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Nanoplastics exposure modulate lipid and pigment compositions in diatoms

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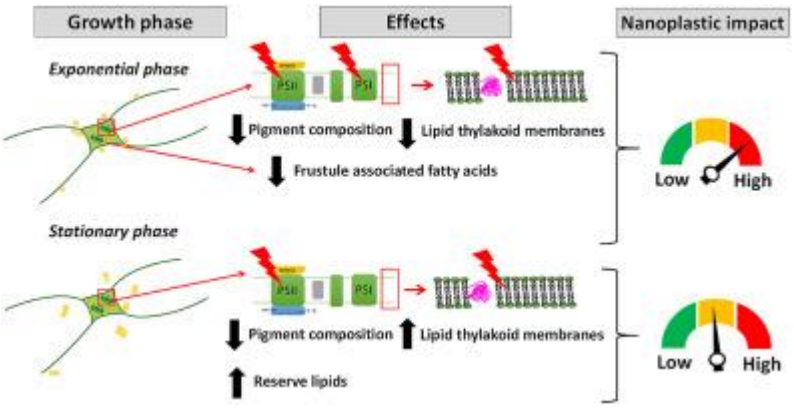
Abstract :

The impact of nanoplastics (NP) using model polystyrene nanoparticles amine functionalized (PS–NH2) has been investigated on pigment and lipid compositions of the marine diatom Chaetoceros neogracile, at two growth phases using a low (0.05 μg mL−1) and a high (5 μg mL−1) concentrations for 96 h. Results evidenced an impact on pigment composition associated to the light-harvesting function and photoprotection mainly at exponential phase. NP also impacted lipid composition of diatoms with a re-adjustment of lipid classes and fatty acids noteworthy. Main changes upon NP exposure were observed in galactolipids and triacylglycerol's at both growth phases affecting the thylakoids membrane structure and cellular energy reserve of diatoms. Particularly, exponential cultures exposed to high NP concentration showed an impairment of long chain fatty acids synthesis. Changes in pigment and lipid content of diatom' cells revealed that algae physiology is determinant in the way cells adjust their thylakoid membrane composition to cope with NP contamination stress. Compositions of reserve and membrane lipids are proposed as sensitive markers to assess the impact of NP exposure, including at potential predicted environmental doses, on marine organisms.

Graphical abstract



Highlights

► Nanoplastics (NP) effect on algae was studied at exponential and stationary phase. ► NP impact pigment and lipid composition of diatoms at both growth phases. ► Algae adjust their thylakoid membrane lipid composition to cope with NP stress. ► Algae physiological state is a determinant factor to evaluate NP impact on diatoms.

Keywords : diatoms, nanoplastics, lipid composition, pigments

Introduction

Plastics, with their many useful physical and chemical properties, are widely used in various industries and activities of daily living. With an increasing year production, plastics exceed 300 million tons annually (Plastics Europe, 2016). Polystyrene (PS) is one of the most largely used plastics worldwide, in food and industry packaging, disposable cutlery, medical products and toys, between other applications (Plastics Europe, 2016). Plastic materials pose a serious threat to the marine environment when not properly disposed or recycled. Approximately the 10% of the annual plastic production ends up into the oceans (Avio et al., 2015). Once in the environment, plastic debris fragments into smaller particles such as microplastics (MP; < 5 mm) and presumably nanoplastics (NP; < 100 nm; Amaral-Zettler et al., 2016). Because of its fundamental role in marine ecosystem's net primary productivity, an increasing number of studies have paid attention to the ecological impact of MP in phytoplankton (Bhattacharya et al., 2010; Lagarde et al., 2016; Long et al., 2017, 2015; Sjollema et al., 2016; Zhang et al., 2017). Nevertheless, when particle size reached millimeter-scale, MP had fewer impacts on growth of marine microalgae (Zhang et al., 2017). Stronger effects were observed when plastic particle size decreased down to nano-size (González-Fernández et al., 2019; Manfra et al., 2017; Mao et al., 2018; Nolte et al., 2017). Furthermore, the small size of NP can favor their pass through the lipid bilayer of biological membranes and affect the functioning of cells, including lipid metabolism (Bergmann et al., 2015). Indeed, due to nano-properties (nano-size, high surface-to-volume ratio), NP can induce high toxicity notably on early life stages (e.g. Balbi et al., 2017; Della Torre et al., 2014; Pinsino et al., 2017 ; Tallec et al., 2018).

Diatoms are unicellular, mainly photosynthetic, eukaryotes living within elaborate silicified cell walls and claimed to be responsible for around 40% of global primary

productivity in the oceans (Prihoda et al., 2012). Lipids are the major constituents of diatom cells and the average lipid content in diatoms could account up to 25% of dry weight (Levitan et al., 2014), although the production of lipids in diatoms can vary according to culture conditions (Yi et al., 2017). Among the lipids produced by diatoms, polyunsaturated fatty acids (PUFA) such as eicosapentaenoic acid (EPA, 20:5n-3), are essential in the nutrition of benthic and pelagic animals. Feeding on phytoplankton, marine arthropods and vertebrates incorporate diatom´ PUFA into their own lipid composition, and thus become an important source of these lipids in human nutrition (Dolch and Marechal, 2015).

It is generally accepted that the ability of algae to adapt to environmental conditions is reflected in a great variety of lipid patterns as well as with their ability to synthesize a number of unusual lipidic compounds (Guschina and Harwood, 2006). These fluctuations in microalgae lipid composition and the resulting change in their membrane physical properties can have significant influence on their physiology and later affecting higher trophic levels. Considering the importance of lipid production by diatoms and their relevance in trophic energy transfer and ecosystem functioning, the aim of this work was to identify the impact of small NP (50 nm PS-NH_2) on lipid metabolism and composition of microalgae at two growth phases. The presence of NH_2 groups on micro- and nanoplastics in natural environments seems unlikely as plastic debris tends to have more carboxylated surfaces than anionic surfaces due to the oxidation processes following UV radiations (Fotopoulou and Karapanagioti, 2012; Ter Halle et al ., 2016). Nevertheless PS-NH_2 constitutes excellent model particles to study the interactions between nanoplastics and live cells as they remain stable in seawater and their cationic surfaces promotes interactions with negatively charged biological membranes (Tallec et al., 2019).

According to model predictions, by the 2060s, concentrations of MP will reach around 1 µg mL^{-1} for MP higher than 0.3 millimeters (Isobe et al., 1957; Lenz et al., 2015). Although the real concentrations of small NP in the marine environment is unknown, for NP around 50 nm it has been predicted to be environmentally relevant concentrations below 0.015 µg mL^{-1} (Al-sid-cheikh et al., 2018). In our study, *C. neogracile* cultures at exponential and stationary growth phases were exposed for 96 hours to two NP concentrations 0.05 and 5 µg mL^{-1}. The effects of NP were evaluated on membrane and reserve lipids (lipid class and fatty acid composition).

