Do transparent exopolymeric particles (TEP) affect the toxicity of nanoplastics on Chaetoceros neogracile?

Carmen González-Fernández, Jordan Toullec, Christophe Lambert, Nelly Le Goïc, Marta Seoane, Brivaëla Moriceau, Arnaud Huvet, Mathieu Berchel, Dorothée Vincent, Lucie Courcot, et al.

Environmental Pollution
July 2019, Volume 250, Pages 873-882
https://doi.org/10.1016/j.envpol.2019.04.093
https://archimer.ifremer.fr/doc/00491/60235/

Do transparent exopolymeric particles (TEP) affect the toxicity of nanoplastics on *Chaetoceros neogracile*?

González-Fernández Carmen [5], Toullec Jordan [1], Lambert Christophe [5], Le Goïc Nelly [5], Seoane Marta, Moriceau Brivaela [5], Huvet Arnaud [2], Berchel Mathieu [3], Vincent Dorothée [4], Courcot Lucie [4], Soudant Philippe [5], Paul-Pont Ika [5, *]

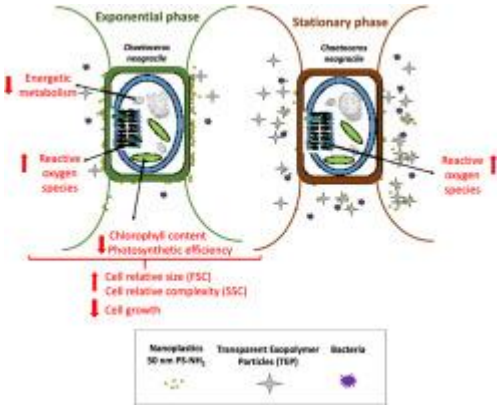
[1] Laboratoire des Sciences de L'Environnement Marin (LEMAR), UMR 6539 CNRS/UBO/IRD/IFREMER, Institut Universitaire Européen de La Mer (IUEM), Rue Dumont D'Urville, 29280, Plouzané, France
[2] Ifremer, Laboratoire des Sciences de L'Environnement Marin (LEMAR), CS 10070, 29280, Plouzané, France
[3] CEMCA, UMR CNRS 6521, IBSAM, UFR Sciences, Université de Bretagne Occidentale, 6 Avenue Victor Le Gorgeu, 29238, Brest, France
[4] Univ. Littoral Côte D'Opale, Univ. Lille, CNRS, UMR 8187, LOG, Laboratoire D'Océanologie et de Géosciences, 32 Avenue Foch, F-62930, Wimereux, France
[5] CNRS

* Corresponding author : Paul-Pont Ika, email address : ika.paulpont@univ-brest.fr

Abstract :

The potential presence of nanoplastics (NP) in aquatic environments represents a growing concern regarding their possible effects on aquatic organisms. The objective of this study was to assess the impact of polystyrene (PS) amino-modified particles (50 nm PSNH2) on the cellular and metabolic responses of the diatom Chaetoceros neogracile cultures at two essential phases of the growth cycle, i.e. exponential (division) and stationary (storage) phases. Both cultures were exposed for 4 days to low (0.05 µg mL−1) and high (5 µg mL−1) concentrations of PS-NH2. Exposure to NP impaired more drastically the major cellular and physiological parameters during exponential phase than during the stationary phase. Only an increase in ROS production was observed at both culture phases following NP exposures. In exponential phase cultures, large decreases in chlorophyll content, esterase activity, cellular growth and photosynthetic efficiency were recorded upon NP exposure, which could have consequences on the diatoms life cycle and higher food-web levels. The observed differential responses to NP exposure according to culture phase could reflect i) the higher concentration of Transparent Exopolymer Particles (TEP) at stationary phase leading to NP aggregation and thus, probably minimizing NP effects, and/or ii) the fact that dividing cells during exponential phase may be intrinsically more sensitive to stress. This work evidenced the importance of algae physiological state for assessing the NP impacts with interactions between NP and TEP being one key factor affecting the fate of NP in algal media and their impact to algal' cells.

Graphical abstract



Highlights

► Interaction of NP and diatoms was studied at exponential and stationary phases. ► Transparent Exopolymer Particles (TEP) alter NP fate by aggregating NP. ► NP impairs major physiological traits of diatoms at exponential phase. ► Whatever the aggregation state, NP promotes oxidative stress at both growth phases.

Keywords : nanoplastics, diatoms, physiology, transparent exopolymer particles

1. Introduction:

Marine plastic pollution is a major environmental concern. Plastics have an increasing global production (>320 million tons of plastics; Plastic Europe 2017) and a part of this production ends up as waste in the Oceans. Small plastic particles (diameter < 5mm) called microplastics (MP) have been found in waters and sediments around the world, reaching concentrations of 8.5 mg L^{-1} and 33.0 mg kg^{-1} respectively (MP sizes ranging between 1 and 275µm) in the most polluted areas (Bergmann et al., 2017; Dubaish and Liebezeit., 2013). However, as a result of the technical limitation for their identification, little attention has been paid to small microparticles, and notably the nanoplastics fraction (GESAMP, 2016). Nanoplastics (NP) were considered as plastic particles that are <100 nm in the definition of engineered nanomaterials (Mattsson et al., 2015; GESAMP, 2016). Primary NP are produced for a wide range of applications such as biosensors (Velev and Kaler, 1999), photonics (Rogach et al., 2000), nanocomposites (Merinska and Dujkova, 2012) and drug delivery tools and cosmetics (Hernandez et al., 2017) as well as pharmaceutical products (FAO, 2017) while secondary NP may originate as demonstrated experimentally from mechanical fragmentation (Lambert and Wagner, 2016), photo-degradation (Gigault et al., 2016) or biodegradation (Dawson et al., 2018) of larger items. NP have been estimated as one of the largest share of nanomaterials on the market (Fabra et al., 2013 andtheir first report in the aquatic environment was argued in the North Atlantic Gyre (Ter Halle et al., 2017).

Amongst phytoplankton, diatoms represent one of the most abundant group, recognized to be responsible for around 40% of global primary productivity in the oceans (Malviya et al., 2016) and influencing the global carbon cycle (Field et al., 1998). The interaction between MP and marine diatom aggregates has already been shown experimentally (Long et al., 2015; 2017) modifying both algae and MP settlement rates in the water column. Due to their intrinsic characteristics, nanomaterials, and more often NP, may have higher impact than their micro-scale counterparts (Mao et al., 2018). Phytoplankton cell walls constitute the first barrier for uptake encountered by any substances including nanoparticles (Khowala et al., 2008). Nanoplastics can attach to the cell surfaces as suggested for the green microalgae *Dunaliella tertiolecta* (Bergami et al., 2017; Nolte et al., 2017) while crossing the cell wall will depend on the size: nanoplastics with larger size than cell wall pore (> 20 nm for diatoms) will not easily cross cell wall (Navarro et al., 2008).

In recent years, the impact of NP on freshwater algae physiology (e.g. photosynthesis) has raised controversial results. On the one hand, studies revealed a decrease in algal photosynthesis upon polystyrene nano-beads exposure (Bergami et al., 2017; Bhattacharya et al., 2010; Mao et al., 2018). On the other hand, other studies reported decreased algal growth with no impact on algal photosynthetic rate (Sjollema et al., 2016; Yokota et al., 2017). Due to technical limitation for its characterization, the assessment of NP behavior in natural environments is still a challenge. Nevertheless, interactions of NP amongst themselves and with other macromolecules such as polysaccharide, organic matter, proteins between others, have been already evidenced under laboratory conditions (Canesi et al., 2017; Galloway et al., 2017) and it is directly related to their toxicity for organisms (reviewed in Paul-Pont et al., 2018). Diatoms (and bacteria) are well known for excreting large quantities of Exopolymeric Substances (EPS) during their growth. One type of EPS called Transparent Exopolymer Particles (TEP) has received increasing attention because of their high stickiness (Passow, 2002). Thus, TEP may interact with NP in natural environments. In fact, differential production of carbohydrates by micro-algae was already observed (Lagarde et al., 2016) depending on the chemical composition of the MP to which they were exposed. Hence, higher carbohydrate production was related to high density polyethylene compared to polypropylene. In addition, TEP is not species-specific (Allard and Tazi, 1993) and rather depends on the age of the strain and/or external conditions such as the presence of bacteria (Corzo et al., 2000). In this context, we hypothesized that physiological state of microalgae may play a role in NP fate and toxicity depending on TEP concentration in the culture media and their interactions with NP.

In this work, we studied the effects of NP (50 nm) exposure on the cellular and metabolic responses of the diatom *Chaetoceros neogracile* during exponential and stationary culture phases via a single experimental design. This has never been assessed before and is of major importance to advance knowledge in the impacts of plastic debris in marine ecosystems. Two concentrations of NP were used: a high concentration (5 µg mL^{-1}) established to compare results with other laboratory ecotoxicological studies (*e.g.* Bergami et al., 2017; Nolte et al., 2017) and a 100 times lower concentration (0.05 µg mL^{-1}) to get closer to suspected concentrations in highly contaminated environment (Huvet et al., 2016; Lenz et al., 2016). Taking into account the relevance of the surrounding media on NP fate and their potential toxicity (Canesi et al., 2017; Galloway

et al., 2017), TEP concentration in the culture media was quantified and NP aggregation in algae' media was characterized.

