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Impact of polystyrene nanoparticles on marine diatom *Skeletonema marinoi* chain assemblages and consequences on their ecological role in marine ecosystems☆

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ABSTRACT

Marine diatoms have been identified among the most abundant taxa of microorganisms associated with plastic waste collected at sea. However, the impact of nano-sized plastic fragments (nanoplastics) at single cell and population level is almost unknown. We exposed the marine diatom *Skeletonema marinoi* to model polystyrene nanoparticles with carboxylic acid groups (PS–COOH NPs, 90 nm) for 15 days (1, 10, 50 μg/mL). Growth, reactive oxygen species (ROS) production, and nano-bio-interactions were investigated. No effect on diatom growth was observed, however Dynamic light scattering (DLS) demonstrated the formation of large PS aggregates which were localized at the diatoms' fultoportula process (FPP), as shown by TEM images. Increase production of ROS and reduction in chain length were also observed upon PS NPs exposure ($p < 0.005$). The observed PS-diatom interaction could have serious consequences on diatoms ecological role on the biogeochemical cycle of carbon, by impairing the formation of fast-sinking aggregates responsible for atmospheric carbon fixation and sequestration in the ocean sea floor.

S. marinoi exposure to PS NPs caused an increase of intracellular and extracellular oxidative stress, the reduction of diatom's chain length and the adhesion of PS NPs onto the algal surface.

1. Introduction

Due to its conformation, highly populated coastlines and tourism, the Mediterranean sea is widely recognized to be severely impacted by plastic pollution (Suaria et al., 2016). Recent simulations estimate that the Mediterranean basin retains between 5% and 10% of the global plastic mass present at sea (Van Sebille et al., 2015). Being the most commonly used polymer for packaging and disposable items (PlasticsEurope, 2015), polystyrene (PS) is frequently found as waste in marine waters (Wan et al., 2018). Weathering of plastic leads to fragmentation into ever smaller particles (Wright and Kelly, 2017; Song et al., 2017). Laboratory studies have demonstrated that PS fragmentation occurs down to submicron (100–1000 nm) and nanoscale particles (1–100 nm) in water media, and environmental weathering is considered the main driver (Lambert and Wagner, 2016a; Lambert and Wagner, 2016b; Gigault et al., 2016; Ekvall et al., 2019). Recently, the occurrence of submicron plastic fragments was confirmed in the North Atlantic subtropical gyre (Ter Halle et al., 2017). Plastic fragments of various size floating on the sea surface can be colonized by bacteria and microalgae (Ye and Andrady, 1991; Lobelle and Cunliffe, 2011; Fazey and Ryan, 2016), which often differ among polymers and generally referred to as the "plastisphere" (Oberbeckmann, Löder and Labrenz, 2015; Carson et al., 2013,

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Muthukrishnan et al., 2018; Zettler, Mincer and Amaral-Zettler, 2013; Reisser et al., 2014). Diatoms are the most abundant taxa found on plastic fragments collected at the sea surface (Masó et al. 2003, Zettler et al., 2013; Masó et al., 2016; Carson et al., 2013; Reisser et al., 2014; Muthukrishnan et al., 2018) which are thus able to spread both harmful species and toxins, as recently documented in our previous study on the Adriatic Sea (NE Mediterranean Sea) (Casabianca et al., 2019). Diatoms play an important ecological role as primary producers, a significant part of the basic constituents of marine food chains (Harris, 2012), and one of the main bloom-forming and exudate producing groups of marine algae (Passow and Alldredge, 1994). Their exudates, known as exopolymeric substance (EPS), represent an important carbon source for the marine environment, playing a fundamental role in marine ecosystem ecology and functioning (Xiao and Zheng, 2016; Middelburg et al., 2000).

The formation of so called marine snow, made of macroscopic aggregates of detritus, living organisms and organic matter, mainly depends on the presence of phyto- and zooplankton and their exudates, with an important role played by algal blooms (Alldredge and Silver, 1988; Turner, 2002). Furthermore, the sinking of these organic aggregates contributes to carbon fluxes from the surface to the deep-sea (Harding, 1974).

The incorporation of plastic particles (both nano and micro) into natural marine aggregates has been studied in the laboratory and observed in the natural environment (Ward and Kach, 2009; Zhao et al., 2017; Summers, Henry and Gutierrez, 2018), while the biofouling of plastic is considered responsible for sinking and disappearance of small microplastic ($\leq$1 mm) from the sea surface (Cózar et al., 2014; Kooi et al., 2017; Fazey and Ryan, 2016). The interaction of microorganisms and their exudates with plastics is hypothesized to affect carbon fluxes, by modifying the sinking rates of marine snow and the bioavailability of small plastic particles for marine organisms (Long et al., 2015; Kooi et al., 2017; Ward and Kach, 2009).

