter (SSC) values were measured and used as  
173 estimates of morphological changes. Natural red autofluorescence, related to chlorophyll *a*  
174 content, was also analysed.

##### 175 **2.5.3. Esterase activity**

176 Esterase activity in *C. neogracile* cells was studied using the fluorescein diacetate (FDA)  
177 cytometric assay previously described in Seoane et al. (2017b). Cells were incubated with FDA  
178 at a final concentration of 6 µM for 10 min at room temperature in the dark. FDA is a non-  
179 fluorescent, non-polar lipophilic molecule that diffuses across cell membranes. After entering  
180 the cell, its acetate residues are cleaved off by non-specific esterases and the polar hydrophilic  
181 fluorescent product fluorescein is retained by cells with intact plasma membranes. Since  
182 fluorescein is accumulated by viable and active cells, esterase activity is measured by means of  
183 the green fluorescent intensity emitted (Prado et al., 2009).

#### 184 **2.5.4. Oxidative stress: intracellular levels of reactive oxygen species**

185 Reactive oxygen species (ROS) content was measured using 2,7-dichlorofluorescein diacetate  
186 (DCFH-DA) as described in González-Fernández et al. (2018). The cells of *C. neogracile* were  
187 incubated with DCFH-DA at a final concentration of 10  $\mu$ M for 50 min at room temperature in  
188 the dark. DCFH-DA is a cell-permeable non-fluorescent probe that is hydrolysed (de-esterified)  
189 intracellularly to form the highly green fluorescent DCF upon oxidation with ROS. Thus, the  
190 green fluorescence measured is quantitatively related to the ROS content in cells.

#### 191 **2.5.5. Cytoplasmic membrane potential**

192 Potential changes in cytoplasmic membrane potential were determined using the bis-(1,3-  
193 dibutylbarbituric acid) trimethine oxonol (DiBAC<sub>4</sub>(3)) following the protocol previously  
194 described by Seoane et al. (2017a). Cells were incubated with DiBAC<sub>4</sub>(3) at a final  
195 concentration of 2  $\mu$ M for 10 min at room temperature in the dark. DiBAC<sub>4</sub>(3) can enter  
196 depolarized cells where it binds to intracellular proteins or membranes showing green  
197 fluorescent emission (Wolff et al., 2003).

#### 198 **2.5.6. Neutral lipid content**

199 Neutral lipid content was assessed using the specific lipid droplet stain BODIPY 493/503. This  
200 highly lipophilic neutral dye easily goes through cell and organelle membranes and  
201 accumulates in intracellular oil-containing organelles, known as lipid bodies, showing green  
202 fluorescence. BODIPY has proven to be very effective for lipid measurement in microalgae  
203 (Govender et al., 2012; Rumin et al., 2015). Cells of *C. neogracile* were incubated with BODIPY  
204 493/503 at a final concentration of 10  $\mu$ M for 10 min at room temperature in the dark.

#### 205 **2.6. Photosynthetic efficiency**

206 The effective quantum yield (QY) of photochemical energy conversion in photosystem II (PSII)  
207 was measured by Pulse Amplitude Modulation (PAM) fluorometry using an AquaPen-C AP-  
208 C 100 fluorometer (Photon Systems Instruments, Czech Republic) equipped with a blue (455  
209 nm) LED excitation light. Aliquots of each culture were dark-adapted for 30 minutes before  
210 measurements.

#### 211 **2.7. Statistical analysis of results**

212 Statistical analysis was performed using IBM SPSS Statistics software 21.0. Mean fluorescence  
213 values and standard deviation (SD) of the three biological replicates were determined for  
214 exposed and control cultures. Data were checked for normal distribution (Shapiro-Wilk test)  
215 and homogeneity of variance (Levene test). One-way analysis of variance (ANOVA) was  
216 performed to test for differences among treatments at each sampling time. When significant  
217 differences were observed, multiple comparisons among treatments were made using the  
218 Tukey's *post hoc* test. For DLS data, pairwise comparisons were also made to compare particle

219 behaviour (size and charge) between the three media (Supp. Table 1). A  $p$  - value  $< 0.05$  was  
220 considered statistically significant for all the analyses.

### 221 **3. Results and discussion**

#### 222 **3.1. Particle characterization: actual size, charge and aggregation**

223 DLS analysis of the MP suspended in Milli-Q water confirmed the approximate size indicated  
224 by the commercial supplier for the two particle sizes used, with Z-averages of  $602.5 \pm 27.3$  and  
225  $2274.0 \pm 100.9$  nm, which correspond to the nominal sizes of  $0.5 \mu\text{m}$  and  $2 \mu\text{m}$ , respectively  
226 (Table 1). Data also showed an optimal dispersion and stability in Milli-Q water since  
227 aggregation was negligible for both particles, as suggested by a Pdl  $< 0.2$  (Table 1). However,  
228 plastic particles showed a significant increase in Z-average reaching values of  $2899.0 \pm 96.0$  nm  
229 and  $5932.0 \pm 694.4$  nm when suspended in FSW and values of  $3268.0 \pm 64.3$  nm and  $4342.3 \pm$   
230  $294.2$  nm in microalgae medium for  $0.5 \mu\text{m}$  and  $2 \mu\text{m}$  PS-NH<sub>2</sub> beads, respectively (Table 1;  
231 Supp. Table 1). These data indicated an increase in particle size congruent with the observed  
232 Pdl values ( $>0.2$ ) that indicated aggregation (Table 1).

233 Regarding the charge, both  $0.5 \mu\text{m}$  and  $2 \mu\text{m}$  PS-NH<sub>2</sub> MP showed negative  $\zeta$ -potential in the  
234 three media assayed (Table 1) although a positive charge was expected since the beads used  
235 presented amino surface modifications. Therefore, particle characteristics must be assessed  
236 prior to performing laboratory exposure to properly interpret particle behaviour and toxicity.  
237 Such difference of charge has also been reported by Sun et al. (2018) with  $1 \mu\text{m}$  PS-NH<sub>2</sub> beads  
238 and by González-Fernández et al. (2018) and Lundqvist et al. (2008) with  $100$  nm PS-NH<sub>2</sub> NP.  
239 These discrepancies may derive from the manufacturing process which may change  
240 according to the commercial suppliers. More information and measurements regarding  
241 physico-chemical properties of MP is thus a prerequisite for a better interpretation of their  
242 behaviour and biological impact in different solutions (González-Fernández et al., 2018).