2. Material and methods

2.1. Batch culture setups

Batch cultures of non-motile diatom *Chaetoceros neogracile* (width: 4µm, length: 7µm; strain CCAP 1010-3) were done in 500 mL flasks containing 400 mL of sterilized filtered (0.2 µm) seawater (FSW) including 1 mL L^{-1} of silica and Conway´s medium (Walne, 1966) in a 24 h light cycle with a constant intensity of 100 µmoles photons m^{-2} s^{-1} at adjusted room temperature of 20ºC. Flasks were kept in constant aeration and CO_2 was supplied to keep the pH between 7.5-7.9. Two sets of cultures were prepared at 7 days interval in order to expose simultaneously *C. neogracile* in exponential phase (3 days old) and in stationary phase (10 days old) to NP. The growth phases were validated previously thanks to a calculation of a growth curve under same conditions of the experiment (data not shown).

2.2. Nanoplastics characterization

Fluorescent-blue 50 nm polystyrene amino (PS-NH_2) nanoparticles were purchased from Sigma-Aldrich (Saint Louis, USA) with an Excitation/Emission: 358 nm/410 nm. Physico-chemical characteristics of NP were confirmed in ultrapure water. Size (Z-average), charge and aggregation state (polydispersity index, PdI) of NP were determined by Dynamic Light Scattering (DLS) using a nano-Zetasizer (Malvern instruments, United Kingdom). Measurements were performed in triplicates, each containing 13 runs for the Z-average and 40 runs for Z-potential. In addition, NP shape was also characterized in ultrapure water by transmission electronic microscopy (JeolJEM 100 CX II). In brief, nanoplastics were diluted in ultrapure water (100 µg mL^{-1}) and placed on copper grid (400 nm mesh) with a carbon-coated Formvar film (Polysciences) and marked with 2% (wt/vol) uranyl acetate to be measured.

DLS analyses of NP were also performed in FSW and algae' spent media of both cultures (exponential and stationary cultures). Algae' spent media were obtained after centrifugation of an algal culture (stationary or exponential) at 550 g for 5 min and supernatant was collected. Data were analyzed using Zetasizer Nano Series software, version 6.20. Results are presented as mean (n=3) ± standard deviations.

143 2.3. Exposure

144 To keep cells in the same culture exposure conditions (i.e. in exponential and stationary
145 growths), concentrations of both batch cultures were adjusted at 4×10^5 cells mL^{-1} by
146 centrifugation (550 g for 5 minutes, final volume 300 mL) and resuspended in their
147 respective media (exponential or nutrient replete vs. stationary media or nutrient
148 limited). This was performed to obtain the same cell/NP ratio without having to dilute
149 algae culture with FSW which could have introduced a bias (dilution of nutriments,
150 exudates, etc.).

151 For each culture phase (stationary and exponential), three experimental treatments were
152 designed: control (no addition of NP), low NP concentration (0.05 µg mL^{-1}) and high
153 NP concentration (5 µg mL^{-1}). All treatments (control, low and high) were performed in
154 triplicates for each culture phase (exponential and stationary). *Chaetoceros neogracile*
155 cultures were sampled after 4, 24, 48, 72 and 96 h exposure to perform the following
156 analyses.

157 2.4. Analyses

158 2.4.1. Scanning electron microscope visualization of NP–diatoms interactions

159 For scanning electron microscopy (SEM) observations, only cultures in exponential
160 phase, which were significantly affected by NP, were analyzed. After 72 h exposure,
161 one mL sample of each replicate from control and high NP treatments was fixed in
162 pseudo-lugol (2% final) according to Verity et al. (2007) protocol, which had proved to
163 be a good fixative for diatoms´ cells and store at 4ºC until analysis. In parallel,
164 additional tests were performed to compare the quality of SEM analyses between fresh
165 and pseudo-lugol fixed samples as we had no clue on the potential effect of pseudo-
166 lugol on the physical interaction between NP and diatom cells.

167 Nevertheless, gradual or direct dehydration with ethanol from Lugol's solution caused
168 gross shrinkage (Bistricki and Munawar, 1978). Thus, both, fresh and pseudo-lugol
169 fixed samples were fixed and post-fixed, respectively, in 0.1 M sodium cacodylate
170 buffer (1.75% w/v of NaCl, pH 7.2) for 10 min at 4 °C before being filtered through
171 polycarbonate filters with a 2 µm pore size. Later, filters were rinsed with a solution of
172 sodium cacodylate 0.1 M (2% w/v of NaCl) in ultrapure water. Samples were then
173 dehydrated by successive immersions in alcoholic hexamethyldisilazan (HMDS) (v:v):

absolute ethanol−HMDS (3:1), absolute ethanol−HMDS (1:1), absolute ethanol−HMDS (1:3), and pure HMDS. Finally, samples were coated with gold palladium before being observed by SEM (Hitachi S-3200N).

2.4.2. Algae descriptive and physiological parameters and bacteria concentration

Flow cytometric analyses were performed in triplicates in each experimental treatment and algal stage using an EasyCyte Plus cytometer (Guava Technologies, Millipore, Billerica, MA), equipped with a 488-nm argon laser and three fluorescence detectors: green (GRN 525/30 nm), yellow (YLW 583/26 nm), and red (RED 680/30 nm). Parameters measured by flow cytometry (cell number and descriptive parameters, microalgal chlorophyll a content, esterase activity, intracellular Reactive Oxygen Species (ROS) production and Bacteria concentration) were measured on separated aliquots according to the following protocols:

Cell number and descriptive parameters. Cells were detected on the flow-cytometer by their optical characteristics obtained at small angle (Forward scatter, FSC) and large angle (Side Scatter, SSC). The flow-cytometer is equipped with an absolute counting system (peristaltic pump, flow rate) allowing calculation of the algae' concentration, expressed in cell mL^{-1}.

Microalgal chlorophyll a content was estimated using chlorophyll autofluorescence from the selected microalgal population (RED detector of the flow-cytometer, 680/30 nm; Long et al. 2017). Data was expressed as the mean of red fluorescence per cell in arbitrary units (A.U.).

Esterase activity which is considered as a proxy of primary metabolism (Garvey et al., 2007) was measured using the fluorescein diacetate staining (FDA; Invitrogen # F1303; final concentration 6 µM) incubating samples for 10 min at 18 ºC in the dark. FDA is a nonpolar compound that can permeate into the cells. Once inside the cells, FDA is cleaved by esterases into acetate and fluorescein molecules (fluorescent green) which are retained within the cells. Intracellular fluorescence increases with both metabolic activity and time. Esterase activity was estimated by measuring the level of green fluorescence (GRN detector of the flow-cytometer) and expressed as mean of fluorescence per cell in arbitrary units (A.U.). Esterase activity was measured at all time

for the stationary phase culture and after 4, 24, 48, 96 h for the exponential phase culture.

Intracellular Reactive Oxygen Species (ROS) production was measured using 2,7-dichlorofluorescein diacetate (DCFH-DA; Sigma-Aldrich, D6883; final concentration 10 µM) after 50 min of incubation at 18 ºC in the dark (Long et al. 2017). DCFH is hydrolyzed (esterase) intracellularly to form DCF which produces green fluorescent upon oxidation with ROS. The green fluorescence measured (GRN detector of the flow-cytometer) is quantitatively related to the intracellular ROS production and is expressed as the mean of green fluorescence per cell in arbitrary units (A.U.).

Bacteria concentration (adapted from Marie et al., 1997) was measured in each culture flask (n=3 for each treatment) in both exponential and stationary cultures, at the beginning and the end of the experiment, by fixing 500 µL of algae culture with formaldehyde (0.35% final) for 10 minutes at room temperature in the dark before freezing the samples (-20 ºC) until analysis. Samples were 1/10 diluted in filtered sterile seawater and incubated for 10 min in the dark after addition of 5 µL of SYBR® Green I commercial solution (Molecular Probes, #S7563) diluted at 1/100 in Milli-Q water. SYBR® Green I was used to detect bacteria as it stains DNA which fluorescence green and was detected by FL1 detector. Results are given as concentration of bacteria in cells per mL. Additionally, bacteria load (bacteria by cell) was also calculated dividing bacteria concentration by cell concentration.

Photosynthetic efficiency (F_v / F_m) which is a proxy of PSII photochemical efficiency, was measured by pulse amplitude modulation (PAM) fluorometry with a handled AquaPen-C AP-C 100 (Photon Systems Instruments, Drassov, Czech Republic) equipped with a blue light (455 nm). For each sampling time, 3 mL sample of each culture were dark-adapted for 30 minutes before measurement of fluorescence variables (F_0, F_m, F_v). F_0 is the initial fluorescence intensity, F_m the maximal intensity under saturating light conditions, and $F_v = F_m - F_0$ (Strasser et al., 2000).

Transparent Exopolymer Particles (TEP) concentration was measured following the method developed by Passow and Alldredge (1995). Three to five replicates were sampled from each culture before the exposure. Later, samples were prepared for exposure as described in section 2.3. At the end of the experiment (96 h exposure), a concentration of 1×10^4 cells mL^{-1} of each culture flask were filtered onto 0.4 µm

polycarbonate filters under low vacuum pressure and stained with 0.5 mL Alcian blue solution (0.02 % in aqueous solution, 0.06 % acetic acid, pH 2.5, filtered at 0.2 µm before use). Polycarbonate filters were then soaked for 2 h in 6 mL of 80 % H_2SO_4 solution under agitation. The absorption of the obtained solution was measured at 787 nm in a 1 cm cuvette and was converted in gram of Gum Xanthan equivalent per litter (g Xeq L^{-1}) using a Gum Xanthan calibration curve fitting our working Alcian blue solution.

