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Baseline

# Microplastics do not affect standard ecotoxicological endpoints in marine unicellular organisms

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ABSTRACT

In this study, the acute toxicity of microplastics (MPs) on unicellular organisms as marine decomposers and microalgae was assessed, by evaluating standards endpoints included in International Standard Organization (ISO) protocols. The bacteria *Vibrio fischeri* and the diatom *Phaeodactylum tricornutum* were exposed to different sizes (1–500 μm) of polyethylene MPs in order to evaluate bioluminescence inhibition and microalgal growth. No acute toxicity was found on bacteria or microalgae in an order of magnitude above environmentally relevant concentrations, suggesting that tested MPs did not affect the investigated biological processes. In conclusion, standard ecotoxicological endpoints are not sufficiently sensitive to assess the potential effects of MPs on decomposers and primary producers, conversely to nanoplastics. These findings highlight that the current approach for MP risk assessment in unicellular species should be revised, by providing alternative endpoints to be included in standardized protocols, able to monitor the fate and biological effects of MPs.

Plastic debris is an anthropogenic contaminant extensively found in the aquatic environment worldwide (Cozar et al., 2014). Global plastic production is rising rapidly, thus plastic debris accumulation and fragmentation in the marine environment have become a global issue (GESAMP, 2015). Microplastics (referred to as MPs hereafter) are small plastic fragments, fibers and beads (< 5 mm, Thompson et al., 2004) manufactured to be microscopic in size or derived from degradation of larger plastic debris (Cole et al., 2011). MPs are widely dispersed in the marine environment and recognized as an emerging contaminant of marine pollution (Gago et al., 2016). MPs affect a diverse array of marine organisms across trophic levels (Christaki et al., 1998; Lusher et al., 2013; Setala et al., 2014; Batel et al., 2016; Santillo et al., 2017; Beiras et al., 2018). Decomposers (bacteria) and primary producers (microalgae) play an important role in the marine environment, being involved for vital processes in marine ecosystems (Azam et al., 1983). They inhabit almost all aquatic environments, being involved in nutrient cycling and energy flow to higher trophic levels, and being the food of filter-feeding organisms (Han et al., 2016). Moreover, any disturbance to these food web components may result in an indirect 'bottom-up' impact on species at higher trophic levels through alteration of the nutrient/food/prey balance (Van Dam et al., 2008; Trenfield et al., 2015). However, when assessing environmental toxicity, investigations about the effects of MPs on them are still very limited. Nano-sized plastics do not induce any ecotoxicological effects in the bacteria *Vibrio fischeri* (Booth et al., 2016), differently from micro-sized particles that are responsible for a decrease in bacteria luminescence at high concentrations (3600 mg/L, Gagnè, 2017). Regarding primary producers, nano and micro-sized plastics do not affect growth and photosynthesis of several marine microalgal species (Davarpanah and Guilhermino, 2015; Sjollema et al., 2016) at environmentally relevant concentrations (~0.5 mg/L, Koelmans et al., 2015). Conversely, a growth inhibitory effect occurs at extremely high concentrations, ranging from 10 mg/L up to 250 mg/L (Sjollema et al., 2016; Prata et al., 2018). There is the need to expand our knowledge on the effects of MPs in unicellular organisms essentials for the marine ecosystem, also because of limited data available in the literature. Since no standard methods for MP toxicity assessment in unicellular organisms are available, the aim of this study was to verify the possibility to use

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endpoints included in International Standard Organization (ISO) protocols, to evaluate MP acute toxicity in bacteria and microalgae. The potential toxicity of polyethylene (PE) MPs with a wide range of sizes was assessed on bacteria luminescence inhibition and microalgal growth. The marine bacterium *V. fischeri* and the marine diatom *Phaeodactylum tricornutum* were selected, since standard methods are available for these species (ISO 11348-3:2007, ISO 21338: 2010 and ISO 10253: 2006), as well as for their physiological significance, and ease and rapidity of measurement (Pérez et al., 2010).

Non-fluorescent and irregularly low and high density PE MPs, with 0.93–0.99 g/cm$^3$ density and particle sizes ranging from 1 to 500 μm, were purchased from Micro Powders Inc. (USA), Cospheric (USA) and Rotogal (Spain). PE was chosen since it is a common plastic polymer worldwide and the most common in sea water (Phuong et al., 2016; Brate et al., 2018). Commercial PE-MPs included CPMS-0.96 (size range supplied by the manufacturer 1–4 μm), MPP-635XF (4–6 μm), Aquamatte 26 HD (6–8.5 μm), MPP-635G (11–13 μm), Aquatex 325 (11–15 μm), MPP-1241 (20–25 μm), Aquatex 230 (36–43 μm), Aquatex 100 (80–100 μm) and micronized PE Rotogal (125–500 μm).