243 Significant lower absolute values of  $\zeta$ -potential (closer to zero) were obtained for both particles  
244 in FSW and in the culture medium as compared to the values in Milli-Q water (Table 1; Supp.  
245 Table 1). These results are in accordance with the results of size and particle aggregation  
246 obtained. The  $\zeta$ -potential is a key indicator of the stability of colloidal dispersions. The  
247 magnitude of this value indicates the degree of electrostatic repulsion between particles.  
248 Particle solutions with high  $\zeta$ -potential (negative or positive) are more stable and greatly  
249 dispersed, while particles with a  $\zeta$ -potential between  $-10$  and  $+10$  mV are less stable and tend  
250 to aggregate. The high concentration of NaCl and other ions in FSW as well as the presence of  
251 proteins or other natural organic matter in the filtered microalgae medium are likely  
252 interacting with the particle surface group eliminating the repulsion forces that maintain  
253 particles isolated and promoting aggregation (Canesi et al., 2017; Paul-Pont et al., 2018). These

254 surface interactions can explain particle behaviour changes (surface charge, size and  
255 aggregation state) observed in FSW and filtered microalgae medium as compared to particles  
256 in Milli-Q water (Supp. Table 1). In environmental media, surface charge neutralization and  
257 increases in particle size are often observed, due to the formation of a protein coating (called  
258 ecocorona; Galloway et al., 2017) on the particle surface (Marques-Santos et al., 2018).  
259 Therefore, the medium and related parameters should be always taken into account for  
260 particle characterization (Della Torre et al., 2014).

### 261 **3.2. Scanning electron microscopy observations**

262 Micrographs of *C. neogracile* cells exposed to both MP sizes were presented, evidencing intact  
263 frustules, similar to control cells, without MP attached at the surface cell (Fig. 1A, B, C). Most  
264 cells showed broken setae, located close to the cell, likely because of the force exerted during  
265 the filtration process required for sample preparation for SEM observation. Several studies  
266 showed the adsorption of plastic micro and nanoparticles onto the microalgal cell surface  
267 (Bhattacharya et al., 2010; Mao et al., 2018) and this adhesion was found to be stronger with  
268 positively charged NP than with the negatively charged ones (Bergami et al., 2017; Nolte et al.,  
269 2017). The observed increase in MP aggregates and the change in MP charge (Table 1) could  
270 be responsible for the absence of particle attachment to the cell wall and its associated  
271 impacts. However, it cannot be excluded that a previous interaction between MP and  
272 microalgae occurred. Although the beads tested showed negative charges, MP could be  
273 adsorbed on microalgae by weak chemical bonds and SEM preparation could have detached  
274 them from the cell wall. Nevertheless, Long et al. (2017) showed that aggregation between *C.*  
275 *neogracile* cells and 2  $\mu\text{m}$  uncharged PS MP is rare during exponential growth (about 2%) and  
276 mainly occurred during the stationary growth phase (<20%).

277 Micrographs also evidenced free small MP aggregates and bacteria attached to the filter  
278 and/or associated to MP aggregates (Fig. 1D, E, F). Bacteria could interact with plastic particles  
279 and colonize them, as it has been shown by Foulon et al. (2016) with 5  $\mu\text{m}$  PS MP. Accordingly,  
280 bacteria concentration during the experiment was measured by flow cytometry and no  
281 significant differences were found among control and exposed cultures (Supp. Table 2).

### 282 **3.3. Little effect of MP exposure on cell growth**

283 Only a slight but significant decrease in growth rate was detected in cultures exposed to 2  $\mu\text{m}$   
284 MP for 72 h (ANOVA;  $F_{(2,6)} = 13.25$ ;  $p < 0.01$ ) (Table 2). The strong aggregation pattern of the  
285 MP in the microalgal medium observed in this study (Table 1) could reduce their bioavailability  
286 and explain their limited impact on growth, as previously hypothesized in other studies with PS  
287 particles (Bergami et al., 2017; Della Torre et al., 2014; Gambardella et al., 2018). Previous  
288 studies that tested different MP concentrations of similar size range, reported no effect on the

289 growth of microalgae. Long et al. (2017) did not observe adverse effects on the growth and  
290 chlorophyll fluorescence of *C. neogracile* cells exposed to 0.04  $\mu\text{g mL}^{-1}$  of uncharged 2  $\mu\text{m}$  PS  
291 MP. Davarpanah and Guilhermino (2015) did not found significant effects on the growth of the  
292 marine green microalgae *Tetraselmis chuii* exposed for 96 h to concentrations ranging from  
293 0.046 to 1.472  $\mu\text{g mL}^{-1}$  of red fluorescent polyethylene microspheres (1-5  $\mu\text{m}$  diameter).  
294 Sjollem et al. (2016) analyzed the growth and photosynthetic capacity of the marine green  
295 microalgae *Dunaliella tertiolecta* exposed to three sizes (50 nm, 0.5 and 6  $\mu\text{m}$ ) of uncharged PS  
296 beads and also observed that these beads had negligible effects on microalgae growth, except  
297 for nano-sized particles (50 nm) at high exposure concentrations (250  $\mu\text{g mL}^{-1}$ ), suggesting that  
298 the effect on microalgal growth increases with decreasing bead size.

299 Deleterious effects of MP on microalgal growth were only detected at very high  
300 concentrations, even higher than the concentration used in this study (2.5  $\mu\text{g mL}^{-1}$ ). However,  
301 influence of particle size was rarely considered and controlled which are making comparisons  
302 difficult. Mao et al. (2018) showed that 10, 50 and 100  $\mu\text{g mL}^{-1}$  of 0.1 and 1  $\mu\text{m}$  PS MP caused a  
303 dose-dependent negative effect on the growth and photosynthetic activity of the freshwater  
304 green microalgae *Chlorella pyrenoidosa* during its logarithmic growth phase. The mechanism  
305 associated to the toxicity was attributed to the physical damage and oxidative stress.  
306 Gambardella et al. (2018) exposed the marine microalgae *D. tertiolecta* to a wide range of  
307 0.1  $\mu\text{m}$  PS MP concentrations (0.001-0.01-0.1-1-10  $\mu\text{g mL}^{-1}$ ) for 72 h and also observed a dose-  
308 dependent growth inhibition. At the highest MP concentration they tested (10  $\mu\text{g mL}^{-1}$ ) about  
309 40% of growth inhibition was observed. Authors suggested that this growth inhibition was due  
310 to the fact that microalgae energy sources were used in detoxification processes, such as the  
311 generation of extracellular polysaccharides. It is also noteworthy that most of the published  
312 works assessing the effects of MP on phytoplankton were made with green microalgae, and  
313 scarce data about the effects of the small MP fraction on other phytoplankton taxonomic  
314 groups is available.

#### 315 **3.4. MP did not cause significant alterations of cell morphology and photosynthesis-related** 316 **parameters**

317 Potential changes in the structural properties of the diatom *C. neogracile* exposed to the MP  
318 were studied by FCM based on light diffraction. Neither of the two particle sizes significantly  
319 altered the morphology of microalgal cells, since no changes were detected in the FSC and SSC  
320 signals (Supp. Table 3). Similarly, Long et al. (2017) did not observed FSC and SSC changes in *C.*  
321 *neogracile* cells exposed to 0.04  $\mu\text{g mL}^{-1}$  of uncharged 2  $\mu\text{m}$  PS MP for all duration of culture  
322 growth. The fact that 0.5 and 2  $\mu\text{m}$  PS-NH<sub>2</sub> MP aggregate and become negatively charged may  
323 have affected the effectiveness of physical adsorption of these MP onto the cell wall.

