1 **Are the primary characteristics of polystyrene nanoplastics responsible for toxicity**
2 **and ad/absorption in the marine diatom *Phaeodactylum tricornutum*?**

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19 **Graphical abstract**
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Abstract

Nowadays, the occurrence of a large volume of plastic litter in oceanic and coastal zones has increased concern about its impacts on marine organisms. The degradation of plastic polymers leads to the formation of smaller fragments at both micro and nano scale (<5 mm and <1 µm respectively). Nanoplastics (NPs), due to their smaller size and high specific surface area can establish colloidal interactions with marine microalgae, therefore potential toxicity can be led. . To assess this hypothesis, the aim of the present study is to examine the behaviour of polystyrene nanoparticles (PS NPs) of different sizes (50 and 100 nm) in marine water and their possible effects at different physiological and cellular levels in the marine diatom *Phaeodactylum tricornutum*. Different biomarkers and stress responses in *P. tricornutum* were analysed when organisms were exposed to environmentally relevant PS NPs concentrations between 0.1-50 mg·L⁻¹. Our results showed significant differences between controls and

exposure microalgae, indicating toxicity. After 24h, an increase in oxidative stress biomarkers, damage to the photosynthetic apparatus, DNA damage and depolarization of mitochondrial and cell membrane from 5 mg·L⁻¹ were observed. Further after 72 h the inhibition of population growth and chlorophyll content were observed. Examining effects the effects related to PS NPs size, the smallest (50 nm) induced greater effects at 24 h while bigger PS NPs (100 nm) at 72 h. This bigger particles (100nm) showed more stability (in size distribution and spherical form) in the different culture media assayed, when compared with the rest of particles used. Strong adsorption and/or internalization of PS NPs was confirmed through changes in cell complexity and cell size as well as the fluorescence of 100 nm fluoresbrite PS NPs after washing cell surface.

Keywords: Plastic particles, toxicity, accumulation, phytoplankton, *P. tricornutum*

Highlights:

- Agglomeration of Polystyrene nanoplastics is unstable in seawater
- *P. tricornutum* showed toxicity responses when it was exposed to Polystyrene nanoplastics.
- Microalgae was more affected by the biggest plastic particles (100 nm).
- Internalization or strong adsorption of plastics in/on algal cells was stated.

1. Introduction

Pollution by plastics is known to be a worldwide threat to marine ecosystems due to the accumulation in certain areas of the open ocean and coasts; however, it mostly accumulates in the convergence zones of each of the subtropical gyres (Cózar et al., 2014). Among the types of material that form marine litter, plastic represents 60-80%, around 4.8-12.7 million tons (Derraik, 2002; Jambeck et al., 2015)..

Polystyrene (PS) has been recognized as one of the most widespread plastic polymers found in marine litter during plastic surveys worldwide (Long et al., 2017). Mainly, PS is used in plastic cutlery, in spectacle frames and egg trays, in building insulation and packaging. In fact, like most plastics, PS is extremely stable, so in marine environments PS is degraded very slowly through processes of photodegradation, oxidation or mechanical abrasion, turning it into a pseudo-persistent material (Lambert and Wagner, 2016), furthermore is one of MPs more likely

found in marine environments associated to organic pollutants (Browne et al., 2008). These degradation processes lead to the formation of "micro-" <5 mm; (MPs) and "nano-plastics" < 1 µm; (NPs) (Andrady, 2011; da Costa et al., 2016; Koelmans et al., 2015). The concentration of plastics in surface water can reach up 0.5 and 8.5 mg·L⁻¹ in South Pacific gyre and southern North Sea respectively (Eriksen et al., 2013; Dubaish and Liebezeit, 2013) while in sediment the highest concentration recorded was 32.9 mg·Kg⁻¹ in Arctic sediment (Bergmann et al., 2016).

Research about the negative effects and bioaccumulation of plastic litter by accidental ingestion and entanglement in the aquatic environments has increased in recent years (Gall and Thompson, 2015). Numerous studies are found about the effect of MPs and NPs in aquatic organisms including crustaceans, bivalves, echinoderms, annelids rotifers and fish (Bergami et al., 2016; Booth et al., 2016; Cole et al., 2013; Chen et al., 2017; Della Torre et al., 2014; Jemec et al., 2016; Wan et al., 2018; Watts et al., 2016; Wegner et al., 2012).

Despite such abundant literature, only a few studies have focused on primary producers which are, potentially, most in danger from the presence of the smallest plastic litter (Wan et al., 2018). Therefore, any attempt to understand the behaviour and effects of MPs and NPs on microorganisms has become a plausible subject to be investigated (Chae and An, 2017; Nel et al., 2009).

The colloidal interactions between NPs (most studies were performed with metal nanoparticles) and microorganisms such as microalgae can trigger direct and indirect toxicity. Such interactions can damage cellular barriers, such as membranes or cell walls, change settlement rates in the water column, cause shading effects, block algae pores, inhibit gaseous exchange, provoke DNA damage and cause oxidative stress and physiological damage (Bhattacharya et al., 2010; Long et al., 2015; Ma et al., 2015; Moore, 2006; Navarro et al., 2008; Sendra et al., 2017).