For counting and sizing, PE powders were suspended in filtered (0.45 μm) natural seawater (36‰ salinity), according to the test media, at a concentration of 100 mg/L. A dispersant, Tween 20, was used to facilitate particle dispersion, at 17 μg/mg MP, meanwhile all suspensions were agitated with a magnetic stirrer for 30 min. Preliminary tests were performed to check that actual concentrations in the bioassays corresponded to nominal ones. Three replicates of each MP suspension were analysed. Particle size distribution was analysed with an electronic Coulter Counter, model Multisizer III by Beckman. The Multisizer provides both volume and number size (in μm) distributions, although only volume distribution is shown. Measures from preliminary tests were converted to mass concentrations according to the MP density established by the manufacturers. Several descriptive statistics were obtained: mean ( ± standard deviation from the 3 replicates) particle size and three deciles D10, D50 (median) and D90 in order to describe the distribution shape. Two aperture tubes were used, 100 (2–60 μm) and 1000 μm (30–600 μm) depending on PE powder size. Moreover, MP behavior in seawater was checked by using a particle size analyzer (Mastersizer, Malvern refractive index).

Acute toxicity of oxidized and not oxidized PE MPs (ranged from 0.625 mg/L up to 10 mg/L) to the bioluminescent marine bacterium *V. fischeri* was determined according to ISO 11348-3: 2007 and ISO 21338: 2010. All Microtox reagents and lyophilized *V. fischeri* bacteria (NRRL B-11177) were obtained from Modern Water Ltd. (USA), using 90% Basic Test (BT) and Solid Phase Test (SPT, Azur Environmental, 1998). MP toxicity was measured in terms of relative bioluminescence by Microtox™ 500 luminometer after 30-minute incubation. Bioluminescence inhibition was determined using the Microtox Toxicity Analyzer (SDI); data were analysed using MicrotoxOmni software.

The diatom *P. tricornutum* (strain A and strain B) was cultured in seawater with complete F/2 culture medium (Guillard and Ryther, 1962) at 20 ± 0.5 °C with a 12–12 h light-dark period or continuous illumination and light intensity of 6000–10,000 lx (Sbrilli et al., 1998) until they reached exponential growth phase. Tests were performed according to ISO 10253 (2006) test method, with particular differences between laboratories detailed in Table 1 using glass flasks for MP solutions (from 0.01 up to 25 mg/L). Microalgae were inoculated into flasks to reach a density of 10,000 cells/mL. Three replicates for each dilution, including control, were prepared. After 72 h, culture growth was stopped using Lugol's solution and algal growth inhibition was evaluated (referred to the control) by counting cells with a haemocytometer (using an inverted microscope) or Coulter Counter.

Statistical analyses were conducted using SPSS statistical software version 20 (Statistical Package for the Social Sciences). Normal data distribution and homoscedasticity were checked using the Shapiro-Wilk's and Levene's tests, respectively. When significant differences ($p < .05$) among groups were found using ANOVA, then each treatment was compared to the control using Dunnett's *post hoc* test to calculate the lowest no observed adverse effects concentration (NOEC) and the lowest observed adverse effects concentration (LOEC). Non-parametric *post hoc* tests were used for heteroscedastic data. Median effective concentrations ($EC_{50}$) were defined as the concentration that produced 50% light reduction after 30 min of contact time for bacteria and that induced 50% algal growth inhibition after 72 h of exposure. $EC_{50}$ and related 95% confidence limits were calculated using MicrotoxOmni and ProbAlg software.

Chemical characterization and particle size distribution of MPs used in this study show that measurements matched the particle diameter provided by the commercial companies, except for PE-MP size II and oxidized PE-MP size I-III (Table 2, Fig. 1). Regarding MP behavior, only non-oxidized PE MPs aggregated rapidly in sea water, conversely to oxidized MPs of the same size range (Supplementary Figure). A conclusive result was not possible for 125–500 μm PE given their larger size (out of range for the equipment used). Results on *V. fischeri* exposed to PE-MPs showed that none of MPs resulted to have toxic effects (Table 3). In each case, $EC_{50}$, NOEC and LOEC values were above tested concentration range (0.001–10 mg/L) irrespective of the method used. Likewise, exposure to different sizes of PE-MPs did not affect microalgal growth (Table 4) and it was not possible to calculate $EC_{50}$, nor NOEC and LOEC.

