Environmental Pollution
August 2019, Volume 251, Pages 363-371
https://doi.org/10.1016/j.envpol.2019.04.142
https://archimer.ifremer.fr/doc/00492/60415/

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

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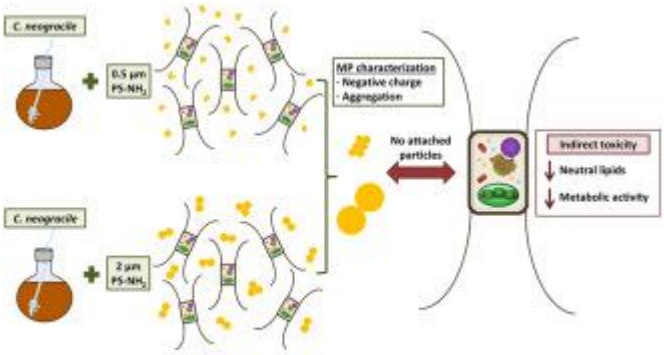
Abstract :

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−1 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_2 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 : icroalgae, microplastic, flow cytometry, cytotoxicity, scanning electron microscopy.

1. Introduction

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

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

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

The aim of this study was to assess the potential effects of two different sizes (0.5 µm and 2 µm) of unlabelled polystyrene (PS) beads on the marine diatom *Chaetoceros neogracile*, trying to disclose differential impacts depending on particle size, as smaller particles are expected to be more toxic (Sjollema et a., 2016). To perform the experiments, PS beads with amino (-NH_2) surface modifications were used at 2.5 µg mL^{-1}, concentration set to compare with the 72 h EC_{50} value of 50 nm PS-NH_2 NP (data not shown). PS is one of the most used plastics worldwide and it is one of the plastic polymers type most frequently detected as micro-debris in marine environments (Andrady, 2011). PS-NH_2 beads have been amply used as model particles in ecotoxicology and have been shown to cause severe damages on different organisms of the aquatic trophic chain (Bergami et al., 2017; Bhattacharya et al., 2010; Canesi et al., 2017; Manfra et al., 2017; Marques-Santos et al., 2018; Pinsino et al., 2017; Tallec et al., 2018). Particle behaviour was measured in Milli-Q water, seawater and in the culture medium using Dynamic Light Scattering (DLS) to assess potential particle aggregation or changes in the particle surface charge. To assess PS-NH_2 MP effects on *C. neogracile* several physiological and biochemical parameters such as cell morphology, autofluorescence, esterase activity, reactive oxygen species (ROS) levels, cytoplasmic membrane potential and neutral lipid content were monitored by flow cytometry (FCM) in addition to traditional growth and photosynthesis endpoints. Furthermore, potential structural damages and disposition or adsorption of unlabelled PS-NH_2 beads on microalgal surface were determined using scanning electron microscopy (SEM).

2. Materials and methods

2.1. Microalgal cultures

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

(frustules), covered with an organic coating (Hecky et al., 1973). This species was selected based on its predominance in marine phytoplankton communities (Malviya et al., 2016). Stock microalgal cultures were maintained in filtered (pore size: 0.2 µm) and autoclaved natural seawater (FSW) supplied with 1 mL L^{-1} of Conway medium (Walne, 1966) and enriched with silica ($Na_2SiO_3 \cdot 9H_2O$) (1.07 10^{-4} M). Flasks were kept at 20 °C, with continuous aeration and light at 100 µmol photon $m^{-2}s^{-1}$. CO_2 was supplied to keep the pH between 7.5 and 7.9.

2.2. Particle characterization

Unlabelled 0.5 µm and 2 µm PS-NH_2 particles were purchased from Micromod (Micromod Partikeltechnologie GmbH). Both particles were characterized by Dynamic Light Scattering (DLS) using Zetasizer Nano Series ZS (Malvern instruments) as conducted by Tallec et al. (2018). Size (Z-average in nm), charge (ζ-potential in mV) and aggregation state (Polydispersity Index, PdI in arbitrary units (a.u.)) were measured in Milli-Q water, FSW and also in filtered (0.2 µm) microalgal culture medium. When PdI exceeds 0.2 a.u. particles were considered to be aggregated. Measurements were carried out in triplicate, each containing 13 runs of 10 s for Z-average and 20 runs, 3 s delay for ζ-potential following the protocol described in Della Torre et al. (2014). Data were analyzed using Zetasizer Nano Series software, version 6.20.