NPs can pose a serious threat to marine ecosystems, altering the physiological balance of the phytoplankton, an element at the base of marine trophic networks and with a fundamental importance for the global carbon cycle. Most of the previous studies tested the toxicity of MPs and NPs in freshwater microalgae (Besseling et al., 2014; Bhattacharya et al., 2010; Casado et al., 2013; Nolte et al., 2017; Yokota et al., 2017). However, the studies performed on marine microalgae in the literature are scarce. Zhang et al. (2017) assessed the effects of PVC from 1 µm and 1 mm at the concentration 0-50 mg·L⁻¹ on *Skeletonema costatum*. These authors found a growth inhibition of 39.7% and negative effects in microalgae photosynthesis (Zhang et al., 2017). Sjollem et al. (2016) studied the toxicity of PS of 0.05, 0.5 and 6 µm at two

concentration 25 and 250 mg·L⁻¹ on *Dunaliella tertiolecta* (Sjollema et al., 2016). For this microalgae, the only effects on growth inhibition were found at the highest concentration. In the research of Davarpanah and Guilhermino (2015) the toxicity of copper was tested in the presence and absence of 1 and 5 µm of PS MPs on *Tetraselmis chui*, no significant effects were found between the presence or absence of MPs. Most of the literature about the toxicity of plastics in marine microalgae is found with PS MPs. In order to fill the gap of information about the toxicity of PS NPs, this study assesses the effects of PS NPs with different nominal size in a marine diatom.

The hypothesis of this work is that the size of NPs determines colloidal forces, NPs-cells interfaces and their toxicity to microorganisms. To check this hypothesis, the following goals were: i) to establish sensitivity of *P. tricornutum* to PS NPs at two different sizes (50 and 100 nm) using environmental concentrations. The size of these particles were chosen to be in a nanoplastics range size and can establish differences in its effects cause one of NPs is double size than the other ii) To reduce the gap of knowledge of the effects of PS NPs over time for different endpoints (cellular and molecular responses): algal growth rate, inherent cell properties, biomarkers of oxidative stress, photosynthetic stress, cytoplasmic membrane effects, pre-apoptotic process and genotoxicity). Currently, most of published data are limited to growth inhibition and photosynthesis damage. Finally, the last objective was iii) to clarify the ad/absorption process of PS NPs onto microalgae.

2. Materials and methods

2.1 Reagents:

PS NPs of 50 nm (cat#08691), 100 nm (cat#00876), 100 nm fluoresbrite ex/em: 488/520 nm (cat# 17150-10) were supplied by Polyscience, Inc. **2.2 Suspensions of NPs**

Stocks of all PS NPs were prepared in ultrapure water immediately before the experiments. Suspensions were sonicated with a tip sonicator (UP 200S Dr. Hielscher GmbH) for 10 min with pulses each 0.5 seconds and a 50% of amplitude of the maximum of the tip sonicator.

2.3 Primary and secondary characterization of the PS NPs

Particle size, shape and structure were analysed by Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM) following methodology of Sendra et al. (2017a). , The composition corroboration of PS NPs was performed by Fourier-transform infrared spectroscopy (FTIR) through the Bruker Alpha System, (Figure S2).

Size distribution and zeta potential in the water suspensions [ultrapure water (MQ) and artificial marine water (ASTM)] were studied (at 0, 10, 60 and 180 min). These measurements were performed using Dynamic Light Scattering; DLS (Zetasizer Nano ZS90, Malvern Instruments, equipped with software version 7.10) at 1 mg·L⁻¹. Due to the high polydispersity index (PDI) found in ASTM (PDI<0.7), the agglomeration stage of PS NPs was evaluated using Mastersizer

2000, Malvern Instruments at $26 \text{ mg}\cdot\text{L}^{-1}$ over time 0, 1, 3, 24 and 72 h in different media: ultrapure water (MQ) and artificial marine water (ASTM). SEM images were performed at 0, 24 and 72 h in MQ and ASTM in order to assess structural changes in PS NPs surface.

2.4 Test organisms.

The marine microalgae selected was *Phaeodactylum tricornutum*, BACILLARIOPHYCEAE (Bohlin, 1897), strain CCMM 07/0402, obtained from the ICMAN Marine Microalgae Culture Collection (CCMM). The microalgae selected was chosen because it is a standard species used in toxicology tests (ISO, 1995). Cells were grown in a filtered ($0.2 \text{ }\mu\text{m}$) f/2 marine medium for two weeks prior to the assays (Guillard and Ryther, 1962). The synthetic marine water used was ASTM Substitute Ocean Water D1141-75 (ASTM, 1975). ASTM preparation found in Supplementary Information.

2.5. Toxicity test

Bioassays were carried out using PS NPs selected at six different and increasing concentrations ($0.1, 1, 5, 10, 20$ and $50 \text{ mg}\cdot\text{L}^{-1}$) for 50, 100 and 100 nm fluoresbrite PS NPs. This range of concentration was chosen to be environmentally relevant concentration in surface water (Eriksen et al., 2013; Dubaish and Liebezeit, 2013).