Biosynthesis and homeostasis of lipids strongly affect chloroplast development (Kobayashi, 2018) potentially impacting the photosynthetic machinery. The function of the photosynthetic process in diatoms is the capture of light energy by pigments (Lavaud, 2007). Most of them are localized in the thylakoid membranes (Kuczynska et al., 2015). Taking into account the importance that lipid have on formation of membrane structures and photosensitive pigments, the pigment composition of *C. neogracile* upon exposure to NP was also investigated.

In the context of NP pollution, the presence of colloids and natural organic matter in the media can affect NP behavior and potential toxicity (Manfra et al., 2017; Galloway et al., 2017). Particularly diatoms are recognized for producing exopolysaccharides (EPS) that may rearrange to form larger particles, the transparent exopolymeric particles (TEP; Mari et al., 2017). NP behaviour in exponential and stationary cultures as well as its impact on cellular and metabolic responses of *C. neogracile* cells were studied in González-Fernández et al. (2019). The present study take part of the same experiment and aims to go further evaluating the effect of 50 nm PS-NH_2 on lipid and pigment compositions of diatom cells.

2. Material and methods

2.1. Microalgae culture and exposure

Batch cultures of *Chaetoceros neogracile* (strain CCAP 1010-3), were grown in 500 mL balloons (24 h light cycle, 100 µmoles photons m^{-2} s^{-1} at 20 ºC) in constant aeration within CO_2 supplementation. For cultures inoculation, two sets of cultures were prepared with a 7 days interval in order to expose them in exponential growth phase and in stationary growth phase to NP (González-Fernández et al., 2019).

For exposure, concentrations of both batch cultures were adjusted at 4×10^5 cells mL^{-1} by centrifugation (550 g during 5 minutes) and resuspension of cells in a final volume of 300 mL of their corresponding media (exponential *vs.* stationary media). Cultures were exposed to fluorescent-blue 50 nm amino (PS-NH_2) polystyrene nanoparticles purchased from Sigma-Aldrich (Saint Louis, USA) with an excitation/emission: 358 nm/ 410 nm. Commercial nanoplastics are suspended in ultrapure water with Tween-20© surfactant (<0.1%) to limit aggregation (Sigma-Aldrich). Before exposure, NP were vortexed to prevent particle aggregation and insure good suspension homogenization before use. An intermediate solution was prepared in ultrapure water (concentration of 1 mg mL^{-1}) and a final volume of 1.5 mL of NP were add to a final volume of 300 mL of culture that was kept in continuous aeration to ensure good dispersion. Final concentration of Tween-20© in the cultures were < 0.00002% in the higher NP treatment. Non-ionic surfactants had previously been demonstrated to be innocuous for marine microalgae at this dose (Pavlic et al., 2005) so no effect is expected owing to its residual concentration in all samples (Douglas et al., 1985). For each batch culture (stationary and exponential), three experimental treatments were tested: control (no NP), low NP concentration (0.05 µg mL^{-1}) and high NP concentration (5 µg mL^{-1}). All treatments (control, low and high) were performed in

triplicates for each growth phase (exponential and stationary) and microalgae were exposed for 96 hours. NP characteristics were determined by Dynamic light scattering (DLS) in ultrapure water and in filtered spent media of algae cultures as described in González-Fernández et al. (2019). NP resuspended in ultrapure water and seawater showed expected sizes (50 nm) and no aggregation (PdI < 0.2). 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 > 0.2) and 162.4 ± 3.3 nm (PdI > 0.2) for exponential and stationary media respectively.

2.2. Analyses

2.2.1. Pigments extraction and composition

At the end of the experiment, 3.5 mL and 25 mL corresponding to a total of 2×10^7 cells of diatom cultures at exponential and stationary phases were filtered over glass fiber filters (preliminary burned for 6 h at 450 °C; 0.2 µm, Whatman GF/F) and stored at -80 ºC. Pigments were then extracted in methanol and analyzed by HPLC (Ras et al., 2008). All the pigment standards were purchased from DHI (HØRSHOLM, Denmark). A total of 11 pigments were identified at both growth phases: chlorophyll a (Chl *a*) and sub-products of chlorophyll a (allo and epi Chl *a*), chlorophyll c (Chl *c2*), fucoxanthin (Fx), β-carotene (β-car), diadinoxanthin (Ddx), diatoxanthin (Dtx), violaxanthin (Vx), neoxanthin (Nx) and pheophytin (Phx).

2.2.2. Lipid class and fatty acid analyses

For lipid class and fatty acid analyses, 4×10^7 cells (7 mL of exponential cultures and 50 mL of stationary cultures) were filtered over glass fiber filters (preliminary burned for 6 h at 450 °C; 0.2 µm, Whatman GF/F). Boiling water was immediately passed through the filter after filtration to inhibit lipase activity and prevent lipid degradation.

Lipids were then extracted with chloroform:methanol (2:1; v:v). Lipid extracts were stored at −20 °C under nitrogen (N_2 (g)) until analysis. Lipid class analyses were performed by high-performance thin layer chromatography (HPTLC) using a CAMAG auto-sampler to spot the sample on HPTLC glass plates pre-coated with silica gel (Merck & Co., Ltd., Darmstadt, Germany). In *C.neogracile* cultures, the following lipid classes were measured: within the phospholipids (phosphatidylinositol (PI), phosphatidyl choline (PC) and Phosphatidylserine (PS)), within the glycolipids (monogalactosyl-diacylglycerol (MGDG) and digalactosyl-diacylglycerol (DGDG)), globally the sum of phosphatidyl ethanolamine (PE), phosphatidyl glycerol (PG) and sulfoquinovosyl-diacylglycerol (SQDG), free sterols (FST), free fatty acids (FFA) and triglycerides (TAG).

In this study, neutral lipid (NL) classes, which mostly contains the reserve lipids and polar lipid (PL) classes, which contains most of the membrane lipids, were analyzed (Moutel et al., 2016). For polar and neutral lipid fatty acid (FA) analysis, an aliquot of the lipid extract was dried under N_2 (g) and then resuspended in chloroform:methanol (98:2; v:v). Separation of neutral and polar lipids was realized by solid phase extraction(Le Grand et al., 2013). Each fraction (PL and NL) was then dried under N_2 (g) and transesterified. The resulting fatty acid methyl esters (FAME) were analyzed and quantified by gas chromatography (GC-FID) (Le Grand et al., 2013) and FA identification controlled using standards (Long et al., 2018). FA compositions were expressed as relative mass percentage of total FA in PL and NL. Fatty acids of diatoms at both growth phases with values higher than zero are listed in supplementary Tables 1 and 2. Two fatty acids iso-15:0 and iso-17:0, specific markers of prokaryote´ presence (Suroy et al., 2014a) were identified, but they were excluded from analysis due to they are not specific markers of diatoms.