324 Environmental stressors could affect the function of photosynthetic systems, thereby affecting  
325 the fluorescence emission (Geoffroy et al., 2007; Juneau et al., 2002). Natural red  
326 autofluorescence measured by FCM and effective quantum yield (QY) measured by PAM in *C.*  
327 *neogracile* cells did not show significant changes upon exposure to 2.5  $\mu\text{g mL}^{-1}$  of 0.5 and 2  $\mu\text{m}$   
328 PS-NH<sub>2</sub> MP as compared to control condition (Supp. Table 3). Mao et al. (2018) showed that  
329 upon exposure of 0.1  $\mu\text{m}$  and 1  $\mu\text{m}$  PS MP at 10  $\mu\text{g mL}^{-1}$ , adsorption of MP onto the surface of  
330 the freshwater microalgae *Chlorella pyrenoidosa* during its exponential growth phase could  
331 provoke shading effects, hindering algal photosynthesis. However, this phenomenon appears  
332 to be negligible for the particles tested, as shown in SEM micrographs, probably due to the  
333 electrostatic repulsion exerted by its observed negative charge on microalgal cell membrane  
334 (Table 1).

### 335 **3.5. Decrease in esterase activity as an early response to MP exposure**

336 A significant decrease in esterase activity of *C. neogracile* cells exposed to the MP tested with  
337 respect to control cells was observed after 24 h (ANOVA;  $F_{(2,6)} = 7.07$ ;  $p < 0.05$ ) and 48 h  
338 (ANOVA;  $F_{(2,6)} = 4.96$ ;  $p < 0.05$ ) (Fig. 2A). This reduction was more pronounced after 24 h of  
339 exposure, followed by a slight recovery at 48 h. After 72 h, esterase activity appeared fully  
340 recovered as significant differences were not observed anymore among treatments (ANOVA;  
341  $F_{(2,6)} = 1.35$ ;  $p > 0.05$ ) (Fig. 2A). Recovery from the detrimental effects caused by MP on  
342 microalgae was also observed by Mao et al. (2018) when cultures started the stationary phase.  
343 Representative flow cytometric histograms showing shifts in the green fluorescence intensity  
344 related to the esterase activity of *C. neogracile* cells in control cultures and cultures exposed to  
345 both MP are shown in Supp. Fig. 1.

346 The lack of effects on key parameters such as growth, morphology or photosynthesis was  
347 explained due to the absence of physical adsorption of MP onto diatom's cell wall. However,  
348 the decrease in esterase activity observed could be attributed to the contact between the MP  
349 and the microalgae during the culture. Contact, even temporal, could be detected and  
350 considered as a stress by the cells and may be translated into biochemical signals, triggering a  
351 response to deal with. It could be described as the "billiard ball effect". As documented in  
352 humans, cells may sense mechanical cues, although the details underlying how cells respond  
353 to mechanical forces are not well understood yet (Yusko et al., 2014). The potential release of  
354 chemicals from MP could be another explanation to the indirect toxicity detected. Monomers  
355 and/or additives incorporated during manufacture could be toxic to microalgae and could  
356 interfere with biological processes, explaining the decrease observed in the general metabolic  
357 activity of cells (Fig. 2A). Many exposure experiments reported significant toxicity of plastic  
358 leachates on aquatic organisms (reviewed in Hermabessiere et al., 2017). Virgin MP, frequently

359 used as model materials in aquatic toxicity laboratory studies, are supposed to be free of  
360 additives or residual monomers. However, some studies suggest that commercial virgin plastic  
361 pellets may also leach toxic unknown chemicals (Nobre et al., 2015). Martínez-Gómez et al.  
362 (2017) reported significant toxicity of virgin and aged PS MP on the fertilization and larval  
363 development of sea urchins, and plastic leachates were found to have higher toxicity than the  
364 virgin and aged materials themselves. Toxicity of leachates from plastic products obtained  
365 after 72 h of leaching was also observed in the copepod *Nitocra spinipes* (Bejgarn et al., 2015).  
366 Moreover, it cannot be excluded the potential repercussions that amino functional groups  
367 could have on cells. MP are coated with -NH<sub>2</sub> groups by chemical bonds and during laboratory  
368 exposure, light or other environmental factors could lead to bond cleavage.

369 As compared to the other parameters analysed, the FDA assay appeared more sensitive to  
370 detect physiological changes in cells exposed to these 0.5 and 2 µm (nominal sizes) particles  
371 after a short period of time. Esterases involved in the FDA assay turn over on a time frame of  
372 several hours (Jochem, 2000). Therefore, this technique seems appropriate to detect changes  
373 in metabolic activity on a day-to-day or even shorter basis, which makes it well suited to  
374 monitor short-term phytoplankton responses to environmental changes or to diverse  
375 pollutants (Esperanza et al., 2015; Franklin et al., 2001; Prado et al., 2009; Seoane et al., 2017a;  
376 Seoane et al., 2017b). Our results showed that, with more subtle measurements, we can  
377 detect the impact of MP. This brings out the suitability and sensitivity of this assay with the  
378 flow cytometry technique to assess the effects of plastic debris on marine phytoplankton.

### 379 **3.6. Unaltered ROS production and membrane potential during MP exposure**

380 Microalgae may have higher levels of ROS as a result of changes in environmental conditions  
381 or the presence of contaminants. When there is an imbalance between the production of ROS  
382 and the cellular antioxidant defence mechanisms, oxidative stress increases leading to several  
383 cellular damages. In the present study, ROS production in *C. neogracile* cells was not  
384 significantly affected by the presence of MP in the medium (Supp. Table 4). ROS  
385 overproduction has been previously observed by Mao et al. (2018) during the exponential  
386 growth phase of the freshwater microalgae *Chlorella pyrenoidosa* exposed to 0.1 µm and 1 µm  
387 PS particles at very high concentrations (10, 50 and 100 µg mL<sup>-1</sup>). However, it seems that with  
388 the 0.5 µm and 2 µm MP we tested (nominal sizes), the concentration used (2.5 µg mL<sup>-1</sup>) was  
389 not high enough to alter the intracellular equilibrium of *C. neogracile* ROS levels.