The tests were performed in a culture chamber at $20\pm 0.5 \text{ }^{\circ}\text{C}$ under continuous light ($300 \text{ }\mu\text{E}\cdot\text{s}^{-1}$), and following OECD (1994) Guidelines: 72 h of exposure with the sampling time every 24 h. Controls and treatments (different size of PS NPs) were developed in triplicate.

2.6 Flow Cytometry analysis

All measurements by flow cytometry were performed at 24, 48 and 72 h with an Accuri C6 flow cytometer with a laser excitation of 488 nm. The microalgae population was determined using side scatter (SSC), forward scatter detector (FSC) and a long pass filter of 670 nm (FL3) related to intracellular complexity, cell size and autofluorescence of chlorophyll *a* (ChlA) respectively (Shapiro, 2005). The growth population, cell complexity, cell size and autofluorescence of ChlA were recorded for all concentrations of the PS NPs tested. However, the study of the biomarkers and toxicological responses were selected at 5, 20 and $50 \text{ mg}\cdot\text{L}^{-1}$. All the fluorophores used in this study were prepared in DMSO and all samples were set to $10^5 \text{ cells}\cdot\text{mL}^{-1}$.

Information about toxicological responses measured by fluorophores through flow cytometry is recorded in Table S1.

2.6.1 Oxidative biomarkers

Production of intracellular ROS was quantified using the 2'-7'-dichlorofluorescein diacetate (DCFH-DA) following the procedures of Stachowski-Haberkorn et al. (2013) and Sendra et al. (2017b). H_2O_2 was analysed using dihydrorhodamine 1, 2, 3 (DHR 123). Samples were incubated at a final concentration of $29 \text{ }\mu\text{M}$ and in darkness at room temperature for over 60 min (Qin et al., 2008). The H_2O_2 present in the cells was measured by an FL1 detector [band pass (BP) 533/30 nm].

Hydroethidine (HE) or dihydroethidium has been widely used to detect intracellular O_2^- in cells (Zhao et al., 2003). Samples were incubated with HE at a final concentration of 16 μ M and in darkness at room temperature for over 30 min. The O_2^- present in the cells was measured by an FL2 detector [band pass (BP) 585/40 nm].

The percentage of cells marked with DCFH-DA, DHR 123 and HE was calculated as a proportion of the total cell population.

The positive controls to corroborate the validation method of the oxidative biomarkers were developed through incubation with 0.1 mM of H_2O_2 .

2.6.2 Cytoplasmic membrane potential

Cytoplasmic membrane potential was determined using the lipophilic anionic oxonol dye bis-(1,3-dibutylbarbituric acid) trimethine oxonol (DiBAC₄(3)); (Ehrenberg et al., 1988; Plášek and Sigler, 1996). This response was measured following the procedures of Prado et al. (2012a). The positive control for this response was done with 120 mM de KCl incubated 10 min.

2.6.3 Mitochondrial membrane potential

Mitochondrial membrane potential was evaluated using the lipophilic cationic probe JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl benzimidazole carbocyanine iodide) following the procedures of Prado et al. (2012b). To verify the specificity of JC-1 staining, cells were treated with carbonyl cyanide m-chlorophenylhydrazone (CCCP) (final concentration of 49 μ M during 15 min)

2.6.4 Viability or membrane cell damage

Membrane integrity or viability was also quantified, following the Propidium Iodide (PI) method (Xiao et al., 2011). The procedures are described in Sendra et al. (2017b). Thermal shock dead cells by microwave heating were used as a positive control.

2.6.5 DNA Damage

Syto 13 can cross cytoplasmic membrane and intercalate stereometrically with nucleic acids. This fluorophore is detected by an FL1 [band pass (BP) 533/30 nm]. In the flow cytometer used in this work, syto 13 do not interact with RNA with excitation at 491 nm. Syto 13 was added 1 μ M and incubated in darkness for 10 min (Guindulain et al., 1997).

2.6.6 Cell protein content

Cell protein content was analysed by fluorescein isothiocyanate (FITC) following the procedures of Prado et al. (2012a). FITC signal was collected by an FL1 detector, [band pass (BP) 533/30 nm]. The positive control for this response was done with digitonin to permeabilize the membrane cells (Kuin et al., 2000).

2.7 Photosynthetic apparatus analysis.

The effective quantum yield of photosynthetic energy conversion in PSII (EQY), chlorophyll concentration (ChlA $\mu\text{g}\cdot\text{L}^{-1}$) and apparent electron transport rate (aETR) were measured fluorometrically by a Phyto-PAM instrument (Heinz Walz GmbH) equipped with an ED-101 US/MP Optical Unit (Schreiber, 2004). The samples were acclimated in darkness for 15 min before the analysis.

2.8 Ad/absorption studies of PS NPs on *P. tricornutum*.

Cell complexity (SSC) and cell size (FSC) were assessed every 24 h through flow cytometry to carry out the ad/absorption study of the PS NPs on/in *P. tricornutum*. These inherent cell properties were monitored following the procedures of Suzuki et al. (2007) and Sendra et al. (2017b).