2.5. Statistical analyses

Statistical analysis was performed using STATGRAPHICS centurion XV.II (Statpoint Technologies, Inc. Warrenton, Virginia, USA). Differences were considered significant at $p < 0.05$. Normality and homogeneity of variances of the data distribution were tested by standardized skewness and standardized kurtosis and Levene's test, respectively. One-way-ANOVA analysis was performed to establish significant differences between treatments at each time. A Tukey's post hoc test was used to test differences among the different exposure treatments. Arcsine or log transformation was performed when necessary to meet the normality and homoscedasticity criteria. Non-parametric analysis (Kruskal-Wallis test) was also performed when the variables did not meet the requirements of a parametric ANOVA.

3. Results

3.1. Nanoplastics behavior and fate in culture media

DLS analysis confirmed the expected size of NP in ultrapure water, with a Z-average of 53.5 ± 0.2 nm (Figure 1A). Nanoparticle geometric diameter was also confirmed in ultrapure water using transmission electron microscopy (Fig. 1E). In FSW, size of NP was similar (55.1 ± 0.5 nm; Fig. 1B). Aggregation was negligible as suggested by a PdI<0.2 for both ultrapure and FSW conditions.

Results of NP size and aggregation state monitored in the filtered spent media of algae cultures revealed a moderate aggregation, showing a size of 70.5 ± 2.2 nm (PdI= 0.3 ± 0.0) and 162.4 ± 3.3 nm (PdI= 0.6 ± 0.0) at exponential and stationary phases, respectively (Fig. 1C and 1D).

Regarding the charge, NP resuspended in ultrapure water had a strong positive charge 45.4 ± 0.2 mV but was reduced to 14.3 ± 3.5 mV when resuspended in FSW. Similarly to FSW, NP resuspended in both exponential and stationary spent media had a positive charge reduced down to 11.0 ± 4.4 mV and 17.5 ± 4.3 mV, respectively.

3.2. Adhesion of nanoplastics to algae cells.

SEM observations were performed to visualise the diatom cells structure and assess potential impacts of NP exposure on cell shape and integrity. Fresh cells of control cultures of *Chaetoceros neogracile* in exponential phase presented intact silica frustule and setae (Figure 2A; 2B). Sometimes, the setae were broken and situated close to the cell as a consequence of the force exerted during filtration process (sampling preparation; e.g. in Fig.2B). Control samples fixed in pseudo-Lugol evidenced more deteriorated frustules compared to fresh samples (Supplementary Figure 1).

After exposure to nanoplastics, adsorption of nanoplastics onto algal' cell surface was observed in both fresh (Fig. 2C and 2D) and pseudo-lugol´ fixed (Supp. Fig 1) samples. Pseudo-lugol fixed samples evidenced large amount of aggregated debris in cells exposed to NP (Supp. Fig 1), but not in controls. However, this amount of debris was not observed in any of the fresh samples regardless of the experimental condition considered (including high NP concentration), suggesting that fixation promoted higher debris aggregation when NP are present.

3.3. Phytoplankton responses to nanoplastics exposure

3.3.1. Cell growth

In control treatments (no addition of NP), culture growth was higher in exponential phase in comparison with stationary phase, with final cellular concentrations being 2 and 19 times higher than at the beginning of the experiment in stationary and exponential growth phases, respectively (Supplementary table 1).

Exposure to high NP concentration significantly reduced cell growth of culture in exponential phase. Growth rate was reduced by 49, 57 and 62 % compared to controls after 48, 72 and 96 h of exposure, respectively (Figure 3A; ANOVA; $p < 0.01$). No significant effect on cell growth was observed at low NP concentration (Figure 3A).

The low growth of cultures in stationary phase was not affected by NP exposure regardless of the NP concentration and exposure duration (Supp. Table 1).

3.3.2. Changes in cell's optical characteristics

Exposure of culture in exponential phase to high NP concentration resulted in up to 100 % increase of cells' optical characteristics at large angle (Side Scatter, SSC). After 4 h exposure, SSC increased by 35% compared to controls. This increase was also observed during the rest of the experiment (ANOVA; $p < 0.001$ all exposure durations; Fig. 3B). Conversely, cell exposed to low NP concentration did not show any modification of their SSC, although a significant 5 % increase of Forward Scatter (FSC) as compared to controls was detected after 96 h exposure (ANOVA; $p < 0.01$; Fig. 3C).

3.3.3. Chlorophyll a content

In cultures in exponential phase, an 8 % decrease in chlorophyll a content compared to controls was observed after 4 and 24 h exposure at both NP concentrations (ANOVA; $p < 0.05$). For respectively low and high NP exposures, the reductions reached 9 and 24 % after 48 h exposure, 6 and 33 % after 72 h exposure and 14 and 32 % after 96 h exposure. For cultures in stationary phase, no significant effect of NP exposure was observed on chlorophyll a content regardless of the NP concentrations and exposure duration (Fig 3D; Supp. Table 2).

3.3.4. Photosynthetic efficiency

Photosynthetic efficiency of cells in exponential phase decreased after 4 h exposure with a reduction of 2 and 3 % for low and high NP concentrations, respectively (ANOVA; $p < 0.01$ both NP concentrations). After 48 h exposure, the photosynthetic efficiency was reduced by 13% only at high NP concentration until the end of the experiment (ANOVA; $p < 0.001$; Fig. 3E). In cultures in stationary phase, a significant decrease in photosynthetic efficiency was observed at low NP concentration after 4 h exposure (ANOVA; $p < 0.01$) being 6 % lower than control. No significant effect of NP exposure was recorded on photosynthetic efficiency thereafter (Supp. Table 2).

3.3.5. Esterase activity

A significant decrease in esterase activity was observed in cultures in exponential phase showing values of 8 % and 21 % reductions after 4 h (ANOVA; $p < 0.05$) for low and

high NP exposure, respectively. A reduction of this parameter was also observed after 24 h exposure (24 % and 52 % decrease at low and high NP concentration, respectively), 48 h exposure (17 % and 69 % decrease at low and high NP concentration, respectively), and 96 h exposure (19 % and 38 % decrease at low and high NP concentration, respectively) (ANOVA; $p < 0.001$ all times; Fig 3F). No significant effect of NP exposure on esterase activity of culture in stationary phase was observed (Supp. Table 2).

3.3.6. Intracellular ROS production

Nanoplastics exposure triggered a significant increase in intracellular ROS production of *C. neogracile* at both culture phases and both NP concentrations (Supp. Table 2). Cultures at exponential phase exposed to high NP concentration showed an increase in ROS production by 48% after 4 h exposure (ANOVA; $p < 0.05$) and reaching 22 % after 48 and 72 h exposure (ANOVA; $p < 0.001$ both exposure times; Fig 3G).

Cultures in stationary phase evidenced an increase in ROS production at both NP concentrations, around 12 % and 14 % at low and high NP concentrations, respectively, compared to the control after 4 h, 48 h and 72 h of exposure (ANOVA; $p < 0.05$ all exposure times).

3.3.7. Bacteria concentration in the cultures

At the beginning of the experiment the bacteria load was 14 times higher in cultures in stationary phase (values bacteria $cell^{-1}$) than in exponential phase (values bacteria $cell^{-1}$) (ANOVA; $p < 0.001$).

At the end of the experiment, bacteria concentration was still twice higher in culture in stationary phase (ANOVA; $p < 0.01$) as compared to exponential ones. At this time, a significant reduction of bacteria concentration by 30.5 % was observed at high NP concentration in the culture in exponential phase (ANOVA; $p < 0.01$) compared to control. However, when normalized by the diatom´ cell concentration in this culture, bacteria load at high NP concentration (4 bacteria $cell^{-1}$) was two times higher than in controls (ANOVA; $p < 0.01$) (Fig. 3H). Neither bacteria concentration nor bacteria-diatom ratio in stationary cultures were significantly affected by NP exposures (Fig. 3H).

354 3.3.8. Concentration of Transparent Exopolymer Particles (TEP)

355 At the beginning of the experiment, TEP concentration was 5 times higher in *C.*
356 *neogracile* cultures in stationary phase than in cultures in exponential phase (5.5×10^4 ±
357 1.2×10^3 and 1.1×10^4 ± 1.3×10^3 µg Xeq L^{-1} in stationary and exponential phases,
358 respectively) as presented in Figure 4A. When normalized by the diatom cell number
359 (Fig. 4B), TEP were 2 times more concentrated in *C. neogracile* cultures in exponential
360 phase than in stationary phase, (~6 µg and ~3 µg Xeq $Cell^{-1}$ in exponential and
361 stationary phases, respectively).

362 At the end of the experiment, TEP concentration in exponential phase exposed to high
363 NP concentration was 2.3 times reduced as compared to controls (1.5×10^4 and 3.5×10^4
364 µg Xeq L^{-1} for high NP exposure and control conditions, respectively, Figure 4C).
365 However, when normalized by the diatom cell concentration, the same final TEP
366 concentration per cell was observed in exponential cultures for all treatments (control,
367 low NP, high NP: ~ 3 µg Xeq $Cell^{-1}$, Figure 4D). At this time for cultures in stationary
368 phase, no differences in TEP concentration between treatments were observed either in
369 µg Xeq L^{-1} (~1.56×10^4 ± 3.1×10^2 µg Xeq L^{-1}) or normalized per diatom cell (~18 µg
370 Xeq $Cell^{-1}$) (Fig. 4C; 4D).
371

372 **4. Discussion:**

373 The main objective of this work was to identify the effect of small nanoplastics (50 nm)
374 on the cellular and metabolic responses of a diatom, *Chaetoceros neogracile,* at two
375 growth phases (exponential and stationary growth phases) via a single experimental
376 design. This experimental approach is innovative and has been never performed before.
377 Moreover, the present study include the measurement of bacteria and TEP
378 concentrations in algae media, as well as characterization of NP behavior in algal
379 cultures, thus providing useful information about the impact of NP on diatoms at
380 different growth phases.