186 2.2.3. Lipid reserves monitoring by flow cytometry

187 Changes of in the reserve cell content after 96 h exposure to NP were monitored using a
188 green-fluorescent dye (BODIPY 505/515 FL; Thermo Fisher scientific). BODIPY can
189 be used as a proxy of lipid reserves as it stains lipid droplets (Govender et al., 2012).
190 We validated that, presence of NP, did not interfere the measurement of BODIPY in
191 algae. Samples were incubated with BODIPY at a final concentration of 10 mM for 10
192 min at 18 ºC in the dark. Analysis were performed for 30 sec at 0.12 µl sec^{-1} flow rate
193 using an EasyCyte Plus cytometer (Guava Technologies, Millipore, Billerica, MA).
194 Additionally, culture cell concentration (cell mL^{-1}) was recorded during analysis.
195 Results were expressed in arbitrary units (A.U).

196 2.3. Statistical analyses

197 Statistical analysis of pigment composition and main lipid classes were performed using
198 STATGRAPHICS centurion XV.II (Statpoint Technologies, Inc. Warrenton, Virginia,
199 USA). Data normality and homogeneity of variances were tested. Student's t-test was
200 used to compare variables between exponential *vs.* stationary phases. For each culture
201 phase, one-way-ANOVA analysis was performed to establish significant differences
202 between treatments (control, low and high) using a Tukey's post hoc test to differentiate
203 treatments. A simple regression analysis was used to link cellular responses already
204 reported in this cells (González-Fernández et al., 2019) and results from the present
205 study. Differences were considered significant at $p<0.05$.

206 Fatty acid data were statistically analyzed using PRIMER.V6 with a similarity
207 percentage analysis (*SIMPER*) being performed on the relative FA percentage data to
208 demonstrate the differences in FA according to growth phases. Only FA showing values
209 higher than 0.5 were included in the analysis. *SIMPER* identifies the FA that contribute

most to the variations in the assemblage patterns recorded. The FA that cumulatively contributed up to 80% of the dissimilarities recorded were selected to characterize the differences in the FA profile of *C. neogracile* at different growing phases. Due to significant differences of the FA profile in *C. neogracile* at exponential and stationary phases, analysis of NP effects were performed independently for each growth phase.

For analysis, data on the relative FA percentages of the samples were (log [x + 1]) transformed and converted into a *Bray-Curtis* similarity matrix to start the multivariate analyses. Later, the Bray-Curtis similarity matrix was used for one-way ANOSIM to test whether algae exposed to NP (within NL or PL fractions) were significantly clustered according to exposure treatments. ANOSIM calculates a global R statistic that assesses the differences between groups, with $R = 1$, $R = 0.5$ and $R = 0$ indicating a perfect, satisfactory and poor separation of the clusters, respectively (Clarke et al., 2008).

Principal component analysis (PCA) was carried out in order to establish relationships between treatments and individual FA. For this study, the components whose cumulative variance explained > 60% of variance were considered.

3. Results

3.1. Pigment and lipid compositions of *C. neogracile* according to growth phase

Pigment composition of *C. neogracile* at the two different growth phases is presented in Table 1. Significant differences in all pigment cell contents were observed between exponential and stationary phases (*T-student test;* $p<0.001$ all cases) presenting higher values at exponential phase.

Significantly higher total lipid concentration per cell were observed at stationary phase than at exponential phase (*T-student test;* $p<0.05$; Supplementary Figure 1) mainly due

234 to differences in reserve lipids (TAG, *T-student test; p*<0.001). Significantly higher
235 content of DGDG and FST were also observed at stationary phase compared to
236 exponential phase (Supp. Figure 2).

237 Furthermore, influence of growth phase on FA composition of polar and neutral lipids
238 was also investigated (Supp. Tables 1 and 2, respectively). At exponential phase, higher
239 proportions of polyunsaturated fatty acids (PUFA) were observed compared to
240 stationary phase for both lipid fractions (*T-student test; p*<0.01). A very good separation
241 of FA between growing phases was observed in both lipid fractions: polar lipids'
242 composition (*Stress*=0.00, NMDS) and neutral lipids' composition (*Stress*=0.00,
243 NMDS). The main differences observed came from SFA (such as 14:0, 16:0 and 18:0),
244 MUFA (18:1n-7; 18:1n-9, 18:1n-5) and PUFA (20:5n-3; 16:2n-7; 16:3n-4; 22:6n-3 and
245 20:4n-6) showing a dissimilarity between growth phases of 19.7% and 19.8% for polar
246 and neutral lipids, respectively (*SIMPER* analysis).

247 3.2. Changes in pigment and lipid compositions after exposure to NP

248 At exponential phase, a decrease (-75%) in the quantity of pigments composing the
249 fucoxanthin-chlorophyll protein (FCP) complex was observed upon high NP exposure:
250 Chl *c2*, Chl *a* and sub-products allo and epi Chl *a* as well as Fx (Table 1). Additionally,
251 a significant reduction of β-car and Phx (-63.7% and -75.5% respectively) in this
252 condition was observed whereas the ratio Dtx/Ddx increased (+1316% equivalent to 13
253 times higher than controls). At stationary phase, Chl *c2*, β-car and Phx were
254 significantly reduced by both NP concentrations (Table 1).

255 When analyzing lipid classes by HPTLC, a decrease in DGDG and MGDG cell content
256 (-28.5% and -46.1%, respectively) was observed in exponential cultures after exposure
257 to high NP concentration (Figure 1A). Moreover, a reduction of the ratio

MGDG/DGDG (-24.8%) was observed (Figure 2) at high NP exposure. The meantime, increases of PS (+41.7%) and FFA (+242%) cell contents were observed at high NP concentrations as well as increase of TAG (+77.6% and +53.3%) at low and high NP exposures, respectively (Fig. 1A). *C. neogracile* at stationary phase showed significant increases of MGDG and TAG (+45.4% and +34.7%, respectively) at high NP concentration (Figure 1B). A dose-dependent increase in the ratio MGDG/DGDG was also observed (Figure 2).

Additionally, neutral lipid content of cells was measured by flow cytometry after 96 h exposure to NP using BODIPY staining (Figure 3). At exponential phase, lipid reserves were increased at low but not at high NP concentration. Conversely, at stationary phase a dose-response increase of lipid reserves was observed upon NP exposure (Fig. 3).