A limited number of studies investigated the impact of nanoplastics on marine microalgae, and even less focused on their effects on diatoms. Available studies mainly consider acute effects at very high exposure concentration, which are probably not environmentally relevant (e.g., $\geq$50–100 mg/L) (Nolte et al., 2017; Bergami et al., 2017; Besseling et al., 2014; Sjollema et al., 2016). Particle adhesion has been documented, as well as the production of reactive oxygen species (ROS) and the reduction of photosynthetic yield (Nolte et al., 2017; Bergami et al., 2017; Chae et al. 2018; Bellingeri et al., 2019; Bhattacharya et al., 2010). Growth inhibition has been documented for the diatom *Skeletonema costatum* upon exposure to 1 mg/L of micro-polyvinylchloride (Zhang et al., 2017), and plastic adhesion has been considered the main driver of the observed toxicity. Predicted environmental concentrations of nanoplastics are in the range of μg/L and are expected to increase in areas showing significant particle accumulation, such as for instance the Mediterranean Sea (Al-Sid-Cheikh et al., 2018).

As nanoscale particles are very reactive and subject to transformations in aquatic media, their biological effects are often non-linear, and data interpretation becomes challenging (Peijnenburg et al., 2015; Rist and Hartmann, 2018). In an earlier study (Bellingeri et al., 2019), we suggested that standard ecotoxicological endpoints and time exposure may not be fully adequate to describe the effects of nanoplastics to aquatic organisms, especially microalgae. First, a detailed physico-chemical characterization of nanoplastics in exposure media is mandatory for assessing exposure conditions. Furthermore, long-term (*e.g.*, 15 days) as well as short-term studies should be used to better mimic environmentally relevant exposure. Moreover, sub-lethal endpoints (*e.g.*, biochemical, physiological, morphological up to behavioural alterations), rather than mortality, should be investigated (Bellingeri et al., 2019; Seoane et al., 2019).

Therefore, based on such scientific gaps, the present study investigated the impact of model polystyrene nanoparticles (PS NPs, 90 nm) functionalized with carboxylic groups (-COOH) on the marine diatom *S. marinoi*, among the most abundant on the Adriatic Sea (Penna, Capellacci and Ricci, 2004; Totti et al., 2019), by chronic toxicity in term of algal growth and sub-lethal responses as reactive oxygen species (ROS) production and chain assemblages at 15 days.

2. Materials & methods

2.1. Materials

Carboxylated polystyrene nanoparticles (PS–COOH NPs, subsequently referred to as PS NPs) were provided by the Physical Chemistry and Soft Matter Department in collaboration with the Food and Biobased Department of Wageningen University (The Netherlands) (Redondo-Hasselerharm et al., 2019; van Weert et al., 2019). The original stock solution was 41.91% w/w of PS NPs containing 0.4% w/w of covalently bound dye (rhodamine B methacrylate) and 1.2% w/w of sodium dodecyl sulfate (SDS). The distribution of SDS in the exposure medium was calculated in order to rule out that aqueous SDS concentrations could contribute to observed effects, if any (provided as Supporting Information). Nanoparticles leachates were not expected since the batch was synthetized without additives and therefore considered inert.

The SDS free aqueous concentration in our system was estimated to be between 0.16 and 0.95 mg/L at the highest PS NPs concentration tested. Literature data report that SDS has no effect on growth of the green alga *Scenedesmus obliquus*, up to 10 mg/L (Besseling et al., 2014), and is able to induce colony formation at concentrations higher than 5 mg/L (Lürling and Beekman, 2002). These threshold effect concentrations are one to two orders of magnitude higher than the predicted SDS concentration in our system, which thus suggests that SDS is not likely to interfere with effects of Nano-PS identified. However, we cannot completely exclude a possible interference of SDS in the observed effects as no literature data is available concerning the effect threshold for *S. marinoi*. Furthermore, stock was bubbled with clean air for 24 h to eliminate potential remaining styrene monomers and was diluted with MilliQ water (mQW) prior to the preparation of test solutions. Before use, each PS NP test solution was vortexed and bath sonicated for 2 min.

2.2. PS NPs characterization

PS NP behaviour in diatom exposure medium (F/2) was characterized by Dynamic Light Scattering (DLS, Malvern instruments), combined with the Zetasizer Nano Series software (version 7.02, Particular Sciences). Z-average (nm) and z-potential (mV) were determined at 50 μg/mL in mQW used for preparing PS NP stock solutions, and in F/2 used for algal exposure study.