390 Regarding cytoplasmic membrane potential, no significant alterations were observed in cells  
391 exposed to MP (Supp. Table 4). Interactions between MP and biological membranes are driven  
392 by particle size, since small particles are suspected to interact more with biological membranes  
393 (Nel et al., 2006; Verma and Stellacci, 2010), and by particle surface properties, notably the net

394 surface charge (Nolte et al., 2017). Taking into consideration the particle size, internalization of  
395 MP used (0.5 and 2  $\mu\text{m}$ ) was discarded on intact cells. Algal cell walls are semipermeable and  
396 the diameter of their pores determines its sieving properties (Navarro et al., 2008). Diatoms'  
397 wall pores are typically between 3 and 50 nm (Sanka et al., 2017). As long as there are no holes  
398 in the cell wall or loss of viability, only particles with a size smaller than that of the largest pore  
399 are expected to pass through the cell wall. Thus, the algal cell wall pore size is too small to  
400 transport MP used through the cell. Gambardella et al. (2018) evaluated MP internalization in  
401 the green microalgae *D. tertiolecta* using 0.1  $\mu\text{m}$  fluorescent PS MP. Although MP caused algal  
402 growth inhibition, they also discard MP internalization into cells, since all fluorescence beads  
403 were observed as aggregates in the medium, out of the microalgal cell surface. With regard to  
404 the charge, it is possibly related to differences in effectiveness of physical adsorption onto the  
405 cell wall, since cationic particles interact with membranes more easily than anionic ones  
406 (Bhattacharya et al., 2010; Nel et al., 2009). In the present study, the aggregation of MP and  
407 the negative charge measured seems to minimize MP effects. MP concentration and time  
408 exposure are also important factors for MP impacts. Mao et al. (2018) showed membrane  
409 damages such as cell wall thickening and loss of viability in *C. pyrenoidosa* exposed to PS MP at  
410 the higher concentration they tested ( $100 \mu\text{g mL}^{-1}$ ) in long-term exposure (30 days). However,  
411 at the concentration used in our short-term study, we did not observed effects on membrane  
412 potential.

### 413 **3.7. Diminished neutral lipid content in MP-exposed cells**

414 Exposure to 0.5 and 2  $\mu\text{m}$  PS-NH<sub>2</sub> MP resulted in a significant decrease in the cellular neutral  
415 lipid content with respect to control cells at all tested times (ANOVA 24 h;  $F_{(2,6)} = 37.50$ ;  
416  $p < 0.001$ ) (ANOVA 48 h;  $F_{(2,6)} = 12.08$ ;  $p < 0.01$ ) (ANOVA 72 h;  $F_{(2,6)} = 18.46$ ;  $p < 0.01$ ) (Fig. 2B).  
417 After 72 h, the lipid content of MP-exposed cells was reduced by half with respect to control.  
418 Representative flow cytometric histograms showing shifts in the green fluorescence intensity  
419 related to the neutral lipid content of *C. neogracile* cells in control cultures and cultures  
420 exposed to both MP are shown in Supp. Fig. 2.

421 Microalgae generally accumulate neutral lipids, mainly triacylglycerol (TAG), in specific  
422 organelles called lipid bodies, upon stresses such as nutrient limitation, elevated  
423 temperatures, unfavourable light intensities, alkaline pH, high salinity or dehydration  
424 (Zienkiewicz et al. 2016). In laboratory cultures, microalgae begin to accumulate lipids in the  
425 stationary phase of growth, when shortage of nutrients arrives (Huerlimann et al., 2010; Xu et  
426 al., 2008). Previous studies found disturbances in lipid metabolism after exposure to MP in  
427 other species such as marine fishes and mussels (Von Moos et al., 2012; Yin et al., 2018).  
428 Although exposure to MP does not usually cause mortality in marine organisms, it has been

429 observed that it can affect them by altering their feeding behaviour and reducing their energy  
430 reserves, with consequences for growth and reproduction (Galloway et al., 2017). Microalgal  
431 oil bodies have a dynamic nature and appear to function as transient reservoirs, as the storage  
432 lipids are quick catabolized in response to environmental changes (Maeda et al., 2017). In the  
433 present study, oxidative stress was not detected in MP-exposed cells; therefore, the decrease  
434 in lipids observed cannot be associated with its oxidation, but to modulation of energy  
435 metabolism to properly acclimate to the stress conditions, maintaining a healthy status. As  
436 discussed previously, despite not having observed MP attached to the cell surface it cannot be  
437 excluded an interaction between MP and microalgae and an indirect toxicity due to i) the  
438 contact between MP and cells during the culture and ii) the potential toxic compounds  
439 (monomers, oligomers, additives or other chemicals) that could be leached from MP. The  
440 “consumption” of lipid reserves could be interpreted as a cell response to overcome the stress  
441 provoked by MP exposure and for the maintenance of the normal growth, photosynthesis and  
442 even the membrane integrity. Thus, microalgal oil bodies could serve as an energy source for  
443 their recovery. This decrease in the lipid content could also have ecological implications for  
444 food web trophic functioning by reducing microalgae nutritional quality for primary consumers  
445 and onward.

#### 446 **4. Conclusions**

447 MP aggregated in FSW and in filtered culture medium, producing secondary particles with  
448 different properties, which make difficult assessing influence of size on MP toxicity. In  
449 addition, their  $\zeta$ -potential in FSW and in the culture medium outlined low negative values, fact  
450 that could have influenced their aggregation state and their interaction with the cell surface. It  
451 highlights the necessity to characterize behaviour of plastic particles in assay media before  
452 exposure to avoid bias in results interpretation.

453 Direct toxicity on key parameters such as cell growth, morphology, photosynthesis, ROS  
454 content and membrane potential was not observed and SEM micrographs showed that MP  
455 were not attached to the microalgal cell wall. However, indirect toxicity was detected because  
456 we unravelled a significant decrease in the esterase activity and the lipid reserves of MP-  
457 exposed cells. Results suggest that microalgal oil bodies could serve as an energy source for  
458 the maintenance of the normal cellular growth, photosynthesis and membrane integrity to  
459 overcome the stress produced by MP exposure.

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#### 470 **References**

471 Andrady, A.L., 2011. Microplastics in the marine environment. *Mar. Pollut. Bull.* 62, 1596–  
472 1605.

473 Andrady, A.L., 2015. Persistence of plastic litter in the oceans. In: Bergmann, M., Gutow, L.,  
474 Klages, M. (Eds.), *Marine Anthropogenic Litter*. Springer International Publishing,  
475 Cham, pp. 57-72.

476 Barnes, D.K., Galgani, F., Thompson, R.C., Barlaz, M., 2009. Accumulation and fragmentation  
477 of plastic debris in global environments. *Philos. T. Roy. Soc. B.* 364, 1985–1998.

478 Bejgarn, S., MacLeod, M., Bogdal, C., Breitholtz, M., 2015. Toxicity of leachate from  
479 weathering plastics: An exploratory screening study with *Nitocra spinipes*.  
480 *Chemosphere* 132, 114–119.

481 Bergami, E., Pugnali, S., Vannuccini, M. L., Manfra, L., Faleri, C., Savorelli, F., Dawson, K.A.,  
482 Corsi, I., 2017. Long-term toxicity of surface-charged polystyrene nanoplastics to  
483 marine planktonic species *Dunaliella tertiolecta* and *Artemia franciscana*. *Aquat.*  
484 *Toxicol.* 189, 159–169.