3.3. Changes in FA profiles after exposure to nanoplastics

Changes in their FA profile after exposure to the NP concentration was evidenced at exponential growth (Figure 4). In the PL fraction, differences were observed after exposure to high NP concentration (Global R=0.8, p<0.01; *ANOSIM*) but not at low concentration. The main FA responsible for differences between control and high NP exposure were: 18:1n-7 (accounting for 22.8% of the dissimilarity); 22:6n-3 (7.8%); 18:2n-6 (7.4%); 18:0 (7.0%); 18:4n-3 (5.6%); 16:1n-7 (5.0%); 16:2n-7 (4.5%); 16:0 (4.0%); 16:3n-4 (3.7%); 18:3n-6 (3.5%); 16:2n-4 (3.5%); 20:4n-6 (3.4%) and 18:1n-9 (3.2%), accounting together for 81.5% of dissimilarity (*SIMPER* analysis; DS=7.02).

Significant differences were also evidenced in NL fraction at both NP concentrations (Global R=1, p<0.01; *ANOSIM*). Differences between control and low NP exposure were associated to the percentage of 16:2n-6 (accounting for 15.9% of the dissimilarity); 16:4n-3 (13.3%); 18:0 (10.6%); 16:1n-9 (6.6%); 18:1n-9 (6.3%); 18:4n-3

(5.9%); 16:1n-7 (5.5%); 14:1n-5 (4.5%); 18:3n-6 (4.1%); 16:0 (3.0%); 14:0 (2.8%) and 16:2n-4 (2.7%) accounting for the 81.2% of dissimilarity (*SIMPER* analysis; DS=6.80). Differences between control and high NP exposure were determined by the percentage of 16:3n-4 (accounting for 15.3% of dissimilarity); 16:2n-6 (11.9%); 16:4n-3 (8.5%); 16:0 (7.1%); 16:2n-7 (5.2%); 16:2n-4 (5.1%); 18:0 (4.8%); 18:2n-6 (4.5%); 20:4n-6 (4.2%); 14:1n-5 (4.2%); 16:1n-7 (3.7%); 16:1n-9 (3.6%) and 18:1n-9 (3.2%) accounting for the 81.4% of dissimilarity (*SIMPER* analysis; DS=7.94).

In the PL fraction, the 2 first principal components of the PCA explained 86% of the variability in the original data, respectively explaining 50.7% and 34.8% of the combined variance The representation of PC1 and PC2 (Fig. 4A) distinguished an increase in the proportions of 18:0, 18:1n-7, 16:2n-7, 16:2n-4, 16:3n-4 and 22:6n-3 at high NP concentration while 16:0, 16:1n-7, 18:1n-9, 18:2n-6, 18:3n-6, 18:4n-3 and 20:4n-6 decreased at this condition showing higher values at control and low NP exposure treatments (Fig. 4A; 4B).

In the NL fraction, the PC1 and PC2 explained respectively 48% and 38.9% of the total variance (Fig. 4C). PC1 separate low and high NP exposures while PC2 revealed differences between control and NP exposed treatments. The PC1-PC2 factorial plan (Fig. 4D) distinguished an increase in the proportions of 16:2n-6, 18:1n-9 and 20:4n-6 in control compare to NP treatments. Cultures exposed to high NP concentrations evidenced higher proportions of 16:0, 18:0, 16:2n-7, 16:2n-4 and 16:3n-4 while culture exposed to low NP concentrations was characterized by higher proportions of 14:0, 14:1n-5, 16:1n-7, 18:3n-6 and 18:4n-3. The rest of FA that accounted for >80% differences between treatments was not determinant to establish differences between control and NP exposures due to the low factor loading obtained (<0.1).

306 At stationary growth, no significant differences between treatments were observed in
307 both lipid fractions analyzed.

308 **4. Discussion:**

309 **Characterization of pigment and lipid composition of *C. neogracile***

310 In general and in our study, most of the pigments in diatoms showed lower cellular
311 concentrations during stationary growth and the senescence, particularly, the light-
312 harvesting pigments Chl *a* and sub-products, Chl *c2*, Fx and β-car, as a result of
313 nutritional limitations in the media (Carreto and Catoggio, 1976; Klein, 1988;
314 Kuczynska et al., 2015). We observed accumulation of triacylglycerol (TAG) at
315 stationary phase, a general response of diatoms to nutrient limitation (Li et al., 2014;
316 Pratiwi et al., 2009). FA acid composition of *C. neogracile,* showed high proportions of
317 14:0, 16:0: 16:1n-7, 20:4n-6, 20:5n-3 (EPA) and 22:6n-3 (DHA), characteristic of
318 diatoms (González-Fernández et al., 2016; Zulu et al., 2018). In the present study,
319 proportions of SFA and MUFA increased while the proportion of PUFA decreased
320 during stationary phase. Nutrient limitation promotes re-adjustment of lipid
321 compartment. SFA and MUFA increase at the cost of PUFA under phosphate limitation,
322 possibly in relation to the need for NADP during desaturation and elongation
323 (Jónasdóttir, 2019). We previously demonstrated that culture age influences the impact
324 of NP, indicating higher cellular toxicity at exponential growth phase than at stationary
325 phase (González-Fernández et al., 2019). As a continuation of this work, we evaluated
326 lipid composition of diatoms at exponential and stationary growth phases exposed to
327 two NP concentrations (0.05 and 5 µg mL^{-1}).

328 **Impairment of the photosynthetic machinery after exposure to NP**

In the aquatic environment, pigments may undergo degradation in response to chemical, photochemical and biological processes being chlorophylls the most labile compounds (Zigmantas et al., 2004). Changes in photosynthetic pigment levels are usually a fast response to stressful conditions because they are engaged in basic processes such as photosynthesis and photoprotection essential for cell life (Kuczynska et al., 2015). Our results reveal a drastic impact of NP on the photosynthetic machinery of diatoms. The concentrations of pigments associated with the fucoxanthin-chlorophyll protein (FCP) complexes (Chl *a*, Chl *c2* and Fx) were significantly reduced after exposure to high NP concentration in exponential cultures. The decrease in photosynthetic efficiency in this cells were previously observed upon 96 h exposure to 0.05 µg mL^{-1} and 5 µg mL^{-1} NP (González-Fernández et al., 2019) and showed a close positive relationship with the decrease of Chl *a* observed in this study (Regression, R^2=0.89, p<0.05). The decrease of photosynthetic efficiency may result from an inactivation of photosystem (PSII) (Lelong et al., 2011). Thus, photons become excessive and may induce an increase in ROS production by the cell. To avoid this, algae have developed photo-protective mechanisms such as non-photochemical quenching (NPQ) as well as the activation of the xanthophyll cycle (Kuczynska et al., 2015). Diadinoxanthin (Ddx) and diatoxanthin (Dtx) are the main xanthophyll cycle pigments (Grouneva et al., 2009). In the present study, an increase of Dtx at the expense of Ddx (Dtx/Ddx ratio) was observed upon exposure to NP at exponential growth. The deepoxidation of Ddx to Dtx is an essential prerequisite of the thermal dissipation of excess excitation energy and it is considered the most important photoprotective mechanism in diatoms (Goss and Jackob, 2010; Lavaud et al., 2007). Using simple regression, Dtx/Ddx ratio had a positive linear relationship with ROS production at exponential stage (R^2= 0.43, p<0.05). It seems that

353 diatoms at this stage used NPQ strategy to dissipate the excess of energy produced by
354 NP presence.