2.3. Algal culture conditions and exposure study

Skeletonema marinoi CBA4 was maintained in F/2 medium (Guillard, 1975), at 16 ± 1 °C under a standard 12:12 h light-dark cycle; light was provided by cool-white fluorescent bulbs (photon flux of 100 $\mu Em^{-2}\ s^{-1}$). All exposure experiments were performed in 50 mL glass bottles containing *S. marinoi* at initial concentration of 1.0×10^4 cells/mL in artificial seawater (ASPM, Artificial Seawater Provasoli-McLachlan) (Guillard, 1975) enriched with F/2 medium components. Before exposure, PS NPs were briefly vortexed and

bath sonicated (Bandelin, Germany) for 2 min at room temperature. Diatoms were exposed to PS NPs at the following concentrations: 0 (control), 1, 10, 50 μg/mL, and exposure was carried out for 15 days (15-d). Each concentration was tested in triplicate and the experiment was repeated three times. Both exposure and control conditions were performed in triplicate. *S. marinoi* growth was determined by cell density. Samples were harvested at intervals of 3–4 days and fixed with Lugol's iodine solution and stored at +4 °C. Cell density was determined using an inverted microscope (ZEISS Axiovert 40 CFL) at 400x magnification using a Sedgewick Rafter counting chamber. Both growth rate (μ) and inhibition of growth rate (Iμi) were determined. In particular, growth rate, defined as the instantaneous rate of increase, was calculated on the basis of the longest possible period of exponential growth using the equation: $\mu = \ln(Nt/N0)/\Delta t$, where N is the number of cells/mL, Δt is the time interval (Wood, Everroad and Wingard, 2005). Inhibition of growth rate (Iμi) was then determined following a standard guideline (ISO, 2006) and considering the same growth rate time interval.

Diatom chain length was determined with the aid of imageJ software on pictures taken with an optical microscope (Olympus BX51 coupled with Olympus DP-software) of Lugol fixed samples. We counted the number of cells composing each chain over 100, randomly selected, chains for each replicate. The relative frequency of each group (chain composed by 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 cells) was then calculated.

2.4. Sub-lethal effects

2.4.1. S. marinoi-PS NPs interaction

The physical interaction between algal cell and PS NPs at the end of the exposure period (15-d), was imaged through high resolution environmental scanning electron microscopy (ESEM, Quanta 400 (FEI)), and transmission electron microscopy (TEM, Tecnai G2 Spirit (FEI)). At the same time, light microscope Zeiss Axiophot equipped with interference contrast was used to record micrographs from algal samples using a AxioCam MRm fitted with AxioVision software. Different techniques were applied in an attempt to find the most suitable in describing PS NPs-diatom interaction and avoid the creation of artefacts due to sample preparation (Mourdikoudis, Pallares and Thanh, 2018; Tiede et al., 2008). To obtain ESEM images, unaltered samples of PS NPs exposed diatoms and controls were used. For TEM images, instead, diatoms were processed following two different procedures: a) diatoms were fixed in glutaraldehyde (1.5%) and then washed with mQW water and centrifuged (7000 *g* for 15 min at 20 °C) twice, b) diatoms were kept fresh without fixation. For optical microscopy (both brightfield and differential interference contrast) diatoms were fixed with glutaraldehyde (1.5%) and washed using mQW before observation.

2.4.2. Quantification of ROS

The production of reactive oxygen species (ROS) was measured by following the conversion of the non-fluorescent dihydrodichlorofluorescein diacetate (H_2DCF-DA) to the highly fluorescent compound 2′, 7′,-dichlorofluorescein (DCF) as described by Wang and Joseph (1999), recently adapted for algal cells by Morelli et al. (2018). Algae samples (2 mL) were spiked with 20 μL of a 1 mM DCF-DA solution and kept under constant shaking at room temperature for 1 h in the dark. Each replicate was tested in triplicate so nine measurements for each exposure concentration were obtained.

Fluorescence was determined in triplicate at 520 nm emission wavelength (λ_{ex} = 485 nm) using a Victor 3 1420 multilabel Counter (PerkinElmer) and used for total ROS estimation. Thereafter, the remaining volume (1420 μL) was centrifuged (10,000 *g*, 15 min, 20 °C), supernatant was discarded, and the pellet was resuspended in fresh F/2 to a final volume of 1420 μL; fluorescence was measured again and used for intracellular ROS estimation. Tested blanks were F/2, F/2 + H_2DCF-DA and F/2 + H_2DCF-DA + 10 and 50 μg/mL PS NPs. No interference in fluorescence was recorded in the presence of PS NPs. Background value (F/2 + H_2DCF-DA) was subtracted from the obtained fluorescence value of the samples. By subtracting the fluorescent value of intracellular ROS to the fluorescent value of total ROS, we estimated the extracellular ROS value. Fluorescent data were normalized to the cell density and expressed as fluorescence/cell density.

2.5. Statistical analysis

Statistical analyses were performed with non-parametric Mann-Whitney and Kruskal Wallis tests using PAST ver. 3.14 with a *p*-value <0.05 determining significance for growth inhibition, and with an unpaired *t*-test using R with a *p*-value <0.005 for ROS levels.