381 **Transparent Exopolymer Particles (TEP) concentration influences NP fate**

382 According to recent findings, components present in water such as colloids and natural
383 organic matter, under a variety of physico-chemical conditions including pH and ionic
384 strength, affect significantly NP behavior, fate and toxicity (Manfra et al., 2017;

reviewed in Paul-Pont et al., 2018). Plastics has been proven to rapidly develop in its hydrophobic surface diverse microbial community of heterotrophs, autotrophs, predators, and symbionts not found in other substrates in the upper layer of oceans (Datta et al., 2016). This specific plastic associated communities were termed the "*plastisphere*" by Zettler et al. (2013) and evidenced the high adhesion capacity of plastics in natural environments.

Diatoms are particularly well known for excreting significant quantities of exopolysaccharides (EPS) that may rearrange to form larger particles, the TEP (Mari et al., 2017). TEP are essentially constituted by polysaccharides highly surface-active, enriched in deoxysugars and covalently bound sulfate (Mopper et al., 1995; Zhou et al. 1998), with a strong tendency to form metal ion bridges and hydrogen bonds (Passow 2002). These highly sticky particles trigger aggregation of various organic and mineral solid particles from natural or anthropogenic origin (Mari et al. 2017; Long et al. 2015; Passow and De La Rocha, 2006; Zhao et al. 2017). Despite TEP' low density (lower than sea-water) that sometimes lead to form ascending particles, TEP adhesion capacity are so high that most aggregates formed in the natural environment contained a large proportion of ballasted particles associated to strong sinking and export to deeper layer (Azetsu-Scott and Passow, 2004; Mari et al. 2017; Long et al. 2015). This explained why TEP can easily interact with NP, as it has been evidenced with MP (Long et al. 2017; Zhao et al. 2017). In our work, a moderate aggregation of NP was observed in diatoms spent media, but neither in filtered seawater nor in ultrapure water. This may be linked to the formation of an environmental coating, or 'eco-corona' around NP as previously described (Canesi et al. 2017; Galloway et al., 2017). Although the nature of the eco-corona was not investigated, the influence of TEP in the formation of moderate aggregates is suggested here in accordance with previous findings (Long et al. 2015; Lagarde et al. 2016). TEP appear to act as binding agent, holding plastic particles together into agglomerates as it was previously reported for 50 nm polystyrene particles in the presence of exopolymeric substances (EPS; Summers et al., 2018). In our work, total TEP concentration at the beginning of the experiment at stationary phase was 5 times higher than in exponential phase. TEP concentration generally increase when nutrient limit cell growth as also occurred in natural environment where TEP accumulate in surface waters at the end of bloom (Passow 2000, 2002, Mari et al. 2017). As EPS are also excreted by bacteria, the increase of TEP measured in our experiment may also be due to the high bacteria load observed at stationary growth. This bacteria

load not only can participate in TEP production but also get involved in the "*plastisphere*" of NP depending on plastic type and mostly on environmental conditions (Summers et al., 2018).

Regardless of their origin, presence of TEP in the media promoted aggregation of NP. The resulting increase in particle size may have affected their toxicity (Paul-Pont et al., 2018). Indeed, previous studies reported that micro-scale (>1µm) particles had less impact on microalgae than nano-scale plastics (Mao et al., 2018; Sjollema et al., 2016). In addition to the particle size, significant lower values of NP zeta potential were observed in both FSW and algae media. Decrease in positive charges of NP in FSW has been already reported (Bergami et al., 2016) due to interaction of the surface groups with high number of ions (Cole and Galloway, 2015). However, the presence of TEP did not appear to further alter the behavior and fate of NP as compared to FSW.

Altogether, NP aggregation and charge reduction could have minimized NP impacts as observed in this study for cultures in stationary phase.

NP affected the concentration of Transparent Exopolymer Particles (TEP)

The presence of exopolymeric substances (EPS) produced by microalgae and bacteria is associated to diatom cell stickiness and subsequent aggregation, which have an important role in the redistribution and export of organic carbon (Thornton, 2002, Mari et al. 2017).

As a biological response, it is expected than NP alter total TEP concentration as previously reported in presence of MP (Lagarde et al., 2016). Recently, Bergami et al. (2016) suggested that presence of NP promotes the formation of rounded aggregates of PS-NH_2 around algae´ cells probably due to the presence of organic matter and/or the EPS in the media. Nevertheless, this hypothesis was based on scanning electronic microscopy (SEM) images of glutaraldehyde fixed cells not taking into account TEP measurements. Based on the microscopic observations performed in this study on fresh and pseudo-lugol fixated cells, it seems that pseudo-lugol fixation promotes aggregation in the media probably associated to the presence of TEP, not only in NP exposed cultures but also in control ones. Then, microscopy approach could be misleading to evidence NP influence on TEP presence and we advocate quantifying TEP as done here by spectrophotometric measurement. Our data revealed a significant decrease in total

TEP (µg Xeq L^{-1}) at high NP concentration during exponential growth. Such decrease could be explained by a general decrease of the EPS producer diatoms and bacteria: i) we observed reduction (62 % down) of algae´ cellular growth in this treatment and ii) we observed reduction (30.5 % down) in bacteria concentration in comparison to control. Indeed, it is difficult to discriminate the origin of TEP. Some carbohydrates are diatoms-specific, and measurements of dissolved carbohydrates in the culture would be a way to discriminate diatom TEP from bacterial one. However the observed difference between the total TEP concentrations at the end of the experiment for high NP exposure disappeared when TEP concentration is normalized to diatom cell concentrations, suggesting that in our experiment diatom may be the main producer of TEP.

NP impair major physiological traits of diatoms at exponential growth

Exponential cultures are characterized by a fast growth which leads to intense duplication of chloroplasts, nucleus, Golgi apparatus and mitochondria requiring high amounts of energy (Tanaka et al., 2015). They also duplicate their frustules from which two new cells are produced (Pickett-Heaps, 1998) therefore promoting membrane exhibition to NP which may increase their susceptibility to NP exposure. The adsorption of PS-NH_2 onto *Dunaliella tertiolecta* membranes has been reported previously (Bergami et al., 2017). Furthermore, higher adsorption ratio of PS-NH_2 as compared to other functionalized groups such as PS-COOH or non-functionalized polystyrene particles has been reported (Nolte et al., 2017). Such high adsorption of PS-NH_2 also evidenced *in vitro* with mammalian cells (Anguissola et al., 2014; Varela et al., 2012) was justified by the attraction of positively charged particles with negatively charged membrane lipids (Rossi and Monticelli, 2016). Nevertheless, diatoms are covered by a silica frustule, which can protect the membranes against potential external stressors such as NP. Recently, Walsh et al. (2017) have reported that -NH_2 groups have high affinity to frustule's silica in diatoms. This fits with the observed increase in FSC and SSC suggesting cell surface changes upon NP exposure in exponential cultures. It may be explained by a rapid adhesion of NP to algal cell surface as evidenced by SEM. Indeed, increase in light diffraction upon NP adsorption on the cell surface was previously reported in other cells types, *i.e.* gametes (González-Fernández et al., 2018). Particles adhered onto the algae´ cells could also enhance light attenuation and reduce the availability of nutrient and gas exchange, which may consequently trigger adverse

484 effects on algae respiration and photosynthesis (Bergami et al., 2016; Bhattacharya et
485 al., 2010).

486 In this work, NP significantly impacted the photosynthetic machinery of microalgae
487 cultures in exponential phase. A decrease in photosynthesis activity in microalgae upon
488 exposure to NP was already reported (Bergami et al., 2017; Bhattacharya et al., 2010;
489 Mao et al., 2018). Particularly, decrease in photosynthetic efficiency was expected after
490 exposure to positively charged NP because they are tightly absorbed on cellulosic
491 membranes of microalgae (Nolte et al., 2017). The excessive cell surface coverage by
492 PS-NH_2 is likely to inhibit photosynthesis and/or disrupt cell wall (Nolte et al., 2017).
493 Loss of membrane integrity could also explain the decrease in algal metabolic activity
494 (esterase activity). Plant and other photosynthetic organisms use chlorophyll to absorb
495 light and convert it into chemical energy. The dose-dependent reduction of chlorophyll
496 content in cultures in exponential phase suggests a high sensitiveness of thylakoid
497 photosynthetic machinery with however only a moderate impact on photosynthetic
498 efficiency at high NP exposure in our experimental conditions. Overall, it is proposed
499 that NP exposure may impair the diatoms energy intake by affecting chloroplast
500 integrity.