The three types of PS NPs were studied, furthermore fluorescence of 100 nm fluoresbrite PS NPs was evaluated after washing by an FL1 detector, [band pass (BP) 533/30 nm].

Each wash (0, 1, 2 and 3) was analysed by a confocal Axion Observer Z1 laser scanning fluorescence inverted optical microscope with LSM 880 and ZEISS AiryScan super-resolution module. The objective used was 63x (glycerol-specific dip oil immersion). The laser of excitation was 488 nm and emission 670 and 520 nm for the microalgae and 100 nm fluoresbrite for the PS NPs respectively. The assembly of samples were prepared in 0.17 mm thick samples of ZEISS thickness and PBS-Glycerol (in a proportion 1:9) was the mounting medium.

SEM images of the microalgae were taken when *P. tricornutum* was exposed to 100 nm PS NPs at wash 0 and after wash 2 after 72 h of exposure. Samples were prepared in thick samples covered with poly-lysine. Then the samples were fixed for 2h at 4 °C in 0.1M sodium cacodylate buffer containing 2.5% glutaraldehyde, and then rinsed three times in a cacodylate buffer for 15 min. Three dehydrations of 15 min. were performed with ethanol at 70, 90 and 100%. Finally, the samples were coated with Au film and examined with a Nova NanoSEM 450, (Figure S9).

2.7 Statistical analysis

All tests with their controls were performed in triplicate. Data are shown in the document as average $\pm$ standard deviation. Statistical analysis was completed using IBM SPSS, statistics 23 program. Normality distribution was checked previous to the statistical analyses. To assess the toxicological endpoints a General Linear Model (GLM) was constructed (McCullagh and Nelder, 1989; SPSS., 2005). In the GLM all factors such as time, tested PS NPs (treatments) and

different concentration were studied as principal factors and the interaction among factors was also evaluated. These results are shown in supplementary information (Table S2, S3, S4 and S5). EC₅₀ (effective concentration that causes an inhibition or increment of 50% in the response measured) values were computed using PriProbit (Sakuma, 1998). EC₅₀ was calculated for those endpoints with a clear trend dose-response and a range of at least 5 increasing concentrations. The percentage variation with respect to the controls of each endpoint analysed was calculated after 24 h in the case of acute toxicity responses (cell size, EQY and aETR) and after 72 h in the case of chronic responses (cell complexity and ChIA). The EC₅₀ was not calculated for the rest of the responses for two main reasons: i) some responses studied in a wide range of concentration did not show a dose-response curve, and ii) most of the cellular responses measured by flow cytometry were only analysed at three concentrations.

3. Results

3.1 Primary and secondary characterization of PS NPs

The primary sizes of the PS NPs and material corroboration are shown in Figure S1 (panel A) and Figure S2. The nominal sizes from the suppliers are in agreement with the results analysed in the laboratory (4.1±8, 93.5±10.6, 94.1±12.1 nm for 50, 100 and 100 fluoresbrite PS NPs). The size distribution through DLS was studied over 180 min. Furthermore, no differences were found between the mean and main peak of the size distribution for 50, 100 and 100 nm fluoresbrite PS NPs in MQ [151.7 and 113.1±38.3 nm (mean and peak of 50 nm PS NPs), 121 and 106.4±18.1 nm (mean and peak of 100 nm PS NPs) and 107.3 and 107.3±22.8 nm (mean and peak of 100 nm fluoresbrite PS NPs)]. On the other hand, mean and peak in ASTM at time 0 showed differences [1426 and 431.2±31.1 nm (mean and peak of 50 nm PS NPs), 914.5 and 406.1±41.8 nm (mean and peak of 100 nm PS NPs) and 1068 and 454.8±48.3 nm (mean and peak of 100 nm fluoresbrite PS NPs)]. (Figure S1, panel B). The PDI values showed the size distribution appropriately with values <0.3 for MQ; however, PDI >0.88 in ASTM, so agglomeration stage in MQ and ASTM were analysed through Mastersizer at 0, 1, 3, 24 and 72 h (Figure 1). Practically no agglomeration stages were found in MQ over time, all curves of size distribution were similar (around 100 µm). On the other hand, shifts in the size distribution of the PS NPs in ASTM were found among the sampling times. At time zero both PS NPs (50 and 100 nm PS NPs) showed that the maximum percentage of volume of PS NPs was recorded around 100 µm, however the trend was different after 72 h, where 50 nm PS NPs showed a maximum percentage of PS NPs for 22.9 µm while 100 nm PS NPs the agglomeration size was 8.7 µm. Agglomerate sizes in ASTM were lower than MQ for PS NPs studied. The analysis of SEM at 0, 24 and 72 h in different media showed that after 24 and 72 h, 100 nm PS NPs in MQ

and ASTM had more defined spherical forms with a diameter more stable than 50 nm PS NPs (Figure 1).

With respect to the zeta potential, it showed values located in an unstable zone (from -30 to 30 mV) due to the agglomeration process (Figure S1, panel B).

Toxicological responses

In the Figure 2 of this work are showed the most remarkable toxicological responses for *P. tricornutum* exposed to 50 and 100 nm PS NPs. The others toxicological responses analysed and data related to 100 nm fluoresbrite PS NPs are recorded in the supplementary material.