485 Bhattacharya, P., Lin, S., Turner, J. P., Ke, P. C., 2010. Physical adsorption of charged plastic  
486 nanoparticles affects algal photosynthesis. *J. Phys. Chem. C* 114, 16556–16561.

487 Canesi, L., Balbi, T., Fabbri, R., Salis, A., Damonte, G., Volland, M., Blasco, J., 2017.  
488 Biomolecular coronas in invertebrate species: implications in the environmental  
489 impact of nanoparticles. *NanoImpact* 8, 89-98.

490 Cole, M., Lindeque, P., Halsband, C., Galloway, T.S., 2011. Microplastics as contaminants in  
491 the marine environment: A review. *Mar. Pollut. Bull.* 62, 2588-2597.

492 Davarpanah, E., Guilhermino, L., 2015. Single and combined effects of microplastics and  
493 copper on the population growth of the marine microalgae *Tetraselmis chuii*. *Estuar.*  
494 *Coast. Shelf Sci.* 167, 269–275.

495 Dawson, A. L., Kawaguchi, S., King, C. K., Townsend, K. A., King, R., Huston, W. M., Bengtson  
496 Nash, S. M., 2018. Turning microplastics into nanoplastics through digestive  
497 fragmentation by Antarctic krill. *Nat. Commun.* 9, 1001.

498 Della Torre, C., Bergami, E., Salvati, A., Faleri, C., Cirino, P., Dawson, K.A., Corsi, I., 2014.  
499 Accumulation and embryotoxicity of polystyrene nanoparticles at early stage of  
500 development of sea urchin embryos *Paracentrotus lividus*. *Environ. Sci. Technol.* 48,  
501 12302–12311.

502 Dubaish, F., Liebezeit, G., 2013. Suspended microplastics and black carbon particles in the  
503 Jade system, southern North Sea. *Water Air Soil Pollut.* 224, 1352-1359.

504 Eriksen, M., Lebreton, L.C.M., Carson, H.S., Thiel, M., Moore, C.J., Borerro, J.C., Galgani, F.,

- 505 Ryan, P.G., Reisser, J., 2014. Plastic Pollution in the World's Oceans: More than 5  
506 Trillion Plastic Pieces Weighing over 250,000 Tons Afloat at Sea. *PLoS One* 9, 1–15.
- 507 Esperanza, M., Seoane, M., Rioboo, C., Herrero, C., Cid, Á., 2015. *Chlamydomonas reinhardtii*  
508 cells adjust the metabolism to maintain viability in response to atrazine stress. *Aquat.*  
509 *Toxicol.* 165, 64–72.
- 510 Filella, M., 2015. Questions of size and numbers in environmental research on microplastics:  
511 methodological and conceptual aspects. *Environ. Chem.* 12, 527–538.
- 512 Foulon, V., Le Roux, F., Lambert, C., Huvet, A., Soudant, P., Paul-Pont, I., 2016. Colonization of  
513 polystyrene microparticles by *Vibrio crassostreae*: Light and electron microscopic  
514 investigation. *Environ. Sci. Technol.* 50, 10988–10996.
- 515 Franklin, N.M., Stauber, J.L., Lim, R.P., 2001. Development of flow cytometry-based algal  
516 bioassays for assessing toxicity of copper in natural waters. *Environ. Toxicol. Chem.*  
517 20, 160–170.
- 518 Galloway, T. S., Cole, M., Lewis, C., Atkinson, A., and Allen, J. I., 2017. Interactions of  
519 microplastic debris throughout the marine ecosystem. *Nat. Ecol. Evol.* 1, 116.
- 520 Gambardella, C., Morgana, S., Bramini, M., Rotini, A., Manfra, L., Migliore, L., Piazza, V.,  
521 Garaventa, F., Faimali, M., 2018. Ecotoxicological effects of polystyrene microbeads in  
522 a battery of marine organisms belonging to different trophic levels. *Mar. Environ. Res.*  
523 141, 313–321.
- 524 Geoffroy, L., Gilbin, R., Simon, O., Floriani, M., Adam, C., Pradines, C., Cournac, L., Garnier-  
525 Laplace, J., 2007. Effect of selenate on growth and photosynthesis of *Chlamydomonas*  
526 *reinhardtii*. *Aquat. Toxicol.* 83, 149–58.
- 527 Gigault, J., Halle, A. Ter, Baudrimont, M., Pascal, P. Y., Gauffre, F., Phi, T. L., El Hadri, H.,  
528 Grassl, B., Reynaud, S., 2018. Current opinion: What is a nanoplastic? *Environ. Pollut.*  
529 235, 1030-1034.
- 530 Gigault, J., Pedrono, B., Maxit, B., Ter Halle, A., 2016. Marine plastic litter: the unanalyzed  
531 nano-fraction. *Environ. Sci. Nano* 3, 346–350.
- 532 González-Fernández, C., Tallec, K., Le Goïc, N., Lambert, C., Soudant, P., Huvet, A., Suquet,  
533 M., Berchel, M., Paul-Pont, I., 2018. Cellular responses of Pacific oyster (*Crassostrea*  
534 *gigas*) gametes exposed in vitro to polystyrene nanoparticles. *Chemosphere* 208, 764-  
535 772.
- 536 Govender, T., Ramanna, L., Rawat, I., Bux, F., 2012. BODIPY staining, an alternative to the Nile  
537 Red fluorescence method for the evaluation of intracellular lipids in microalgae.  
538 *Bioresour. Technol.* 114, 507–511.
- 539 Hecky, R.E., Mopper, K., Kilham, P., Degens, E.T., 1973. Amino-acid and sugar composition of  
540 diatom cell-walls. *Mar. Biol.* 19, 323-331.
- 541 Hermabessiere, L., Dehaut, A., Paul-Pont, I., Lacroix, C., Jezequel, R., Soudant, P., Duflos, G.,  
542 2017. Occurrence and effects of plastic additives on marine environments and  
543 organisms: a review. *Chemosphere* 182, 781–793.
- 544 Hernandez, L.M., Yousefi, N., Tufenkji, N., 2017. Are there nanoplastics in your personal care  
545 products? *Environ. Sci. Technol. Lett.* 4, 280-285.
- 546 Huerlimann, R., Rocky, N., Heimann, K., 2010. Growth, lipid content, productivity and fatty  
547 acid composition of tropical microalgae for scale-up production. *Biotechnol. Bioeng.*  
548 107, 245-257.
- 549 Jochem, F. J., 2000. Probing the physiological state of phytoplankton at the single-cell level.