355 At stationary phase, only few pigments were impacted by NP exposure: β-car which is
356 one of the main photo-protective carotenoids, Chl *c2* that participates effectively in
357 photosynthesis as an accessory pigment (Kuczynska et al., 2015) and Phx which
358 derivate of chlorophyll and has been recently identified as the primary electron acceptor
359 of PSII (Klimov, 2014). Nevertheless, the decrease observed on pigment composition
360 upon NP exposure highlights that diatoms are susceptible to NP exposure also at
361 stationary phase.

362 **NP modulates thylakoid and membrane lipid composition**

363 Analysis of lipid classes revealed that NP exposure impacted proportions and ratios of
364 MGDG and DGDG, which are the main constituents of thylakoid membranes,
365 accounting for about 50 and 25% of total thylakoid lipids, respectively (Dorney, 2013).
366 Surprisingly, MGDG/DGDG ratio of cells at exponential and stationary growth changed
367 in opposite direction in a dose-dependent manner upon exposure to NP. Fluctuations in
368 the MGDG/DGDG ratio could affect the properties and structures of thylakoid
369 membranes by altering the lipid organization from hexagonal inverse to lamellar phases
370 (Dorney, 2013). Thylakoid lipids not only provide a lipid bilayer matrix but also form
371 part of the structural components in PSII and photosystem I (PSI) complexes
372 (Kobayashi, 2018). The deepoxidation of Ddx occurs in presence of MGDG (Goss et
373 al., 2007). Decreases in the MGDG cell content observed in exponential cultures upon
374 NP exposure suggest that MGDG has been used for Ddx deepoxidation. The re-
375 arrangement of thylakoid lipid composition can be related to the maintenance of
376 functional chloroplasts preventing photo-oxidative damage from intermediate

complexes or unbalanced electron transfer systems (Fujii et al., 2014). Thus, we can speculate that MGDG are being used by cells to reduce superoxide radicals accumulated in cells (Kuczynska et al., 2015) as consequence of NP presence, as previously discussed in (González-Fernández et al., 2019).

On the other hand, increase in MGDG/DGDG ratio was observed upon NP exposure at stationary phase that may indicate changes in thylakoid membrane´ structure. Changes in membranes of microalgae´ cells under MP exposure have been previously reported showing a thickening of algal cell walls, which could effectively block the invasion of MP and thus avoid cell damage (Mao et al., 2018).

Changes in membrane fatty acid (FA) profile upon NP exposure were also observed. In exponential phase, at high NP concentration, an increase in proportions of polar lipid FA 18:1n-7, 16:2n-4, 16:3n-4 at the expenses of 16:0, 18:2n-6, 18:3n-6, 18:4n-3 and 20:4n-6 suggested some metabolic responses/adjustments in PUFA pathways. Synthesis of the n-7 and n-4 PUFA series occur in chloroplast and used 16:1n-7 as precursor while n-3 and n-6 synthesis may occur from 16:0 in chloroplast and 18:0 in chloroplast and endoplasmic reticulum synthesized through fatty acid synthase (Liang et al., 2013). The synthesis of *de novo* FA and galactolipids is linked with photosynthetic electron transport of chloroplasts. The reduction observed at exponential phase of MGDG and DGDG could impair the PSII photochemical reaction and disrupt energy coupling between reaction centers and antenna complexes (Fujii et al., 2014). Since MGDG and DGDG from diatoms are synthesised in the “prokaryotic” pathway, and are enriched in 16-carbons fatty acids in the sn–2 position (Bromke et al., 2015), a significant reduction of MGDG and DGDG may result in changes of fatty acid biosynthesis and metabolism (Zulu et al., 2018). Similar changes of PUFA have been previously revealed after algae exposure to pesticides (Filimonova et al., 2016)

decreasing the level of 18:2, 18:3 but increasing the level of EPA (20:5n-3) and MUFA (16:1 and 18:1) (Filimonova et al., 2016).

Moreover, the decrease in 14:0 and 16:0 at high NP concentration may reflect some changes in cell´ frustules as these two FA were identified as main saturated FA conforming diatoms´ frustule-associated lipid fractions (Suroy et al., 2014b). The attachment of 50 nm PS-NH_2 to diatoms cell´ frustules has been previously demonstrated by scanning electron micrographs (SEM) at exponential phase (González-Fernández et al., 2019). In addition, absorption of PS-NH_2 NP on algae´ cell membranes has been also reported in green algae (Bergami et al., 2017; Nolte et al., 2017). Nevertheless, the presence of a silica frustule in diatoms could protect from the negative effects of NP in membranes. It has been reported that –NH_2 have high affinity to frustule's silica in diatoms (Walsh et al., 2017). In this sense, although the impact of NP on diatoms´ frustule has not been identified yet, the attachment of NP to frustules could reduce light availability and nutrient and gas exchange, potentially impacting the photosynthesis activity (González-Fernández et al., 2019) and affecting its machinery as reported above (the decrease in FCP pigments and activation of Ddx cycle).

Contrary to the effect observed at exponential phase, membrane lipid composition was not affected by NP at stationary phase. Cultures at stationary phase present 6 times higher TEP concentration by cell than at exponential phase (González-Fernández et al., 2019). Presence of TEP in culture media act as an adhesive, binding particles together (Summers et al., 2018) increasing aggregation. This aggregation promotes the increase of NP size and may reduce their associated impacts. Secondly, cells at exponential phase are more susceptible to stress due to the fast growth which requires energy and promote membrane exhibition (Tanaka et al., 2015).

Readjustment of lipid reserves upon NP exposure

The increase in reserve lipids in microalgae is an acclimation mechanism to cope with the decrease of nutrient availability or the presence of anthropogenic pollution (Guschina and Harwood, 2006). In our study, cells exposed to NP concentration displayed higher TAG than non-exposed cells. TAG increased not only after exposure to high NP concentration but also upon low NP concentration. This fact, indicates that lipid measurements are sensitive enough to evaluate the impact of NP exposure even at environmental concentrations. TAG can be temporarily stock-piled in essentially unlimited amounts and be utilized as an energy-rich carbon source when favorable conditions come back. Some algal species make dual use of excess lipid stores, both as a reserve for future growth and as a mean of reducing photosynthesis by directly absorbing part of the incoming light (Thompson, 1996). The reduction of content of principal pigments associated to light-harvesting functions observed in exponential cultures, together with the accumulation of ROS previously observed (González-Fernández et al., 2019) may result in promoting the accumulation of TAG for survival at both growth phases.