3.2 Growth and inherent cell properties of microalgae population.

Growth data indicated that after 48 h of exposure to PS NPs of 50, 100 and 100 nm fluoresbrite, a significant inhibitory response in *P. tricornutum* was induced in relation to the controls ($p < 0.05$); (Figure 2, panel A; Figure S3, panel A). Growth inhibition of *P. tricornutum* after 72 h exposed at $10 \text{ mg} \cdot \text{L}^{-1}$ for the different treatments was 67.1 ± 13.0 , 54.1 ± 12.6 and 47.1 ± 5.8 % for 50, 100 and 100 nm fluoresbrite PS NPs ($p < 0.05$). Thus, there is not a clear dose-response for cell growth when populations are exposed to the selected PS NP in the experimental conditions (Figure 2, panel A; Figure S3 panel A).

The *P. tricornutum* population showed a decrease in cell complexity from the first 24 h to 72 h. The highest values of cell complexity were found after 24 h of exposure for almost all the PS NPs tested from $10 \text{ mg} \cdot \text{L}^{-1}$ ($p < 0.0001$); (Figure 2, panel B; Figure S3, panel B). EC_{50} for cell complexity at 72 h was: 1.1, 1.3 and $1.9 \text{ mg} \cdot \text{L}^{-1}$ for 50, 100 and 100 nm fluoresbrite respectively (Table S6). A similar trend was observed when the alterations in cell size were studied. The highest changes in cell size were found in the first 24 and 48 h ($p < 0.05$); however, no significant differences were found in this response after 72 h (Figure S3, C). EC_{50} was calculated after 24 h of exposure, the values found were: 8.7 (3.4-21.3), 18.5 ($5.7\text{-}1.227 \cdot 10^4$) and 59.3 (52.1-70.5) $\text{mg} \cdot \text{L}^{-1}$ for 50, 100 and 100 nm fluoresbrite respectively (Table S6).

3.3 Effects on photosynthetic apparatus.

All variables used to analyse the deleterious effects on the photosynthetic apparatus such as autofluorescence of ChlA, EQY, total ChlA content and aETR were affected from the first 24 h of exposure to PS NPs, and can be seen in Figure S4.

In the analysis of the autofluorescence of ChlA, a significant increase persisted even in subsequent sampling times (after 48 and 72 h) when *P. tricornutum* was exposed to 50, 100 and 100 nm fluoresbrite PS NPs; (Figure S4, panel A).

In most cases this response as growth inhibition did not seem to follow a dose-response trend. In fact, the "intermediate" concentrations had a greater effect (Figure S4, panel A)).

In the analysis of the EQY, a significant decrease ($p < 0.05$), compared to the control, was detected in all the sampling times in the samples treated with the highest concentration of the

different PS NPs (Figure 2, panel C; Figure S4, panel B). However, there was a significant decrease ($p<0.05$) in the response respect to the controls at the first 24 h of exposure for all the PS NPs sizes and concentrations tested. At this time, a clear dose-response trend was detected and values of EC_{50} were calculated at 24 h with values of: 32.6 (18.2-90.6), 3.8 (1.5-8.0) and 2.7 (1.1- 5.7) $mg\cdot L^{-1}$ for 50, 100 and 100 nm fluoresbrite, respectively (Table S6). In subsequent sampling times (48 and 72 h), a decrease in this response was observed respect to the controls, but significant differences were only found at the highest concentrations for microalgae exposed to 100 nm PS NPs (Figure 2, panel C).

A dose-response trend was recorded in the analysis of the total concentration of ChlA. A significant variation with respect to the controls was more accentuated after 72 h of PS NPs exposure (Figure S4, panel C). The EC_{50} values were: 44.9 (39.8-52.3), 11.5, and 41.4 for 50, 100 and 100 nm fluoresbrite PS NPs, (Table S6).

Finally, with regard to aETR, a significant inhibition ($p<0.05$) was present in all the sampling times at the highest exposure concentrations (Figure S4, panel D). The changes were more marked after 24 h of exposure, for this reason the EC_{50} was calculated at this sampling time. The EC_{50} values for aETR were: 31.9 (26.8-39.7), 5.5 (2.2-10.8) and 3.9 (0.8- 13.4) for 50, 100 and 100 nm fluoresbrite PS NPs; (Table S6).

3.4 Stress biomarkers and toxicological responses.

3.4.1 Oxidative biomarkers

The total percentage of intracellular ROS (Figure 2, panel D), $O_2^{\cdot -}$ and H_2O_2 were analysed to study the stress oxidative biomarkers (Figure S5).

A significant increase in the percentage of ROS, $O_2^{\cdot -}$ and H_2O_2 was recorded at 24, 48 and 72 h when microalgae were exposed to PS NPs ($p<0.05$) (Figure 2; panel D and Figure S5). All the treatments showed differences in the percentage of the oxidative biomarkers with respect to the controls ($p<0.05$); without differences among the concentrations used. The highest values of ROS and $O_2^{\cdot -}$ were found in the first hours of exposure (after 24 h) for both PS NPs. On the other hand, 100 nm PS NPs after 72 h of exposure was the only treatment with a significant increase in oxidative biomarkers respect to the controls ($p<0.05$) (Figure 2; panel D and Figure S5).