- 550 Sci. Mar. 64, 183-195.
- 551 Juneau, P., El Berdey, A., Popovic, R., 2002. PAM fluorometry in the determination of the  
552 sensitivity of *Chlorella vulgaris*, *Selenastrum capricornutum* and *Chlamydomonas*  
553 *reinhardtii* to copper. Arch. Environ. Contam. Toxicol. 42, 155–164.
- 554 Lambert, S., Wagner, M., 2016. Characterisation of nanoplastics during the degradation of  
555 polystyrene. Chemosphere 145, 265-268.
- 556 Long, M., Paul-Pont, I., Hégaret, H., Moriceau, B., Lambert, C., Huvet, A., Soudant, P., 2017.  
557 Interactions between polystyrene microplastics and marine phytoplankton lead to  
558 species-specific hetero-aggregation. Environ. Pollut. 228, 454–463.
- 559 Lundqvist, M., Stigler, J., Elia, G., Lynch, I., Cedervall, T., Dawson, K. A., 2008. Nanoparticle  
560 size and surface properties determine the protein corona with possible implications  
561 for biological impacts. Proc. Natl. Acad. Sci. 105, 14265–14270.
- 562 Lusher, A. L., Hollman, P. C. H., Mendoza-Hill, J. J., 2017. Microplastics in Fisheries and  
563 Aquaculture: Status of Knowledge on Their Occurrence and Implications for Aquatic  
564 Organisms and Food Safety. FAO Fisheries and Aquaculture Technical Paper. No. 615,  
565 p. 147.
- 566 Maeda, Y., Nojima, D., Yoshino, T., Tanaka, T., 2017. Structure and properties of oil bodies in  
567 diatoms. Phil. Trans. R. Soc. 372, 20160408.
- 568 Malviya, S., Scalco, E., Audic, S., Vincent, F., Veluchamy, A., Poulain, J., Wincker, P., Iudicone,  
569 D., de Vargas, C., Bittner, L., Zingone, A., Bowler, C., 2016. Insights into global diatom  
570 distribution and diversity in the world's ocean. Proc. Natl. Acad. Sci. 113, E1516-  
571 E1525.
- 572 Mao, Y., Ai, H., Chen, Y., Zhang, Z., Zeng, P., Kang, L., Li, W., Gu, W., He, Q., Li, H., 2018.  
573 Phytoplankton response to polystyrene microplastics: Perspective from an entire  
574 growth period. Chemosphere 208, 59–68.
- 575 Marques-Santos, L. F., Grassi, G., Bergami, E., Faleri, C., Balbi, T., Salis, A., Damonte, G.,  
576 Canesi, L., Corsi, I., 2018. Cationic polystyrene nanoparticle and the sea urchin  
577 immune system: biocorona formation, cell toxicity, and multixenobiotic resistance  
578 phenotype. Nanotoxicology 0, 1–21.
- 579 Martínez-Gómez, C., León, V. M., Calles, S., Gomáriz-Olcina, M., Vethaak, A. D., 2017. The  
580 adverse effects of virgin microplastics on the fertilization and larval development of  
581 sea urchins. Mar. Environ. Res. 130, 69-76.
- 582 National Oceanic and Atmospheric Administration (NOAA), 2008. Proceedings of the  
583 International Research Workshop on the Occurrence, effects, and fate of Microplastic  
584 Marine debris. C Arthur, J. Baker, and H. Bamford (eds). NOAA Technical  
585 Memorandum NOS-OR&R-30.
- 586 Navarro, E., Baun, A., Behra, R., Hartmann, N.B., Filser, J., Miao, A.J., Quigg, A., Santschi, P.H.,  
587 Sigg, L., 2008. Environmental behavior and ecotoxicity of engineered nanoparticles to  
588 algae, plants, and fungi. Ecotoxicology 17, 372–386.
- 589 Nel, A., Xia, T., Mädler, L., Li, N., 2006. Toxic potential of materials at the nano level. Science  
590 311, 622-627.
- 591 Nel, A.E., Mädler, L., Velegol, D., Xia, T., Hoek, E.M.V., Somasundaran, P., Klaessig, F.,  
592 Castranova, V., Thompson, M., 2009. Understanding biophysicochemical interactions  
593 at the nano-bio interface. Nat. Mater. 8, 543-557.
- 594 Nobre, C. R., Santana, M. F. M., Maluf, A., Cortez, F. S., Cesar, A., Pereira, C. D. S., et al., 2015.

- 595 Assessment of microplastic toxicity to embryonic development of the sea urchin  
596 *Lytechinus variegatus* (Echinodermata: Echinoidea). *Mar Pollut. Bull.* 92, 99–104.
- 597 Nolte, T. M., Hartmann, N. B., Kleijn, J. M., Garnæs, J., van de Meent, D., Jan Hendriks, A.,  
598 Baun, A., 2017. The toxicity of plastic nanoparticles to green algae as influenced by  
599 surface modification, medium hardness and cellular adsorption. *Aquat. Toxicol.* 183,  
600 11–20.
- 601 OECD 201, 2011. Alga Growth Inhibition Test (201). OECD Guideline for Testing of Chemicals.  
602 Organization for Economic Cooperation and Development, Paris, France.
- 603 Paul-Pont, I., Tallec, K., Gonzalez-Fernandez, C., Lambert, C., Vincent, D., Mazurais, D.,  
604 Zambonino-Infante, J.L., Brotons, G., Lagarde, F., Fabioux, C., Soudant, P., Huvet, A.,  
605 2018. Constraints and priorities for conducting experimental exposures of marine  
606 organisms to microplastics. *Front. Mar. Sci.* 5, 252.
- 607 Peeken, I., Primpke, S., Beyer, B., Gütermann, J., Katlein, C., Krumpfen, T., Bergmann,  
608 M., Hehemann, L., Gerdt, G., 2018. Arctic sea ice is an important temporal sink and  
609 means of transport for microplastic. *Nat. Commun.* 9, 2041–1723.
- 610 Pham, C. K., Ramirez-Llodra, E., Alt, C.H.S., Amaro, T., Bergmann, M., Canals, M., Company,  
611 J.B., Davies, J., Duineveld, G., Galgani, F., Howell, K.L., Huvenne, V.A., Isidro, E., Jones,  
612 D.O., Lastras, G., Morato, T., Gomes-Pereira, J.N., Purser, A., Stewart, H., Tojeira, I.,  
613 Tubau, X., Van Rooij, D., Tyler, P.A., 2014. Marine litter distribution and density in  
614 European seas, from the shelves to deep basins. *PLoS ONE* 9(4), e95839.
- 615 Phuong, N. N., Zalouk-Vergnoux, A., Poirier, L., Kamari, A., Châtel, A., Mouneyrac, C., Lagarde,  
616 F., 2016. Is there any consistency between the microplastics found in the field and  
617 those used in laboratory experiments? *Environ. Pollut.* 211, 111–123.
- 618 Plastics Europe, 2017. Plastics – the facts 2017. In: An Analysis of European plastics  
619 production, demand and waste data. Technical Report.
- 620 Prado, R., García, R., Rioboo, C., Herrero, C., Abalde, J., Cid, Á., 2009. Comparison of the  
621 sensitivity of different toxicity test endpoints in a microalga exposed to the herbicide  
622 paraquat. *Environ. Int.* 35, 240–247.
- 623 Rosenwasser, S., van Creveld, G.S., Schatz, D., Malitsky, S., Tzfadia, O., Aharoni, A., Levin, Y.,  
624 Gabashvili, A., Feldmesser, E., Vardi, A., 2014. Mapping the diatom redox-sensitive  
625 proteome provides insight into response to nitrogen stress in the marine  
626 environment. *Proc. Natl. Acad. Sci. U. S. A.* 111, 2740–2745.
- 627 Ruiz-Orejón, L.F., 2016. Floating plastic debris in the central and western mediterranean sea.  
628 *Mar. Environ. Res.* 120, 136–144.
- 629 Rumin, J., Bonnefond, H., Saint-Jean, B., Rouxel, C., Sciandra, A., Bernard, O., Cadoret, J.P.,  
630 Bougaran, G., 2015. The use of fluorescent Nile red and BODIPY for lipid  
631 measurement in microalgae. *Biotechnol. Biofuels* 8, 42.
- 632 Ryan, 2015. A Brief History of Marine Litter Research. In: Bergmann, M., Gutow, L., Klages, M.  
633 (Eds.), *Marine Anthropogenic Litter*. Springer International Publishing, pp. 1–25.
- 634 Ryan, P.G., Moore, C.J., van Franeker, J.A., Moloney, C.L., 2009. Monitoring the abundance of  
635 plastic debris in the marine environment. *Philos. Trans. R. Soc. B* 364, 1999–2012.
- 636 Sanka, I., Suyono, E.A., Alam, P., 2017. The effects of diatom pore-size on the structures and  
637 extensibilities of single mucilage molecules. *Carbohydr. Res.* 448, 35–42.
- 638 Seoane, M., Esperanza, M., Cid, Á., 2017a. Cytotoxic effects of the proton pump inhibitor  
639 omeprazole on the non-target marine microalga *Tetraselmis suecica*. *Aquat. Toxicol.*