501 **NP triggered an increase of ROS production by algae at both growing phases**

502 In photosynthetic organisms, ROS are continuously produced as by-products through
503 various metabolic pathways localized in mitochondria, chloroplasts and peroxisomes,
504 (Liu et al., 2007). Enhancement of ROS production after exposure to NP has been
505 previously observed (Bhattacharya et al., 2010; Mao et al., 2018), as well as in this
506 study at two culture phases strengthening its status of ubiquitous stress response.
507 Impairment of the electron transport chain in chloroplasts may results in electron
508 accumulation increasing the ROS level. It is also noteworthy that ROS production was
509 the unique parameter affected in stationary cultures, even at low NP dose. This suggests
510 that ROS generation upon NP exposure may constitute a predominant mechanism
511 leading to nano-toxicity, as reported by Fu et al. (2014). The impairment of the
512 photosynthetic machinery together with the increase in ROS levels could lead to
513 membrane lipid peroxidation as previously described by Mao et al. (2018) promoting
514 cell damage and reducing cell growth.

515 **5. Conclusion**

In natural environments, all growing states coexist, making difficult to predict the impact of NP on diatoms community and on organisms at higher trophic levels. Ecological effects of NP may indeed occur higher up in the food web, with algae-particle interaction as the first step in the biomagnification (Nolte et al., 2017 ; Navarro et al., 2008) as previously shown in suspension-feeding bivalves (Ward and Kach, 2009). Differences in TEP concentration according to growth phase can be a key parameter affecting the fate and toxicity of NP on marine diatoms. This work using fairly realistic environmental scenario of nanoplastics pollution highlights the importance of taking into account algae growing phases when evaluating the impact of plastic in phytoplankton ecotoxicological studies. Further research focusing on the interaction of NP with cellular membranes as well as the impairment of the photosynthesis machinery are of great interest for deeply understanding the global diatoms' responses to plastic contamination.

Acknowledgements

The authors are grateful to Philippe Miner from Ifremer for his assistance during phytoplankton culture, to Dr. Philippe Elies for the microscopy observations and Olivier Lozach from the COSM team at the University of Western Brittany for DLS measurements. We thank Helen McCombie for her help in editing the English.

Funding sources

This work was supported by the NANO Project (ANR-15-CE34-0006-02) funded by the French Agence Nationale de la Recherche (ANR).

References

Allard, B., Tazi, A., 1993. Influence of growth status on composition of extracellular polysaccharides from two *Chlamydomonas* species. Phytochemistry 32, 41-47.

Anguissola, S., Garry, D., Salvati, A., O'Brien, P.J., Dawson, K.A., 2014. High content analysis provides mechanistic insights on the pathways of toxicity induced by amine-modified polystyrene nanoparticles. PLoS One 9. https://doi.org/10.1371/journal.pone.0108025

Azetsu-Scott, K., Passow, U. 2004. Ascending marine particles: Significance of transparent exopolymer particles (TEP) in the upper ocean. Limnol. Oceanogr. 49,

741–748.

Bergami, E., Pugnalini, S., Vannuccini, M.L., Manfra, L., Faleri, C., Savorelli, F., Dawson, K.A., Corsi, I., 2017. Long-term toxicity of surface-charged polystyrene nanoplastics to marine planktonic species Dunaliella tertiolecta and Artemia franciscana. Aquat. Toxicol. 189, 159–169. https://doi.org/10.1016/j.aquatox.2017.06.008

Bergmann, M.,Wirzberger, V., Krumpen, T., Lorenz, C., Primpke, S., Tekman, M.B., et al. 2017. High quantities of microplastic in Arctic deep-sea sediments from the HAUSGARTEN Observatory. Envir. Sci. Technol. 51, 11000–11010.doi: 10.1021/acs.est.7b03331

Bistricki, T., Munawar, M. 1978. A rapid preparation method for scanning electron microscopy of Lugol preserved algae. J. Microsc. 114, 215-218.Bhattacharya, P., Lin, S., Turner, J.P., Ke, P.C., 2010. Physical adsorption of charged plastic nanoparticles affects algal photosynthesis. J. Phys. Chem. C 114, 16556–16561. https://doi.org/10.1021/jp1054759

Canesi, L., Balbi, T., Fabbri, R., Salis, A., Damonte, G., Volland, M., Blasco, J., 2017. Biomolecular coronas in invertebrate species: Implications in the environmental impact of nanoparticles. NanoImpact 8, 89–98. https://doi.org/10.1016/j.impact.2017.08.001

Cole, M., Galloway, T.S., 2015. Ingestion of nanoplastics and microplastics by Pacific oyster larvae. Environ. Sci. Technol. 49, 14625 -14632. https://doi.org/10.1021/ acs.est.5b04099.

Corzo, A., Morillo, J.A., Rodríguez, S., 2000. Production of transparent exopolymer particles (TEP) in cultures of *Chaetoceros calcitrans* under nitrogen limitation. Aquat. Microb. Ecol. 23, 63–72. https://doi.org/10.3354/ame023063

Datta, M. S., Sliwerska, E., Gore, J., Polz, M. F., and Cordero, O. X. 2016. Microbial interactions lead to rapid micro-scale successions on model marine particles. Nat. Commun. 7:11965. doi: 10.1038/ncomms11965

Dubaish, F., and Liebezeit, G. 2013. Suspended microplastics and black carbon particles in the Jade system, southernNorth Sea.Water. Air. Soil. Poll. 224. 1352.doi:

576 10.1007/s11270-012-1352-9

577 Erni-Cassola, G., Gibson, M.I., Thompson, R.C., christie-oleza, J., 2017. Lost, but
578 found with Nile red; a novel method to detect and quantify small microplastics (20
579 µm–1 mm) in environmental samples. Environ. Sci. Technol., 2017, 51 (23),
580 13641–13648

581 Fabra, M.J., Busolo, M.A., Lopez-Rubio, A., Lagaron, J.M., 2013. Nanostructured
582 bilayers in foodpackaging. Trends Food Sci. Technol. 31, 79–87

583 FAO. 2017. Microplastics in fisheries and aquaculture.

584 Field, C.B., Behrenfeld, M.J., Randerson, J.T., Falkowski, P., 1998. Primary production
585 of the biosphere: integrating terrestrial and oceanic components. Science 281
586 (5374), 237–240

587 Fu, P.P., Xia, Q., Hwang, H., Ray, P.C., Yu, H., 2014. Mechanisms of nanotoxicity :
588 Generation of reactive oxygen species. J. Food Drug Anal. 22, 64–75.
589 https://doi.org/10.1016/j.jfda.2014.01.005

590 Galloway, T.S., Cole, M., Lewis, C., 2017. Interactions of microplastic debris
591 throughout the marine ecosystem. Nat. Ecol. Evol. 1, 1-16.
592 https://doi.org/10.1038/s41559-017-0116

593 Garvey, M., Moriceau, B., and Passow, U. 2007. Applicability of the FDA assay to
594 determine the viability of marine phytoplankton under different environmental
595 conditions. Mar. Ecol. Prog. Ser. 352, 17–26. doi:10.3354/meps07134.

596 GESAMP. 2016. “Sources, fate and effects of microplastics in the marine environment:
597 part two of a global assessment” (Kershaw, P.J., and Rochman, C.M., eds).
598 (IMO/FAO/UNESCO-IOC/UNIDO/WMO/IAEA/UN/UNEP/UNDP Joint Group
599 of Experts on the Scientific Aspects of Marine Environmental Protection). Rep.
600 Stud. GESAMP No. 93, 220 p.

601 Gigault, J., Pedrono, B., Maxit, B., Ter Halle, A., 2016. Marine plastic litter: the
602 unanalyzed nano-fraction. Environ. Sci. Nano 3, 346–350.
603 https://doi.org/10.1039/C6EN00008H

Gigault, J., Ter Halle, A., Baudrimont, M., Pascal, P.-Y., Gauffre, F., Phi, T.-L., et al. 2018. Current opinion: what is a nanoplastic? Environ. Polllut. 230, 1030–1034. doi: 10.1016/j.envpol.2018.01.024

González-Fernández, C., Tallec, K., Le Goïc, N., Lambert, C., Soudant, P., Huvet, A., Suquet, M., Berchel, M., Paul-Pont, I., 2018. Cellular responses of Pacific oyster (*Crassostrea gigas*) gametes exposed *in vitro* to polystyrene nanoparticles. Chemosphere 208, 764–772. https://doi.org/10.1016/j.chemosphere.2018.06.039

Hernandez, L. M., Yousefi, N., Tufenkji, N. 2017. Are There Nanoplastics in Your Personal Care Products? Environmental Science & Technology Letters, 4(7), 280–285. https://doi.org/10.1021/acs.estlett.7b00187

Huvet, A., Paul-Pont, I., Fabioux, C., Lambert, C., Suquet, M., Thomas, Y., … Sussarellu, R. (2016). Reply to Lenz et al.: Quantifying the smallest microplastics is the challenge for a comprehensive view of their environmental impacts. Proceedings of the National Academy of Sciences, 113(29), E4123–E4124. https://doi.org/10.1073/pnas.1607221113

Khowala, S., Verma, D., Banik, S.P., 2008. Biomolecules: Introduction, Structure & Function -Carbohydrates[online]. Indian Institute of Chemical Biology http://www.academia.edu/1114817/Biomolecules Introduction Structure and Functions - Carbohydrates.