In addition to that, fatty acid composition of reserve lipids were also affected by NP exposure at both concentrations. However, fatty acid re-arrangement differed according to the concentration of NP used. Some of the changes observed in cultures exposed to high NP concentration may be related to the impairment of long chain fatty acids synthesis by affecting the fatty acid elongase and therefore reducing the proportion of C18 and C20 fatty acids (the conventional pathway to EPA) (Filimonova et al., 2016). As consequence of this interference, an accumulation of precursors of the fatty acid elongase, 16:0 was observed(Filimonova et al., 2016). Most of microalgae naturally synthesize significant quantity of free fatty acids (FFA) that are further converted to

acyl-CoA thioesters for lipid biosynthesis and accumulation (Hao et al., 2018). Nevertheless, interruption of fatty acid elongation cycle can promote the release of FFA as observed in our study in cultures exposed to high NP exposure.

Cultures exposed to low NP concentration also evidenced a high percentage of 16:1n-7. Desaturation steps starting from 16:1n-7 are linked with the glycerol-backbone of galactolipids, usually MGDG (Zulu et al., 2018). Although MGDG were not affected at this low NP concentration, the ratio MGDG/DGDG showed a dose-dependent reduction with NP concentration. This may serve as an alternative way to compensate the decrease of C18-20 PUFA observed at upon NP exposure. Particular attention should be drawn to the 16:2n-6 and 16:3n-4 fatty acids, which were PUFA determinant for the discrimination of control and NP exposed cultures. In diatoms, plastidial desaturases have been identified as enzymes involved in the generation of 16:3n-4, which represents an unusual Δ6-harboring C16-PUFA (Zulu et al., 2018). The incorporation of some PUFA into TAG may result from membrane lipid remodeling by the release of PUFA from galactolipids (Zulu et al., 2018). The increase of TAG at low and high NP exposure, together with the decrease of MGDG/DGDG ratio observed upon exposure to NP in exponential cultures may suggest a major readjustment of lipids to cope the stress exerted by NP.

In this work, we measured TAG concentration through HPTLC. Additionally, we monitored changes in the reserve cell content after 96 h exposure to NP by flow cytometry, using a fluorescent dye (BODIPY). Our results showed that measurements of lipid reserves by flow cytometry and HPTLC displayed the same tendency being a good indicator of lipid reserves in algae. These results are of great interest not only for laboratories studies but also for field works since it is a fast and economic measurement that will provide a proxy of the energetic status of the microalgae. This technique

revealed high sensitivity to detect reserve lipid changes in cells even upon potential environmental concentrations of NP being a good indicator of environmental stress.

5. Conclusion

In conclusion, our results bring new insights of NP effects on diatoms and indicate how NP impair their photosynthetic machinery and affect lipid structures. Diatoms´ pigment cell content was drastically reduced upon NP exposure at both physiological states evidencing the impact of NP mainly on the FCP associated pigments at both concentrations. A readjustment of thylakoid´ lipid class and fatty acid compositions was also induced by NP exposure. Interestingly, although the impact of NP on diatoms was dependent of culture growth, being more sensitive at exponential culture than at stationary one, cells in different physiological phases were able to develop different acclimation mechanisms to cope with the NP induced stress. Altogether, compositions of reserve and membrane lipids are new and sensitive markers to assess the impact of NP exposure, including at low dose, on marine organisms. Finally, we validated the measurement of reserve lipid content in cells by flow cytometry as a tool for identifying changes associated to lipid reserves in diatoms.

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Figure captions

Figure 1. Main lipid classes of C. *neogracile* after exposure to two concentrations of nanoplastics for 96 hours, expressed as µg lipid 10^{-6} cells (mean ± standard deviation, n=3). A: exponential growth phase and B: stationary growth phase. Letters indicate significant differences between treatments (*1-Way-ANOVA* analysis) with significant values as $p<0.05$. PC: phosphatidyl choline, PE+PG+SQDG: sum of phosphatidyl ethanolamine, phosphatidyl glycerol and sulfoquinovosyl-diacylglycerol, PI: phosphatidylinositol, PS: phosphatidylserine, MGDG: monogalactosyl-diacylglycerol, DGDG: digalactosyl-diacylglycerol, FST: free sterols, FFA: free fatty acids and TAG: triglycerides.

Figure 2. Ratio of monogalactosyl-diacylglycerol and digalactosyl-diacylglycerol (MGDG/ DGDG) of *C. neogracile* after exposure to two concentrations (0.05 and 5 µg mL^{-1}) of nanoplastics for 96 hours (mean ± standard deviation, n=3). Letters indicate significant differences between treatments (*1-Way-ANOVA* analysis) with significant values as $p<0.05$

Figure 3. Neutral lipid content of *C. neogracile* after 96 hours exposure to 0.05 and 5 µg mL^{-1} PS-NH_2 expressed as arbitrary units (mean ± standard deviation, n=3). Letters indicate significant differences between treatments (*1-Way-ANOVA* analysis) with significant values as $p<0.05$

Figure 4. Principal component analysis of *C. neogracile´* fatty acid composition exponential cultures exposed to two concentrations of PS-NH_2 for 96 hours. A, B: polar lipid fraction and C,D: neutral lipid fraction

Table 1. Pigment composition of *C. neogracile* after exposure to two concentrations of PS-NH_2 for 4 days. Pigments are expressed as mean ± standard deviation (n=3) of peak area (sample corresponding to 2×10^7 cells in each condition). Letters show differences between treatments within the same growth stage. Significant levels are considered as $p < 0.05$. Chl *a*: chlorophyll *a*, Allo Chl *a*: allo chlorophyll *a*, Epi Chl *a*: epi chlorophyll *a*, Chl *c2*: chlorophyll *c2*, Fx: fucoxanthin, Nx: neoxanthin Vx: violaxanthin, Ddx: diadinoxanthin, Dtx: diatoxanthin, Phx: phaeophytin and β-car: β-carotene.