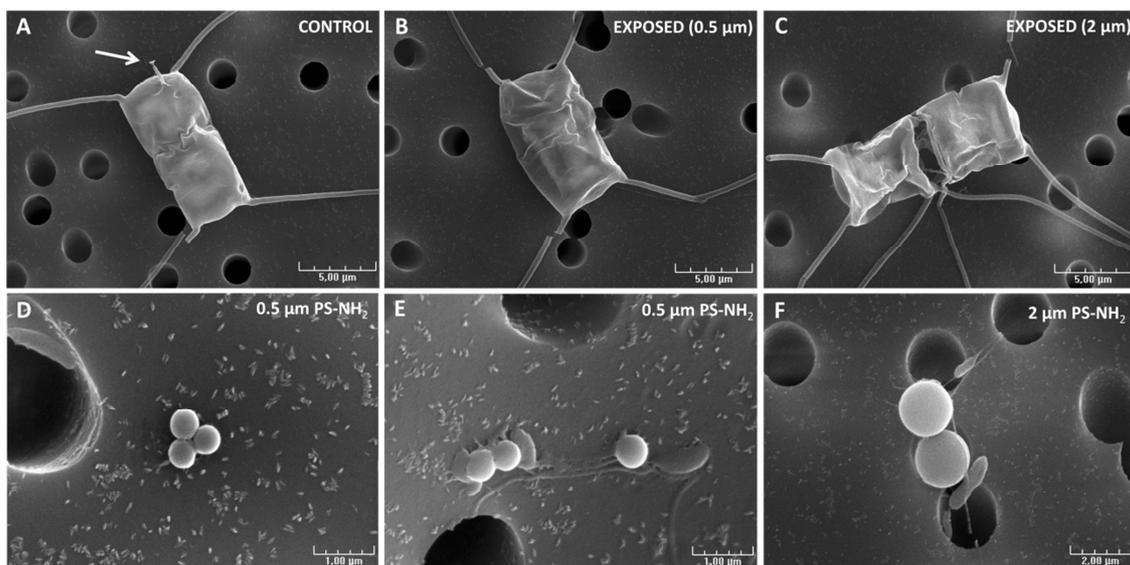
- 640 191, 62–72.
- 641 Seoane, M., Esperanza, M., Rioboo, C., Herrero, C., Cid, Á., 2017b. Flow cytometric assay to  
642 assess short-term effects of personal care products on the marine microalga  
643 *Tetraselmis suecica*. *Chemosphere*, 171, 339–347.
- 644 Sjollema, S.B., Redondo-Hasselerharm, P., Leslie, H. a., Kraak, M.H.S., Vethaak, a. D., 2016.  
645 Do plastic particles affect microalgal photosynthesis and growth? *Aquat. Toxicol.* 170,  
646 259–261.
- 647 Sun, X., Chen, B., Li, Q., Liu, N., Xia, B., Zhu, L., Qu, K., 2018. Toxicities of polystyrene nano-  
648 and microplastics toward marine bacterium *Halomonas alkaliphila*. *Sci. Total Environ.*  
649 642, 1378–1385.
- 650 Tallec, K., Huvet, A., Di Poi, C., González-Fernández, C., Lambert, C., Petton, B., Le Goïc, N.,  
651 Berchel, M., Soudant, P., Paul-Pont, I., 2018. Nanoplastics impaired oyster free living  
652 stages, gametes and embryos. *Environ. Pollut.* 242, 1226–1235.
- 653 Ter Halle, A., Jeanneau, L., Martignac, M., Jardé, E., Pedrono, B., Brach, L., Gigault, J., 2017.  
654 Nanoplastic in the North Atlantic Subtropical Gyre. *Environ. Sci. Technol.* 51, 13689–  
655 13697.
- 656 Verma, A., Stellacci, F., 2010. Effect of surface properties on nanoparticle-cell interactions.  
657 *Small* 6, 12-21.
- 658 Von Moos, N., Burkhardt-Holm, P., Köhler, A., 2012. Uptake and effects of microplastics on  
659 cells and tissue of the blue mussel *Mytilus edulis* L. after an experimental exposure.  
660 *Environ. Sci. Technol.* 46, 11327–11335.
- 661 Walne, P. R., 1966. Experiments in the Large-scale Culture of the Larvae of *Ostrea edulis* L.  
662 Fishery In, Her Majesty's Stationery Office, London.
- 663 Wolff, C., Fuks, B., Chatelain, P., 2003. Comparative study of membrane potential- sensitive  
664 fluorescent probes and their use in ion channel screening assays. *J. Biomol. Screen* 8,  
665 533-543.
- 666 Xu, Z.B., Yan, X.J., Pei, L.Q., Luo, Q.J., Xu, J.L., 2008. Changes in fatty acids and sterols during  
667 batch growth of *Pavlova viridis* in photobioreactor. *J. Appl. Phycol.* 20, 237–243.
- 668 Yin, L., Chen, B., Xia, B., Shi, X., Qu, K., 2018. Polystyrene microplastics alter the behavior,  
669 energy reserve and nutritional composition of marine jacopecover (*Sebastes schlegelii*).  
670 *J. Hazard. Mater.* 360, 97–105.
- 671 Yokota, K., Waterfield, H., Hastings, C., Davidson, E., Kwietniewski, E., Wells, B., 2017. Finding  
672 the missing piece of the aquatic plastic pollution puzzle: Interaction between primary  
673 producers and microplastics. *Limnol. Oceanogr. Lett.* 2, 91–104.
- 674 Yusko, E.C., Asbury, C.L., 2014. Force is a signal that cells cannot ignore. *Mol. Biol. Cell* 25,  
675 3717–3725.
- 676 Zhang, C., Chen, X., Wang, J., Tan, L., 2017. Toxic effects of microplastic on marine microalgae  
677 *Skeletonema costatum*: interactions between microplastic and algae. *Environ. Poll.*  
678 220, 1282-1288.
- 679 Zienkiewicz, K., Du, Z. Y., Ma, W., Vollheyde, K., Benning, C., 2016. Stress-induced neutral  
680 lipid biosynthesis in microalgae — Molecular, cellular and physiological insights.  
681 *Biochim. Biophys. Acta* 1861, 1269–1281.