Kroll, A., Behra, R., Kaegi, R., Sigg, L. 2014. Extracellular polymeric substances (EPS) of freshwater biofilms stabilize and modify CeO2 and Ag nanoparticles. Plos One 9(10), e110709

Lagarde, F., Olivier, O., Zanella, M., Daniel, P., Hiard, S., Caruso, A., 2016. Microplastic interactions with freshwater microalgae: Hetero-aggregation and changes in plastic density appear strongly dependent on polymer type. Environ. Pollut. 215, 331–339. https://doi.org/10.1016/j.envpol.2016.05.006

Lambert, S., Wagner, M., 2016. Characterisation of nanoplastics during the degradation of polystyrene. Chemosphere 145, 265–268. https://doi.org/10.1016/j.chemosphere.2015.11.078

633 Lenz, R., Enders, K., & Nielsen, T. G. (2016). Microplastic exposure studies should be
634 environmentally realistic. Proceedings of the National Academy of Sciences,
635 113(29), E4121–E4122. https://doi.org/10.1073/pnas.1606615113

636 Liu, W.; Au, D. W. T.; Anderson, D. M.; Lam, P. K. S.; Wu, R. S. S. Effects of
637 nutrients, salinity, pH and light:dark cycle on the production of reactive oxygen
638 species in the alga *Chattonella marina*. J. Exp. Mar. Biol. Ecol. 2007, 346, 76-86

639 Long, M., Moriceau, B., Gallinari, M., Lambert, C., Huvet, A., Raffray, J., Soudant, P.,
640 2015. Interactions between microplastics and phytoplankton aggregates: Impact on
641 their respective fates. Mar. Chem. 175, 39–46.
642 https://doi.org/10.1016/j.marchem.2015.04.003

643 Long, M., Paul-Pont, I., Hégaret, H., Moriceau, B., Lambert, C., Huvet, A., Soudant, P.,
644 2017. Interactions between polystyrene microplastics and marine phytoplankton
645 lead to species-specific hetero-aggregation. Environ. Pollut. 228, 454–463.
646 https://doi.org/10.1016/j.envpol.2017.05.047

647 Malviya, S., Scalco, E., Audic, S., Vincent, F., Veluchamy, A., Poulain, J. 2016.
648 Insights into global diatom distribution and diversity in the world's ocean. Proc.
649 Natl. Acad. Sci., 201509523.

650 Manfra, L., Rotini, A., Bergami, E., Grassi, G., Faleri, C., Corsi, I., 2017. Comparative
651 ecotoxicity of polystyrene nanoparticles in natural seawater and reconstituted
652 seawater using the rotifer *Brachionus plicatilis*. Ecotoxicol. Environ. Saf. 145,
653 557–563. https://doi.org/10.1016/j.ecoenv.2017.07.068

654 Marie, D., F. Partensky, S. Jacquet, D. Vaulot. 1997. Enumeration and Cell Cycle
655 Analysis of Natural Populations of Marine Picoplankton by Flow Cytometry Using
656 the Nucleic Acid Stain SYBR Green I. Appl. Environ. Microbiol. 63, 1. 186 93.

657 Mari, X., Passow, U., Migon, C., Burd, A.B., Legendre, L., 2017. Transparent
658 exopolymer particles: Effects on carbon cycling in the ocean. Prog. Oceanogr. 151,
659 13–37. https://doi.org/10.1016/j.pocean.2016.11.002

660 Mao, Y., Ai, H., Chen, Y., Zhang, Z., Zeng, P., Kang, L., Li, W., Gu, W., He, Q., Li, H.,
661 2018. Phytoplankton response to polystyrene microplastics: Perspective from an

entire growth period. Chemosphere 208, 59–68. https://doi.org/10.1016/j.chemosphere.2018.05.170

Merinska, D., Dujkova, Z., 2012. Polystyrene (nano) composites with possible anti-bacterial effect. Mathematical Methods and Techniques in Engineering and Environmental Science (ISBN: 978-1-61804-046-6).

Mopper, K., Zhou, J., Ramana, K. S., Passow, U., Dam, H. G., & Drapeau, D. T. 1995. The role of surface-active carbohydrates in the flocculation of a diatom bloom in a mesocosm. Deep-Sea Research II, 42,47–73

Navarro, E., Baun, A., Behra, R., Hartmann, N.B., Filser, J., Miao, A.J., Quigg, A., Santschi, P.H., Sigg, L., 2008. Environmental behavior and ecotoxicity of engineered nanoparticles to algae, plants, and fungi. Ecotoxicology 17, 372–386. https://doi.org/10.1007/s10646-008-0214-0

Nolte, T.M., Hartmann, N.B., Kleijn, J.M., Garnæs, J., van de Meent, D., Jan Hendriks, A., Baun, A., 2017. The toxicity of plastic nanoparticles to green algae as influenced by surface modification, medium hardness and cellular adsorption. Aquat. Toxicol. 183, 11–20. https://doi.org/10.1016/j.aquatox.2016.12.005

Passow, U., 2002. Transparent Exopolymer Particles in Aquatic Environments. Prog. Oceanogr. 55, 287–333. https://doi.org/10.1016/S0079-6611(02)00138-6

Passow, U., and Alldredge, A. L. 1995. A dye-binding assay for the spectrophotometric measurement of transparent exopolymer particles (TEP). Limnol. Oceanogr. 40, 1326–1335. doi:10.4319/lo.1995.40.7.1326.

Passow, U., De La Rocha, C.L. 2006. Accumulation of mineral ballast on organic aggregates. Glob. Biogeochem. Cycles.20(1), 10-13.

Paul-Pont, I., Tallec, K., Gonzalez-Fernandez, C., Lambert, C., Vincent, D., Mazurais, D., Zambonino-Infante, J.-L., Brotons, G., Lagarde, F., Fabioux, C., Soudant, P., Huvet, A., 2018. Constraints and Priorities for Conducting Experimental Exposures of Marine Organisms to Microplastics. Front. Mar. Sci. 5, 1–22. https://doi.org/10.3389/fmars.2018.00252

Pickett-Heaps, J.D., 1998. Cell Division and Morphogenesis of the Centric Diatom

Chaetoceros Decipiens (Bacillariophyceae) Ii. Electron Microscopy and a New Paradigm for Tip Growth. J. Phycol. 34, 995–1004. https://doi.org/10.1046/j.1529-8817.1998.340995.x

Peeken, I., Primpke, S., Beyer, B., Gütermann, J., Katlein, C., Krumpen, T., 2018. Arctic sea ice is an important temporal sink and means of transport for microplastic. Nat. Commun. 95, 2041–1723. doi: 10.1038/s41467-018-03825-5

Plastics Europe 2017. An analysis of European plastics production, demand and waste data.Technical Report. https://www.plasticseurope.org/application/files/5715/1717/4180/Plastics_the_facts_2017_FINAL_for_website_one_page.pdf

Rogach, A., Susha, A., Caruso, F., Sukhorukov, G., Kornowski, A., Kershaw, S., Möhwald, H., Eychmüller, A., Weller, H., 2000. Nano- and microengineering: 3-D colloidal photonic crystals prepared from sub-μm-sized polystyrene latex spheres pre-coated with luminescent polyelectrolyte/nanocrystal shells. Adv. Mater. 12, 333–337.

Rossi, G., Monticelli, L., 2016. Simulating the interaction of lipid membranes with polymer and ligand-coated nanoparticles. Adv. Phys. X 1, 276–296. https://doi.org/10.1080/23746149.2016.1177468

Sjollema, S.B., Redondo-Hasselerharm, P., Leslie, H.A., Kraak, M.H.S., Vethaak, A.D., 2016. Do plastic particles affect microalgal photosynthesis and growth? Aquat. Toxicol. 170, 259–261. https://doi.org/10.1016/j.aquatox.2015.12.002

Smith, D. C., Steward, G. F., Long, R. A., & Azam, F. 1995. Bacterial mediation of carbon fluxes during a diatom bloom in a mesocosm. Deep-Sea Research II, 42,75–97

Strasser, J.R., Srivastava, A., Tsimilli-Michael, M., 2000. The fluorescence transient as a tool to characterize and screen photosynthetic samples, in: Probing Photosynthesis: Mechanism, Regulation & Adaptation. pp. 445–483.

Summers, S., Henry, T., Gutierrez, T., 2018. Agglomeration of nano- and microplastic particles in seawater by autochthonous and de novo-produced sources of exopolymeric substances. Mar. Pollut. Bull. 130, 258–267.

https://doi.org/10.1016/j.marpolbul.2018.03.039

Tallec, K., Huvet, A., Di Poi, C., González-Fernández, C., Lambert, C., Petton, B., Le Goïc, N., Berchel, M., Soudant, P., Paul-Pont, I., 2018. Nanoplastics impaired oyster free living stages, gametes and embryos. Environ. Pollut. 242, 1226–1235. https://doi.org/10.1016/j.envpol.2018.08.020

Tanaka, A., De Martino, A., Amato, A., Montsant, A., Mathieu, B., Rostaing, P., Tirichine, L., Bowler, C., 2015. Ultrastructure and Membrane Traffic During Cell Division in the Marine Pennate Diatom *Phaeodactylum tricornutum*. Protist 166, 506–521. https://doi.org/10.1016/j.protis.2015.07.005

Ter Halle, A., Jeanneau, L., Martignac, M., Jardé, E., Pedrono, B., Brach, L., Gigault, J., 2017. Nanoplastic in the North Atlantic Subtropical Gyre. Environ. Sci. Technol. acs.est.7b03667. https://doi.org/10.1021/acs.est.7b03667

Thornton, D.C.O., 2002. Diatom aggregation in the sea: Mechanisms and ecological implications. Eur. J. Phycol. 37, 149–161. https://doi.org/10.1017/S0967026202003657

Varela, J.A., Bexiga, M.G., Åberg, C., Simpson, J.C., Dawson, K.A., 2012. Quantifying size-dependent interactions between fluorescently labeled polystyrene nanoparticles and mammalian cells. J. Nanobiotechnology 10, 39. https://doi.org/10.1186/1477-3155-10-39

Velev, O.D., Kaler, E.W., 1999. In situ assembly of colloidal particles into miniaturized biosensors. Langmuir 15, 3693–3698. http://dx.doi.org/10.1021/la981729c.