3. Results and discussion

3.1. PS NPs characterization in exposure media

DLS measurements showed a good dispersion of PS NPs in mQW, with a hydrodynamic diameter of 88.2 ± 2.9 nm and a Z-potential of −42 mV (Table 1). In F/2, a negative surface charge was still preserved (−22.8 mV) while the formation of large PS NP aggregates (hydrodynamic diameter 1793 ± 56.9 nm) was observed, in agreement with previous characterizations done in algal medium in artificial sea water (Bergami et al., 2017; Bergami et al., 2016).

3.2. Growth

No effects on diatom growth were observed upon exposure to PS NPs (1, 10 and 50 μg/mL) for 15 days (Hc = 0.63, p = 0.89). Growth rates were in the range of 0.61–0.66 per day, similarly in controls and exposed diatoms (Hc = 2.131, p = 0.5457) (Fig. S1). These findings are in agreement with previous studies in which PS NPs did not cause any effect on algal growth, both in fresh water and in sea water (Bergami et al., 2017; Sjollema et al., 2016; Besseling et al., 2014; Bellingeri et al., 2019). In a long-term (30-d) exposure study with *Chlorella pyrenoidosa* Mao et al. (2018) reported a growth phase-dependent inhibitory effect of PS NPs: a significant initial inhibition (38.5%) disappeared after 22 days, while at the end of exposure period (30-d) exposed algae showed an even higher cell density than control. On the contrary, *S. marinoi* exhibited a constant growth similar to controls during 15 days of exposure until the beginning of stationary phase (6-d) and at the end of exposure period (15-d). Regarding the SDS present in PS NPs stock according to our calculation it results below probable effect threshold concentrations for phytoplankton.

3.3. Sub-lethal effects

TEM images clearly show PS aggregates interacting with the

Table 1
DLS measurements of hydrodynamic diameter (z-average), polydispersity index (PDI) and surface charge (z-potential) of PS NPs (50 μg/L) in mQW and F/2 medium at 25 °C.

	z-average (nm)	PDI	Z-potential (mV)
MilliQ	92.9 ± 4.65	0.052	−42
F/2	1933 ± 525	0.697	−22.2

S. marinoi cell surface. Both in fixed and fresh (not fixed) diatom cells (Fig. 1 b, d, f and Fig. 2, respectively), the adhesion seems to be mainly localized in the terminal fultoportula processes (TFPP), the elongated structures responsible for chain formation and maintenance in this marine diatom (Fig. 1 a, arrow).

Images obtained from fresh diatoms clearly show the adhesion of PS aggregates to the diatom cell surface (Fig. 2), while in samples fixed with glutaraldehyde and further washed in mQW, such interaction is far less evident (Fig. 1 b, d, f). Since TEM images are obtained when electrons are transmitted through the sample, in order to avoid any disturbance due to the presence of salts and organic matter, marine organisms are commonly washed in mQW before TEM analysis. However, based on our findings, fixation and washing significantly change the interaction of PS aggregates with diatom cell surface, producing an artefact which does not resemble the natural interaction occurring between *S. marinoi* and PS. Both sample-processing steps might alter the chemical composition of the medium and contribute to the removal of natural organic matter (*e.g.,* algal exudates).

Morphological alterations in diatom cells are also evident in both PS-exposed and controls, probably as a consequence of preparative methods, which is further confirmed by their absence in