**Table 1.** Characterization of PS-NH<sub>2</sub> MP in Milli Q water, filtered natural seawater (FSW) and microalgae medium using DLS analysis. Z-average (nm), polydispersity index - Pdl (a.u.) and  $\zeta$ -potential (mV), referred to a PS-NH<sub>2</sub> MP solution concentration of 100  $\mu\text{g mL}^{-1}$  are reported. Pdl > 0.2 showing aggregation is marked by an asterisk (\*). Values are shown as mean  $\pm$  standard deviation of the three measurements.

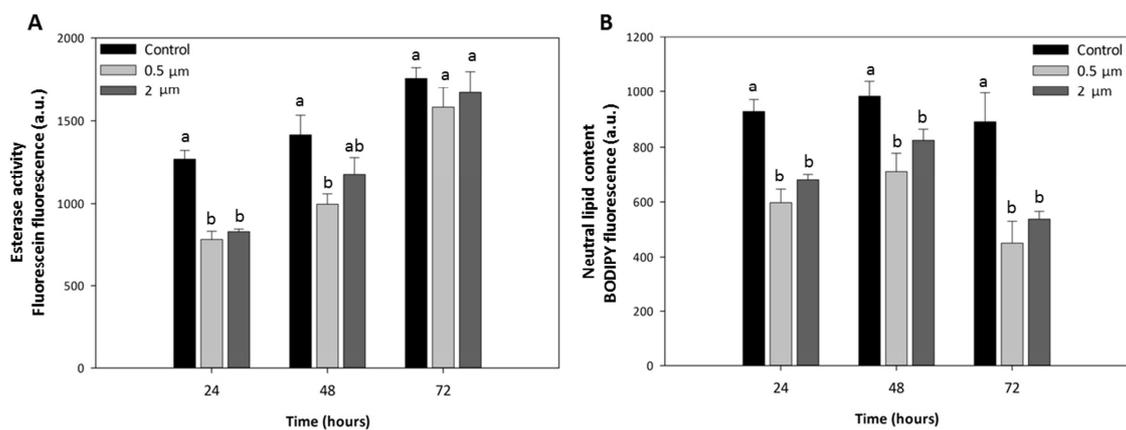
Nominal size	Milli Q			FSW			Filtered (0.2 $\mu\text{m}$ ) microalgal culture medium		
	Size Z-average (nm)	Pdl (a.u.)	Charge $\zeta$ -potential (mV)	Size Z-average (nm)	Pdl (a.u.)	Charge $\zeta$ -potential (mV)	Size Z-average (nm)	Pdl (a.u.)	Charge $\zeta$ -potential (mV)
0.5 $\mu\text{m}$	602.5 $\pm$ 27.3	0.16 $\pm$ 0.02	-11.9 $\pm$ 0.6	2899.0 $\pm$ 96.0	0.46 $\pm$ 0.10*	-7.3 $\pm$ 0.9	3268.0 $\pm$ 64.3	0.40 $\pm$ 0.02*	-6.9 $\pm$ 1.7
2 $\mu\text{m}$	2274.0 $\pm$ 100.9	0.16 $\pm$ 0.08	-12.8 $\pm$ 0.3	5932.0 $\pm$ 694.4	0.89 $\pm$ 0.10*	-4.8 $\pm$ 0.5	4342.3 $\pm$ 294.2	0.67 $\pm$ 0.15*	-5.0 $\pm$ 2.0

**Table 2.** Growth rates  $\mu$  ( $\text{day}^{-1}$ ) of *C. neogracile* cultures exposed to 0 and  $2.5 \mu\text{g mL}^{-1}$  of  $0.5 \mu\text{m}$  and  $2 \mu\text{m}$  PS-NH<sub>2</sub> MP at each sample time. Data are given as mean values  $\pm$  standard deviation of three replicates. Significant differences with respect to control at a level of significance of 0.05 ( $p < 0.05$ ) are represented by an asterisk (\*).

Particle type	Growth rate $\mu$ ( $\text{day}^{-1}$ )		
	24 h	48 h	72 h
Control	$1.56 \pm 0.06$	$1.59 \pm 0.05$	$1.44 \pm 0.02$
0.5 $\mu\text{m}$	$1.63 \pm 0.02$	$1.58 \pm 0.02$	$1.44 \pm 0.01$
2 $\mu\text{m}$	$1.61 \pm 0.09$	$1.55 \pm 0.03$	$1.39 \pm 0.01^*$



**Figure 1.** Scanning electron micrographs showing a control cell (A) and cells exposed to 0.5 μm (B) and 2 μm PS-NH<sub>2</sub> MP (C). 0.5 μm (D, E) and 2 μm (F) PS-NH<sub>2</sub> beads micro-aggregates and bacteria are also shown. Arrow indicates a rimoportula (tubular process through the valve of some diatoms). Scale bars: 5 μm (A, B, C); 1 μm (D, E); 2 μm (F).



**Figure 2.** Esterase activity (A) and neutral lipid content (B) of *C. neogracile* cells in control cultures and cultures exposed to 2.5 μg mL<sup>-1</sup> of 0.5 and 2 μm PS-NH<sub>2</sub> MP for 24, 48 and 72 h. Significant differences between treatments are marked with lowercase letters (p < 0.05).

**Highlights**

1. Effects of 0.5 and 2  $\mu\text{m}$  PS-NH<sub>2</sub> microplastics (MP) were evaluated on *C. neogracile*
2. MP showed negative charge, were aggregated and were not attached to the cell wall
3. Exposure to MP decreased the cellular metabolic activity and neutral lipid content
4. Cells modulate their energy metabolism to properly acclimate to the stress conditions
5. Microalgal oil bodies serve as an energy source for maintaining a healthy status