The PE-MPs used in this study were not toxic to the unicellular marine organisms exposed at environmentally relevant concentrations or even at higher concentrations. MP loads in sea water are within the μg/L range and the highest MP loads reported in the sea are around 0.08 and 0.3 mg/L (Lusher et al., 2014; Beiras, 2018). In this study, bacteria luminescence inhibition and micro algal growth were not affected by either the virgin or the oxidized PE-MPs at concentrations of up to 25 mg/L. These results confirm previous data on the absence of ecotoxicological effects included in standard test guidelines in decomposers and primary producers exposed to MPs. Booth et al. (2016) and Gagnè (2017), investigated MP ecotoxicological effects in the same bacterium and did not find any toxic effect at environmental concentrations. Likewise, no inhibition on the growth of *P. tricornutum* was observed after PE-MP exposure up to 25 mg/L, confirming previous findings on several marine microalgae (*D. tertiolecta*, *T. chuii*, *Skeletonema costatum*) exposed to a wide range of micro-sized plastic polymers (Davarpanah and Guilhermino, 2015; Sjollema et al., 2016; Zhang et al., 2017). In order to find the toxicity threshold on microalgae cellular growth, Sjollema et al. (2016) tested even higher concentrations, reporting algal growth inhibition by MPs at 250 mg/L, a concentration 3 orders of magnitude above maximum concentrations recorded in natural marine waters (reviewed by Beiras, 2018).

In this study, polyethylene MPs with a wide size range – from 1 to

**Table 1**
Parameters used by the three laboratories for microalgae bioassays.

| | Microalgae Strain | Container | Volume | Aeration | Medium | Exposure | Dynamic conditions |
|---|---|---|---|---|---|---|---|
| Laboratory 1 | *P. tricornutum*: B | Flask | 10 mL | No | FNSW | Photoperiod (16 light: 8 dark) | Orbital shaker (50 rpm) |
| Laboratory 2 | *P. tricornutum*: A | Vials | 25 mL | No | ASW | Photoperiod (16 light: 8 dark) | Rotatory wheel (1 rpm) |
| Laboratory 3 | *P. tricornutum*: A | Flask | 500 mL | Yes | FNSW | Continuous light | Air point |

FNSW: filtered natural seawater; ASW: artificial seawater.

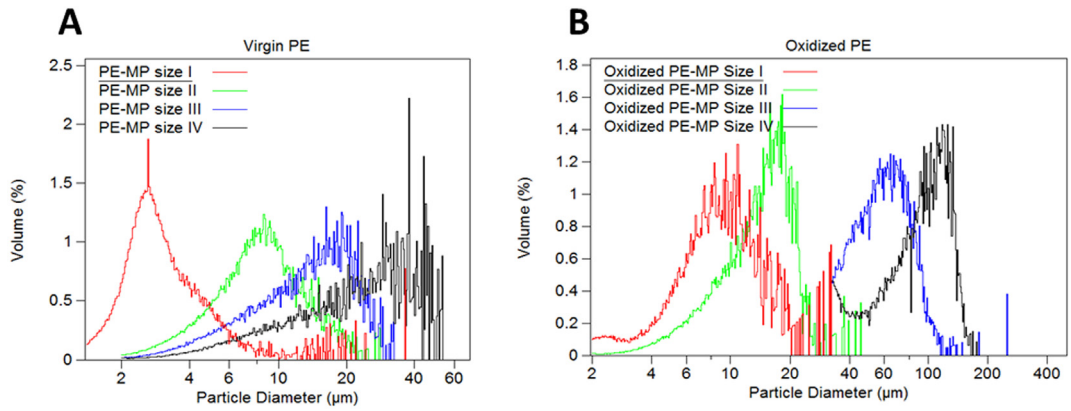


**Fig. 1.** Coulter Counter profiles of particle size distributions for PE-MP (A) and oxidized PE-MP (B).

500 µm – did not affect bacteria nor microalgal growth. These findings suggest that only MPs of smaller size (< 1 µm) would cause toxicity, since pore size of the cell wall is likely to prevent larger MPs to get through (Zhang et al., 2017). Standard endpoints included in the ISO guidelines can still be useful to detect ecotoxicological responses in decomposers and primary producers exposed to nanoplastics rather than MPs. In this regard, literature data show how nano-sized plastics affect marine bacteria and microalgae growth at high concentrations (Sun et al., 2018; Bergami et al., 2017; Gambardella et al., 2018). The growth of the bacterium *Halomonas alkaliphila* was affected under the stress of polystyrene nanoplastics (20 mg/L, Sun et al., 2018). Similarly, different studies on the microalgae *D. tertiolecta* demonstrated that nano-polystyrene (50 nm, 100 nm) inhibited its growth up to 45% at high concentrations (> 10 mg/L, Bergami et al., 2017; Gambardella et al., 2018). Similar results on microalgae have been also found in freshwater environment. Thus, Besseling et al. (2014) demonstrated that polystyrene nanoparticles (1 g/L) affected the population growth of the green alga *Scenedesmus obliquus*. These findings suggest the urgent need to identify and investigate alternative responses, different from the standard ones included in international guidelines, able to detect the potential risk of MPs in unicellular organisms.