3.4.2 Deleterious effect on membranes

The results for cell viability showed an increase in the % of non-viable cells since the first hours of exposure (24 h) to PS NPs from 20 $mg\cdot L^{-1}$ ($p < 0.05$) (Figure S6). However, after 72 h non-significant variations were found (Figure S6).

Mitochondrial membrane depolarization showed a significant increase ($p<0.05$) in almost all

exposure concentrations (5, 20 and 50 mg·L⁻¹ for 50 and 100 nm PS NPs) and sampling times, less in the case of 50 nm PS NPs at 5 mg·L⁻¹ after 72 h of exposure (Figure 2, panel E).

Regarding the percentage of depolarization of the cell membrane, significant differences were found after the first 24 h of PS NPs exposure at tested concentrations ($p<0.05$) (Figure S6). In subsequent sampling times (48 and 72 h), although there was a clear decrease in this response with respect to the first 24 h, a significant increase was recorded at the highest concentration tested ($p<0.05$).

3.4.3 Protein cell content

In general, this response did not show a very marked dose-response trend for almost all the PS NPs selected over time (Figure S7). Significant differences in the percentage of cell protein content were found at 24 and 72 h when *P. tricornutum* was exposed to 50 mg·L⁻¹ of 100 nm PS NPs ($p<0.05$).

3.5 Genotoxicity

The values for the percentage of DNA damaged after 24 h of exposure- showed a significant increase for PS NPs tested at almost all the concentrations ($p<0.05$) (Figure 2, panel F). In subsequent sampling times, the magnitude of the response tends to decrease, although significant differences were found at the highest concentration for 50 and 100 nm PS NPs after 48 and 72 h of exposure ($p<0.05$).

3.6 Ad/absorption PS NPs in *P. tricornutum*.

Confocal images showed adsorbed PS NPs onto microalgae cell surface (Figure 3, and Figure S8 and S9). After the specific protocol of washing, inherent properties of the microalgae such as cell complexity, cell size and fluorescence were recorded by an FL1 detector for 100 nm fluoresbrite PS NPs (Figure 3).

A significant increase in cell complexity was observed between the controls and the microalgae exposed to the PS NPs after 24 h before wash protocol ($p<0.05$). Significant differences were also found between controls and wash 1 when cells were exposed to 100 nm ($p<0.05$). However, no significant differences were found in cell complexity between the controls and microalgae exposed to the PS NPs after two consecutive washes (Figure 3, panel A).

A significant increase in cell complexity after the first washes for 50, 100 and 100 nm fluoresbrite at 20 and 50 mg·L⁻¹ was observed ($p<0.05$) after 48 h of exposure. After 72 h of microalgae exposure to the PS NPs, the variation in cell complexity was significant ($p<0.05$) after the first wash at the highest concentration (50 mg·L⁻¹) for 50, 100 and 100 nm fluoresbrite ($p<0.05$).

With regard to cell size, significant differences were found after 24 h with the three consecutive washes for microalgae exposed to 5 and 50 mg·L⁻¹ of 100 nm fluoresbrite PS NPs (p<0.05). After 72 h of exposure, the increase in cell size remains significant at mostly until the second wash (p<0.05). Significant differences (p <0.05) were detected between the second and third washings in all samples that showed a significant increase in volume after the second wash with PBS (Figure 3, panel B).

Finally, a significant adsorption and/or internalization of PS NPs was observed for the exposure of *P. tricornutum* to 100 nm fluoresbrite PS NPs (Figure 3, panel C). A significant increase in the fluorescence emitted by these PS NPs, with respect to the controls, was recorded after the three consecutive washes at all the different concentrations tested (p<0.05) for all the sampling times; except at 5 mg·L⁻¹ after 48 and 72 h. However, no significant difference in intensity of fluorescence was observed among the consecutive washes.

Confocal images of *P. tricornutum* exposed to the PS NPs after 72 h showed a release of the different PS NPs tested after consecutive washes (Figure 3, panel D and Figure S8). The particle agglomerate most frequently found onto the cell surface after three consecutive washes was 100 nm PS NPs (Figure S8). The attachment of PS NPs onto cell surface was also observed by SEM (Figure S9).

4. Discussion

4.1 Influence of seawater in the fast agglomeration and deagglomeration of PS NPs.

The ionic strength of the marine culture media significantly affected the behaviour of the PS NPs. From the results obtained, a stability of the PS NPs was observed in MQ when compared to those in ASTM. PS NPs suspended in ASTM displayed an immediate agglomeration although no differences were found among the hydrodynamic radii by DLS of the three NPs used. The fast agglomeration of the PS NPs in ASTM was probably due to the presence of a large quantity of ions in solution. These ions enhance the formation of agglomerates/aggregates of PS NPs due to the compression of the electro-double layer among the PS NPs. This high ionic strength produces a shielding effect on the surface of the NPs, screening surface charges which determine the stability due to the electrostatic repulsion (Ma et al., 2015). In addition, when PS NPs were studied over time, it was observed that after 3 h the PS NPs showed smaller agglomerates with respect to time zero in ASTM. Due to these singular results, they were double checked by DLS and SLS techniques showed the same behaviour, following this trend after 24 and 72 h. These findings about agglomeration/deagglomeration over time in ASTM have not been reported before. This provides a new insight about the perpetual bioavailability of PS NPs in marine environments.