Verity, P.G., Whipple, S.J., Nejstgaard, J.C., Aldrekamp, A.C. 2007. Colony, size, cell number, carbon and nitrogen content of *Phaeocystis pouchetiii* from western Norway. J. Plankton. Res. 4, 359-367.

Walne, P. R., 1966. Experiments in the Large-scale Culture of the Larvae of *Ostrea edulis* L. 848 Fishery In, Her Majesty's Stationery Office, London.

Walsh, P.J., Clarke, S.A., Julius, M., Messersmith, P.B., 2017. Exploratory Testing of Diatom Silica to Map the Role of Material Attributes on Cell Fate. Sci. Rep. 7, 1–13. https://doi.org/10.1038/s41598-017-13285-4

750 Ward, J.E., Kach, D.J., 2009. Marine aggregates facilitate ingestion of nanoparticles by
751 suspension-feeding bivalves. Mar. Environ. Res. 68, 137e142. http://
752 dx.doi.org/10.1016/j.marenvres.2009.05.002.

753 Yokota, K., Waterfield, H., Hastings, C., Davidson, E., Kwietniewski, E., Wells, B.,
754 2017. Finding the missing piece of the aquatic plastic pollution puzzle: Interaction
755 between primary producers and microplastics. Limnol. Oceanogr. Lett. 2, 91–104.
756 https://doi.org/10.1002/lol2.10040

757 Zhao, S., Danley, M., Ward, J. E., Li, D., and Mincer, T. J. 2017. An approach for
758 extraction, characterization and quantitation of microplastic in natural marine snow
759 using Raman microscopy. Anal. Methods 9, 1470–1478.
760 doi:10.1039/C6AY02302A.

761 Zhou, J., Mopper, K., Passow, U. 1998. The role of surface-active carbohydrates in the
762 formation of transparent exopolymer particles (TEP) by bubble adsorption of
763 seawater. Limnol. Oceanogr. 43, 1860-1871

764 Zettler, E. R., Mincer, T. J., and Amaral-Zettler, L. A. 2013. Life in the “Plastisphere”:
765 microbial communities on plastic marine debris. Envir. Sci. Technol. 47, 7137–
766 7146. doi: 10.1021/es401288x

FIGURE CAPTIONS

Figure 1. Nanoplastics characterization using Dynamic Light Scattering in ultrapure water (A), filtered seawater (B), filtered algal media at exponential phase (C) filtered algal media at stationary phase (D) and Transmission Electronic Microscopy (TEM) imaging in ultrapure water (E). Size distribution of 50 nm PS-NH_2 measured in triplicates (corresponding to the different colors: green, red, blue) using a Zetasizer Nano Series software 6.2. TEM observations of 50 nm PS-NH_2 are also presented. Scale bar: 20 nm.

Figure 2. Scanning electron micrographs of *Chaetoceros neogracile* exponential cells in control (A, B) and after 72h exposure to high NP concentration (5 µg mL^{-1} PS-NH_2) (C, D). Samples were processed in fresh. Exposed cells presented high amount of PS-NH_2 particles attached onto the cell surface. Scale bar: 5µm (A, C) and 1µm (B, D).

Figure 3. Diatom cellular parameters affected by PS-NH_2 exposure at exponential phase: cellular growth (A), relative cell complexity (Side scatter SSC) (B), relative cell size (forward scatter FSC) (C), chlorophyll content (red fluorescence) (D), photosynthesis activity (E), esterase activity (green fluorescence) (F), intracellular ROS production (G) and bacteria concentration (H). Data are presented as means (n=3) ± SD. Significant level marked at $p< 0.05$. Lowercase letters indicate differences between treatments within the same exposure time time (1-way-ANOVA analysis).

Figure 4. Transparent Exopolymer Particles (TEP) concentration measured before exposure (A, B) and after 96 h exposure to nanoplastics (C, D). A,C: TEP expressed by microgram of Gum Xanthan equivalent per litter (µg Xeq L^{-1}) and B,D: TEP expressed by microgram of Gum Xanthan equivalent per cell (µg Xeq $cell^{-1}$). Data are presented as means (n=3) ± SD. Significant level marked at $p< 0.05$. Lowercase letters indicate differences between treatments within the same exposure time (1-way-ANOVA analysis).

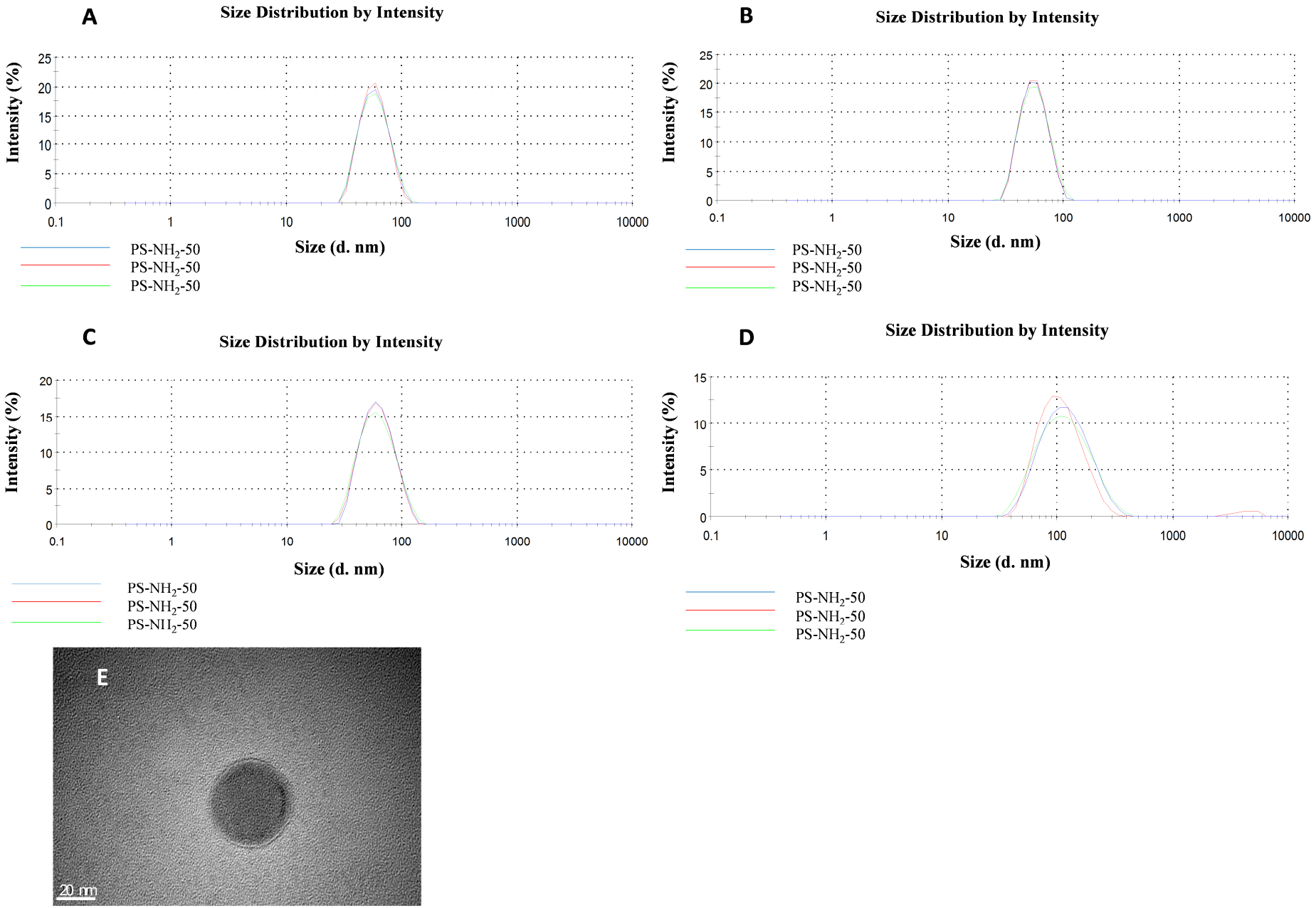

A
Size Distribution by Intensity
Intensity (%)
Size (d. nm)
PS-NH2-50
PS-NH2-50
PS-NH2-50
B
Size Distribution by Intensity
Intensity (%)
Size (d. nm)
PS-NH2-50
PS-NH2-50
PS-NH2-50
C
Size Distribution by Intensity
Intensity (%)
Size (d. nm)
PS-NH2-50
PS-NH2-50
PS-NI I2-50
D
Size Distribution by Intensity
Intensity (%)
Size (d. nm)
PS-NH2-50
PS-NH2-50
PS-NH2-50
E
20 nm

Figure 1.