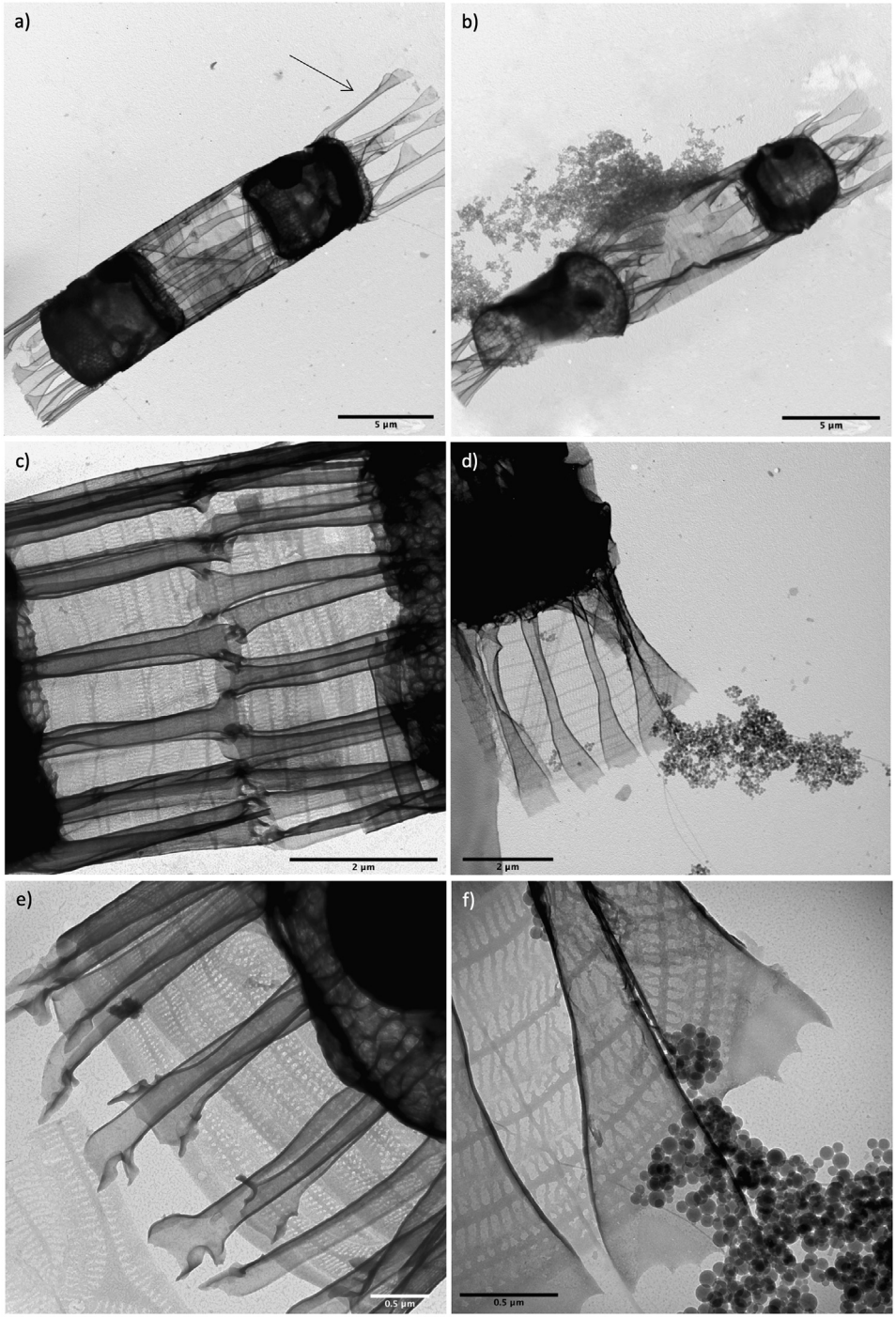

Fig. 1. TEM images of *S. marinoi* samples fixed with glutharaldheyde (1.5%) and further washed with mQW; a, c, e) control cells, **b, d, f)** PS NPs exposed cells.

