Microplastics effects in *Scrobicularia plana*

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Abstract

One of the most common plastics in the marine environment is polystyrene (PS) that can be broken down to micro sized particles. Marine organisms are vulnerable to the exposure to microplastics. This study assesses the effects of PS microplastics in tissues of the clam *Scrobicularia plana*. Clams were exposed to 1 mg L^{-1} (20 μm) for 14 days, followed by 7 days of depuration. A qualitative analysis by infrared spectroscopy in diffuse reflectance mode period detected the presence of microplastics in clam tissues upon exposure, which were not eliminated after depuration. The effects of microplastics were assessed by a battery of biomarkers and results revealed that microplastics induce effects on antioxidant capacity, DNA damage, neurotoxicity and oxidative damage. *S. plana* is a significant target to assess the environmental risk of PS microplastics.

Key words: ecotoxicology, biomarkers, neurotoxicity, oxidative stress, genotoxicity, bivalves, peppery furrow shell

Main findings

S. plana is an important target of PS microplastics ecotoxicity.

S. plana can be a suitable biomonitor for assessing PS microplastics environmental risk.

Introduction

Plastics are used in everyday life and in several items: cars, electronic equipment, furniture, footwear, construction, food packages, among others. The largest plastics producers are the sectors of packaging (39%) and construction (21%), followed by transportation, agriculture, household and electronics (Pinto, 2012). The annual production of plastics increased considerably from 1.5 million tons in 1950 (decade where the commercial development of polyolefins, polypropylene and polyethylene started), to approximately 322 million tons in 2015 (PlasticsEurope, 2015; Wright *et al.*, 2013b), representing an increase of 9% per year, approximately. This production volume, coupled

with their high durability, resistance to degradation, low weight and low recycled volume, leads to the widespread and accumulation of discarded plastics in landfills and, as litter, in terrestrial and aquatic habitats worldwide (Derraik, 2002; Moore, 2008; Thompson *et al.*, 2004). The consumption of plastics in many European countries indicates that the plastic resins most used since 2007 are low density polyethylene (LDPE), high density polyethylene (HDPE), polypropylene (PP), polyvinyl chloride (PVC), polyethylene terephthalate (PET) and polystyrene (PS).

It is not possible to obtain reliable estimates of the amount of plastic debris reaching the marine environment, but they are, however, quite substantial (Derraik, 2002). The major sources of plastic materials and debris in the sea are fishing fleet (Cawthorn, 1989), and marine recreational activities (Pruter, 1987; Wilber, 1987). Plastic also reaches the sea as litter from land-based sources, carried by rivers and municipal drainage systems (Derraik, 2002; Williams & Simmons, 1997). In the sea, these versatile and non-biodegradable polymers are found in the form of larger items (macroplastics), including hulls of boats and fishing nets many meters long, and tiny fragments (Browne *et al.*, 2008; Canesi *et al.*, 2015). When exposed to UV-B radiation, to the oxidative properties of the atmosphere and to the hydrolytic properties of seawater, these plastics brittle and break into smaller pieces, until they reach micrometres in length (microplastics), and potentially, also the nano-scale level (nanoplastics) (Browne *et al.*, 2008; Canesi *et al.*, 2015).

Microplastics are defined as particles with less than 5 mm in diameter, according to the National Oceanic and Atmospheric Administration of the United States of America (NOAA, 2015). Their presence in the ocean is distributed according to the currents (Lusher, 2015). The distinction between primary and secondary microplastics is based on whether these particles were originally manufactured to be that size (primary) or whether they resulted from the breakdown of larger items (secondary) (Kershaw, 2015). The primary source of microplastics includes different typology; polyethylene (PE), polypropylene (PP) and polystyrene (PS) from cleaning products or cosmetics (Fendall & Sewell, 2009), or from industries or industrial effluents (Lusher, 2015). The secondary source is the degradation of plastics under marine conditions, that dramatically reduces the molecular weight of these polymers (Andrady, 2011). Ideally, these particles may also undergo further degradation by microbial action, releasing carbon (Andrady, 2011). Although microplastics greatly exceed large plastic items in marine systems, they are still only a small proportion of the total mass of plastics in the ocean. Therefore, microplastics

100 became a growing issue in such a way that the Marine Strategy Framework Directive
101 (MSFD Nº 2008/56/EC) highlights microplastics and their associated chemicals as one
102 of the major policy descriptors whose impact need to be assessed in the marine
103 environment (Zarfl *et al.*, 2011).

104 The presence of microplastics is documented in most habitats in the open ocean,
105 seas and beaches, surface waters, the water column and in the deep ocean (Lusher, 2015),
106 and recently, in freshwater systems (Eerkes-Medrano *et al.*, 2015). In Portugal, 62% of
107 microplastics were identified in the North Atlantic by trawling, with a density of 580 000
108 particles per km^2. 61% of the water samples collected in the Portuguese coast contained
109 microplastics and the concentration was higher in the Vicentina Coast and Lisbon (0.036
110 and 0.033 particles m^{-3}, respectively) than in the Algarve and Aveiro areas (0.014 and
111 0.002 particles per m^3, respectively) (Lusher, 2015).

112 The occurrence of plastic in the ocean and the potential impact to marine
113 organisms are of growing concern (Canesi *et al.*, 2015). The fact that microplastics have
114 such a small size and different shapes actively contributes to their bioavailability and
115 accumulation in organisms of lower trophic levels. As the particles interact with plankton
116 and sediments, both organisms that feed on suspended particles and the ones that feed on
117 the bottom are at risk of, accidentally or selectively, ingesting plastic (Lusher, 2015).
118 Particles with less than 20 μm are likely to be ingested and egested (Lee *et al.*, 2013) by
119 small organisms (Thompson *et al.*, 2004; Wright *et al.*, 2013b). Microplastics with size
120 between 1 and 5 mm can compromise the nutrition and digestion (Codina-García *et al.*,
121 2013). The ingestion of plastics with a greater size can cause serious external and internal
122 injuries, ulcers, digestive tract blockage, false sense of fullness, loss of feeding capacity,
123 impairment and inability to avoid predators or death (Gall & Thompson, 2015).

124 Microplastics ingestion was documented for a wide range of marine vertebrates
125 and invertebrates for wild populations (Table 1). Whilst it is apparent that microplastics
126 have become widespread and ubiquitous in the marine environment, the information on
127 accumulation, mode of action and biological impact of this emerging contaminant in
128 marine organisms is still scarce (Wright *et al.*, 2013b). Microparticles are inert and, after
129 ingestion, pass through the cell membrane and are incorporated into tissues, particularly
130 in the gut cavity (Wright *et al.*, 2013b). After passing to the circulatory system they are
131 transferred to other organs, via hemolymph, where they are retained for several weeks in
132 several organs and cause adverse effects (Browne *et al.*, 2008). Experiments with

different sizes (Mazurais *et al.*, 2015), shapes (Graham & Thompson, 2009) and types (Green *et al.*, 2016) were conducted in order to mimic a more realistic scenario and truly assess the effects of microplastics. Table 2 summarizes the information about the ecotoxicological effects of microplastics in several bivalves that include weight loss, reduced feeding activity, increased phagocytic activity, transference to the lysosomal system, accumulation and inhibition of acetylcholinesterase (AChE) activity (e.g. Avio *et al.*, 2015a; von Moos *et al.*, 2012; Van Cauwenberghe *et al.*, 2015).

The aim of this study was to investigate the presence and mode of action of polystyrene microparticles (one of the most largely used plastic worldwide, with a density of 1.09 g cm^{-3}) in different tissues of the peppery furrow shell *Scrobicularia plana* and assess the potential ecotoxicological risk of this emerging contaminant in this species. The presence of microplastics in gills and digestive gland of *S. plana* was evaluated by infrared spectroscopy in diffuse reflectance mode and their effects by using a battery of biomarkers of oxidative stress (superoxide dismutase (SOD), catalase (CAT), glutathione peroxidases (GPx), biotransformation (glutathione-S-transferases (GST)), genotoxicity, neurotoxicity and oxidative damage.

Materials and methods

Microplastics characterization

Monodisperse PS microplastics were obtained from Sigma-Aldrich (Germany) with the particle size 20 µm and density 1.05 g cm^{-3}. Two stock solutions (100 mg L^{-1}) were prepared: one in ultrapure water (18 MΩ/ cm) and another in natural seawater (S = 35), both maintained in constant aeration.

The microplastics size was determined by optical microscopy (OM) and dynamic light scattering (DLS), and the surface charge (zeta potential) by electrophoretic light scattering (ELS), for both PS solutions. The zeta potential of the microparticles was determined by electrophoresis mobility measurements at 25ºC using a DLS particle sizer (ZetaSizer Nano ZS90, Malvern Inc.) in a disposable polycarbonate capillary cell (DTS1061).

164 The sedimentation rate (SR) was measured by the change of turbidity with time
165 (0-24 h), as described in Sousa and Teixeira (2013). The SR relates to the normalized
166 microparticle turbidity C/C_0, where C is the turbidity at time t and C_0 the initial turbidity
167 at time 0. Then, the SR is given by the expression $\delta(C/C0)/\delta t$, estimated from the decrease
168 in turbidity (C/C_0), which occurred within the first two hours for the fast sedimentation
169 (fast SR) conditions and within 3-24 h for slow sedimentation (slow SR) conditions
170 (Keller *et al.*, 2010).
171

172 **Laboratory exposure assay**

173 *Scrobicularia plana* (38 ± 5 mm shell length) were collected in Cabanas de Tavira,
174 Ribeira do Almargem (South of Portugal) (N 37º7'59.75" W 7 36'34.95") and transferred
175 to the laboratory, where they were acclimated for 7 days at constant aeration, with a
176 photoperiod of 12h light and 12h darkness. Three replicate aquaria were used for each
177 control and exposed group to 1 mg L^{-1} of PS microplastics which corresponded to around
178 4 particles ml^{-1}. This value is below the concentrations used in previous laboratory
179 exposure experiments (summarized in Table 2) although higher than environmentally
180 relevant concentrations of microplastics found in seawater in regions highly
181 contaminated, e.g. ~0.5 particles mL^{-1} in South Korea (Song *et al.*, 2014) or 0.1 particles
182 mL^{-1} (Norén, 2007).
183 Sixty clams were placed in each glass aquaria filled with 20 L of natural seawater
184 with constant aeration and no sediments were added. Glass Pasteur pipettes were used to
185 provide aeration and the use of plastic material was avoided during the experiment.
186 Exposure ran for 14 days, followed by 7 days of depuration. The water was changed every
187 24 hours with subsequent addition of PS microplastics. Immediately before addition of
188 PS microplastics, the 100 mg L^{-1} stock solution in ultra-pure water was sonicated for 30
189 minutes (Ultrasonic bath VWR International, 230 V, 200 W, 45 kHz frequency). During
190 the experiment abiotic parameters were checked in all tanks by measuring temperature
191 (18.0 ± 1 ºC), salinity (35 ± 0.2), percentage of oxygen saturation (93.0 %) and pH (7.8),
192 with the multiparametric probe TRIPOD (from PONSEL). Clams were not fed to avoid
193 any interaction of microplastics and food.
194 Unexposed and exposed clams were collected after 0, 3, 7 and 14 days of
195 exposure, and after the 7 days of depuration. The hemolymph was immediately collected
196 by gently prying the shell open approximately 2 to 3 mm with a scalpel and the shell was

held open with forceps. Then, the posterior adductor muscle of the *S. plana* was gently penetrated with a sterile hypodermic syringe 1 ml (12 mm x 12:33) and the hemolymph easily collected using intermittent suction. To avoid potential contamination of the haemolymph, the water inside the shell was drained from each clam prior to hemolymph extraction, and a new syringe was used for each replicate of each treatment and each sampling day. Gills and digestive gland were dissected and stored at -80 ºC until analysis. No significant mortality was observed between treatments, during the accumulation and depuration periods (p >0.05).

Qualitative assessment of microplastics accumulation

A qualitative assessment of microplastics accumulation and transport to different tissues was conducted by optical microscopy (OM) analysis of the hemolymph of control and exposed clams at day 14. In addition, gills and digestive gland tissues collected at different times of exposure were lyophilized at -40 ºC, during approximately 48 hours with a Modulyo freeze dryer and analysed by infrared spectroscopy. This analysis was performed in a Mattson RS1 Fourier transform infrared spectrophotometer, with a wide band MCT (mercury cadmium telluride) detector, in the range 400-4000 cm^{-1}, at 4 cm^{-1} resolution. Since the samples were powders, in order to avoid compressing the tissues to the high pressures needed to prepare disks, the most convenient mode for obtaining the spectra was in diffuse reflectance (DRIFT). Each lyophilized sample was diluted (~1:4) in KBr (from Aldrich, FTIR grade) and finely grinded in an agate mortar, to reduce particle size and thus decreases diffuse Fresnel reflectance. This mixture was placed in a 11 mm diameter sample cup and pressed to obtain a very smooth surface. The cup was filled, in order to attain a so-called infinite thickness (all the light is reflected or absorbed by the sample) and mounted in a Graseby/Specac Selector accessory, to collect all the diffusely reflected radiation, excluding specular reflection. Each DRIFT spectrum resulted from the ratio of 500 single-beam scans obtained for the sample to the same number of background scans for pure KBr.

The spectra in reflectance (R_∞) were transformed into Kubelka-Munk units [$f(R_\infty)$] using the FIRST software, according to the Kubelka-Munk equation (Mitchell, 1993; Stuart, 2005)

$$f(R_\infty) = \frac{(1 - R_\infty)^2}{2R_\infty}$$

The spectra were baseline corrected, normalized to a typical band of the tissues not overlapped with a PS band, and the average of 10 samples was calculated.

Condition index

To assess the physiological status of control and PS exposed clams, soft tissues and shells were weighted, and the condition index (CI) determined as the percentage (%) of the ratio between drained weight of the soft tissues (g) and total weight (g), according to Gomes *et al.* (2013).

Biomarker analysis

Antioxidant enzymes

Prior to the analysis of the enzymatic activities, the tissues (gills and digestive glands) of control and microplastic exposed clams (6 replicates of individual tissues) were weighed and rapidly buffered in Tris-HCl buffer (50 mM Tris-HCl, 250 mM Sucrose, 5mM $MgCl_2$, 1mM DTT, pH=7.6) (the tissue-to-buffer ratio was 1:3 wet weight tissue/volume of buffer), homogenized in an ice bath and centrifuged at 10000 *g*, for 10 minutes, at 4 ºC, using a biofuge stratus 230 V centrifuge (Thermo scientific, Germany). Both the soluble and pellet fractions were stored at -80 ºC for future analysis. Enzyme activities were measured in the cytosolic fraction. To determine SOD activity, the reduction of cytochrome *c* by the system xanthine oxidase/hypoxanthine was measured at 550nm (McCord & Fridovich, 1969) and results expressed in U mg^{-1} of total protein concentration. CAT activity was determined by the decrease in absorbance at 240nm due to H_2O_2 consumption, with a molar extinction coefficient of 40 M^{-1} cm^{-1} (Greenwald, 1987) and results expressed as µmol min^{-1} mg^{-1} of total protein concentration. GP_X activity was measured through NADPH oxidation in the presence of excess glutathione reductase, reduced glutathione and hydroperoxide as substrate, at 340nm (Lawrence & Burk, 1978) and results expressed as nmol min^{-1} mg^{-1} of total protein concentration. GST activity was measured by the conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) with

reduced glutathione (GSH) and the increase of absorbance measured at 340 nm (Ɛ 340 (CDNB) = 9.6 mM^{-1} cm^{-1}) (Habig *et al.*, 1974). The results are expressed in µmol CDNB min^{-1} mg $protein^{-1}$.

Oxidative damage

Before the analysis of oxidative damage, gills and digestive gland (6 replicates of individual tissues per treatment) were weighed and rapidly buffered in 0.02M Tris-HCl (0.1 M HCl, 0.2 M Tris, pH=8.6) (the tissue-to-buffer ratio was 1:3 wet weight tissue/volume of buffer). Then, 10 µl of BHT (Butylated hydroxytoluene) was added, per each ml of Tris-HCl [0.02M]. The samples were homogenized in an ice bath and centrifuged at 30000 g, for 45 minutes, at 4 ºC, using a biofuge stratus 230 V centrifuge (Thermo scientific, Germany). Both the cytosolic and mitochondrial fractions were stored at -80 ºC for future analysis. Lipid peroxidation (LPO) was quantified based on the method described by Erdelmeier *et al.* (1998) - determining malondialdehyde (MDA) and 4-hydroxyalkenals (4-HNE) concentrations upon the decomposition by polyunsaturated fatty acid peroxides. The tissue supernatant (200 µL) was incubated at 45 ºC, for 60 minutes, with 650 µL of 1-methyl-2-phenylindone diluted in methanol and 150 µL of methanesulfonic acid. The absorbance was measured at 586 nm and LPO levels are expressed as nmol malondialdehyde (MDA) + 4-HNE g^{-1} per mg of protein.

AChE activity

Gills (6 replicates of individual tissues per treatment) were homogenized on ice in five volumes of a Tris-HCl buffer (100 mM, pH 8.0) containing 10% Triton X-100 and centrifuged at 12000g, for 30 minutes, at 4º C. Anti-cholinesterase activity was measured by the modified Ellman's colorimetric method (Ellman *et al.*, 1961), assessed by the addition of Ellman's reagent – DTNB - using AChEI (acetylcholine) as substrate, for the estimation of respective thiocholine (ChE). The absorbance is measured, at 405 nm (coefficient of extinction of $\varepsilon = 13.6$ $mM^{-1}.cm^{-1}$), to estimate the amount of ChE liberated by the reaction, which is proportional to the AChE activity (Colovic *et al.*, 2013). The results are expressed by nmol AChEI min^{-1} mg $protein^{-1}$.

293 **Genotoxicity**

294 Genotoxicity was estimated using the comet assay in a slightly modified version
295 of Singh *et al.* (1988) and described in Almeida *et al.* (2011). Microscopic slides were
296 coated with 0.65% normal melting point agarose (NMA), in Tris-acetate EDTA. After
297 collection, hemolymph cells were centrifuged at 3000 rpm for 3 min (4 ºC), and the pellets
298 with isolated cells suspended in 0.65% low melting point agarose (LMA) in Kenny' s salt
299 solution, and casted on the microscope slides. Afterwards, the slides with the embedded
300 cells were immersed in a lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1%
301 Triton X-100, 10% Dimethylsulfoxide, 1% Sarcosil, pH 10, 4 ºC), for the diffusion of
302 cellular components and DNA immobilization in agarose. Following the lysis step, slides
303 were placed in an electrophoresis chamber containing electrophoresis buffer (300 mM
304 NaOH, 1 mM EDTA, adjusted at pH 13, 4 ºC), gently submerged and left in this solution
305 for 15 minutes, to permit DNA unwinding. The electrophoresis was carried out and, once
306 concluded, the slides were removed and immersed in a neutralizing solution (0.4 mM
307 Tris, pH 7.5) and rinsed with ultrapure water. Then, the slides were allowed to dry for 48
308 hours, from which the analysis in fluorescence microscope was possible. The slides were
309 stained with 4,6-diamidino-2- phenylindole (DAPI, 1 mg mL^{-1}) and the presence of
310 comets analysed using an optical fluorescence microscope (Axiovert S100) coupled to a
311 camera (Sony). Fifty randomly chosen cells for each slide (25 in each gel from each
312 organism) of nine organisms were scored with the Komet 5.5 image analysis system
313 (Kinetic Imaging Ltd) at a total magnification of x400. Different parameters of the comet,
314 including the olive tail moment (OTM), comet tail length and amount of DNA in the
315 comet tail (Tail DNA %) were measured, and results are expressed as mean ± STD.
316

317 **Statistical analysis**
318

319 Statistical differences between treatments, time of exposure/depuration and
320 tissues were assessed using parametric tests (two-way ANOVA, followed by the Tukey's
321 test) and non-parametric tests (Kruskal-Wallis, followed by multiple comparisons), after
322 normality (Shapiro-Wilk W test) and homogeneity of variances verified (Levene's test).
323 Principal component analysis (PCA) for gills and digestive gland was used to evaluate
324 the influence of PS microplastics in the determined biomarkers, in exposed clams, along

the period of exposure and assess the overall results. Statistical significance was defined at $p < 0.05$.

Results

PS microplastics characterization

PS microplastics characterization is summarized in Table 3. DLS measurements show that d_h (hydrodynamic diameter) of PS microplastics is 18.4 ± 1.33 μm, which is similar to the size specified by the manufacturer. ζ - potential measurements show that PS microplastics have highest negative surface charge in seawater (-12.4 ± 2.36 mV) than in Milli-Q water (-52.6 ± 2.34 mV) ($p < 0.05$), showing the tendency of these PS microplastics to aggregate in seawater.

Turbidity (C/C_0) of PS microplastics suspension was measured to assess the sedimentation rate (SR) (Figure 1). The SR is faster during the first two hours, estimated from the initial 5% decrease in normalised particle turbidity and slows down after this period of time. No significant differences in turbidity decrease were observed between Mili-Q water (97.94%) and seawater (94.65 %) over time (24 h) ($p > 0.05$). However, a sharp decrease in turbidity is observed in MQ water during the first 2 hours (1.68×10^{-1} h-1) although higher when compared to seawater (1.04×10^{-1} h^{-1}) ($p < 0.05$), while for the last 22 hours there is a higher decrease in SR for MQ water (3.05×10^{-4} h^{-1}) compared to seawater (1.16×10^{-3} h^{-1}) indicating that, after the initial time, PS microplastics tend to sediment faster in natural seawater ($p < 0.05$).

Condition index

The condition index at the beginning of the experiment (time 0) was 36.01 ± 4.04 %. No significant changes were observed between unexposed and exposed organisms after 14 days (unexposed: 33.05 ± 4.76 %; exposed: 31.53 ± 5.30 %; $p>0.05$) and in the elimination period (unexposed: 31.31 ± 4.58 % exposed: 31.83 ± 4.72 %; $p>0.05$), indicating that the organisms were in good health throughout the duration of the experiment.

Qualitative assessment for microplastics accumulation

In order to corroborate the presence of PS in the clams exposed to the aquatic environment containing microplastic, optical microscopy and infrared data were analysed.

The PS microplastics present in the hemolymph observed under the OM are in Figure 2. The presence of a small aggregate of PS microplastics is observed in image A and two polystyrene particles in image B (highlighted by circles) indicating that, like for seawater, there is a tendency for the PS microplastics to form small aggregates/agglomerates in the hemolymph.

The diversity of the tissue samples implies variations of the infrared spectra from individual specimens in the same conditions. Moreover, due to the amount of PS microplastics expected to accumulate during a 14 days exposure, the spectral analysis is not intended as quantitative, but only as a proof of detection of PS in the gills and digestive gland of the clams. It consisted in a comparison of the average spectra of unexposed clams with clams exposed to PS microplastics.

In Figure 3, the DRIFT spectra of digestive gland and gills from clams not exposed to PS microplastics (DG T0 and Gills T0, respectively) are compared to that of digestive gland from specimens into which microplastics were directly added *in vitro*, in a known amount (50 µl). The spectrum of the same PS microplastics is also included in Figure 3 for comparison. In order to allow the comparison of the relative intensities of the bands, the spectra were normalized to the amide II mode (C-N stretching), at 1543 cm^{-1} (Matthäus *et al.*, 2008), common to all of them, and not overlapped with any PS band or the water deformation mode. The average spectra of the digestive gland and gills are similar, with some features specific to the gills: a small band at 3060 cm^{-1}, a clear splitting of the bands at 1082/1047 cm^{-1} and at 609/588 cm^{-1}. On the other hand, the region below 1200 cm^{-1} is much stronger (relatively to the amide II band) for the digestive gland. The average spectrum of digestive gland with added microplastics (DG Added PS) shows a general increase of relative intensities in the regions overlapped with PS bands; new small bands appear at 696 and 737 cm^{-1} that may be assigned to the strongest bands of the polymer, slightly shifted due to interactions with the specimen tissues. In the spectrum of PS microplastic these bands appear at 700 and 756 cm^{-1}, and are related to out of plane C-H deformation modes of the benzene rings (Holland-Moritz & Siesler, 1976; Koenig, 1999; Liang & Krimm, 1958).

The average DRIFT spectra of samples obtained before exposure to PS microplastics (T0) were compared to those exposed for 14 days (T14) and after 7

depuration days (T21) (Figures 4A and C for gills and digestive gland, respectively). Both for gills and digestive gland the spectral region where PS absorbs mostly is stronger after the exposure period and decreases during depuration, not achieving the initial relative intensities.

The spectral subtractions (T14-T0) and (T21-T0) were calculated and compared to the spectrum of PS microplasctics (Figures 4B and D for gills and digestive gland, respectively). The differences (T14-T0) are generally positive for both tissues, evidencing the accumulation of PS microplastics during exposure. For the gills, the region where PS absorbs more strongly (below 1000 cm^{-1}) corresponds to larger differences between T14 and T0, and some relative maxima observed in the high wavenumber region are coincident with (or slightly shifted from) PS bands. For the digestive gland, clear maxima in T14-T0 differences are coincident with PS bands, at 1725, 1590, 1435, 1360 and 1275 cm^{-1}. In the low wavenumber region, the spectra at 0 and 14 days are both strong, rendering the definition of eventual PS bands more difficult. A partial elimination of PS during the depuration period is unambiguous from the spectral differences (T21-T0) that remain positive for both tissues, although smaller than (T14-T0).

Enzymatic activity

The antioxidant enzymes (SOD, CAT, GPx) and GST activities in the gills and digestive gland of *S. plana* are presented in Figure 5. Only CAT shows a tissue specific response with higher activity in the digestive gland when compared to the gills ($p < 0.05$).

In the gills, the exposure to PS microplastics induces a significant increase in SOD activity after 7 days of exposure that continues in the depuration period ($p<0.05$) (Figure 5A), while for CAT activity there was a significant increase only after three days of exposure ($p<0.05$)(Figure 5B). Similarly, in PS exposed clams, GPx activity increases in clams exposed to PS microplastics after the 3rd day of exposure ($p < 0.05$) and similarly to SOD, GPx continues to increase at the end of the depuration period ($p < 0.05$) (Figure 5C). Moreover, exposure to PS microplastics induces an enhancement of GST activity at the end of the exposure period ($p < 0.05$), but it decreases in the depuration period ($p < 0.05$) (Figure 5D).

In the digestive gland, the SOD activity, like in the gills, increases with time of exposure, but this increase was only significant at the end of the exposure period (day 14, $p < 0.05$). Similarly to the gills, SOD activity in the digestive gland also increases at the end of the depuration period ($p < 0.05$) (Figure 5E). On the other hand, CAT activity in PS contaminated clams decreased ($p < 0.05$) with no differences in the depuration period ($p > 0.05$) (Figure 5F). GPx activity, however, showed a bell shape behaviour with a significant increase after the 3rd day of exposure and a decrease in the remaining period (Figure 5G). In clams exposed to PS microplastics, GST activity only significantly decreased after the 3rd day of exposure ($p < 0.05$). This decrease was similar at the end of the depuration period ($p < 0.05$) (Figure 5H).

Comet assay

Genotoxic effects for both treatments (CTR and PS exposed clams), analysed by the comet assay and expressed as % of tail DNA and Olive Tail Moment are in Figure 6A-B, respectively. No significant changes were observed for the % of tail and OTM between controls and exposed clams except for day 7 where a significant increase occurred for OTM in exposed clams ($p < 0.05$). In the depuration period, significant differences were detected between control and clams previously exposed to PS microplastics ($p < 0.05$) for both parameters.

AChE activity

The activity of AChE in the gills is in Figure 7. In PS exposed group the AChE activity significantly decreased ($p < 0.05$) on day 3 when compared to T0. AChE activity was also significantly lower on day 3 and 14 of exposure and after the depuration period, when compared to non-exposed clams ($p < 0.05$).

Oxidative damage

LPO levels were significantly higher in the digestive gland then in the gills (($p < 0.05$; Figure 8A-B). In the gills of PS exposed clams, although LPO levels remained unchanged through time, they significantly decreased compared to those at the beginning of the experiment (day 0) and to the other sampling days ($p < 0.05$) while in the digestive gland, in PS exposed clams, LPO levels significantly increased after 7 days of exposure

compared to controls (p <0.05). In the elimination period, in the digestive gland of PS exposed clams LPO levels significantly decreased when compared to controls (p <0.05).

Principal Component Analysis

PCA was applied to all the data for the gills and digestive gland to explain the effects of PS microplastics on biomarkers responses (Figure 9). Regarding the gills, the two principal components represent 72.7 % of total variance, with PC1 representing 53.4 % and PC2 19.3 % (Figure 9 A). PCA indicates a clear separation between the initial time of the experiment (T0) and the remaining days, between exposed clams and controls and also a clear separation of the depuration period in exposed clams (T21) comparing to others. SOD, CAT, GPx and the genotoxic parameters are in the positive part of PC1, closely related with PS exposed clams, principally after 7 days of exposure (MICR 7). LPO and AChE are negatively related to the other biomarkers in PC1 and are more influenced by non-exposed clams. In PC2, SOD, LPO, AChE and genotoxicity are in the negative part, with SOD and genotoxic parameters being more influenced by exposed clams on day 7 and after depuration (MICR 21). CAT, GPx and GST are in the positive part of PC2, with particularly GST being more influenced by exposed organisms at days 3 and 14.

In the digestive gland, the two principal components represent 75.2 % of total variance, with PC1 representing 53.4 % and PC2 21.8 % (Figure 9 B). In this case, there is a clear separation between unexposed and exposed clams, where all the non-exposed clams are in the positive part of the PC1. SOD and the genotoxic parameters are in the negative part of PC1 and more related to exposed clams at days 7 and 14 together with the MICR 21, while CAT, GPx, GST and LPO are on the positive side and more related to non-exposed clams. PC2 clearly isolates exposed clams at day 3 (MICR 3) from the rest, although exposed clams from day 7 also fall in the negative part of PC2. Also in the negative part of PC2 are LPO and GPx, with GST, CAT and SOD and Tail DNA in the positive side.

Discussion

The presence and mode of action of polystyrene microparticles in the gills and digestive gland of the peppery furrow shell *S. plana* and the potential ecotoxicological

risk of this emerging contaminant was evaluated by characterizing the type of microplastics used and identifying their presence using infrared spectroscopy in diffuse reflectance mode, and by assessing their effects using a battery of biomarkers. These biomarkers were chosen to evaluate the possible effects that come from the physical damage caused by the microparticles, namely: oxidative stress and oxidative damage (through enzymatic activity and LPO), which in turn can lead to genotoxicity and/or neurotoxicity (analysed through the comet assay and the AChE activity).

One of the questions that arise from laboratory experiments is whether they are able to really mimic the natural environment (Phuong *et al.*, 2016) in terms of environmental relevant exposure concentrations. Higher concentrations of microplastics have been found in sediments, 3.3 particles g^{-1} (Rhine estuary; Leslie *et al.*, 2013) or 62 particles g^{-1} (Wadden sea islands; Liebezeit & Dubaish, 2012). A major concern is the lack of information on the environmental concentrations for plastic particles smaller than 50 μm, with only a few exceptions reported and where it was noted that the smaller particles were much more abundant than the larger ones (Song *et al.*, 2014). For these reasons, it was considered that the concentration of 1 mg L^{-1} (4 particles ml^{-1}) was a good compromise between measured environmental concentrations taking into account, both the water and sediment compartments, acknowledging also the lack of data on the concentration for smaller sized particles and their potential to exist in the environment in higher concentrations than those currently detected for larger particles.

The combination of multiple analytical techniques (OM, DLS, ELS) to characterize PS microplastics using both natural seawater and ultrapure water (Milli-Q) (Table 3 and Figure 1) provide an insight about the hazard and risk of these microparticles in the aquatic environment. Size and density are important proxies for microplastics bioavailability that can also be enhanced by biological factors (Wright *et al.*, 2013b). The PS microplastics used had a density of 1.09 g/cm^3 and took 24 hours to sediment. Data indicated that the particles start reaching the bottom after 2 hours of exposure and thus are available to the organisms present therein. The PS microparticles used in this assay are spherical in shape and of the same size (20 μm) and tend to form small aggregates in seawater (Table 3). Similarly, the ingestion of PS spheres (100 nm) in suspension-feeders bivalve molluscs increased when they were fed with microplastics aggregates generated manually in the laboratory (Wright *et al.*, 2013a). Therefore it is hypothesised that the effects of PS microplastics might be caused by the formation of aggregates when accumulated in clam tissues, although it could be different if the microparticles were

heterogeneous and not of the same shape and size (Frias, 2015). Most of the microplastics found in the marine environment are secondary microplastics, resulting from the degradation of larger pieces, and thus, with different sizes, shape and sharp edges, that may contribute to injuries in the digestive tract (Browne *et al.*, 2008; von Moos *et al.*, 2012).

S. plana is able to accumulate PS microplastics from seawater in the gills and digestive gland (Figures 3 and 4). Bivalves are able to select particles before ingestion but not after ingestion (Wright *et al.*, 2013b). The presence of microplastics in the gills was noted after 14 days of exposure, with a partial recovery during the depuration period. This suggests that PS microplastics are trapped in this organ, the first in contact with the polymer. The microparticles are also ingested through the inhalant siphon and subsequently transported to the mouth and to the digestive gland for intracellular digestion (Hughes, 1969). This was noted by the presence of PS microparticles in *S. plana* digestive gland (Figures 4 C-D), where they are likely not digested, and some of them were eliminated. The presence of small aggregates of microplastics in the haemolymph (Figure 2) indicates that PS microplastics were transported into the circulatory system, indicating a possible translocation, where they can be retained for several weeks and then transported to several tissues where they can cause harm. But, the question is if the translocation really occurred or was it a contamination by microplastic while sampling haemolymph? Lambert *et al.* (2014) discuss whether the presence of microplastics in the circulatory system is due to translocation or contamination due to sampling technique. PS microplastics in *M. edulis* persisted in the circulatory system for 48 days (Browne *et al.*, 2008), giving an evidence of microplastic translocation. The accumulation, upon ingestion, of 2 µm and 4-16 µm PS fluorescently labelled microplastics (0.51 µg L^{-1}) in the gut cavity and digestive tubules of *Mytilus edulis* was also observed by Browne *et al.* (2008), after 12 hours of exposure. The same author identified PS microparticles in the haemolymph and haemocytes of the same mussel species although no toxicity effects were observed. Conversely, in mussels exposed for 48h to microplastics (1-80 µm) an increase in haemocytes and a decrease in lysosomal membrane stability was observed in the same mussel species, indicating the presence of an inflammatory process (Bowmer & Kershaw, 2010). Also, von Moos *et al.* (2012) shows that HDPE particles (0-80 µm) were transported to the digestive gland where they accumulated in the lysosomal system of *M. edulis*. However, in oysters *C. gigas* exposed to fluorescent microplastic beads (of 2 and 6 µm), microplastics were only present in the digestive gland (Sussarellu *et al.*, 2016),

existing no evidence of transfer from the digestive track to the circulatory system. Although there is some controversy about the translocation of microplastic in the haemolymph of bivalve molluscs, the data reported is only on filter-feeders (Browne *et al.*, 2008; von Moos *et al.*, 2012). It might occur that in the case of suspension-feeders like *S. plana*, microplastics tend to translocate in the circulatory system but this needs to be confirmed in future studies. In *S. plana* exposed to gold nanoparticles (Au NPs), nanoparticles were accumulated almost exclusively in the digestive gland, although they were also present in gills. These results highlight the accumulation of both nano and microparticles essentially in the digestive tract of this clam species. The accumulation of microplastics in this tissue might impair the digestive system with a consequent decrease of feeding behaviour. There is very limited information regarding the capacity of aquatic organisms to eliminate microplastics. The present results indicated that after a week of depuration, microplastics were still present in both tissues (Figure 4). Future studies that quantify the amount of microplastics accumulated in clam tissues and assess whether microplastics are eliminated in the pseudo-faeces of *S. plana* are necessary, in order to increase the knowledge about the accumulation, metabolism and elimination of PS microplastics in this species.

A battery of biomarkers was used to assess the biological effects and toxicity of PS microplastics in the gills and digestive gland of *S. plana.* Results indicate an oxidative stress response in gills and digestive gland cells that are tissue and exposure time dependent (Figures 5A-E). SOD is the first defence line to protect these tissues against oxidative stress probably caused by injuries of PS microplastics in the tissues. SOD activity enhanced in both tissues in the presence of PS microplastics reflects the need to balance the excess of superoxide radical ($O_2^{\bullet -}$) into the less damaging hydrogen peroxide (H_2O_2) and thus, contribute to prevent cellular oxidative damage (Jo *et al.*, 2008). The same response occurred in marine mussels *Mytilus spp.* exposed to 32 μg L^{-1} of PS microplastics (2 and 6 μm) after 14 days (Paul-Pont *et al.*, 2016). CAT is involved in the removal of H_2O_2 - the main precursor of hydroxyl radical in aquatic organisms - and acts as a defence mechanism toward the exogenous source of H_2O_2 (Regoli & Giuliani, 2014). However, CAT activity was only enhanced in the gills after three days of exposure and inhibited in the digestive gland after 7 days of exposure (Figure 5B, F) and is apparently not the antioxidant defence mechanism used by *S. plana* to respond to PS microplastics toxicity. Avio *et al.* (2015) also noted an inhibition of CAT activity in the digestive tissue of the marine mussel *M. galloprovincialis* exposed to microplastics. CAT and GPx are

both involved in the removal of H_2O_2. Regarding GPx, an increase in activity after 3 days of exposure, suggests a defence mechanism, but the posterior reduction in GPx activity, particularly in the digestive gland (Figure 5G), may be an indication of an inhibition triggered by excess of ROS and the incapacity to deal with the negative effect of this stressor. Glutathione peroxidases are known to be particularly sensitive in revealing the early onset of a pro-oxidant challenge, even at low levels of environmental disturbance (Regoli & Giuliani, 2014). In *S. plana* exposed to mercury, a decrease in GPx activity in the whole soft tissues occurred due to the higher toxicity of mercury (Ahmad *et al.*, 2011). Such ROS perturbations were also observed in mussels (*M. galloprovincialis*) exposed to PS and PE microplastics alone or in combination with pyrene (Avio *et al.*, 2015). The pro-oxidant challenge induced by microplastics on mussels was supported by the lack of significant variation of malondialdehyde, lipofuscin and neutral lipids in digestive tissues. The ROS production led to an inhibition of CAT and Se-GPx in *M. galloprovincialis* exposed to PS and PE microplastics (Avio *et al.*, 2015). Browne *et al.* (2013) showed that PVC microparticles induced an oxyradical production in *Arenicola marina*, and lugworms that ingested sediments with PVC reduce the capacity by more than 30% to deal with oxidative stress. PS microbeads also led to an increase in ROS production in haemocytes and to the enhancement of anti-oxidant and glutathione-related enzymes in mussel tissues (Paul-Pont *et al.*, 2016).

GST is usually associated with phase II biotransformation, involved in the metabolism of lipophilic organic compounds by catalysing the conjugation of the reduced form of glutathione (GSH) to xenobiotic substrates, and has also a protective role against oxidative stress (Lesser, 2006). *S. plana* may be using this detoxification mechanism to deal with the exposure to PS microplastics, by catalysing the conjugation of the reduced form of glutathione (GSH) to xenobiotic substrates, playing a significant role in the detoxification of the reactive products from lipid peroxidation (Lesser, 2006). Gills showed an increase in GST activity after 14 days of exposure (Figure 5D). Similar results were found in gills of *M. galloprovincialis* after exposure to organic persistent pollutants, such as pp'DDE (2,2-bis-(p-chlorophenyl)-1,1-dichlorethylene) (Hoarau *et al.*, 2002; Khessiba *et al.*, 2001). In the digestive gland, GST activity decreased (Figure 5H), as was previously observed in mussels after PS-exposure for 14 days (Avio *et al.*, 2015).

In the depuration period, SOD activity continued to increase in both organs (Figure 5A, E). The increase in enzymatic activity at the end of the depuration period can be due to an impairment of the filtration activity or to the presence of remaining

624 microparticles in the gills and digestive gland, that still induce an enzymatic response
625 (Paul-Pont *et al.*, 2016), suggesting the inability of *S. plana* to eliminate microplastics,
626 by the mechanical damage caused by the release of the particles from the tissues, or even
627 the lack of capacity to recover after this elimination period. Nevertheless, 7 days of
628 depuration might not be enough for *S. plana* to completely recover from the exposure of
629 PS microplastics as suggested by the present results (Figure 5). In conclusion, it can be
630 hypothesized that ROS are produced as a result of PS microplastics uptake, and possibly
631 cause injury of gills and/or internalization in the digestive gland cells, since it is known
632 that these microparticles are able to cross cell membranes, leading to cell damage
633 (Browne *et al.*, 2008; Rosenkranz *et al.*, 2009; Van Cauwenberghe *et al.*, 2015).
634 The levels of oxidative damage to lipids decreased in the gills in the PS exposed
635 clams (Figure 8A). This could be linked to antioxidant defences, which consequently limit
636 the attack of ROS to membrane lipids. In the digestive gland, there is a tendency of LPO
637 levels to increase (from day 0 to day 7), but no significant differences were noted (Figure
638 8B). The enzymatic activities measured in this tissue were always lower on day 7, with a
639 recovery after one week of depuration, with the exception of GPx. A significant
640 enhancement of ROS in digestive gland haemocytes of *Mytilus spp.* after 7 days of micro-
641 PS exposure (mixture of 2 and 6 μm) was previously reported but no antioxidant markers
642 were activated and no sign of lipid peroxidation was observed (Paul-Pont *et al.*, 2016).
643 Ahmad *et al.* (2011) showed an increase in LPO levels in both gills and digestive gland
644 of *S. plana* exposed to mercury. When enzymatic defences do not actively respond to the
645 presence of PS microplastics this may result in an inflammation response and a lysosomal
646 membrane destabilization, as a cellular response observed in mussels (*M. edulis*) exposed
647 to 2.5 g L^{-1} of a high-density polyethylene (HDPE) particles (> 0−80 μm) for 96 hours
648 (von Moos *et al.*, 2012). Future exposure experiments with *S. plana* should look at this
649 aspect.
650 The Comet assay is a sensitive, rapid and economic technique for the detection of
651 DNA strand breaks and can be regarded as a good method to assess genotoxicity in
652 aquatic species (Jha, 2008). Moreover, Petridis *et al.* (2009) demonstrated that the blood
653 cells of *S. plana* are suitable for screening genotoxic effects, using this method. DNA
654 strand breaks induced in PS exposed haemocytes of *S. plana* (Figure 6 A,B) are similar
655 to mussels treated with PE microplastics where a significant enhancement of DNA strand
656 breaks was detected (Avio *et al.*, 2015). Regarding the elimination period, there was an
657 increase of both Tail DNA (%) and OTM, in PS exposed organisms. The mechanism of

genotoxicity of PS microplastics remains unknown, but it is suggested that it can be related to ROS production and oxidative stress, not handled by the antioxidant defence mechanism, as occurs with nanoparticles (Rocha *et al.*, 2014). Hence, causing damage by covalently binding to DNA (Hossain & Huq, 2002) or by inhibiting DNA synthesis (Hidalgo & Dominguez, 1998) and thus preventing cell division and DNA replication (Singh *et al.*, 1988).

PS microplastics inhibit the AChE activity in clam gills at different times of exposure with this effect remaining even after 7 days of depuration (Figure 7). The ability of microplastics to inhibit AChE activity was previously described in juveniles of the common goby *Pomatoschistus microps* exposed to 18.4 and 184 μg L^{-1} of PE microspheres (1-5 μm) for 96 hours, alone or in combination with pyrene (Oliveira *et al.*, 2013) or in combination with chromium (Luís *et al.*, 2015).

PCA for the gills and digestive gland show different responses that reflect the distinct physiological and metabolic functions of the two tissues (Figure 9A-B). Exposure to PS microplastics in the gills was positively related to an increase in the activity of oxidative stress enzymes and DNA damage, although negatively related to the oxidative damage and to the activity of AChE (inhibition), meaning that there is an inflammation process and a neurotoxic effect and validating the hypothesis that gills had a more effective response against oxidative stress than digestive gland (Figure 9A). In the case of the digestive gland, SOD is the biomarker that best relates to exposure to PS microplastics. As gills are the main tissue involved in filtration, they are in direct contact with the PS microplastics, being more susceptible to oxidative stress than the digestive gland (Figure 9B). Despite existing data about the increasing occurrence of PS microplastics in the marine environment (Andrady, 2011; Cole *et al.*, 2011; Wright *et al.*, 2013b), there is still much to understand about their biological effects. Further studies are needed to investigate the capacity to recover from the potential effects of PS and other microplastics in marine organisms beyond 7 days of depuration. The evaluation of the biological effects of microplastics also requires a molecular-level to understand how they interact with cells in a physiological environment, but up to date the functional implications at cellular level still remains to be elucidated.

Conclusions

PS microplastics were taken up by clams, mainly by the gills where they tend to accumulate, but they were also present in the digestive gland where they seemed to be stored. Tissue-specific sensibility is involved in the clams response to PS exposure by inducing oxidative stress, with the gills providing a more effective response than digestive gland. The genotoxicity of PS microplastics increased with time. Furthermore, the detoxification process of PS microplastics in clams tissues was inefficient for the 7 days duration tested, indicating their potential trophic transfer.

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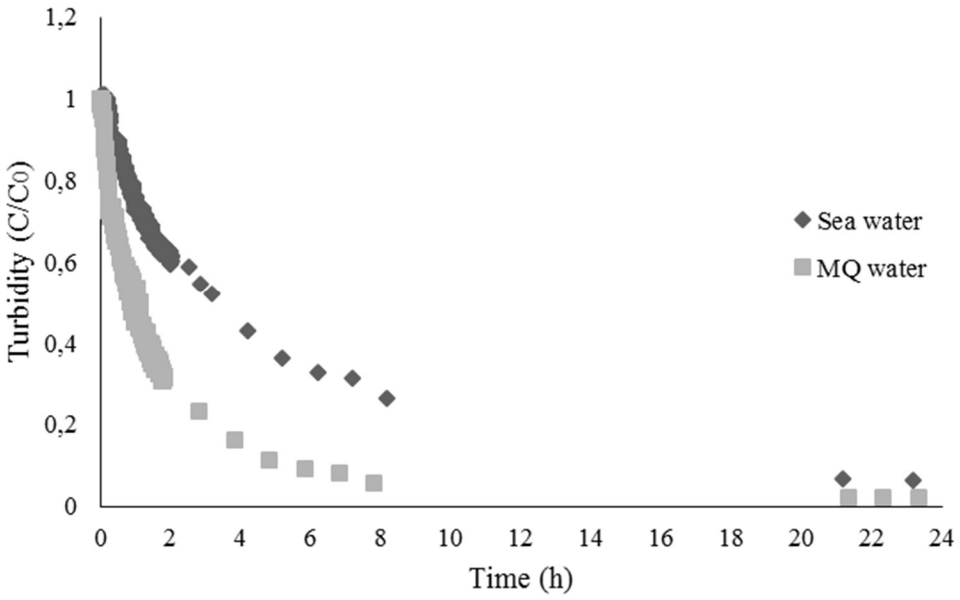

Figure 1. Turbidity of PS microparticles for 24 hours in MQ water and in sea water. C/C_0 is the normalised microparticle turbidity where C is the turbidity at time t and C0 the initial turbidity at time 0.

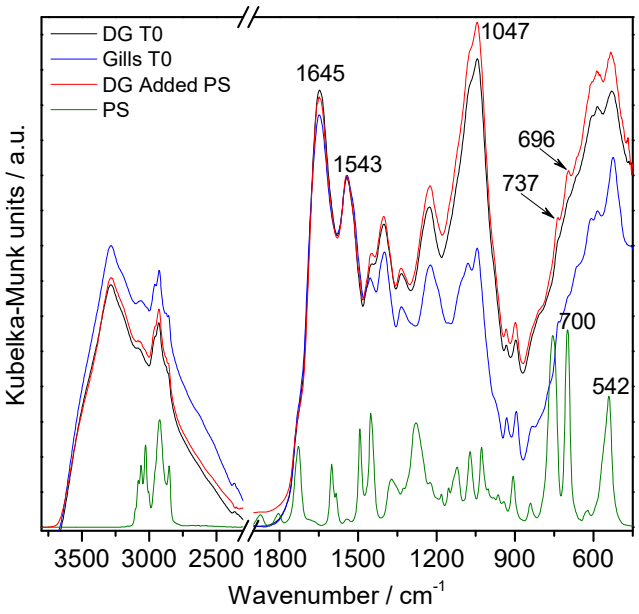

Figure 3. Average DRIFT spectra of: unexposed digestive gland (DG T0) and gills (Gills T0) and digestive gland with added polystyrene (DG Added PS). The spectra are normalized to the band at 1543 cm^{-1}. The spectrum of polystyrene (PS) microparticles is included for comparison.

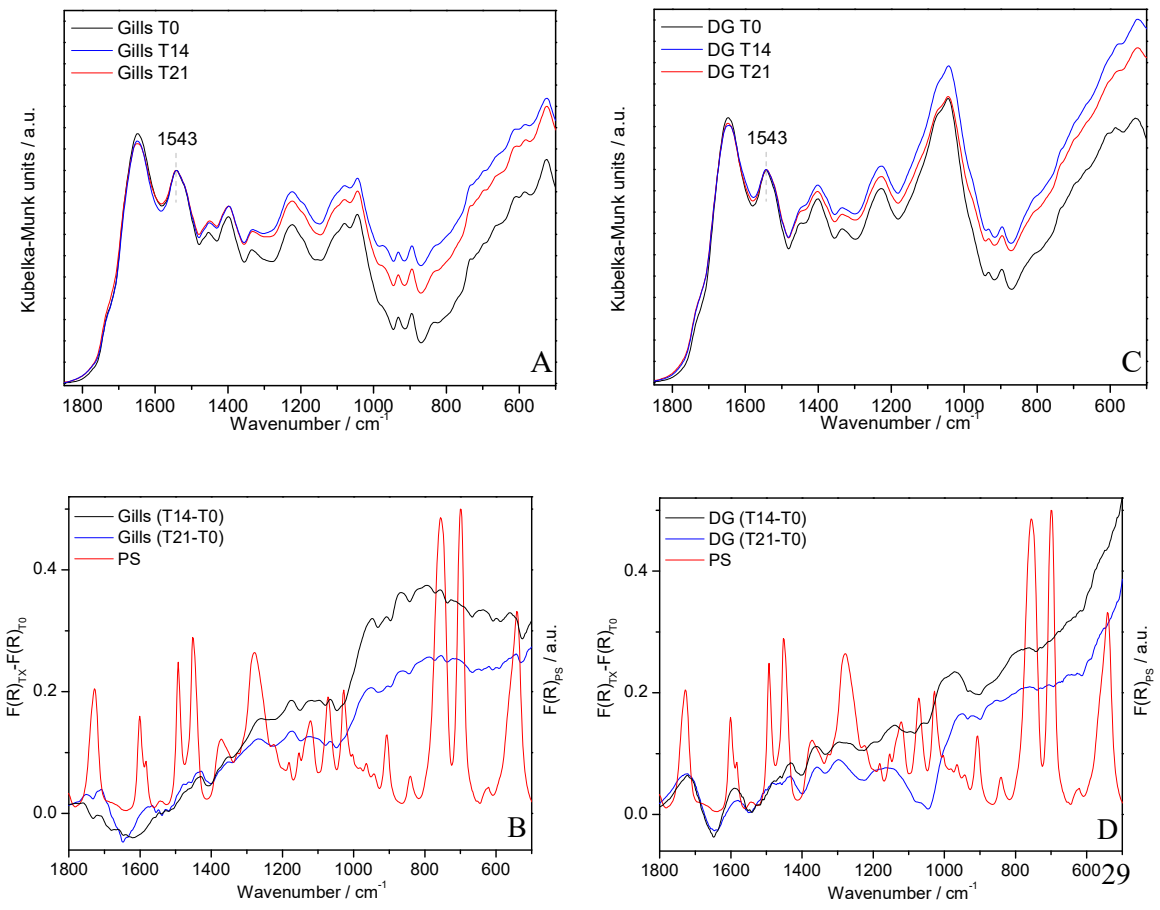

Figure 4. Comparison of the average DRIFT spectra in the 1800-450 cm^{-1} region for gills (A) and digestive gland (C) of *S. plana*, taken at the beginning (T0) and end of the exposure period (T14) and after the depuration (T21); Spectral subtraction for gills (B) and digestive gland (D), as indicated, compared with the PS microparticles spectrum.

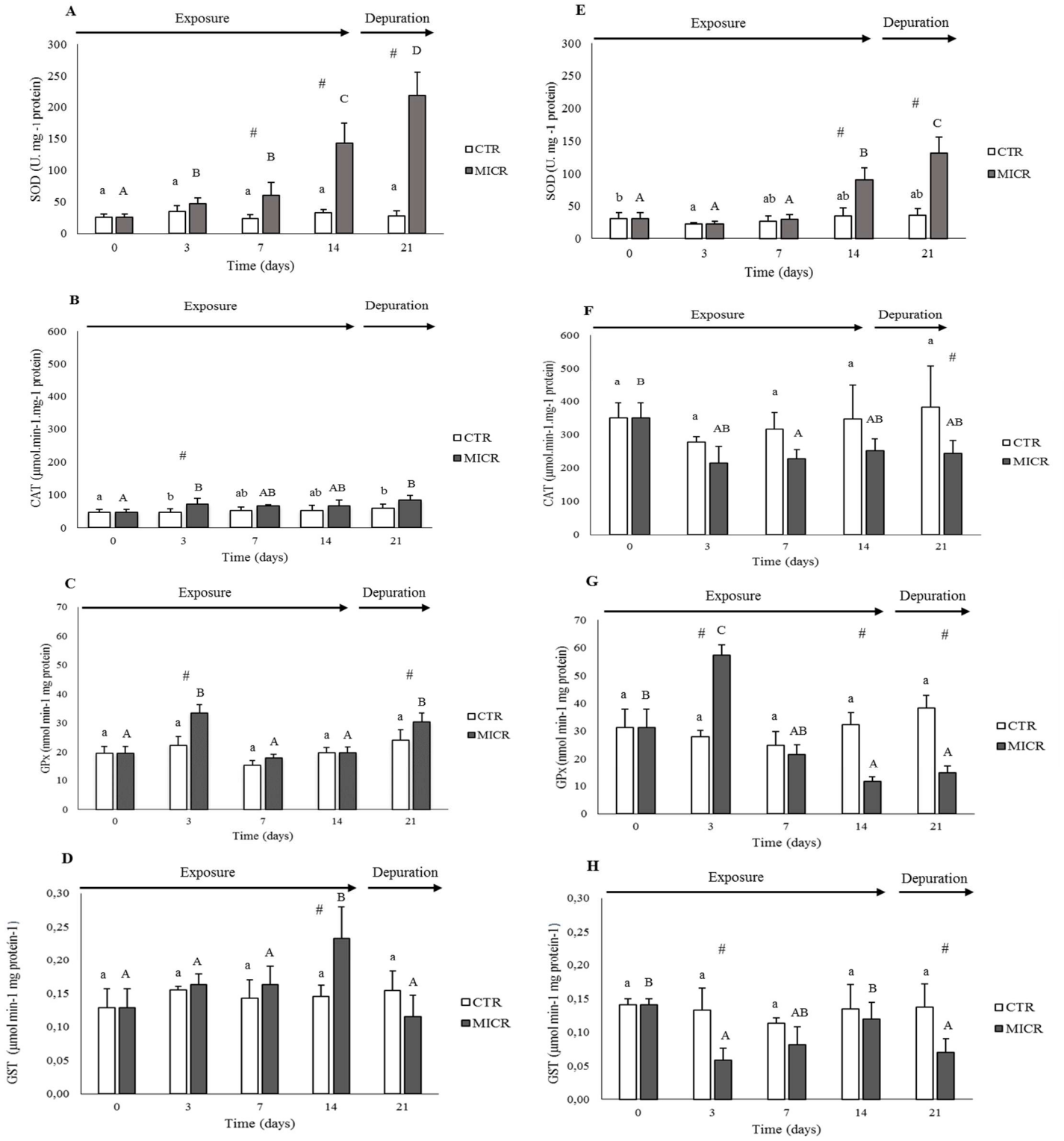

Figure 5. SOD, CAT, GPx and GST activities (mean ± SD) in the gills (A, B, C and D) and digestive gland (E, F, G and H) of *S. plana* for control (CTR) and PS microplastics (MICR 1 mg L^{-1}) during exposure and depuration. Statistically significant differences between treatments at each time of exposure are indicated with #; different lower case letters indicate significant differences between controls over time and capital letters between microplastic exposed (MICR) treatment over time ($p < 0.05$).

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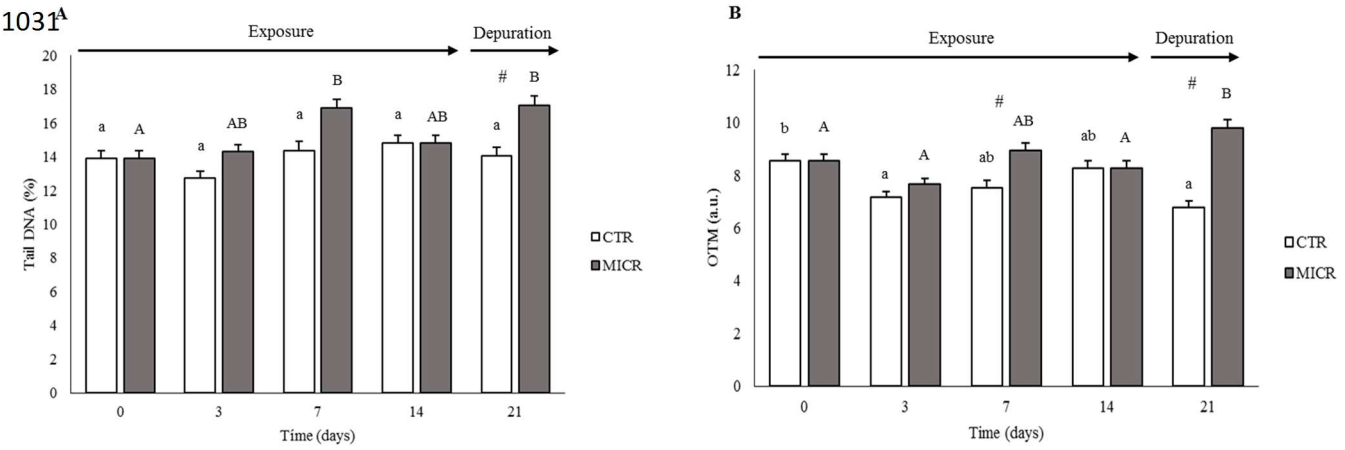

Figure 6. DNA damage (average ± SEM) in the haemocytes of *S. plana* expressed as tail DNA % (A) and OTM (a.u.) (B) for control (CTR) and PS microplastics (MICR). Statistically significant differences between treatments at each time of exposure are indicated with #; different lower case letters indicate significant differences between controls over time and capital letters between microplastic exposed (MICR) treatment over time ($p < 0.05$).

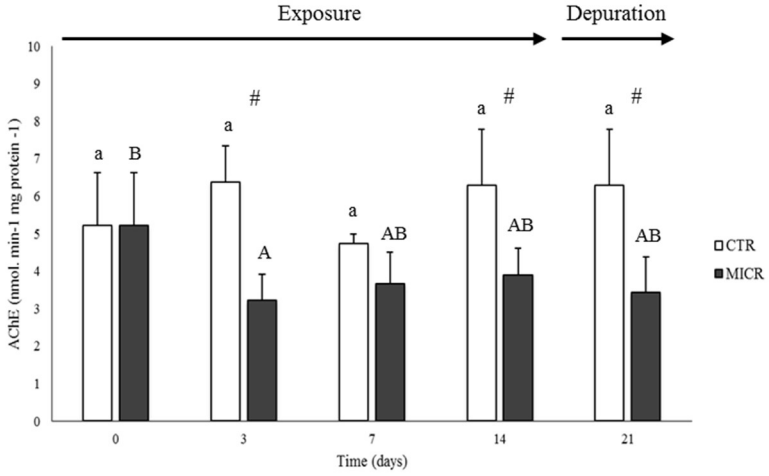

Figure 7. AChE activity in the gills of *S. plana* (average ± SD) for control (CTR) and microplastics (MICR). Statistically significant differences between treatments at each time of exposure are indicated with #; different lower case letters indicate significant differences between controls over time and capital letters between microplastic exposed (MICR) treatment over time ($p < 0.05$).

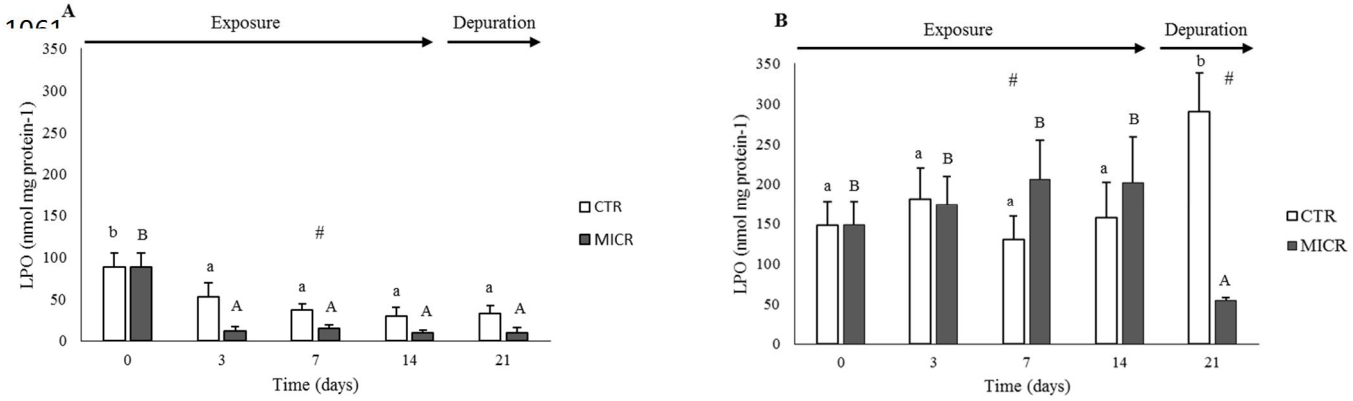

Figure 8. LPO (mean ± SD) in gills (A) and digestive gland (B) of *S. plana* for control (CTR) and microplastics (MICR). Statistically significant differences between treatments at each time of exposure are indicated with #; different lower case letters indicate significant differences between controls over time and capital letters between microplastic exposed (MICR) treatment over time ($p < 0.05$).

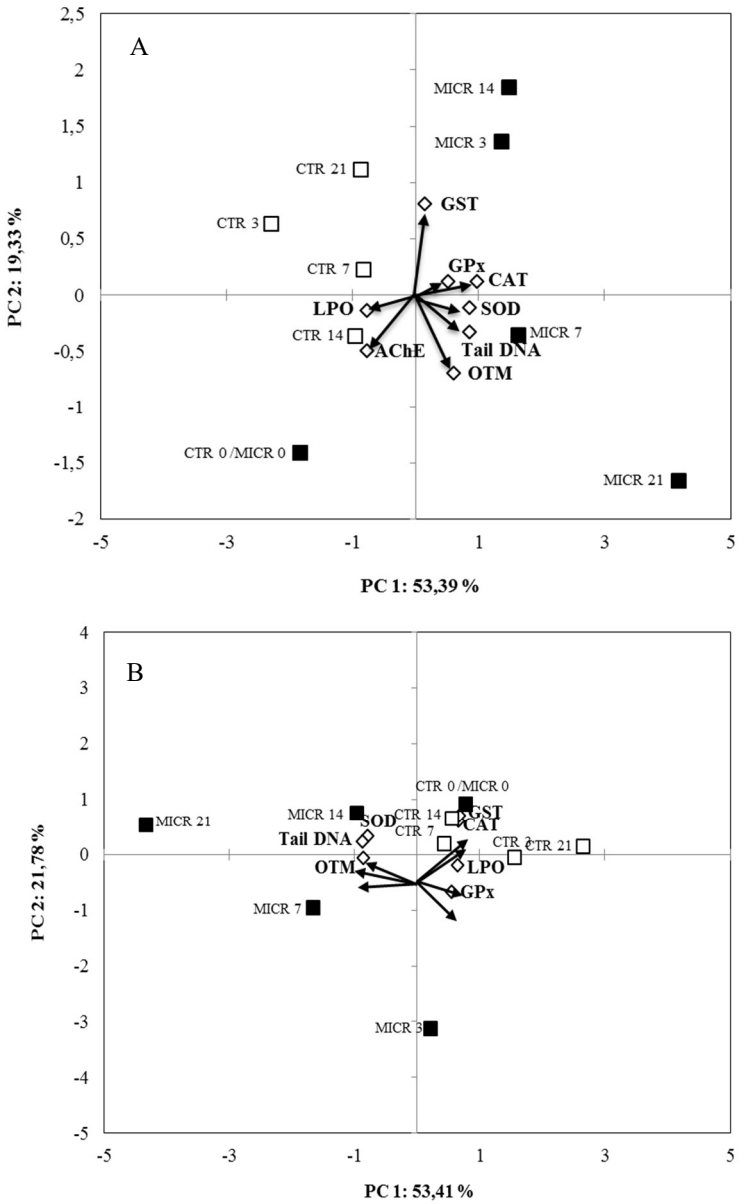

Figure 9. Principal component analysis (PCA) of a battery of biomarkers in the gills (A) and digestive gland (B) of *S. plana* unexposed (□) and exposed to PS microplastics (■)

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1110 **Table 1.** Evidence of microplastics ingestion in marine organisms

Species	Microplastics (%)	Mean (± SD) number of particles/ individual	Type and size (µm)	Reference
Phylum Arthropoda				
Gammarus pulex	_	_	Acrilic 29.5 ± 26	Imhof *et al.* (2013)
Notodromas monacha	_	_	Acrilic 29.5 ± 26	Imhof *et al.* (2013)
Phyllum Annelida				
Lumbriculus variegatus	_	_	Acrilic 29.5 ± 26	Imhof *et al.* (2013)
Phylum Mollusca				
Mytilus edulis	_	3.7 particles per 10 g tissue	Fibres 300-1000	De Witte *et al.* (2014)
Mytilus edulis	_	0.36 (± 0.07) particles g^{-1}	5 - 25	Van Cauwenberghe and Janssen (2014)
Cassostrea gigas	_	0.47 (± 0.16) particles g^{-1}	5 - 25	Van Cauwenberghe and Janssen (2014)
Phylum Crustacea				
Lepas spp.	33.5	1-30 particles/ individual	1.41	Goldstein and Goodwin (2013)
Nephrops norvegicus	83	_	_	Murray and Cowie (2011)
Crangon crangon	_	11.5 fibres per 10 g shrimp	300-1000	Devriese *et al.* (2015)
Phylum Chaetognatha				
Parasagitta elegans	100	_	0.1-3 PS	Carpenter and Smith (1972)

PS (Polystyrene)

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1119 **Table 2.** Effects of microplastics to aquatic organisms

Species	Microplastics		Exposure		Effects	Reference
	Type	Size (µm)	Concentration	Duration		
Phylum Mollusca						
Class Bivalvia						
Mytilus galloprovincialis	PE,PS, PE-PYR e PS_PYR	<100	1.5 g L^{-1}	7 d	Adsorption of pyrene not differ between PS and PE; bioaccumulation in digestive tissues and gills	Avio *et al.* (2015)
Mytilus edulis	PS fluorescently labeled	2	0.51 µg L^{-1}	12 h	Uptake accumulation in gut; and hemolymph after 3 d	Browne *et al.* (2008)
		4-16 3 and 9.6		3 d		
	PS	10	50 particles ml^{-1}	14 d	Greater accumulation of smaller particles; no significant effects on metabolism	Van Cauwenberghe *et al.* (2015)
		30	50 particles ml^{-1}			
		90	10 particles ml^{-1} 110 particles ml^{-1} (Total concentration)			
	HDPE	0-80	2.5 g L^{-1}	3, 6, 12, 24, 48 and 96 h	Uptake; retention in gut and transfer into the lymphatic system; immune response	von Moos *et al.* (2012)
	PS Microspheres fluorescently labeled	0.5	2 x 10^7 particles ml^{-1}	1 h	Uptake; trophic transfer to *Carcinus maenas*	Farrell and Nelson (2013)
	PS	10	2×10^4 particles ml^{-1}	30 min	Intake	Ward and Targett (1989)
	PS	10, 30	3.10×10^5 particles ml^{-1}	_	Intake	Claessens *et al.* (2013)
Mytilus trossulus	PS	10	1000 particles ml^{-1}	_	Intake	Ward *et al.* (2003)

Crassostrea virginica	PS	10	1000 particles ml^{-1}	45 min	Intake and egestion	Ward and Kach (2009)
Crassostrea gigas	PS	2 6	2.06 ± 170 118 ± 15 particles ml^{-1}	2 months	Decreases in oocyte number, diameter, and sperm velocity; decrease of larval development; endocrine disruption	Sussarellu *et al.* (2016)
Placopecten magellanicus	PS	15, 10, 16, 18, 20	5 x 10^3 particles ml^{-1}	1 h	Intake, retention and egestion	Brillant and MacDonald (2002)

PS (Polystyrene) **PE** (Polyethylene) **PP** (Polypropylene) **LDPE** (Low Density Polyethylene) **HDPE** (High-density Polyethylene) **UPVC** (Polyvinyl chloride) **PE-PYR** (Pyrene Treated Polyethylene) **PS_PYR** (Pyrene Treated Polystyrene)

Table 3. Characterization of PS microplastics using different techniques

Particle characterization	Method	PS microplastics
Particle size (μm) [a]	OM	20 ± 0.02
Density (g cm^{-3}) [a]	_	1.05
Mean particle diameter (μm) [c]	DLS	18.4 ±1.33
Zeta (ζ) potential (mV) [b c]	ELS	Sea water: -12.4 ± 2.36 Mili-Q water: -52.6 ± 2.34

a. Original solution of PS microplastics from Sigma Aldrich (4.3 x 10^6 particles ml^{-1})
b. 100 mg L^{-1} of microplastics dispersed in natural seawater
c. 100 mg L^{-1} of microplastics dispersed in ultrapure water

OM. Optical microscope
DLS. Dynamic light scattering
ELS. Electrophoretic light scattering