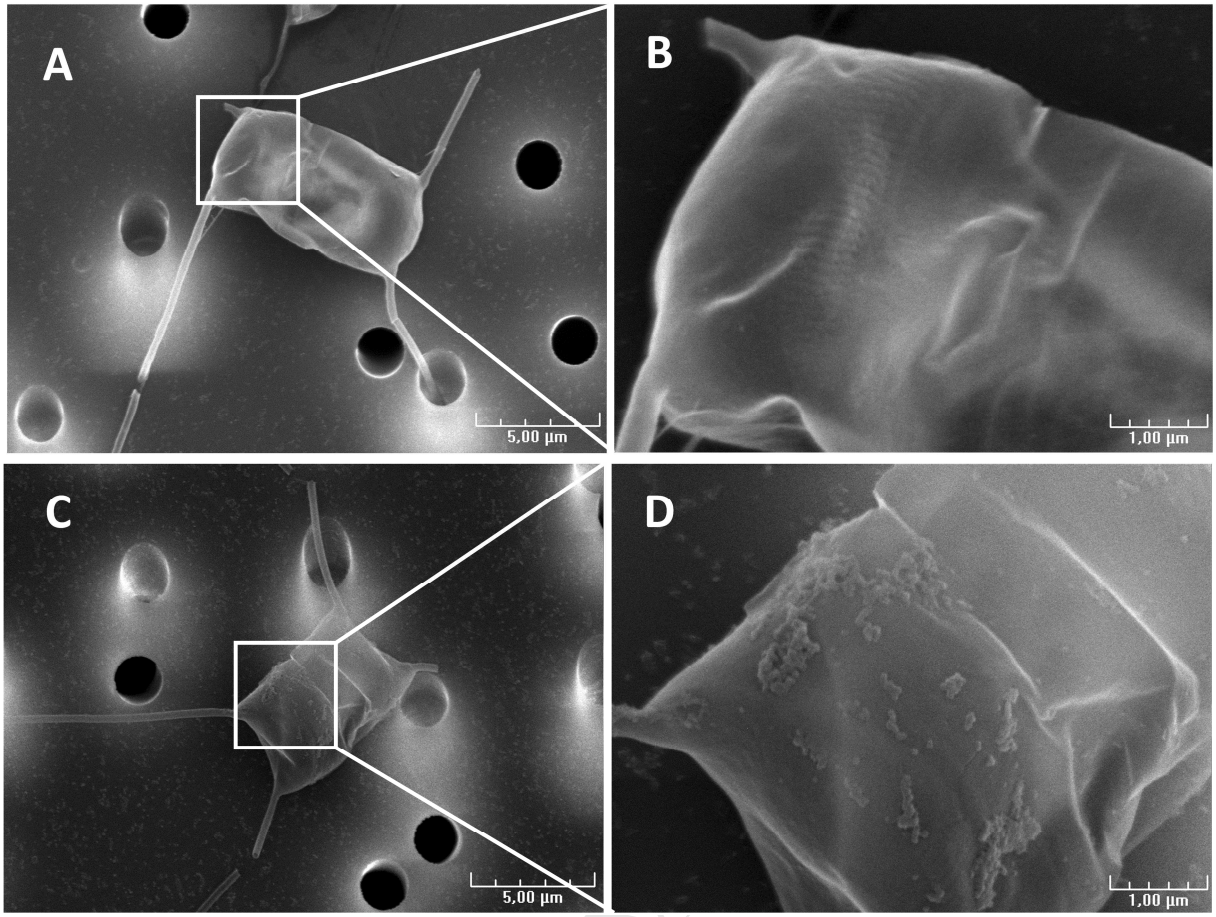


Figure 2.

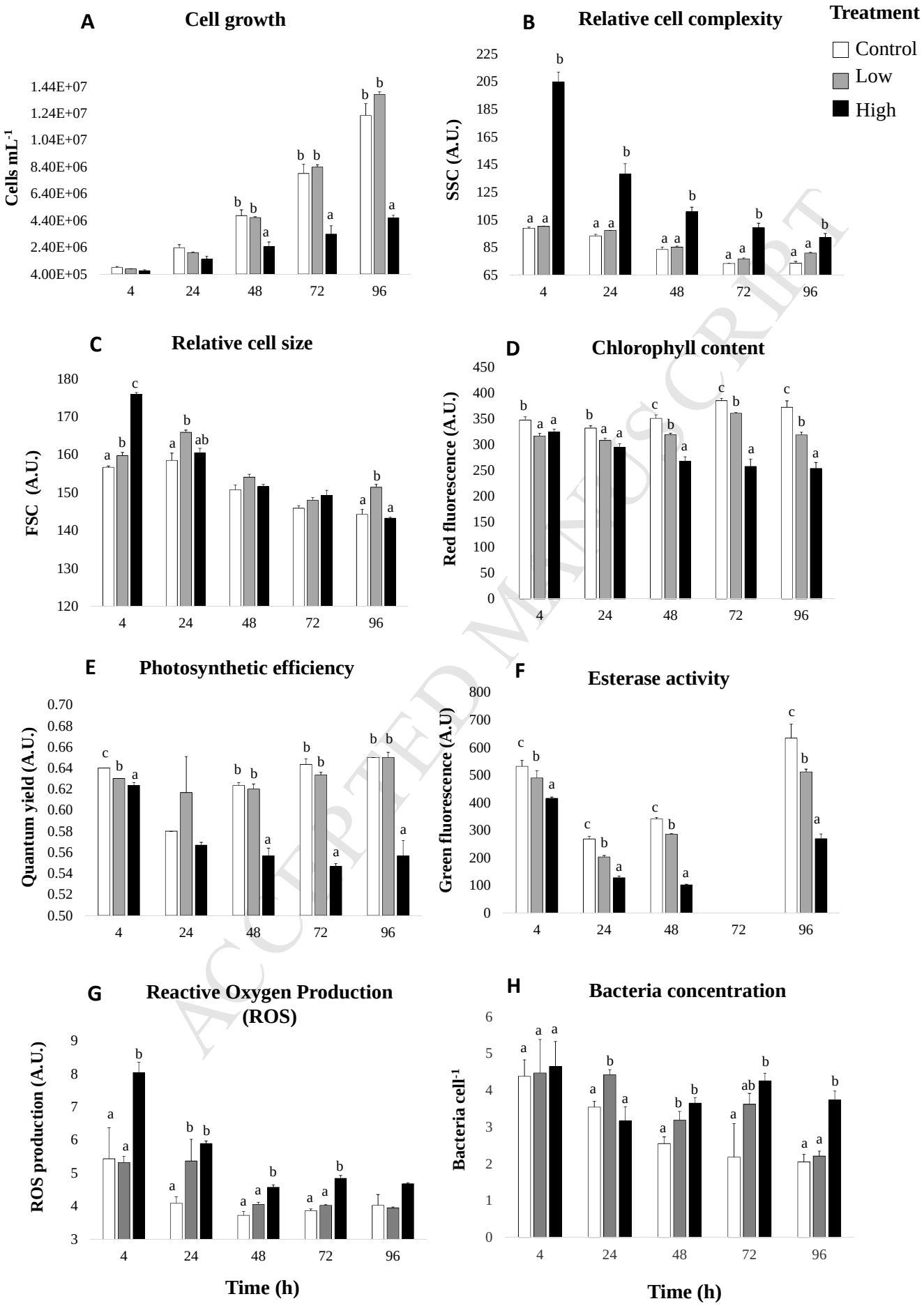


Figure 3.

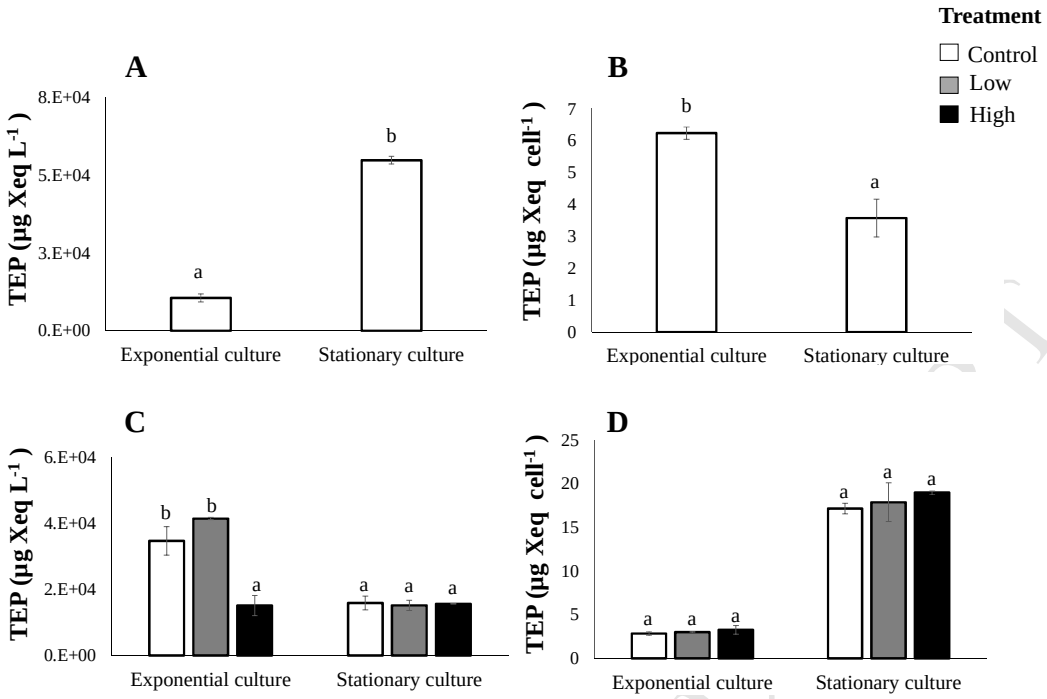


Figure 4.

Highlights

1. Interaction of NP and diatoms was studied at exponential and stationary phases
2. Transparent Exopolymer Particles (TEP) alter NP fate by aggregating NP
3. NP impairs major physiological traits of diatoms at exponential phase
4. Whatever the aggregation state, NP promotes oxidative stress at both growth phases