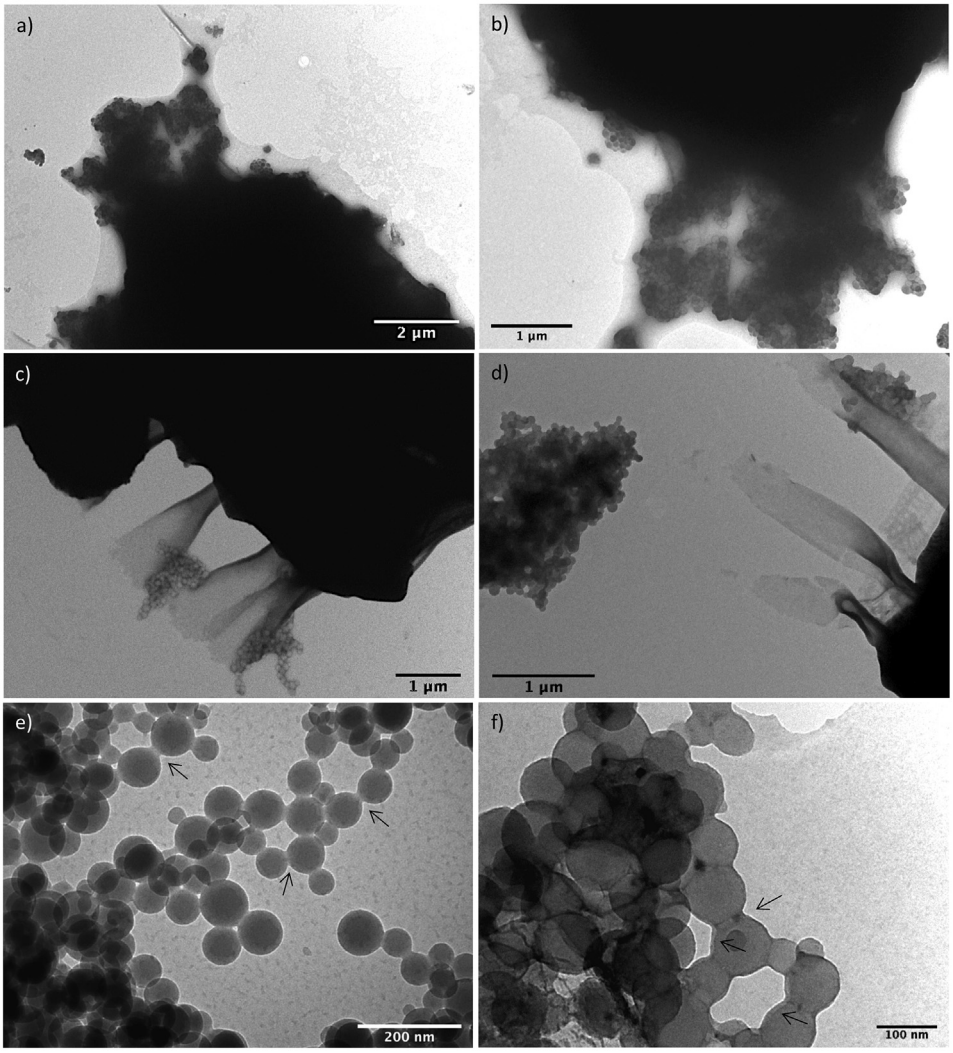

Fig. 2. TEM images of *S. marinoi* fresh (not fixed) samples. **a, b**: PS aggregates entrapped with organic material; **c, d**: details of PS aggregates localized at FFP; **e, f**: higher magnification of PS aggregates embedded in organic materials presumably EPS and details of PS NPs rounded with organic material (see arrows).

optical images (Fig. 3). A further confirmation is obtained also by ESEM images where cells appeared to be altered both in control and exposed diatoms (Fig. S2). Seoane et al. (2019) reported similar morphological alterations in cells of the diatom *Chaetoceros gracile* processed for SEM analysis (fixed with glutaraldehyde and filtrated) both in controls and in those exposed to microplastics.

Sample preparation is a necessary step for TEM analysis, however, studies conducted with NPs, recognized some limitations due to a significant sample alteration (Tiede et al., 2008; Mourdikoudis et al., 2018). Moreover, working under vacuum conditions could also affect sample integrity and produce artefacts (Mavrocordatos, Perret and Leppard, 2007). In the study of nano-bio-interactions, any preparative procedure which might affect the integrity of the sample should be avoided by using for instance ESEM, being recognized as a more conservative process, using fresh unprocessed samples (100% humidity). However, according to our findings, high levels of humidity might have reduced the contrast and made the smaller particles less detectable (Tiede et al., 2008). ESEM images (Fig. S2), in fact, did not allow to detect algal cell-PS NPs interaction, probably because of the small size of the PS NPs and the presence of solution partially masking cell surface. PS NPs were not easy to identify but are probably represented by the brighter and grainy spots on the background of exposed cell images to PS NPs (Fig. S2 c, d), which are not visible in control images (Fig. S2 a, b).

Furthermore, TEM images highlighted an adhesion of diatom EPS to PS NPs as shown in Fig. 2(e and f, see arrows). Chen et al. (2011) and Summers et al. (2018) already described the formation of plastic agglomerates held together by a biopolymer matrix, connecting and trapping the particles. Such process could be even more relevant for microalgae producing high amount of exudates as diatoms, with possible implications for their role on plastic behaviour and fate in the water column. The incorporation of plastics into algal and marine aggregates has been documented and shown to modify the buoyancy and sinking rates of aggregates, and to increase ingestion of plastic particles by suspension-feeding bivalves (Long et al., 2015; Ward and Kach, 2009; Porter et al., 2018).

EPS play many important roles in diatom ecology in terms of motility, adhesion and overall cell protection and colony formation (Hoagland et al., 1993), but more importantly, they play a key role in the formation of the siliceous frustule and also in protection against dissolution (Round, Crawford and Mann, 1990, Simpson and Volcani, 2012). The observed adhesion of PS aggregates to the algal surface could be the result of EPS interaction with PS NPs and be linked to the observed effect on algal chain length.