	Exponential stage			***Stationary stage***		
Pigment	Control	Low	High	Control	Low	High
Chl *a*	4205±301**b**	3641±726 **b**	990±273 **a**	224±73	167±16	150±17
Allo Chl *a*	995±66 **c**	708±81 **b**	33±0.9 **a**	14±5	10±0.3	10±0.2
Epi Chl *a*	66±3 **b**	53±10 **b**	14±4 **a**	4±1	2±0.3	2±0.3
Chl *c2*	695±81 **b**	579±194 **b**	143±63 **a**	21±11 **b**	17±0.9 **a**	15±0.6 **a**
Fx	3079±70 **b**	2461±567**b**	518±192**a**	132±48	103±6	103±17
Nx	11±1	11±2	8±1	1±0.4	0.9±0.1	0.8±0.1
Vx	7±0.3	5±1	6±0.9	0.6±0.2	0.5±0.1	0.5±0.1
Ddx	834±98 **b**	848±169 **b**	269±59 **a**	42±14	35±1	37±6
Dtx	6±0.6 **a**	7±1 **a**	26±4 **b**	15±5	11±1	11±1
Phx	354±42 **b**	278±5 **b**	87±35 **a**	19±5 **b**	3±2 **a**	0.0 **a**
β-car	85±18 **b**	70±10 **b**	31±11 **a**	3±1 **b**	1±0.2 **a**	0.8±0.1 **a**