2.3. Microalgal exposure to microplastics

Microalgae cells were exposed to both particles in different batch cultures for 72 h, during the exponential growth phase, as recommended in most standardized growth inhibition tests with microalgae (OECD 201, 2011). It has also been shown that during exponential growth phase MP impaired more drastically the major cellular and physiological parameters (Mao et al., 2018). Exposures were performed in triplicates in 500 mL glassware balloon flasks filled with 300 mL of microalgal culture under the same environmental conditions as stock cultures. Microalgal cells in early exponential growth phase were used as inoculum and initial cell density was adjusted to 4 x 10^5 cells mL^{-1}. The MP concentration used (2.5 µg mL^{-1}) corresponds to the 72 h EC_{50} value of 50 nm PS-NH_2 NP (data not shown) and was established to compare results with other laboratory studies (Long et al., 2017; Mao et al., 2018; Zhang et al., 2017), but not as high as in most published works, so that we could get closer to worst environmental scenarios (Paul-Pont et al., 2018). This same mass concentration for the two beads tested results in differences in the nominal concentration in number of particles per mL, corresponding to 3.7 x 10^7 particles mL^{-1} for the 0.5 µm MP and to 5.7 x 10^5 particles mL^{-1} for the 2 µm MP. Before the experiment, MP stock solutions were diluted in Milli-Q water. The volume of the diluted MP solutions added to microalgal cultures to reach the final MP concentration used for the assay (2.5 µg mL^{-1}) never exceeded 0.3 % of the final culture volume. Therefore, in the whole process an approximate dilution of 1/20000 of the

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

2.4. Scanning electron microscopy

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

2.5. Flow cytometric analyses

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

2.5.1. Cellular density and growth rate

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

2.5.2. Morphological parameters and chlorophyll *a* fluorescence

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

2.5.3. Esterase activity

Esterase activity in *C. neogracile* cells was studied using the fluorescein diacetate (FDA) cytometric assay previously described in Seoane et al. (2017b). Cells were incubated with FDA at a final concentration of 6 µM for 10 min at room temperature in the dark. FDA is a non-fluorescent, non-polar lipophilic molecule that diffuses across cell membranes. After entering the cell, its acetate residues are cleaved off by non-specific esterases and the polar hydrophilic fluorescent product fluorescein is retained by cells with intact plasma membranes. Since fluorescein is accumulated by viable and active cells, esterase activity is measured by means of 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 µM for 50 min at room temperature in
188 the dark. DCFH-DA is a cell-permeable non-florescent 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 ($DiBAC_4(3)$) following the protocol previously
194 described by Seoane et al. (2017a). Cells were incubated with $DiBAC_4(3)$ at a final
195 concentration of 2 µM for 10 min at room temperature in the dark. $DiBAC_4(3)$ 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 µM 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

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

3. Results and discussion

3.1. Particle characterization: actual size, charge and aggregation

DLS analysis of the MP suspended in Milli-Q water confirmed the approximate size indicated by the commercial supplier for the two particle sizes used, with Z-averages of 602.5 ± 27.3 and 2274.0 ± 100.9 nm, which correspond to the nominal sizes of 0.5 µm and 2 µm, respectively (Table 1). Data also showed an optimal dispersion and stability in Milli-Q water since aggregation was negligible for both particles, as suggested by a PdI < 0.2 (Table 1). However, plastic particles showed a significant increase in Z-average reaching values of 2899.0 ± 96.0 nm and 5932.0 ± 694.4 nm when suspended in FSW and values of 3268.0 ± 64.3 nm and 4342.3 ± 294.2 nm in microalgae medium for 0.5 µm and 2 µm PS-NH_2 beads, respectively (Table 1; Supp. Table 1). These data indicated an increase in particle size congruent with the observed PdI values (>0.2) that indicated aggregation (Table 1).

Regarding the charge, both 0.5 µm and 2 µm PS-NH_2 MP showed negative ζ-potential in the three media assayed (Table 1) although a positive charge was expected since the beads used presented amino surface modifications. Therefore, particle characteristics must be assessed prior to performing laboratory exposure to properly interpret particle behaviour and toxicity. Such difference of charge has also been reported by Sun et al. (2018) with 1 µm PS-NH_2 beads and by González-Fernández et al. (2018) and Lundqvist et al. (2008) with 100 nm PS-NH_2 NP. These discrepancies may derivate from the manufacturing process which may change according to the commercial suppliers. More information and measurements regarding physico-chemical properties of MP is thus a prerequisite for a better interpretation of their behaviour and biological impact in different solutions (González-Fernández et al., 2018).

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

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

3.2. Scanning electron microscopy observations

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

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

3.3. Little effect of MP exposure on cell growth

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

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

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

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

Potential changes in the structural properties of the diatom *C. neogracile* exposed to the MP were studied by FCM based on light diffraction. Neither of the two particle sizes significantly altered the morphology of microalgal cells, since no changes were detected in the FSC and SSC signals (Supp. Table 3). Similarly, Long et al. (2017) did not observed FSC and SSC changes in *C. neogracile* cells exposed to 0.04 µg mL^{-1} of uncharged 2 µm PS MP for all duration of culture growth. The fact that 0.5 and 2 µm PS-NH_2 MP aggregate and become negatively charged may have affected the effectiveness of physical adsorption of these MP onto the cell wall.

Environmental stressors could affect the function of photosynthetic systems, thereby affecting the fluorescence emission (Geoffroy et al., 2007; Juneau et al., 2002). Natural red autofluorescence measured by FCM and effective quantum yield (QY) measured by PAM in *C. neogracile* cells did not show significant changes upon exposure to 2.5 µg mL^{-1} of 0.5 and 2 µm PS-NH_2 MP as compared to control condition (Supp. Table 3). Mao et al. (2018) showed that upon exposure of 0.1 µm and 1 µm PS MP at 10 µg mL^{-1}, adsorption of MP onto the surface of the freshwater microalgae *Chlorella pyrenoidosa* during its exponential growth phase could provoke shading effects, hindering algal photosynthesis. However, this phenomenon appears to be negligible for the particles tested, as shown in SEM micrographs, probably due to the electrostatic repulsion exerted by its observed negative charge on microalgal cell membrane (Table 1).

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

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

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

used as model materials in aquatic toxicity laboratory studies, are supposed to be free of additives or residual monomers. However, some studies suggest that commercial virgin plastic pellets may also leach toxic unknown chemicals (Nobre et al., 2015). Martínez-Gómez et al. (2017) reported significant toxicity of virgin and aged PS MP on the fertilization and larval development of sea urchins, and plastic leachates were found to have higher toxicity than the virgin and aged materials themselves. Toxicity of leachates from plastic products obtained after 72 h of leaching was also observed in the copepod *Nitocra spinipes* (Bejgarn et al., 2015). Moreover, it cannot be excluded the potential repercussions that amino functional groups could have on cells. MP are coated with -NH_2 groups by chemical bonds and during laboratory exposure, light or other environmental factors could lead to bond cleavage.

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

3.6. Unaltered ROS production and membrane potential during MP exposure

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

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

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

3.7. Diminished neutral lipid content in MP-exposed cells

Exposure to 0.5 and 2 μm PS-NH_2 MP resulted in a significant decrease in the cellular neutral lipid content with respect to control cells at all tested times (ANOVA 24 h; $F_{(2,6)} = 37.50$; $p < 0.001$) (ANOVA 48 h; $F_{(2,6)} = 12.08$; $p < 0.01$) (ANOVA 72 h; $F_{(2,6)} = 18.46$; $p < 0.01$) (Fig. 2B). After 72 h, the lipid content of MP-exposed cells was reduced by half with respect to control.

Representative flow cytometric histograms showing shifts in the green fluorescence intensity related to the neutral lipid content of *C. neogracile* cells in control cultures and cultures exposed to both MP are shown in Supp. Fig. 2.

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

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

4. Conclusions

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

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

Acknowledgements

This work was supported by the ANR CESA (ANR-15-CE34-0006-02, NANOPLASTICS project) and by the Unique Inter-ministerial Fund (FUI) as part of the MICROPLASTIC2 project.

M.S. acknowledges a pre-doctoral grant from “Campus do Mar” and all the LEMAR team for their help during the course of this project.

Authors would thank Philippe Miner for his help with microalgal cultures at the Ifremer facilities. We are also very grateful to Philippe Eliès from the PIMM core and to Olivier Lozach from the COSM team at the University of Western Brittany for the scanning electron microscopy observations and for providing us the equipment for DLS measurements, respectively.

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Table 1. Characterization of PS-NH_2 MP in Milli Q water, filtered natural seawater (FSW) and microalgae medium using DLS analysis. Z-average (nm), polydispersity index - PdI (a.u.) and ζ-potential (mV), referred to a PS-NH_2 MP solution concentration of 100 µg mL^{-1} are reported. PdI > 0.2 showing aggregation is marked by an asterisk (*). Values are shown as mean ± standard deviation of the three measurements.

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

Table 2. Growth rates μ (day^{-1}) of *C. neogracile* cultures exposed to 0 and 2.5 μg mL^{-1} of 0.5 μm and 2 μm PS-NH_2 MP at each sample time. Data are given as mean values ± 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 (*).

	Growth rate μ (day^{-1})		
Particle type	**24 h**	**48 h**	**72 h**
Control	1.56 ± 0.06	1.59 ± 0.05	1.44 ± 0.02
0.5 μm	1.63 ± 0.02	1.58 ± 0.02	1.44 ± 0.01
2 μm	1.61 ± 0.09	1.55 ± 0.03	1.39 ± 0.01*

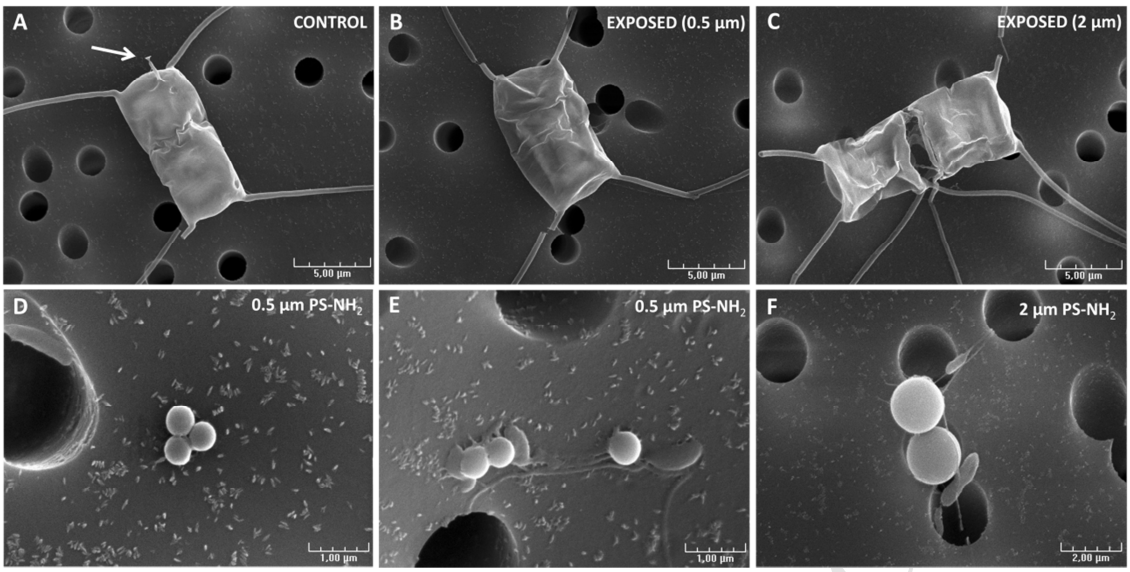

Figure 1. Scanning electron micrographs showing a control cell (**A**) and cells exposed to 0.5 µm (**B**) and 2 µm PS-NH_2 MP (**C**). 0.5 µm (**D, E**) and 2 µm (**F**) PS-NH_2 beads micro-aggregates and bacteria are also shown. Arrow indicates a rimoportula (tubular process through the valve of some diatoms). Scale bars: 5 µm (A, B, C); 1 µm (D, E); 2 µm (F).

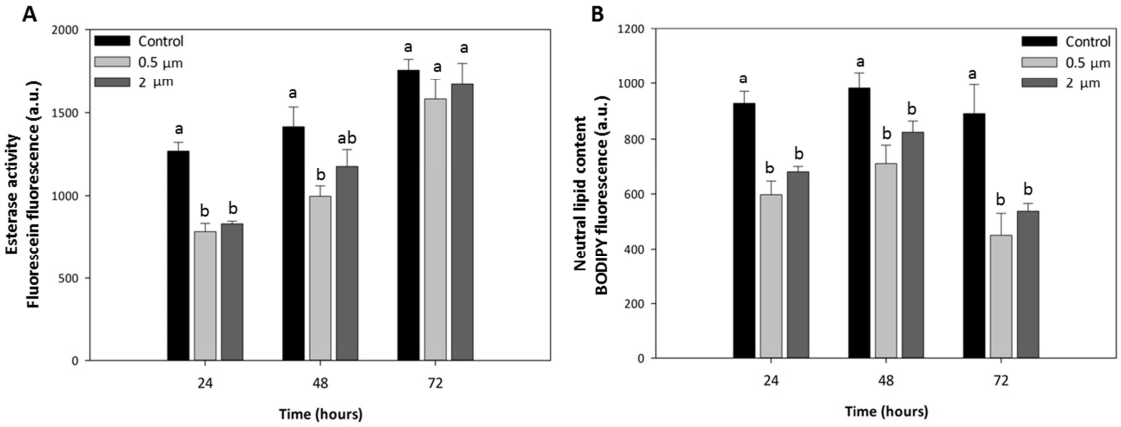

Figure 2. Esterase activity (**A**) and neutral lipid content (**B**) of *C. neogracile* cells in control cultures and cultures exposed to 2.5 µg mL^{-1} of 0.5 and 2 µm PS-NH_2 MP for 24, 48 and 72 h. Significant differences between treatments are marked with lowercase letters ($p < 0.05$).

Highlights

1. Effects of 0.5 and 2 µm PS-NH_2 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