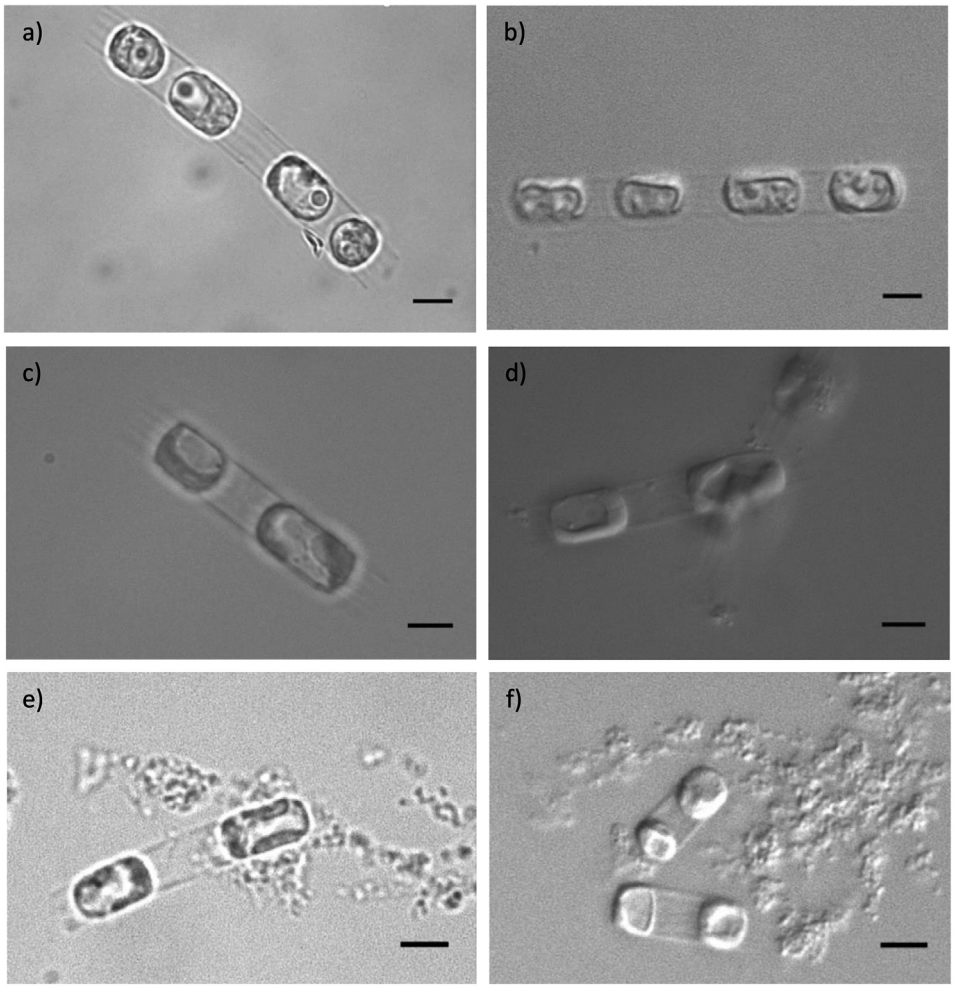

Fig. 3. Optical (left) and differential interference contrast (DIC) microscopy (right) images of *S. marinoi* CTRL (a, b, magnification: 100x and 40x) and exposed to 10 μg PS/mL (c, d, magnification: 100x) and 50 μg PS/mL (e, f, magnification: 40x). Scale bar is 5 μm.

The length of *S. marinoi* chains was significantly affected by PS NPs, at 10 and 50 μg/L exposure. Exposed algae showed a high percentage of single cells and 2-cell chains, altogether accounting for 95% and 84% of 10 and 50 μg/mL exposure, respectively. As opposed to control algae, in which single cells and 2-cells chains accounted for 36% of the observed chains, while 43% was represented by 4- and 8-cell chains (Fig. 4, Table S1). At 1 μg/L exposure no difference in chain length was observed (data not reported). Shorter chain length could have serious consequences on diatoms ecology by impairing their buoyancy and enhancing their sinking rates with potential implications for the maintenance of phytoplankton productivity on the sea surface (Smayda and Boleyn, 1966). The assessment of the floating capacity of algae was beyond the aim of our study, however our findings highlight the

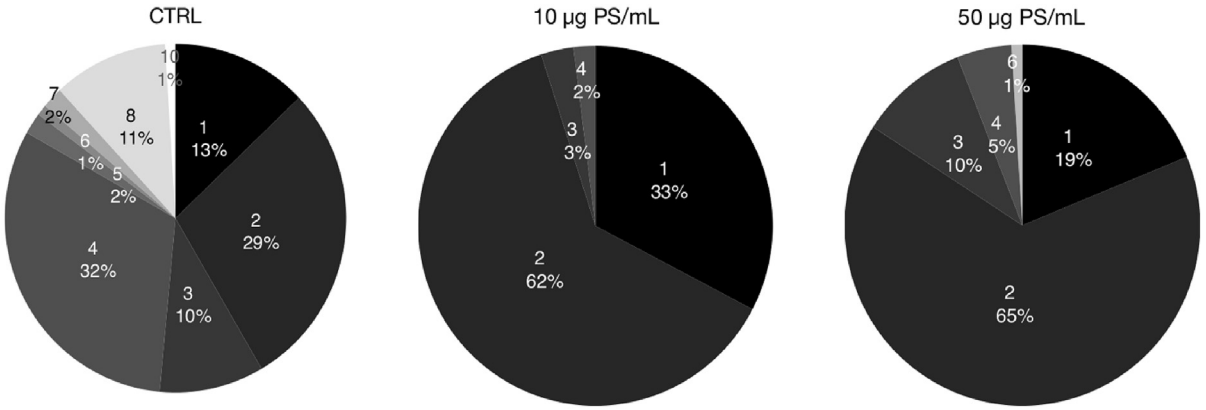

Fig. 4. *S. marinoi* chain length expressed as percentage of different number of cells (1, 2, 3, 4, 5, 6, 7, 8, 9, 10) in control (CTRL) and PS NPs exposed (10 μg PS/mL and 50 μg PS/mL).

need for further investigations in order to better understand which consequences of nanoplastics exposure can be expected for the ecological role of diatoms.