Furthermore SEM images analysis of PS NPs in different culture media over time concluded that 100 nm PS NPs were most stable PS NPs and conserved its original spherical forms after 72 h in MQ and ASTM.

4.2 Selected endpoints to assess acute and chronic toxicity in microalgae exposed to PS NPs.

In this work, almost all the endpoints assessed showed differences over time, both between treatments and controls, and between the selected concentrations. However, one of the objectives of this study was to elucidate what responses are the proper endpoints to assess toxicity after 24 and 72 h in *P. tricornutum* when the microalgae population was exposed to PS NPs. Two clear trends were found when the results were analysed.

One of the trends was that some endpoints such as: growth, autofluorescence of ChlA, total of ChlA concentration and protein content were excellent responses to assess the effects of PS NPs on this marine diatom after 72 h of exposure. Shifts in these responses were found when the microalgae population reached the exponential phase in its growth (from 48 h). On the other hand, another trend was found in most of the endpoints measured such as: inherent cell properties, oxidative biomarkers, damage in photosynthetic apparatus, effects in membrane damage and DNA damage were all candidate responses to study the immediate effects in the first 24 h of PS NPs exposure. After 48 h these responses were mitigated, and completely suppressed in almost all analyses.

One of the reasons to explain the acute toxicity of PS NPs to microalgae at the first 24 h and its mitigation after 48 and 72 h could be explained by the lack of bioavailability of the PS NPs to *P. tricornutum*. On the one hand, heteroagglomeration (PS NPs-Cells) and homoagglomeration (PS NPs-PS NPs) took place simultaneously from the beginning of experiment. Therefore, single PS NPs decreased in the culture media due to sedimentation; thereby the PS NPs-Cells interactions were less likely at 72 h than at the beginning of the experiment. However, the size of agglomerates after 72 h was lower than the first hours, so the lack of bioavailability of PS NPs is not a plausible explanation.

In addition, microalgae when subjected to stress conditions, such as the presence of PS NPs adhering onto the cell surface, can produce a greater quantity of exo-polymeric substances (EPS) (Miao et al., 2009; Mishra and Jha, 2009; Sendra et al., 2017; Steele et al., 2014). The bond between PS NPs-EPS is established by hydrophobic interactions. Previous studies have showed that this over-production of EPS can accelerate assembly kinetics, causing unwanted effects in the cell wall and membrane (Bhattacharya et al., 2010; Chen et al., 2011; Chiu et al., 2017; Long et al., 2017). Although EPS production occurs under stressful situations, it also depends on the phase of the growth cycle of the microalgae and the presence of lysed cells in the culture medium (Long et al., 2017). Therefore, EPS secretion under stress and the exponential phase of the microalgae population could determine the detachment of PS NPs from

the microalgae surface, releasing EPS-PS NPs into the surrounding environment. Therefore, this process could determine again the bioavailability of the material.

Last, the flow cytometry measurement evaluated the percentage of marked cells against the total microalgae population; as the population of *P. tricornutum* grew exponentially after 48 h, thus a larger percentage of new microalgae did not exhibit adherent and/or internalized PS NPs. Therefore, the cellular quota concept and the decrease in the bioavailability of the PS NPs over time will cause a decrease in the percentage of negatively affected cells (Moreno-Garrido et al., 2000).

4.3 Acute and chronic toxicity in relation to the different sizes of PS NPs.

A fundamental goal of this work was to clarify the importance of PS NPs primary characteristics to the sensitivity of *P. tricornutum*. The different sizes of PS NPs did not seem to substantially affect their toxicity. However, in the first 24 h of exposure, 50 nm PS NPs produced a more intense response for some endpoints such as: cell size, DNA damage, ROS, viability, H₂O₂ and the depolarization of mitochondrial membrane respect to 100 nm PS NPs. A different response was detected after 72 h of exposure, 100 nm PS NPs provoked the highest negative effects for oxidative stress biomarkers, depolarization of mitochondrial membrane, ChlA concentration and aETR. Therefore, PS NPs with a smaller nominal size could be key to the acute toxicity of PS NPs to *P. tricornutum*. However, due to the instability of the PS NPs (agglomeration/deagglomeration) and lack of PS NPs structure previously explained, the PS NPs bioavailability and toxicity in seawater environments could also be affected (Figure 4). The characterization of the PS NPs in ASTM performed over time through SEM images and Mastersizer showed that 100 nm PS NPs had a clear spherical form conserved only for 100 nm PS NPs respect to 50 nm PS NPs. 50 nm PS NPs seem to be in incrustated in a complex matrix when were analysed SEM images at 24 and 72 h. This work concludes that agglomeration/aggregation of PS NPs determine the behaviour, the bioavailability and the chronic toxicity in marine diatoms.