This study supports the view that current MP levels in the oceans do not pose a risk to marine heterotrophic bacteria and microalgae. However, further investigations using alternative endpoints better suited to detect MP toxicity on marine bacteria and microalgae are necessary. Therefore, the current approach to MP testing in risk assessment should be corrected (Thompson et al., 2004), by providing alternative and sensitive endpoints to be included in standardized protocols.

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**Table 3**
$EC_{50}$, LOEC and NOEC values (mg/L) obtained in *V. fischeri* after 30 min exposure to the different microplastics (MPs).

| Particle name | MP size | Method | $EC_{50}$ | LOEC | NOEC |
|---|---|---|---|---|---|
| PE-MP size I | 1–4 µm | BT | > 10 | > 10 | > 10 |
| PE-MP size II | 4–6 µm | BT | > 10 | > 10 | > 10 |
| oxidized PE-MP size I | 6–8.5 µm | SPT | > 10 | > 10 | > 10 |
| PE-MP size III | 11–13 µm | SPT | > 10 | > 10 | > 10 |
| oxidized PE-MP size II | 11–15 µm | SPT | > 10 | > 10 | > 10 |
| PE-MP size IV | 20–25 µm | SPT | > 10 | > 10 | > 10 |
| oxidized PE-MP size III | 36–43 µm | SPT | > 10 | > 10 | > 10 |
| oxidized PE-MP size IV | 80–100 µm | SPT | > 10 | > 10 | > 10 |
| PE-MP size V | 125–500 µm | SPT | > 10 | > 10 | > 10 |

BT: Basic Test; SPT: Solid Phase Test; $EC_{50}$: median effective concentration; LOEC: lowest observed adverse effect concentration; NOEC: no observed adverse effects concentration.

**Table 4**
$EC_{50}$, LOEC and NOEC values (mg/L) obtained in *P. tricornutum* after 72 h exposure to the different microplastics (MPs).

| Particle name | MP size | $EC_{50}$ | LOEC | NOEC |
|---|---|---|---|---|
| PE-MP size I | 1–4 µm | > 25 | > 25 | > 25 |
| PE-MP size II | 4–6 µm | > 25 | > 25 | > 25 |
| PE-MP size III | 11–13 µm | > 25 | > 25 | > 25 |
| PE-MP size IV | 20–25 µm | > 25 | > 25 | > 25 |

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**Table 2**
Particle size distribution (in µm) of the MP tested measured by an electronic counter (Multisizer III from Beckman Coulter Counter). Distributions were measured in volume-mode and mean size, decil 10 (D10), decil 50 (50) and decil (D90) are shown. All data are expressed as the mean of three replicates ± standard deviation.

| Particle name | Nominal size (µm) | Volume-based particle size (µm) | D10 (µm) | D50 (µm) | D90 (µm) |
|---|---|---|---|---|---|
| PE-MP size I | 1–4 | 4.25 ± 0.60 | 1.99 ± 0.01 | 2.92 ± 0.03 | 6.31 ± 0.77 |
| PE-MP size II | 4–6 | 9.03 ± 0.29 | 4.31 ± 0.06 | 8.27 ± 0.13 | 14.86 ± 0.83 |
| PE-MP size III | 11–13 | 14.07 ± 0.29 | 5.65 ± 0.06 | 13.79 ± 0.15 | 22.76 ± 0.71 |
| PE-MP size IV | 20–25 | 24.46 ± 1.71 | 7.77 ± 0.49 | 23.65 ± 1.64 | 42.18 ± 3.25 |
| PE-MP size V | 125–500 | n.m. | n.m. | n.m. | n.m. |
| Oxidized PE-MP size I | 6–8.5 | 10.14 ± 0.31 | 4.63 ± 0.01 | 9.04 ± 0.28 | 16.80 ± 0.99 |
| Oxidized PE-MP size II | 11–15 | 14.73 ± 0.69 | 7.06 ± 0.18 | 14.64 ± 0.32 | 21.46 ± 0.72 |
| Oxidized PE-MP size III | 36–43 | 62.14 ± 1.52 | 37.98 ± 0.35 | 59.22 ± 1.20 | 87.35 ± 1.52 |
| Oxidized PE-MP size IV | 80–100 | 90.60 ± 4.79 | 38.67 ± 2.02 | 93.43 ± 4.41 | 131.25 ± 1.34 |

n.m.: not measurable.

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