S. marinoi chains are composed of cells connected to one another by the fultoportula processes (FPP). The documented adhesion of PS aggregates to the FPP might be responsible for the shorter chains, since these structures play an important role by acting as a bridge between diatom cells, thus causing the assembly of the chain. Therefore, we hypothesize that the reduction in chain length is a consequence of PS NPs adhesion to these structures. The adhesion may cause a localized stress and a weakening of the siliceous structures, causing shortening of the chains. Bhattacharya et al. (2010) suggested the occurrence of contact-induced stress following cell-PS NPs interactions, resulting in enhanced ROS production. In fact, a concentration-dependent increase in both intracellular and extracellular ROS levels was observed in our study in diatom exposed to PS NPs (Fig. 5). In particular, intracellular ROS significantly increased ($p < 0.005$) compared to the control for both 10 and 50 μg PS/mL, while for extracellular ROS a significant increase was observed only at 50 μg/mL.

Such findings are in agreement with Liu et al. (2019) and Bhattacharya et al. (2010), who reported an increase in ROS production in microalgae exposed to uncharged and positively charged PS NPs, and with Morelli et al. (2018) and Ševců et al. (2012) reporting similar results with metallic NPs, mainly metal oxides. Liu et al. (2019) also reported an increase in superoxide dismutase (SOD) activity at lower concentration (1 μg/L, 1 mg/L) of PS-COOH probably as a sign of early oxidative stress response.

Concerning the observed chain length reduction, other hypotheses can be formulated. Takabayashi et al. (2006) observed a positive correlation between nutrient availability and longer chains in *Skeletonema costatum*. The presence of PS NPs could cause a reduction in nutrient concentration through adsorption on their surface, thus influencing algal chain length. Alternatively, *S. marinoi* was demonstrated (Bergkvist et al., 2012; Bjærke et al., 2015) to be able to shorten its chains as a response to a size-dependent grazing pressure, by copepod grazing selectively on longer chains. This resulted to be induced by chemical cues, produced either by the grazing copepods or by the algae being grazed. PS NPs could therefore activate the same molecular pathway involved in this predator escaping strategy and resulting in an algae self-induced reduction of chain length.

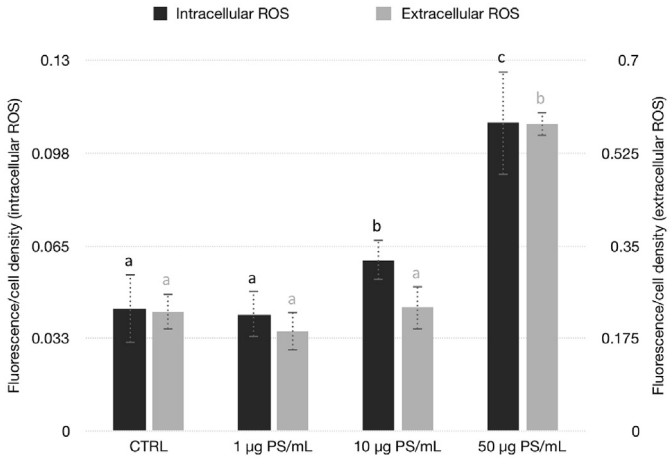

Fig. 5. Intracellular (black, left y-axis) and extracellular (grey, right y-axis) ROS levels in *S. marinoi* exposed to PS NPs (1,10, 50 μg PS/mL) and in controls. Data shown as fluorescence units/cell density (cells/mL) and presented as mean ± standard deviation. Within the same data group, data with different letters are statistically different with $p < 0.005$.

4. Conclusions

Our findings highlighted no lethal effect of PS NPs to the marine diatom *S. marinoi*, while showing an increase in intracellular and extracellular oxidative stress, the adhesion of PS NPs onto the algal surface and a reduction of diatom's chain length. Further studies should focus on potential ecological implications as for instance changes in algal buoyancy as well as the formation and sinking of aggregates.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Arianna Bellingeri: Methodology, Investigation, Conceptualization, Writing - original draft, Writing - review & editing. **Silvia Casabianca:** Methodology, Investigation, Conceptualization, Writing - review & editing. **Samuela Capellacci:** Methodology, Investigation, Conceptualization, Writing - review & editing. **Claudia Faleri:** Investigation. **Eugenio Paccagnini:** Investigation. **Pietro Lupetti:** Investigation. **Albert A. Koelmans:** Methodology, Writing - review & editing. **Antonella Penna:** Supervision, Project administration, Methodology, Conceptualization, Writing - original draft, Writing - review & editing. **Ilaria Corsi:** Supervision, Project administration, Methodology, Conceptualization, Writing - original draft, Writing - review & editing.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2020.114268.

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