4.4 Apoptotic signals in *P. tricornutum* when exposed to PS NPs.

Exposure to PS NPs appears to induce programmed cell death in *P. tricornutum*. The high fragmentation of DNA, the increase in cellular complexity and the depolarization of the mitochondrial membrane are, in fact, all early signs of the induction of the apoptosis process (Henry et al., 2013; Hollville and Martin, 2016; Ziegler and Groscurth, 2004). The decrease in cell size represents an additional signal of induction of programmed cell death (Hollville and Martin, 2016). Although in the early hours of exposure to NPs the cell size was significantly larger compared to the control. It can be assumed that this result is due to the presence of

particles adsorbed on the surface. After 72 h, the difference, with respect to the control, was significantly reduced, probably due to the release of EPS-PSNPs into the culture media; however, a significant increase in cell complexity was still maintained in all probability due to PS NPs internalization. The induction of apoptosis could be due to the physical adsorption of the nanoparticles on the algae surface. Such adsorption could result in a decrease in photosynthetic activity due to light shading effect and pore obstruction for nutrients (Bhattacharya et al., 2010). The reduction of photosynthetic efficiency leads to alterations in the electron transport chain that determines the production of ROS (these causal pathways have been observed in this work).

4. 6 Adsorbed and internalized PSNPs in *P. tricornutum*

PS NPs ad/absorption by the marine diatom *P. tricornutum* has been further accredited through the specific procedures developed in this work. The results obtained for cell size and cell complexity before washing in this study showed a significant increase in cell complexity and cell size compared to the control when *P. tricornutum* was exposed to all types of the PS NPs. After consecutive washing protocols, cell size did not show significant differences with respect to the control samples after 48 h of exposure in the majority of the cases. However, differences were found after 24 h of exposure. Therefore, the increase in cell size after 24 h of exposure of the cells without washing is due to adsorbed PS NPs (confocal images), but the increase in cell size after consecutive washes is probably due to physiological damage. After 72 h, the lack of change in cell size after the consecutive washes is probably due to the release of PS NPs and, the toxicity of some responses is mitigated over time.

Although initially the PS NPs tested in this study would not be expected to become internalized because of the larger size respect to pores of the cell wall; (Navarro et al., 2008), the damage to the cell membrane caused by the attachment of the PS NPs could allow the passage of particles and/or aggregates. In addition, the PS NPs could also be internalized during the reproductive phase, since the neo-synthesized wall is more permeable to nanoparticles (Navarro et al., 2008). In this work we have explained the fluorescence of 100 nm fluoresbrite after consecutive washes in two ways; i) the strong adsorption of the PS NPs onto the cell impossible to remove after three consecutive washes, and ii) the internal uptake of NPs.

5. Conclusions

Despite their fast agglomeration, PS NPs found in the marine environment are bioavailable and are toxic to the marine diatom *P. tricornutum*. Our results show that PS NPs have a acute toxicity on the microalgae tested. Several acute cytotoxic responses occur in the first 24 h of exposure being mitigated after 72 h. Further, after 72 h of exposure, chronic toxic effects are evident with the alteration of responses such as growth, autofluorescence of ChlA, total

concentration of ChlA and protein content. The toxicological responses related to acute toxicity (24 h) showed greater alterations when the microalgae were exposed to the smallest nominal size of PS NPs (50 nm). Additionally, toxicological responses related to chronic toxicity showed more significant alterations when the microalgae were exposed to 100 nm PS NPs. These PS NPs (100 nm) are the most stable particle in size and shape in ASTM among the particles assayed, and this could be a key item about their toxicity due to a higher bioavailability. The fast ad/absorption of PS NPs onto/in *P. tricornutum* have been demonstrated in this study, through an increase in cell complexity, cell size and microalgae fluorescence caused by marked PS NPs. Our results suggest that the PS NPs found in the ocean are a threat to the primary producers and therefore this could represent a serious issue to marine ecosystems. The authors encourage future research about NPs toxicity, the use of techniques as flow cytometry and Phyto-PAM necessary to understand acute toxicity.

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Figure 1. Secondary characterization of PS NPs. Panel A and B agglomeration stage and SEM images of 50 and 100 nm PS NPs over time in ultrapure water and artificial seawater respectively.

Figure 2. Growth, cell complexity, E.Q.Y., % of ROS, % depolarised mitochondrial membrane and % DNA damage for microalgae after 24, 48 and 72 h exposed to 50 and 100 nm PS NPs (panel A, B, C, D, E and F). Data are represented as average $\pm$ SD (n:3). A GLM analysis was performed. Asterisk means $p < 0.05$.

significant differences between wash 1 and 2 and between 2 and 3 respectively; $p < 0.05$. Panel D; confocal images of microalgae after 72 h of exposure 100 nm fluoresbrite PS NPs after 3 consecutive washes. Cells can be identified by fluorescence in green. Black arrows and blue colour represent agglomeration of PS NPs.

Figure 4. Schematically illustration about behaviour of different size of PS NPs in artificial seawater and its effects in *P. tricornutum* related to acute and chronic toxicity.

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