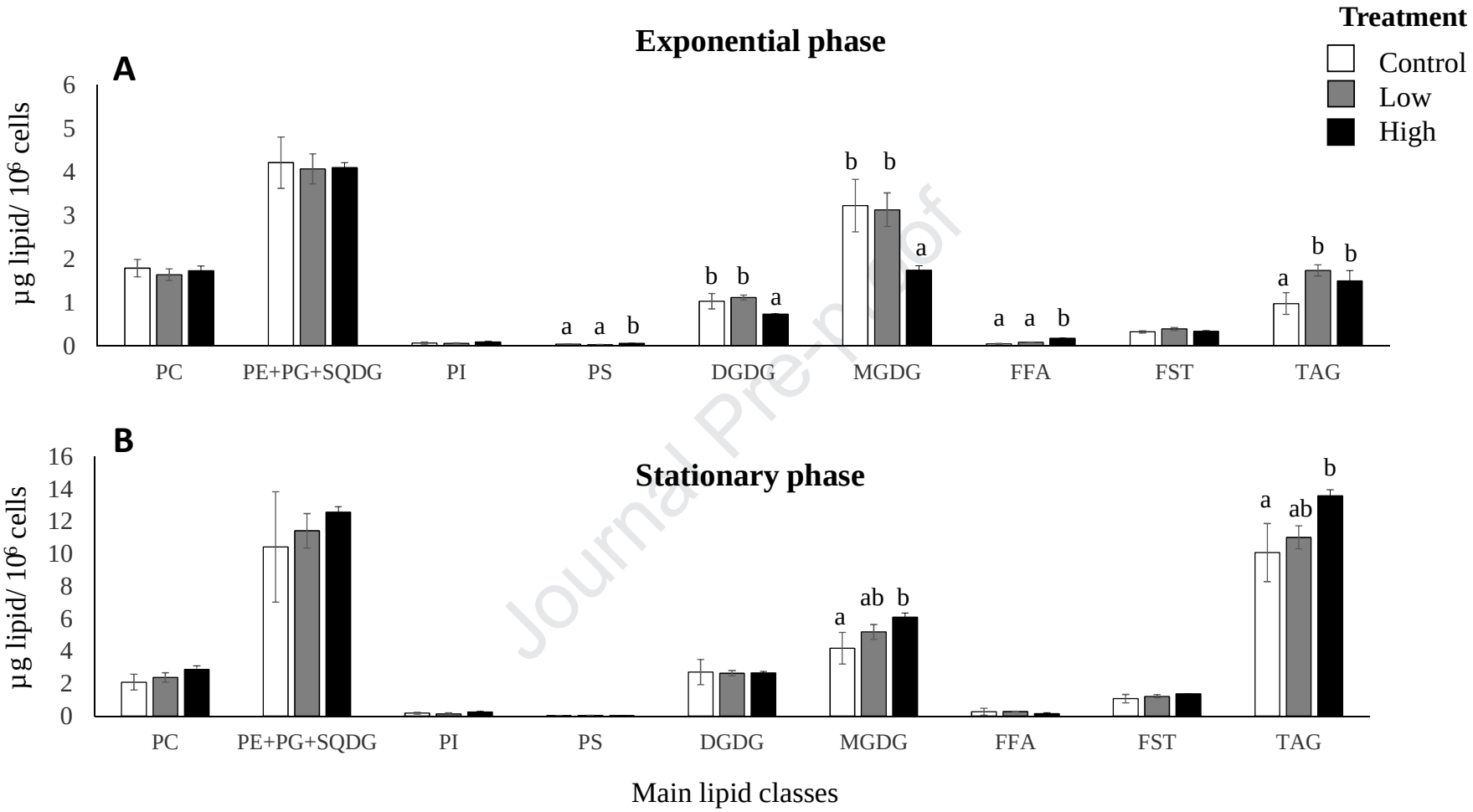

Figure 1

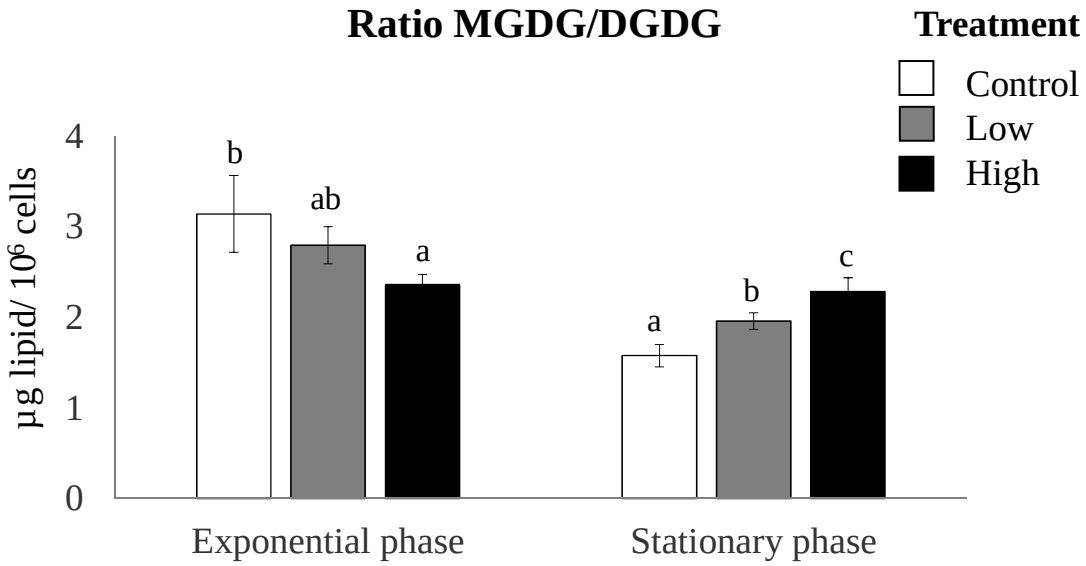

Figure 2

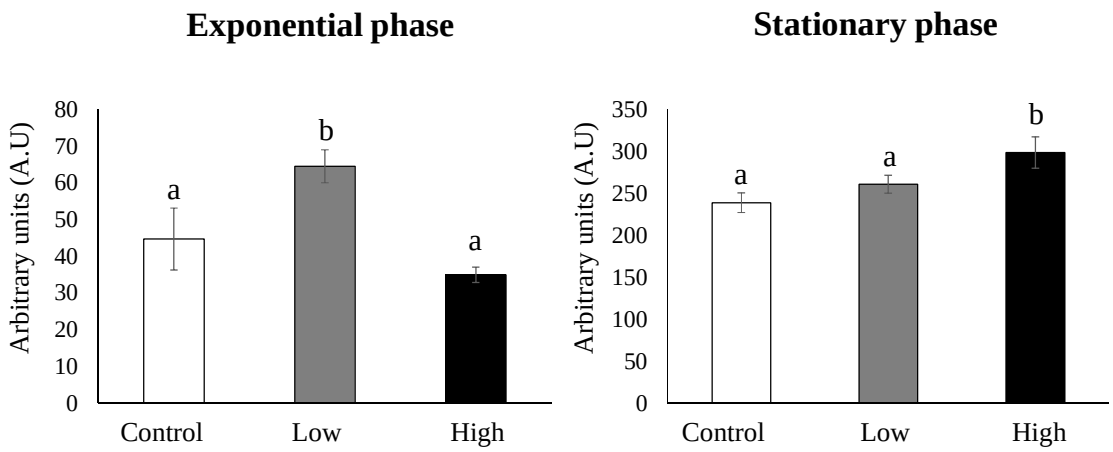

Figure 3

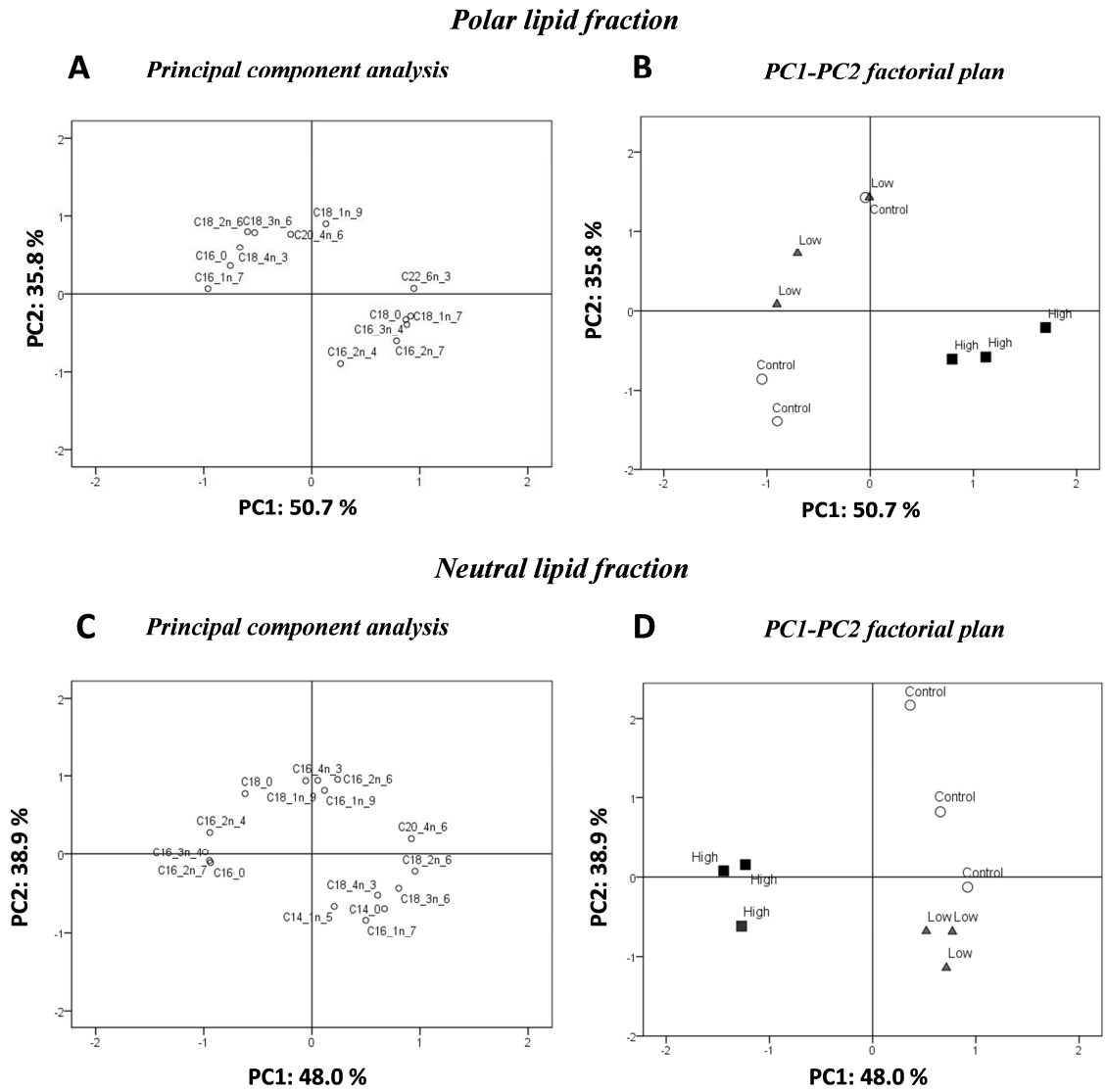
Polar lipid fraction
A
Principal component analysis
B
PC1-PC2 factorial plan
PC2: 35.8 %
PC1: 50.7 %
C18_2n_6
C18_3n_6
C18_1n_9
C20_4n_6
C16_0
C18_4n_3
C16_1n_7
C22_6n_3
C18_0
C18_1n_7
C16_3n_4
C16_2n_4
C16_2n_7
Low
Control
High
Neutral lipid fraction
C
Principal component analysis
D
PC1-PC2 factorial plan
PC2: 38.9 %
PC1: 48.0 %
C16_4n_3
C18_0
C16_2n_6
C18_1n_9
C16_1n_9
C16_2n_4
C20_4n_6
C16_3n_4
C18_2n_6
C16_2n_7
C16_0
C18_4n_3
C18_3n_6
C14_1n_5
C14_0
C16_1n_7
Control
High
Low

Figure 4

Highlights:

1. Nanoplastics (NP) effect on algae was studied at exponential and stationary phase
2. NP impact pigment and lipid composition of diatoms at both growth phases
3. Algae adjust their thylakoid membrane lipid composition to cope with NP stress
4. Algae physiological state is a determinant factor to evaluate NP impact on diatoms

All the authors participated in the elaboration of this research article.

C. González Fernández performed the experimental design, did the experiment and write the original draft. She also participated on the revision process.

A. Bideau performed lipid analysis. F. Le Grand supervised and validated lipid analysis.

A. Huvet and I. Paul-Pont participated in the elaboration of the manuscript and the process of revision.

P. Soudant participate in the validation of the results, elaboration of the manuscript, review process and funding acquisition.

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.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: