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Perfluorooctane sulfonic acid (PFOS) adsorbed to polyethylene microplastics: Accumulation and ecotoxicological effects in the clam *Scrobicularia plana*

Naimul Islam [a], Tainá Garcia da Fonseca [a], Juliano Vilke [a,b], Joanna M. Gonçalves [a], Paulo Pedro [c], Steffen Keiter [d], Sara C. Cunha [e], José O. Fernandes [e], M.J. Bebianno [a,*]

[a] Centre for Marine and Environmental Research (CIMA), Universidade do Algarve, Campus de Gambelas, 8000-139, Faro, Portugal
[b] Fishery Engineering Department, Santa Catarina State University, Laguna, 88790-000, Brazil
[c] Laboratório de Análises Químicas, Faculdade de Ciências e Tecnologia, Universidade do Algarve, Campus de Gambelas, 8000-139, Faro, Portugal
[d] Man-Technology-Environment Research Centre (MTM), School of Science and Technology, Örebro University, Fakultetsgatan 1, S-701 82, Örebro, Sweden
[e] LAQV-REQUIMTE, Laboratory of Bromatology and Hydrology, Faculty of Pharmacy, University of Porto, Rua Jorge de Viterbo Ferreira 228, 4050-313, Porto, Portugal

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ABSTRACT

Microplastics are widespread in the marine environment, whereby the uptake of these tiny particles by organisms, can cause adverse biological responses. Plastic debris also act as a vector of many contaminants, herein depending on type, size, shape and chemical properties, possibly intensifying their effects on marine organisms. This study aimed to assess the accumulation and potential toxicity of different sizes of microplastics with and without adsorbed perfluorooctane sulfonic acid (PFOS) in the clam *Scrobicularia plana*. Clams were exposed to low-density polyethylene microplastics (1 mg L^{-1}) of two different sizes (4–6 and 20–25 μm) virgin and contaminated with PFOS (55.7 ± 5.3 and 46.1 ± 2.9 μg g^{-1} respectively) over 14 days. Microplastic ingestion, PFOS accumulation and filtration rate were determined along with a multi biomarker approach to assess the biological effects of microplastics ingestion. Biomarkers include oxidative stress (superoxide dismutase, catalase, glutathione peroxidases), biotransformation enzymes (glutathione-S-transferases activity), neurotoxicity (acetylcholinesterase activity), oxidative damage and apoptosis. Microplastics ingestion and PFOS accumulation was microplastic size dependent but not PFOS dependent and filtration rate was reduced at the end of the exposure. Reactive oxygen species in gills and digestive gland were generated as a result of exposure to both types of microplastics, confirming the disturbance of the antioxidant system. Larger virgin microparticles lead to stronger impacts, when compared to smaller ones which was also supported by the Integrated Biomarker Responses index calculated for both tissues. An anti-apoptotic response was detected in digestive glands under exposure to any of the MPs treatments.

1. Introduction

Plastics are synthetic organic polymers with highly durable and long lasting properties, whereby they have various additives to give them their specific characteristics (Derraik, 2002; Rios and Moore, 2007; Lee et al., 2013). They have good mechanical properties, low density, and low production cost. These characteristics allow for the wide use of plastics in industries and in everyday life. Over the last 50 years, plastics became more and more used and now million tons of plastic are produced every year (Boucher and Friot, 2017) and it is projected to reach around 1800 million tonnes in 2050 (Plastics - the Facts, 2019). Alarmingly, plastic consumption reached 100 kg per person per year in Western Europe and North America, and 20 kg in Asia (UNEP, 2016). The United Nations and European Union frameworks stated that more than 50% of the plastics are hazardous substances based upon their constituent monomers, additives and by-products (Lithner et al., 2011).

A major portion of plastic products reaches the ocean through various pathways such as indiscriminate disposal of litter, illegal dumping, blown in form the atmosphere and from landfills, fibres from washing, scrubbers and abrasives in cosmetics and commercial cleaning applications, unintentional release during manufacture and transport, etc. (Mato et al., 2001; Moore, 2008; Browne et al., 2011; Leslie et al.,

* Corresponding author.
E-mail address: mbebian@ualg.pt (M.J. Bebianno).

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2011). Therefore, the increasing production of plastics is accompanied by an accumulation of plastic litter in the marine environment (Thompson et al., 2004; Barnes et al., 2009). Plastic litter with a terrestrial origin contributes to around 80% of marine litter and marine sources such as fishing vessels, nets, lines and other items contribute between 20 and 30% (Andrady, 2011).

With time, plastics degrade into microplastics (MPs) defined in the first international MPs workshop (2008), organized by the National Oceanographic and Atmospheric Agency (NOAA), as particles less than 5 mm in diameter (Arthur et al., 2009). There are two types of MPs, primary and secondary. Primary MPs are manufactured to be of a microscopic size and used in consumer products such as cosmetics, ship-breaking industry and as industrial abrasives in synthetic 'sand-blasting' media (Fendall and Sewell, 2009), whilst secondary MPs derive from weathering and breakdown of meso and macroplastics both at sea and on land (Ryan et al., 2009; Thompson et al., 2004). MPs are widely distributed throughout the world ocean in shallow and surface waters (Browne et al., 2011; Hidalgo-Ruz et al., 2012) as well as in deep-sea sediments (Van Cauwenberghe et al., 2013; Woodall et al., 2014; Fischer et al., 2015). Various environmental conditions affect the behaviour and fate of MPs in the ocean such as currents, horizontal and vertical mixing, temperature, wind, biofilm formation and UV exposure (Barnes et al., 2009; Lusher 2015).

The presence of MPs at sea cause harm to a wide range of marine organisms (Thompson et al., 2004; Fendall and Sewell, 2009; OSPAR, 2009), from planktonic (Cole et al., 2013) to fish species (Phillips, 2014), and even large whales (Fossi et al., 2012). Although some species are capable of rapid excretion or egesting MPs, others accumulate, retain, or pass ingested particles into their circulatory system. The gut cavity is the storage for internalized ingested particles, where they can remain in the digestive tract, absorbed into the gut epithelium via phagocytosis, or egested via faeces (Browne et al., 2008).

MPs accumulated in tissues, cause disruption of physiological processes and have effects at the cellular level (Browne et al., 2008). Serious external and internal injuries, ulcers, digestive tract blockage, false sense of fullness, loss of feeding capacity, impairment, and inability to avoid predators or death are MPs size dependent (Gall and Thompson, 2015), so differently sized particles are likely to have different effects. Recent data demonstrate that particle size influences MP impact on bivalves and other marine organisms (Gonzalez-Sotto et al., 2019).

MPs are a threat not only due to their physical stress but also to their ability to adsorb extraneous pollutants. Plastic particles act as a 'cocktail of chemicals' including additives, or compounds produced during manufacturing, and those present in the marine environment that adsorb onto the debris from surrounding seawater. The type and size also affect the sorption behaviour of chemicals adsorbed or accumulated on MPs. Therefore, adherence to MPs and ingestion through gills and a potential source for bioaccumulation can be a vector of other contaminants (Endo et al., 2005; Teuten et al., 2009).

Persistent organic pollutants (POPs) are a group of chemicals that may be adsorbed to MPs. The bioavailability of POPs adsorbed onto MPs poses critical ecological risk by potentially entering the marine food web (Andrady, 2011). Perfluorooctane sulfonic acid (PFOS) is a strong acid which does not hydrolyse, photolyze, or biodegrade under environmental conditions (O'Donovan et al., 2018). It is released into the environment through industrial manufacturing and disposal of PFOS-containing products. Information about the amount of PFOS released into the environment is limited, however empirical oceanographic data estimates that about 235–1770 tons currently reside in oceanic waters (Paul et al., 2009). PFOS are toxic to animals, producing reproductive, neurobehavioral, developmental and systemic effects (Austin et al., 2003, Huang et al., 2012). Therefore, it is important to fill the gap regarding the ecotoxicological effects of MPs, induced by the particle stress as well as the uptake of other contaminants by organisms imported by MPs via the "trojan horse" effect. In order to mimic and truly assess the effects of MPs, the present study pertained to investigate whether detrimental effects provoked by MPs exposure in the clam *Scrobicularia plana* vary according to the size of particles and to the presence or absence of PFOS adsorbed onto MPs of two different sizes. *S. plana* is an environmentally relevant species used as a bioindicator for evaluating the health status of coastal and estuarine ecosystems (Mouneyrac et al., 2008), and particularly to asses biochemical responses related to MPs exposure (Ribeiro et al., 2017; O'Donovan et al., 2018). A set of biological endpoints were employed to elucidate any effects of exposure to MPs' size range, uncontaminated and contaminated with PFOS. The efficiency and outcome of filter feeding, when exposed to these two types of MPs, was measured by ingestion and filtration rates. The biomarkers analysed were the activity of antioxidant enzymes that enable to maintain cellular integrity (superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidases (GPx)); glutathione S-transferases (GST) that participate in biotransformation and protection against oxidative stress; acetylcholinesterase (AChE) activity whose inhibition is indicative of neurotoxicity and cell death mechanism through caspase (CAS) activity. Moreover, levels of lipid peroxidation (LPO) by-products were also determined to assess oxidative damage in lipid membranes. A biomarker index was calculated to assess these effects in an integrated manner.

2. Materials and methods

2.1. Microplastics characteristics

Low-density polyethylene (LDPE) MPs (MPP-635G, 0.96 g cm^{-3}), of two different sizes (4–6 and 20–25 μm) were purchased from Micro Powders Inc. (NY-USA). Chemicals were obtained from Sigma Aldrich. Sorption of the PFOS to MP particles was conducted by the Man-Technology-Environment Research Centre, Department of Natural Science, Örebro University, Sweden. Briefly, 50 g L^{-1} of LPDE MPs of sizes 4–6 or 20–25 μm were weighed and introduced into polypropylene bottles filled with 500 mL of double-deionized water and 20 mg L^{-1} of heptadecafluorooctanesulfonic acid potassium (CAS 2785-37-3, purity ≥98%). The bottles were placed on a rotary shaker at 20 rpm for 7 days and then filtered with a 1.0 μm glass microfiber filter (WhatmanR), rinsed with double-deionized water and dried by vacuum evaporation on a ceramic funnel. MPs were extracted in methanol (>99.9% purity, Fisher Scientific) by ultra-sonication followed by centrifugation. Extracts were filtered with a 0.2 μm filter (AcrodiscGHP, 13 mm) and PFOS analysis performed on an Acquity UPLC system coupled to a Xevo TQ-S quadrupole mass spectrometer (Waters Corporation, Milford, U.S.A.) separated on 100 mm Acquity BEH C18 column (2.1, 1.7 mm). The final concentration of PFOS adsorbed to LDPE (4–6 and 20–25 μm) MPs was 55.7 ± 5.3 and 46.1 ± 2.9 μg g^{-1}, respectively. A detailed description of the methodology used to prepare MPs with PFOS adsorbed are available in O'Donovan et al. (2018) and Eriksson et al. (2016).

Because plastics are present everywhere it is important to be careful whilst handling MPs. To prevent cross-contamination, all equipment used was rinsed with Milli-Q water, dried at room temperature in a fume hood and kept under aluminium foil until used. Throughout the experiment, laboratory coats made of cotton and nitrile gloves were worn and the use of plastic material was avoided, whenever possible.

2.2. Sediment collection and preparation

Sediments were collected from the top 30 cm, during low tide in the Ria Formosa coastal lagoon (37°7′59.75″N 7°36′34.95″ W). After collection, sediments were sieved through a 4 mm mesh to remove macro-organisms and debris and dried at 65 °C for 48 h (Maranho et al., 2014), following re-hydration to the initial moisture content (%). To determine the organic matter content, around 2 g of sediments was weighed and kept at 500 °C for 3 h. After cooling, the sample was weighed, and the organic content determined as a percentage of weight loss.

2.3. Experimental design

S. plana clams (3.0 ± 0.3 cm) were collected at the same time and place of the sediments and transported alive to the laboratory along with seawater. Clams were acclimatised over 5–7 days, at a constant temperature (18 °C) and photoperiod (12:12 light:dark), under continuous aeration. Afterwards, clams (n = 60 per aquarium) were transferred to ten pre-set glass tanks filled with a volume ratio 1:4 (3.5 L sediments and 14 L seawater) of sediments and seawater. The exposure experiment was performed in duplicate, with five treatments: control, virgin LDPE MPs of sizes 4–6 μm (V4) and 20–25 μm (V20), PFOS-contaminated LDPE MPs of sizes 4–6 μm (P4) and 20–25 μm (P20) at a concentration of MPs of 1 mg L-1. Water was changed every 72 h, with the routine application of the different types of MPs. Clams were randomly sampled from each aquarium in the beginning of the experiment and after 7 and 14 days of exposure. Water quality parameters (temperature, salinity, oxygen saturation, pH) were monitored using a multiparametric probe (ODEON V3.3.0) and remained stable during the experiment (19.5 °C ± 0.3, 33.9 ± 1.8 psu, 93.4% ± 0.1, 8.01 ± 0.10 respectively). During the exposure time, clams were only fed with unfiltered natural seawater providing animals with enough food to avoid starvation and to minimise the interaction of MPs with other particles. Care was also taken to avoid and minimise plastic cross contamination by providing aeration with glass tubes.

After collection, clams were weighed, the shell length measured, the whole soft tissues separated from the shells for the MPs and PFOS analysis. For the analysis of enzymes activities (antioxidant, GST, AChE and caspase) gills and digestive gland were dissected and immediately frozen in liquid nitrogen and stored at −80 °C until further analysis. Whole soft tissues collected for determination of MPs and PFOS concentrations were collected under a fume hood, weighed and frozen at −20 °C until further analysis.

2.4. Condition index

The condition index (CI) was assessed in clams (6 per treatment and time of exposure) to determine their physiological status at the beginning (day 0) and after 7 and 14 days of exposure. The clams whole soft tissues were dried at 80 °C for 24 h before being weighed. The CI was calculated as the percentage (%) of the ratio between dry weight of the soft tissues (g) and the weight (g) of the shell.

2.5. Filtration rate

Filtration rate was calculated based on the removal of microalgae cells from clean medium, according to Oliveira et al. (2018) at the beginning and after 14-day exposure. Briefly, each clam was placed into individual glass beakers filled with 250 mL of clean medium containing *Tetraselmis chuii* (algal suspension - 2.2×10^6 cells/mL). Two hours later, each beaker of algal suspension was sampled, followed by an algal cell count under a microscope using an Improved Neubauer haemocytometer. The filtration rate (FR) expressed in mL of algal suspension/h/bivalve was calculated as following:

$$FR = \frac{V}{n \times t} \times ln \frac{Ci}{Cf}$$

where V is the volume of the test medium (mL), n the number of bivalves, t the time (hours), and C_i and C_f the concentrations of microalgae (number of cells/mL) at the beginning and after 2 h, respectively.

2.6. Microplastic quantification in clam tissues

Whole soft tissues were digested with 2 mL of 67% HNO_3 in a glass tube and placed in a hot bath at 60 °C for 24 h. For a complete digestion of the organic matter content of clam tissues, 2 mL of hydrogen peroxide (30% H_2O_2) was added to each tube and kept in a hot bath for another 24 h. After digestion, 3 mL of Milli-Q water was added followed by two drops of Nile Red. The mixture was vortexed for 30 min to allow to dye the MPs. Then a filtered NaCl solution (1.2 g cm^{-3}) was added to collect the surface water by overflowing the tube, as MPs mostly float on the surface. Collected fractions were filtered through 0.45 μm membrane filter (Whatman membrane filter, ME 25/21, diam. 47 mm) and kept in a glass Petri dish for observation and quantification of MPs under a fluorescence microscope (Leica DMLB, 10X/0.25).

2.7. Determination of PFOS content

2.7.1. Sample preparation for LC-MS/MS analysis

PFOS was extracted according to Kwadijk et al. (2010) with some modifications. Briefly, 1 g of whole soft tissue previously triturated in a Precellys Evolution homogenizer (Bertin Instruments, Montigny-le-Bretonneux France) was weighed into a 15 mL polypropylene (PP) tube. Four mL of acetonitrile were added after which the sample was shaken for 30 min and subsequently centrifuged at 1690 *g*, for 10 min. The acetonitrile phase was transferred to a 50 mL PP tube after which the extraction was repeated twice. The extract was concentrated to 5 mL under a nitrogen stream after which 8 mL of hexane were added. The sample was then shaken vigorously for 5 min and centrifuged for 5 min at 1690 *g* after which the hexane layer was discarded. This step was repeated twice, and the extract was concentrated to 700 μL under a nitrogen stream. The remaining extract was transferred to a PP Eppendorf tube, 50 mg of ENVIcarb was added and the sample vortexed for 1 min and centrifuged for 5 min at 1690 *g*. The extract was then stored at 4 °C until LC-MS/MS analysis.

2.7.2. PFOS quantification using LC-MS/MS

Separation and quantification of the target analyte was performed by LC-MS/MS analysis in a high-performance liquid chromatography (HPLC) system Waters 2695 (Water, Milford, MA, USA) coupled to a Micromass Quattro micro API™ triple quadrupole detector (Waters, Manchester, UK). A Kinetex C18 2.6 μm particle size analytical column (150 × 4.6 mm) with a pre-column from Phenomenex (Tecnocroma, Portugal), maintained at 40 °C was used for chromatographic separation. The separation was obtained during 12 min under isocratic conditions with a mobile phase consisting of 80% MeOH and 20% water (18.2 mΩ cm^{-1} was purified by a Milli-Q gradient system from Millipore, Milford, MA, USA) added with 0.2 μM ammonium acetate (p. a. Merck, Darmstadt, Germany).

MS/MS acquisition was operated in a negative-ion mode with multiple reaction monitoring (MRM), the collision gas was Argon 99.995% (Gasin, Portugal) with a pressure of 2.9×10^{-3} mbar in the collision cell. Capillary voltages of 3.0 KV were used in the positive ionization mode. Nitrogen was used as dissolution gas and cone gas with flows of 350 and 60 L h^{-1}, respectively. The dissolution temperature was set to 450 °C and the source temperature to 150 °C. A DA dwell time of 0.1 s/scan was selected. The transitions used for PFOS were m/z 499 > 80 and m/z 499 > 99 (Furdui et al., 2008). Eleven standards were used to perform the calibration curve, ranging from 0.25 to 320 μg L^{-1}. The limit of detection (LOD, defined as a signal-to-noise of 3:1) and the limit of quantification (LOQ, which corresponds to the lowest concentration measured with acceptable accuracy and precision, relative standard deviation (% RSD) < 20%), were 0.04 ng g^{-1} and 0.125 ng g^{-1}, respectively. The data was collected using the software program MassLynx4.1.

2.8. Antioxidant enzymes activities

Gills and digestive glands (n = 6 per treatment and time of exposure) of *S. plana* were individually homogenised in 5 mL of Tris sucrose buffer (sucrose 0.5 M, Tris 20 mM, KCL 0.5 M, DTT 1 M, EDTA 1 mM, and Milli-Q water at pH 7.6), according to the protocol of Géret et al. (2002). The homogenate was centrifuged at 500 *g*, at 4 °C for 15 min. The cytosolic

fraction was separated and centrifuged a second time at 12,000 *g*, at 4 °C for 45 min. Supernatant was divided into aliquots for the determination of the antioxidant enzymes (SOD, CAT and GPx) and GST activities. Aliquots were frozen at −80 °C until further analysis.

SOD activity was determined in the cytosolic fraction by the percentage of inhibition of the reduction of cytochrome *c* by the superoxide anion generated by the xanthine/hypoxanthine system measured by the absorbance at 550 nm using the method described by McCord and Fridovich (1969). Samples were defrosted on ice, vortexed and then 2650 μL of phosphate buffer (50 mM, with EDTA 0.1 mM, at pH 7.8), 100 μL hypoxanthine (1.5 mM), 100 μL of cytochrome *c* oxidase (0.15 mM), 50 μL of sample and 100 μL of xanthine oxidase (56 mU/ml) were added. The absorbance was read for 1 min using a UV spectrophotometer (Jasco V-650). Samples were run in triplicates. SOD activity is expressed in Units (U) mg^{-1} protein.

CAT activity was determined by measuring the absorbance of the consumption of hydrogen peroxide (H_2O_2) at 240 nm following the method described by Greenwald (1987). CAT activity is expressed as μmol min^{-1} mg^{-1} of total protein concentration.

GPx activity was measured based on the colorimetric method described by Lawrence and Burk (1976) using a cumene hydroperoxide probe. GPx converts reduced glutathione (GSH) to oxidized glutathione (GSSG) while reducing the cumene hydroperoxide probe. The generated GSSG is reduced to GSH as NADPH is consumed by glutathione reductase (GR). The absorbance of the decrease in NADPH measured at 340 nm in a microplate reader (Tecan Infinite 200 Pro) is directly proportional to GPx activity. GPx activity is expressed as nmol min^{-1} mg^{-1} of total protein concentration.

2.9. Glutathione-S-transferases activity

GST activity was determined in the cytosolic fraction of gills and digestive glands of clams prepared as detailed in section 2.8, according to the method described by Habig et al. (1974) and McFarland et al. (1999), adapted for microplate. GST catalyses the conjugation of 1-chloro 2,4 dinitrobenzene (CDNB) with reduced glutathione (GSH) resulting in the formation of glutathione-S 2,4-dinitrobenzene (GS-DNB). Dinitrophenyl thioether is produced as a result, the absorbance was measured at 340 nm using a Tecan (Infinite 200 Pro) microplate reader. GST activity is expressed in nmol CDNB min^{-1} mg $protein^{-1}$.

2.10. Oxidative damage

Gills and digestive glands (n = 6 per treatment and time of exposure) were defrosted, weighed and homogenised on ice, in 5 mL of Tris HCL buffer (0.1 M HCl, 0.02 M Tris, pH 8.6) and 50 μL of butylated hydroxytoluene solution (BHT). The homogenate was centrifuged at 30,000 g, at 4 °C for 45 min using a Biofuge Stratus 230 V centrifuge (Thermo scientific, Germany). The supernatant, containing the cytosolic fraction, was divided into two aliquots and stored at −80 °C until further analysis. One of the aliquots was used for the determination of lipid peroxidation and the other for total protein concentrations.

Levels of LPO by-products were quantified following the colorimetric method described by Erdelmeier et al. (1998). 200 μL of supernatant was incubated at 45 °C, for 60 min, with 650 μL of 1-methyl-2-phenylindone diluted in methanol and 150 μL of methane sulfonic acid (15.4 M). After incubation, the mixture was centrifuged at 15,000 *g*, at 4 °C for 10 min. Then 150 μL of the resulting supernatant was added, in quadruplicate to a 96 well microplate and the absorbance read at 386 nm using a Tecan (Infinite 200 Pro) microplate reader. LPO levels were determined by the quantification of the absorbance of malondialdehyde and (2E)-4-hydroxy-2-nonenal. LPO results are expressed as MDA nmol mg^{-1}protein.

2.11. Acetylcholinesterase activity

AChE activity was only assessed in the gills of *S. plana* (n = 6 per treatment and time of exposure) following a modification of the Ellman's colorimetric method (Ellman et al., 1961). AChE hydrolyses to acetylthiocholine and produces thiocoline, which reacts non-enzymatically with DNTB releasing the yellow 5-mercapto-2-nitrobenzoato compound. The increase of the absorbance was measured at 405 nm, using an extinction co-efficient of $\varepsilon = 13.6\ mM^{-1}\ cm^{-1}$, representing the amount of thiocoline produced, which is proportional to the activity of AChE (Colovic et al., 2013).

Gills were defrosted, weighed and homogenised on ice, in 5 mL of Tris HCL buffer (100 mM, pH 8.0) and 50 μL of Triton – X 100 (0.1%). The homogenate was centrifuged at 12,000 *g*, at 4 °C for 30 min. The supernatant was subdivided into two aliquots and stored at −80 °C, until further analysis. One of the aliquots was used for the determination of AChE activity and the other for total protein concentrations. Determination of AChE activity was made in triplicate. 50 μL of blank or non-diluted samples were included in each 96 well microplate and 200 μL of 5,5′-dithio-bis (2- nitrobenzoic acid) (DNTB, 0.75 mM) solution was added to each well and incubated at ambient temperature for 5 min. To trigger the reaction, 50 μL of acetylthiocholine solution (ATC, 3 mM) was added to each well. The absorbance was determined at 412 nm using a Tecan (Infinite 200 Pro) microplate reader, for 5 min, with an interval of 30 s. AChE activity is expressed as nmol ATC min^{-1} mg $protein^{-1}$.

2.12. Total protein concentrations

Total protein concentrations were used to normalize the activity of antioxidant enzymes, GST, AChE, as well as LPO levels. Total protein concentrations were determined in the cytosolic fraction of gills and digestive glands (n = 6 per treatment and time of exposure) following the Bradford method (Bradford, 1976), adapted for microplate reader using bovine serum albumin (BSA) as a standard (Sigma-Aldrich). Absorbance was measured at 595 nm and protein concentrations are expressed as mg protein g^{-1} of tissue.

2.13. Caspase 3/7 activity

Apoptosis was only determined in digestive glands of *S. plana* (n = 6 per treatment) controls and exposed to MPs with and without PFOS over 14 days of exposure using a Caspase-Glo 3/7 assay kit (Promega, Cat. #G8090), according to the method described by Fernández and Albentosa (2019). Tissues were homogenised in an extraction buffer (25 mM HEPES [pH 7.5], 5 mM MgCl2, 1 mM EGTA; 1 μg mL^{-1} pepstatin, 1 μg mL^{-1} leupeptin and 1 μg mL^{-1} aprotinin). Homogenates were centrifuged at 13,000 rpm, for 15 min, at 4 °C. Supernatant (50 μL) was seeded into white-walled 96-well microplates, following addition of an equal volume of Caspase-Glo 3/7 reagent. A negative control of tissue homogenates was prepared, jointly with a blank (i.e. homogenization buffer and reactive Caspase-Glo 3/7 reagent) (50 μL). Samples were incubated for 1 h at room temperature, in the dark. A blank reaction was included to determine the background luminescence from the homogenization buffer and the Caspase-Glo 3/7 reagent along with a negative control of tissue homogenate. Caspase activity was measured with a luminometer (Berthold Sirius L, Germany) and the obtained luminescence expressed as relative light units (RLU) is proportional to caspase 3/7 activity.

2.14. Statistical analysis

The data from filtration rate, MPs ingestion, PFOS accumulation and biochemical biomarkers was compared using One-way ANOVA, in accordance to the distribution of the data and homogeneity of variance (Shapiro-Wilk and Levene's tests, respectively), followed by the Tukey

post-hoc pairwise test. Results were compared between treatment and time using a two-way ANOVA. Statistical analyses were carried out using Graphpad Prism v.8 (GraphPad Inc, San Diego, CA). The statistical significance was set at the $p < 0.05$ level.

2.15. Integrated biomarker response index

Biomarkers data from gills and digestive gland of *S. plana* exposed to virgin (V4 and V20) and PFOS-adsorbed MPs (P4 and P20) were integrated using a biomarker response index version 2 (IBRv2) proposed by Sanchez et al. (2013) that allows to integrate the different biomarker responses into a numeric value. This version was modified from the IBR index defined by Beliaeff and Burgeot (2002) and detailed in Serafim et al. (2011). The IBRv2 index uses the reference deviation concept based on a disturbed and undisturbed state and was developed with the aim to remove the IBR result dependency on arrangement of the biomarkers and on the induction and inhibition for each biomarker (Sanchez et al., 2013). IBRv2 represents a sum of the deviations between unexposed, virgin and PFOS adsorbed LDPE MPs. Briefly, combined data of each individual biomarker (Xi) was compared to the data (X0) of each biomarker from the control group and log transformed (Yi) to reduce variance ($Yi = \log(Xi/X0)$). Then the mean (μ) and standard deviation (s) for Yi was calculated and data of each parameter further standardized, according to the following equation: $Zi = (Yi - \mu)/\sigma$.

To create a baseline centred on controls and to represent parameters variation according to this baseline, the mean of the standardized biomarker response (Zi) and the mean of the unexposed biomarker data (Z_0) were used to define a biomarker deviation index (A):

$$A = Zi - Z0$$

Finally, to obtain the IBRv2 index, the absolute value of A was calculated for each parameter in each experimental condition and summed:

$$IBR = \sum |A_i|$$

3. Results

3.1. Condition index

No significant differences exist in the condition index of *S. plana* among treatments, nor within the same time among treatments nor for the same treatment between sampling times ($p > 0.05$) (Table 1).

3.2. Filtration rate (FR)

At the end of the experiment, a decreased filtration rate was observed in clams *S. plana* compared to the start of the experiment (Table 2). The filtration rate varies among different treatments regarding MP size and PFOS contaminated but only MPs of 4–6 μm size (V4 and P4) had the lowest filtration rate that was significantly different from controls ($p < 0.05$).

Table 1
Condition Index (mean ± S.D.) of *S. plana* exposed to virgin (V4 and V20) and PFOs adsorbed MPs (P4 and P20) (n = 6) at the beginning and end of exposure period.

Time (day)	CT	V4	V20	P4	P20
0	56.0 ± 11.5				
7	44.2 ± 11.2	42.4 ± 6.1	50.8 ± 7.0	41.5 ± 10.4	42.9 ± 9.5
14	44.1 ± 5.9	42.0 ± 4.2	44.7 ± 8.3	42.3 ± 6.8	39.0 ± 4.6

Table 2
Filtration rate (mean ± S.D.; mL/clam/h) of *S. plana* exposed to virgin (V4 and V20) and PFOs adsorbed MPs (P4 and P20) at the beginning and end of exposure period.

Time (days)	Control	V4	V20	P4	P20
0	513.3 ± 57.5				
14	393.2 ± 26.7	146.8 ± 57.5*	261.8 ± 57.5	138.6 ± 41.0*	204.3 ± 115.1

*significantly different from the beginning of the experiment ($p < 0.05$).

3.3. Microplastic accumulation in clam tissues

No MPs were detected in whole soft tissues of unexposed clams. When clams were exposed to MPs, both virgin and PFOS-contaminated, MPs were ingested in whole tissues and the ingestion significantly increase with the time of exposure whatever the size or presence of PFOs (Fig. 1) ($p < 0.05$).

3.4. PFOS accumulation in clam tissues

PFOS accumulation in *S. plana* indicate that PFOS levels in controls and in both sizes of virgin LDPE was below the detection limit. On the other hand, PFOS was significantly accumulated in the whole soft tissues of clams exposed to PFOS-contaminated MPs compared to controls and to clams exposed to both sizes of virgin MPs ($p < 0.05$), and after 14 days of exposure, the accumulation of PFOS was not significantly different between particle sizes (0.79 ± 0.18 and 0.73 ± 0.18 ng g^{-1} for P4 and P20 respectively).

3.5. Activity of antioxidant enzymes activities

3.5.1. Superoxide dismutase

SOD activity in gills and digestive glands exposed to virgin and PFOS-contaminated MPs are presented in Fig. 2A and B. In unexposed clams, SOD activity did not change over the time of exposure, either in gills or in digestive glands ($p > 0.05$). SOD activity significantly increase in the gills of clams exposed to virgin LDPE MPs of sizes 4-6- and 20–25 μm (V4 and V20) until the 7th day ($p < 0.05$). At the 14th day, SOD activity in clam gills exposed to V4 remained while in those exposed to V20, SOD activity decreased to levels similar to those in unexposed individuals (Fig. 2A). However, in clams exposed to PFOS-contaminated MPs (P4 and P20), SOD activity increased significantly, after 14 days of exposure, by 1.7- and 1.8-fold when compared to controls ($p < 0.05$) and was significantly different from clam gills exposed to 20–25 μm virgin MPs

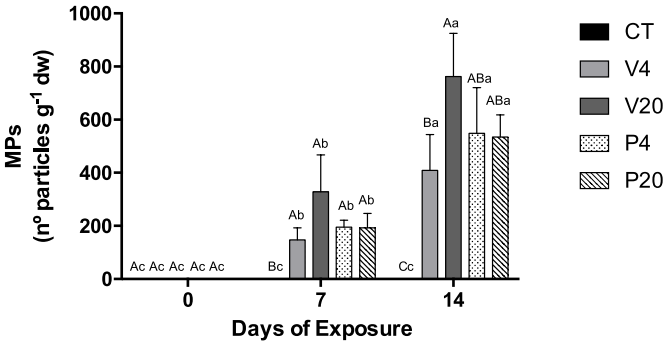

Fig. 1. MPs (mean ± s.d.) in the whole soft tissues of *S. plana* unexposed (CT) and exposed to two sizes of virgin (V4 and V20) and PFOS adsorbed MPs (P4 and P20) for 14 days. Different capital letters indicate significant differences between treatments within the same time. Different lowercase letters indicate significant differences for the same treatment between times (ANOVA; $p < 0.05$).

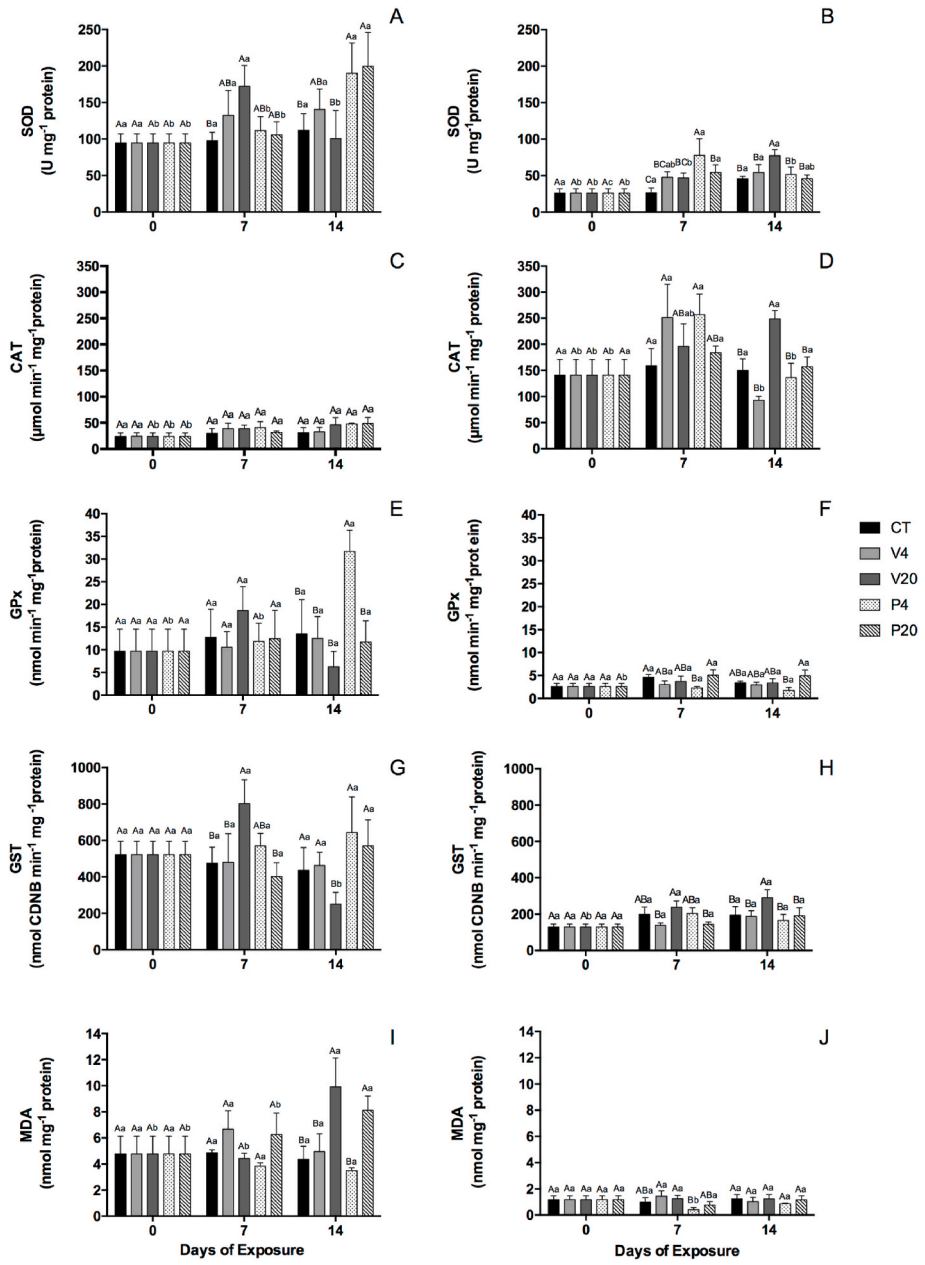

Fig. 2. Biochemical biomarkers in gills (A, C, E, G, I) and digestive glands (B, D, F, H, J) of *S. plana,* unexposed (CT) and exposed to two sizes of virgin (V4 and V20) and PFOS adsorbed MPs (P4 and P20) for 14 days. Different capital letters indicate a significant difference between treatments within the same time. Different lowercase letters indicate a significant difference for the same treatment between times (ANOVA; $p < 0.05$).

(V20) ($p < 0.05$).

SOD activity in digestive glands was significantly lower than in gills ($p < 0.05$) (Fig. 2B). After 7 days of exposure, SOD activity was significantly induced in clams exposed to P4 compared to the respective control, followed by a decrease at the end of the experiment ($p < 0.05$). At the 14th day, V20 MPs elicited a significant increment in SOD activity compared to the respective control, with an overall increasing trend over the exposure period ($p < 0.05$).

3.5.2. Catalase

CAT activity in clams is presented in Fig. 2C and D. CAT activity in gills and digestive gland of control organisms remained unchanged during the experiment ($p > 0.05$), but, CAT activity was 3-fold higher in the digestive gland ($p > 0.05$). From day 7 to the end of the experiment, CAT activity increased in gills of clams exposed to V20, P4 and P20, in comparison to the beginning of the bioassay ($p < 0.05$), but was similar among treatments within the same time of exposure ($p > 0.05$) (Fig. 2C). In the digestive gland, virgin and PFOS-contaminated MPs of size 4–6 µm (V4 and P4) showed a significant increase in CAT activity compared to controls after 7 days of exposure, followed by a significant decrease at the end of the exposure (Fig. 2D). In clams exposed to V20, CAT activity significantly increased in the whole exposure period ($p < 0.05$).

3.5.3. Glutathione peroxidase

GPx activity did not change in both tissues of unexposed clams throughout the experiment ($p > 0.05$) (Fig. 2E and F). Similar to the SOD activity trend, in the gills of V20-exposed clams, GPx activity also showed a slight increase at the 7th day, followed by a decrease at the 14th day, (Fig. 2E). At the end of the bioassay, GPx activity significantly increased in clam gills exposed to P4, in comparison to other treatments ($p < 0.05$). In digestive glands, GPx activity significantly decreased in clams exposed to P4 at the 7th day ($p < 0.05$), and also showed a lower

activity at the 14th day, while in those exposed to P20 significantly increased compared to controls ($p < 0.05$) (Fig. 2F).

3.6. Glutathione-S-transferases

GST activity in gills and digestive glands of *S. plana* are presented in Fig. 2G and H. Results showed no significant variation in GST activity in gills from unexposed clams throughout the bioassay ($p > 0.05$) (Fig. 2G). In contrast, at the 7th day of exposure, a significant induction in the GST activity was observed in gills exposed to V20, followed by a considerable decrease on day 14 ($p < 0.05$). In the digestive gland, the range of GST activity was 3-fold lower than those observed in gills (Fig. 2H). At the 14th day, *S. plana* exposed to V20 presented the highest GST activity in the digestive gland, being significantly different from all other treatments ($p < 0.05$).

3.7. Oxidative damage

LPO levels did not show any difference in gills and digestive glands from unexposed clams ($p > 0.05$) (Fig. 2I and J). Despite the increase in levels of MDA by-products in gills from clams exposed to V4 and P20, no significant alterations were observed among treatments within the 7th day of exposure ($p > 0.05$). At the end of the bioassay, higher LPO levels were detected in the gills of clams exposed to V20 and P20, being significantly different from controls and from their smaller counterparts ($p < 0.05$) (Fig. 3B).

In general, results from digestive glands show a concentration of LPO by-products 3-fold lower than those observed in gills (Fig. 3C), with only significant differences between 4 and 6 μm-sized PFOS MPs (P4) and its virgin counterpart, at the 7th day ($p > 0.05$).

3.8. AChE activity

AChE activity in the gills exposed to the different treatments is presented in Fig. 3. Regarding the controls, AChE activity remained unchanged throughout the experiment ($p > 0.05$). In clams exposed to virgin MP size 4–6 μm (V4), AChE activity was significantly higher at day 14 than at day zero and day 7 ($p < 0.05$). In clams exposed to MPs contaminated with PFOS of size 4–6 μm (P4) AChE activity was significantly inhibited, while in those of size 20–25 μm (P20), there was a significant increase in AChE activity on day 7 compared to the other treatments, followed by a significant decrease on the last day of the experiment ($p < 0.05$).

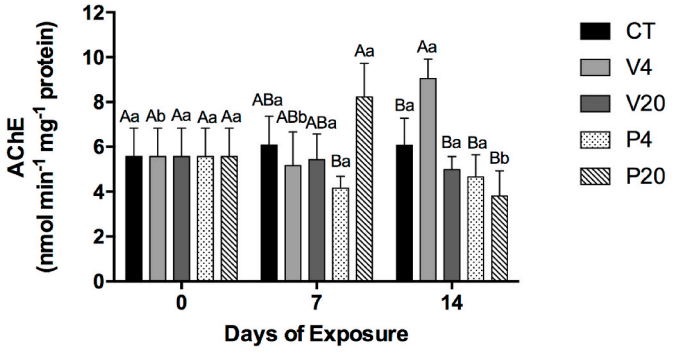

Fig. 3. AChE activity (mean ± standard deviation) in the gills of S. plana. of unexposed S. plana (CT), exposed to two sizes of virgin (V4 and V20) and PFOS adsorbed MPs (P4 and P20) for 14 days. Different capital letters indicate a significant difference between treatments within the same time. Different lowercase letters indicate a significant difference for the same treatment between times ($p < 0.05$).

3.9. Caspase activity

Because not enough samples were available at the beginning of the experiment, caspase activity was only determined at the end of the exposure period. Caspase 3/7 activity in the digestive glands from all MPs-treated clams indicate a sharp and significant decrease in luminescence, with regard to controls ($p < 0.05$) (Fig. 4). *S. plana* exposed to the smallest virgin MPs (V4) presented the lowest luminescence for caspase activity, with significant differences to its respective contaminant and size counterparts, P4, V20 and P20 treatments respectively ($p < 0.05$).

3.10. Integrated biomarker response

The Integrated Biomarker Response (IBR) was calculated for the data on gills and digestive glands biomarkers from all treatments. Graphical representation of IBR and star plots for both tissues are in Fig. 5. The IBR pattern was similar between tissues and IBR was size and treatment dependent with higher values in both tissues of clams exposed to V20 significantly different from P20 ($p < 0.05$) (Fig. 5A) Similarly, the start plots showed that changes in biomarkers were treatment dependent (Fig. 5B).

4. Discussion

The ingestion of MPs, along with a cocktail of contaminants, have biological consequences in bivalves and other marine organisms (Van Cauwenberghe et al., 2015; Sussarellu et al., 2016; Farady, 2019; Dowarah et al., 2020; Yu et al., 2020). Microplastics dimension are a key factor influencing both sorption and desorption rates of chemicals to and from particles (González-Soto et al., 2019), due to their high specific surface area to volume ratio and a short diffusion pathway, ensuring a rapid chemical exchange with the media (Velzeboer et al., 2014; Sikdokur et al., 2020). Moreover, the smaller the particles, the higher potential to be transported between tissues and haemolymph, along with its retention time (Browne et al., 2008; Farrell and Nelson, 2013; Ribeiro et al., 2017). In addition, a marine product of high economical relevance such as the clam *S. plana,* also represent a susceptibility to MPs uptake via filter-feeding and sediment ingestion as well as close interactions to higher trophic levels (Ribeiro et al., 2017; O'Donnovon et al., 2018). Due to the scarce information related to the effects of MPs as vectors of traditional and emerging contaminants in bivalves (Van Cauwenberghe et al., 2015; Sussarellu et al., 2016; O'Donovan et al., 2018), this study investigated the presence and mode of action of 4–6 and 20–25 μm LDPE MPs (1 mg L^{-1}) in the gills and digestive gland of the clam *S. plana* along

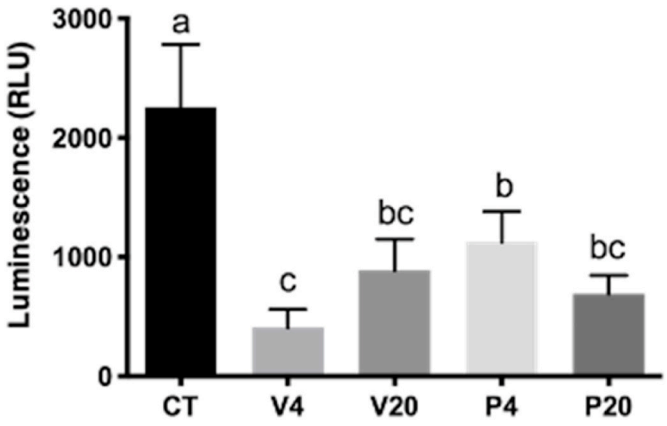

Fig. 4. Caspase activity in digestive glands of S. plana unexposed and exposed to virgin (V4 and V20) and to PFOS adsorbed MPs (P4 and P20) after 14 days (mean luminescence ± SD). Different letters indicate significant differences among treatments (ANOVA, $p < 0.05$).

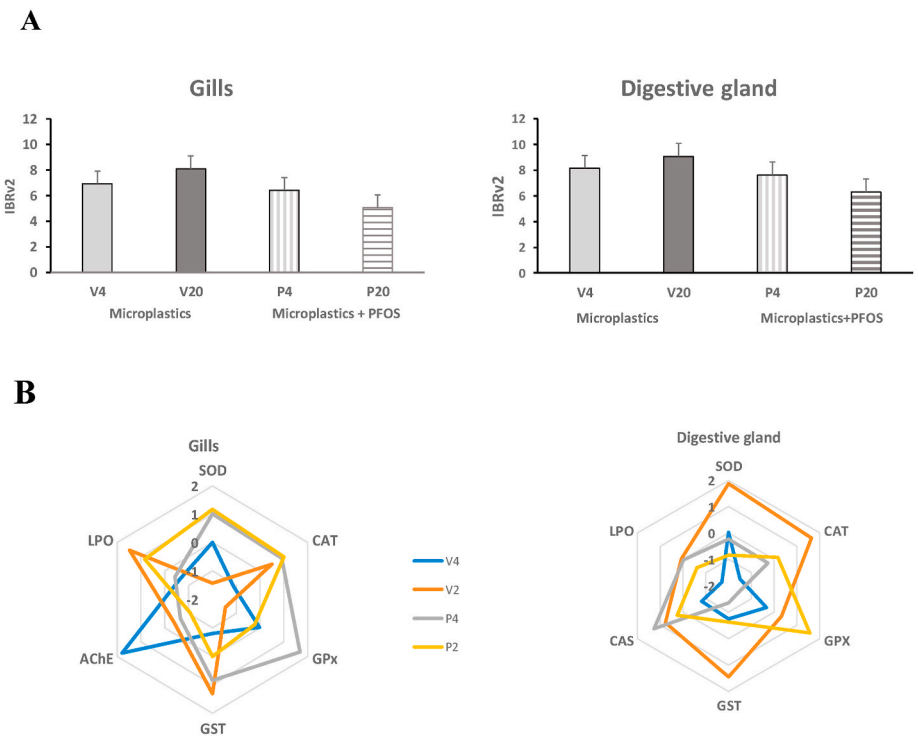

Fig. 5. (A) Integrated biomarker response index version 2 (IBRv2) (mean ± s.d.) and (B) star plots of the gills and digestive gland tissues of *S. plana* exposed to two sizes of virgin (V4 and V20) and PFOS adsorbed MPs (P4 and P20) for 14 days.

with the potential ecotoxicological risk of MPs adsorbed to an emerging contaminant, PFOS, by identifying their presence and assessing their effects using a battery of biomarkers. The concentration of MPs used is considered environmentally relevant, taking into account the data available for marine waters (Li et al., 2019; Ku et al., 2019).

The present findings revealed that *S. plana* ingest both sizes of either virgin or PFOS-adsorbed MPs in whole soft tissues and the ingestion increase with the time of exposure with lower levels for the smaller MPs (V4) (Fig. 2) which are related to the filtration rate detected (Table 2). Curiously, the amount of MPs ingested by both PFOS-sorbed microbeads sizes were similar as well as the levels of PFOS accumulated, which indicate an absence of an ingestion pattern relatively to MPs size or coating, but a combination of both. These results agree with those reported by González-Soto et al. (2019), whereby in the marine mussel *Mytilus galloprovincialis* dietarily exposed to 4.5 μm PS microbeads, with and without benzo-*a*-pyrene (BaP), the mussels accumulated higher quantity of virgin microplastics in gills and lumen of the digestive system, in contrast to mussels exposed to BaP-coated MPs. However, with the increase of the time of exposure, the ingestion of these microspheres coated with BaP were similar like those without the chemical, demonstrating that the final outcomes can also vary with the duration of the exposure (Pittura et al., 2018). Moreover, the accumulation of two different types of plastic (PS and PE) (<100 nm) with pyrene adsorbed was similar in the gills and digestive gland of *M. galloprovincialis* (Avio et al., 2015) indicating that these might be a common pattern of accumulation of organic contaminants when adsorbed on MPs. Nonetheless, the accumulation of PS MPs coated with BaP was considerably higher in tissues of *M. galloprovincialis* after a long-term dietary exposure (26 days) to a smaller size of MPs (0.5 μm) in comparison to the sizes close to the present ones (4.5 μm) (González-Soto et al., 2019), which in the present case did not occur. Besides, Sikdokur et al. (2020) detected in the marine clam *Ruditapes phillipinarum,* the following ranking of MPs body burden of size range 10–45 μm: virgin MPs < MPs co-exposed with Hg < MPs coated with Hg. The presence of Hg adsorbed to MPs seemed to hamper the filtration rate and the uptake of MPs due to the closure of valves, as an adaptative strategy to avoid chemical exposure (Tran et al., 2007; Wagner et al., 2014). The accumulation of PFOS in *S. plana* soft tissues supports the hypothesis that MPs are potential vector of hazardous substances, particularly of persistent organic pollutants (Ferreira et al., 2016; Paul-Pont et al., 2016; O'Donovan et al., 2018; Pittura et al., 2018; Qu et al., 2019; González-Soto et al., 2019; Sikdokur et al., 2020). It is important to bear in mind that longer residence times and ageing of plastics in the natural environment will favour the transference of adsorbed or absorbed chemicals from MPs to the surrounding environment, increasing the threats to marine biota (Wang et al., 2018). It is known that PFOS alone induces a vast array of biological effects, including metabolic disturbance, developmental and reproductive impairments, oxidative stress, and hepatomegaly (Lau, 2012; Chen et al., 2016; Balbi et al., 2017; Du et al., 2017; Kariuki et al., 2017; Sant et al., 2017, 2018). Therefore, in order to assess to what extent uptake and accumulation of the chemical in the water column is altered when binding to MPs (Koelmans et al., 2016; Paul-Pont et al., 2016; Tourinho et al., 2019), it is recognized that the pattern of accumulation of PFOS via MPs sorption should be compared to the single-PFOS exposure scenario.

4.1. Biochemical biomarkers

MPs are known to modulate oxidative stress (Ribeiro et al., 2017; O'Donovan et al., 2018, 2020; Pittura et al., 2018) and therefore a battery of biomarkers of oxidative stress, neurotoxicity, oxidative damage and apoptosis was used to assess the ecotoxicological effects of both types and sizes of MPs.

The enzyme SOD is the first line of defence in protecting tissues against oxidative stress, by means of catalysing the dismutation of superoxide anion radical ($O_2^{\bullet -}$) into hydrogen peroxide to reduce oxidative damage (Jo et al., 2008; Ribeiro et al., 2017). In clams exposed to V20, SOD activity was first induced in the gills and later in the digestive gland indicating an efficient response of this antioxidant enzyme to counteract the toxic by-products generated by physical stress provoked by the accumulation of this MPs size (Fig. 2A and B). Ribeiro et al. (2017) also found a significant increase in SOD activity in the same

biological model exposed to a similar concentration of PS MPs (20 μm). Moreover, consistent with the superoxide scavenging, CAT activity was unable to counter act the removal of the excess of hydrogen peroxide and defence mechanism toward the exogenous source of H_2O_2 (Regoli and Giuliani, 2014) in the digestive gland of V20-exposed clams in the end of the exposure period while in V4-exposed clams the behaviour of CAT activity was the opposite (Fig. 2D) (Catalano et al., 2012; Gomes et al., 2014) indicating an effect of size. This agrees with the fact that MPs are ingested in the gills, through microvilli and endocytosis and transported to the digestive gland and the physical injury of this ingestion generates reactive oxygen species that are size dependent (Von Moos et al., 2012; Kolandhasamy et al., 2018). When peppery furrow shell clams were exposed to PFOS contaminated MPs, the increase of SOD activity in the gills was independent of MPs size evidencing a potential outcome of the toxicity of PFOS adsorbed to these MPs while in the digestive gland this does not occur. CAT activity did not change in clams exposed to PFOS contaminated MPs within this size range in either tissues (O'Donovan et al., 2018).

Glutathione peroxidases are known as particularly sensitive in revealing the early onset of a prooxidant challenge, even at low levels of environmental disturbance and are particularly sensitive to catalyse the metabolically produced H_2O_2 (Regoli and Giuliani, 2014). Compared to the virgin LDPE MPs, GPx activity was only significantly different in the gills of clams exposed to the smaller size of MPs contaminated with PFOS (Fig. 2E and F). O'Donovan et al. (2018) also observed significant differences in GPx activity in the gills between virgin LDPE MPs and LDPE + PFOS MPs of an intermediate size (11–13 μm). These results suggest different mechanism of H_2O_2 production as proposed by a Avio et al. (2015). In the digestive gland however, GPx activity decreased in P4 indicating a higher toxicity of this size of PFOS contaminated MPs. In *S. plana* exposed to mercury, a decrease in GPx activity in the whole soft tissue occurred at the contaminated sites, which contributed to the higher toxicity (Ahmad et al., 2011).

GST is involved in the phase II biotransformation metabolism of organic compounds by catalysing the conjugation of the reduced form of glutathione (GSH) to non-polar compounds that contain an electrophilic carbon, nitrogen or sulphur atom (Hayes et al., 2005). In general, conjugation of xenobiotic compounds with GSH leads to the formation of less reactive products that are readily excreted, ultimately with a protective role against oxidative stress (Hoarau et al., 2002; Hayes et al., 2005). In the current study, the biotransformation induced by GST was not activated in PFOS-exposed clams, at the two MPs size (Fig. 2G and H). Accordingly, the present findings are supported by the results reported by O'Donovan et al. (2018), evidencing no alterations in GST activity of clams *S. plana* under exposure to LDPE MPs + PFOS, at size 11–13 μm, over the same time. Also, no alterations were registered in green mussels *P. viridis* exposed to PFOS only, at any of the concentrations applied (from 0.1 to 1000 μg L^{-1}), which may be attributed to its inert property besides resistance and persistence to biodegradation (Liu et al., 2014). Collectively, these results may be attributed to the lack of biotransformation carried out by these enzymes, corroborating with the evidence gathered for mammals that also denotes the absence of PFOS metabolism (Stahl et al., 2011). In contrast, primary cultures of digestive gland cells of mussels *M. edulis* indicated a sharp stimulation in GST activity under exposure to PFOS at 50 μg L^{-1} (Balbi et al., 2017).

On the other hand, the exposure of clams to virgin MPs of 20–25 μm (V20) was able to induce GST activity first in the gills and then in the digestive gland (Fig. 2G and H). This induction of GST activity was also illustrated in gills from clams *S. plana*, after 14 days of exposure to virgin polystyrene MPs with 20 μm in size (Ribeiro et al., 2017) and virgin LDPE of 11–13 μm (O'Donovan et al., 2018). Therefore, it is hypothesized that GST in *S. plana* may play a central role in the detoxification of virgin MPs polymers related with size regardless of their polymer type. As previously reported the present results confirm that GST seems to be a more sensitive enzyme to MPs exposure with and without an adsorbed organic contaminant (O'Donovan et al., 2018, 2020).

Among the end-products of lipid peroxidation, GSTs conjugate GSH with the 2-alkenals acrolein and crotonaldehyde (Hayes et al., 2005). Accordingly, LPO levels increased in clam gills exposed for two weeks to virgin LDPE 20–25 μm MP with and without PFOS (V20 and P20), revealing an inefficient set of antioxidant mechanisms to counter act the excess of ROS generated either by the physical stress provoked by the particles, or even by the chemical composition of the polymer while no changes were detected in the smaller size (Fig. 2I and J). The present results are in contrast with the findings obtained by Ribeiro et al. (2017) in which *S. plana* exhibited a remarkable significant decrease in LPO by-products under exposure to virgin PS MPs, of 20 μm, as a consequence of feasible antioxidant defences to cope with the attack of ROS onto membrane lipids. Similarly, no oxidative damage was reported in *S. plana* submitted to virgin LDPE MPs (11–13 μm) (O'Donovan et al., 2018), concluding that the combination of chemical composition of MPs and size may provide different biological outcomes in bivalves. Moreover, in PFOS-contaminated 20–25 μm MP (P20), LPO also increased in gills of *S. plana* (Fig. 2I) suggesting that the oxidative effects on lipid membranes were driven by bare large particles, disregarding the contaminant adsorbed. Nevertheless, single PFOS exposure induces cytotoxicity and immunosuppression in the green mussel *P. viridis*, under 100 μg L^{-1} (Liu and Gin, 2018); alterations in the glycolytic metabolism, antioxidant and biotransformation enzymes (Balbi et al., 2017); effects on gene expression alterations on the metabolism of carbohydrate and lipids and in the biosynthesis of amino acids, in addition to the disturbance of the immune system and antioxidant defenses of *R. phillipinarum,* at 6.74 mg L^{-1} (Zhang et al., 2020) but this need to be confirmed in MPs contaminated with PFOS.

Regarding neurotoxicity, AChE activity increased in the gills of the clams exposed to the smaller size of virgin MPs (V4) while there was no alteration in the gills of clams exposed to the bigger size. Similarly, an increase was also detected for the same species exposed to the same virgin MPs with BaP or oxybenzone (PB-3) adsorbed MPs (11–13 μm) (O'Donovan et al., 2018, 2020). The present results seem to indicate that LDPE virgin MPs do not induce neurotoxicity (O'Donovan et al., 2018, 2020). In contrast, AChE activity decreased in the gills of the clams exposed to PFOS contaminated MPs with increasing size indicating that MPs contaminated with PFOS induce neurotoxicity. Polystyrene microplastics (20 μm) also induce a reduction in AChE activity in gill tissues of *S. plana* following two weeks exposure at similar concentrations (Ribeiro et al., 2017) and in mussels *M. galloprovincialis* gills exposed to PS MPs (<100 μm) with and without pyrene adsorbed (Avio et al., 2015) indicating that PS MPs along with size might have an effect on neurotransmission.

The overwhelming prooxidant intracellular status upon MPs exposure could also affect metabolic pathways and cell survival via an apoptotic cascade of events through which the intrinsic pathway is activated (Grütter, 2000). Extensive research on aquatic pollution has reported the induction of caspase activity and upregulation of pro-apoptotic and caspase genes in aquatic organisms exposed to xenobiotics, such as MPs (Liu et al., 2014, 2019; Détrée and Gallardo-Escárate, 2017; Sant et al., 2018; Zhao et al., 2019; Shi et al., 2020). Surprisingly, the present results indicated that the effect on caspases 3/7 was significantly inhibited in the digestive gland of *S. plana* under exposure to any of the MPs treatments (Fig. 4), thus signifying an interruption in apoptotic mechanism. Tang et al. (2020) demonstrated the significant up- and down-regulation of Bcl-2 and caspase-3, respectively, in cockles *Tegillarca granosa*, as a response to single and combined exposures to MPs and organic pollutants.

The overall results of oxidative stress biomarkers indicate that MPs exposure only have a limited effect on the antioxidant capacity of the gills and digestive glands exposed to the higher virgin MPs size (V20) leading to oxidative damage. This was also supported by IBR that showed higher levels in the gills and digestive gland of *S. plana* exposed to the higher size of virgin MPs (Fig. 5). The relatively limited impact on the antioxidant system result from a gradual desorption and leakage of

the PFOS coated on MPs, supported by the accumulation of PFOS in tissues (Fig. 1), indicating that this stressor is a threat to the marine ecosystem, at any conditions of environmental exposure. Considering both sizes, if PFOS MPs become bioavailable, they can penetrate cells and chemically interact with biologically important molecules wherein the clams exposed to the lower size were more affected.

5. Conclusions

S. plana accumulate both types and sizes of MPs with a higher accumulation of larger virgin MPs. PFOS had no observed effect on plastic accumulation indicating that this chemical coating of MPs can alter MPs intake.

Oxidative stress biomarkers showed either induction or inhibition, confirming a limited effect of antioxidant activity that can vary according to virgin exposure or PFOS contaminant MPs conditions and size but PFOS contaminated MPs induced neurotoxicity.

Following 14 days of exposure, time, tissue and size-dependent effects were observed, with the larger virgin LDPE MPs leading stronger impacts, when compared with smaller MPs indicating that *S. plana* respond to the investigated biomarkers. A long-term exposure is required to understand bioavailability of MPs, adsorption and desorption of chemicals.

Considering the adverse ecotoxicological effects of MPs, it is important to raise awareness towards decision makers, scientists and people in general to reduce the level of plastic consumption and elimination along with the presence of organic contaminants with to avoid alterations of the health of marine ecosystems.

Credit author statement

Naimul Islam – Investigation of the accumulation of MPs in the whole soft tissues and gills; Tainá Garcia da Fonseca – investigation of caspase effects and statistical analysis; Juliano Vilke – Investigation of biomarkers effects in the digestive gland; Joanna M. Gonçalves – investigation of neurotoxic and oxidative damage, Paulo Pedro – investigation of microplastic accumulation analysis, Steffen Keiter – preparation and analysis of microplastics uncontaminated and contaminated with PFOS; Sara C. Cunha and José O. Fernandes- PFOS analysis in biological tissues; Maria João Bebianno: Ideas; formulation or evolution of overarching research goals, aims and funding acquisition and determination of biomarker index

Declaration of competing interest

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.

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