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Perfluorooctane sufonic acid (PFOS) adsorbed to Polyethylene Microplastics: Accumulation and Ecotoxicological effects in the clam Scrobicularia plana

--Manuscript Draft--

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Abstract:	Microplastics are widespread in the marine environment, whereby organisms' uptake these tiny particles, presenting adverse biological responses. Plastic debris also act as a vector of many contaminants which intensify their effects to marine organisms that depend on type, size, shape and chemical properties. 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 1 mg L-1 of virgin and PFOS (70.2 ± 12.4 μg g−1) contaminated low-density polyethylene microplastics of two different sizes (4-6 and 20-25 μm) over 14 days. Microplastic and PFOS accumulation and filtration rate were determined along with a multi biomarker approach to access the biological effects of microplastics accumulation. Biomarkers include oxidative stress (superoxide dismutase, catalase, glutathione peroxidases), biotransformation enzymes (glutathione-S-transferases) activity, neurotoxicity (acetylcholinesterase activity), oxidative damage and apoptosis. Microplastics and PFOS accumulated were 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 microparticles lead to stronger impacts, when compared to smaller ones which was also supported by 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 as a probable mechanism associated to carcinogenic responses derived from MPs uptake.
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Response to Reviewers:	

Dear editors,

I am submitting the manuscript entitled Perfluorooctane sufonic acid (PFOS) adsorbed to Polyethylene Microplastics: Accumulation and Ecotoxicological effects in the clam *Scrobicularia plana* Naimul Islam, Tainá Garcia da Fonseca, Juliano Vilke, Joanna M. Gonçalves, Paulo Pedro, Steffen Keiter, Sara C. Cunha, José O. Fernandes, M. J. Bebianno to be considered for publication in the Journal Marine Environmental Research.

All authors are aware of and accept responsibility for the manuscript and state that this is an original work that has not been published or submitted for publication elsewhere. This publication is approved by all authors and tacitly or explicitly by the responsible authorities where the work was carried out, and, if accepted, it will not be published elsewhere in the same form, in English or in any other language without the written consent of the Publisher. This work was funded by the JPI Oceans EPHEMARE project
We believe this manuscript is a valuable contribution to the journal's scope and that the ideas presented in this manuscript will be of significant impact and interest across wide parts of the scientific community.

On behalf of the authors, kind regards,

Maria João Bebianno

Manuscript Number: MERE_2020_656R1

Perfluorooctane sufonic acid (PFOS) adsorbed to Polyethylene Microplastics: Accumulation and Ecotoxicological effects in the clam Scrobicularia plana

Dear Dr Bebianno,

Thank you for submitting your manuscript to Marine Environmental Research.

I have completed my evaluation of your manuscript. As you will see from the enclosed comments, the reviewers appreciated changes you have made to the manuscript but they consider the revisions not satisfactory. Specifically, they point out to a confirmation bias in the discussion and a disconnect between the results (that mostly show no significant effects of the treatments) and the interpretations that often overstate the size and the significance of the effects. I agree with this assessment and recommend that the discussion is shortened and the conclusions limited to those that are directly supported by the data, ovoiding speculations and overstatements. I also agree with the second reviewer that PCA analysis does not provide a value added and should be removed. I invite you to resubmit your manuscript after addressing the comments below. Please resubmit your revised manuscript by Jan 29, 2021.

R: The PCA analysis was removed and the discussion was shortened and the conclusions limited to those directly supported by the data

When revising your manuscript, please consider all issues mentioned in the reviewers' comments carefully: please outline every change made in response to their comments and provide suitable rebuttals for any comments not addressed. Please note that your revised submission may need to be re-reviewed.

To submit your revised manuscript, please log in as an author at https://www.editorialmanager.com/mere/, and navigate to the "Submissions Needing Revision" folder under the Author Main Menu.

Marine Environmental Research values your contribution and I look forward to receiving your revised manuscript.

Kind regards,

Inna Sokolova

Editor

Marine Environmental Research

Editor and Reviewer comments:

Reviewer #1: Authors have taken into account some of the remarks I made. However, if occurrence of overstatement has been reduced however some are still present in results and many in discussion. Results

L404 "At the 7th day, clams exposed to V20 showed a higher amount of particles in whole tissues compared to controls ($p < 0.05$), although not significantly different from any of the other MPs treatments ($p > 0.05$)." Writing is misleading and there is no reason to exemplify V20 here. Sentence could be as "All MPs accumulate in whole tissues whatever the size or presence of PFOS."
R: The sentence was changed as suggested.

L463 "During the bioassay, V20-treated clams had significantly higher GST activity in comparison to controls, to V4 and to P20." During the bioassay likely means at day 7. At this time point V20 is not different from control.

R: We agree with the comments of the reviewer and the sentence was corrected

PCA analyses Please provide Eigen values (or loadings) of each variable and significance of its contribution to each axis. Otherwise (especially considering the very central position of biomarkers) data presented are not convincing.

R: As suggested by the other reviewer the PCA was deleted

Discussion
still appears disproportionate compared to the amount of results, the small changes and for some a lack of consistency over time or between MPs of same size or virgin vs PFOS. A general case is that Authors chose one situation and draw general conclusion (as an example see below the case of GPx L628).
Examples (not complete) of overstatements, approximation :
L550 "However, 20-25 µm virgin MPs (V20) dominate the accumulation of MPs over the whole exposure time." This is not true since there are differences only at D14
R: The sentence was corrected
L577 "In the present study, the MPs accumulation profile agrees with the results obtained for the filtration rate (Table 2), hypothesizing that virgin and larger microbeads ensure a preferential feeding scenario in contrast to the smallest virgin particles and those coated with PFOS, disregarding the MPs dimension." This is not fully true since there is no difference in filtration rate between V4 and P4 and between V20 and P20 so PFOS DOES NOT influence filtration rate.
R: The sentence was corrected

L599 "MPs and PFOS are known to induce oxidative stress" This should be modulated ! Half of papers concluding that MPs do not induce oxidative stress and this largely varies according to polymer PE not being the one triggering stress most often.
R: The sentence was corrected

L621 "… while in the digestive gland exposed to P4, SOD activity exhibited a similar pattern of V4, suggesting a similar pro-oxidant injury in this tissue for this size of MPs." You cannot say this !
At day 7 P4 is higher than V4 while at day 14 P4=V4 AND they are both equal to control so there is no pro-oxidant activity of 4µm MPs
R: The sentence was corrected
L628 "Compared to virgin LDPE MPs, GPx activity was significantly different in the gills of clams exposed to PFOS contaminated MPs of the same size (Figures 2E-F)."
In fact this true for only one situation P4 compared to V4 at only one of the two dates, day 14. So drawing this general conclusion is not accurate.
R: The sentence was changed

So considering intensity of changes, overstatements in discussion appear highly speculative. In addition discussion has been changed by partial reshuffling which finally didn't contribute much to its reduction. Overstatements and generalizations are so numerous that it is not possible to point them all (please check this carefully and throughout the whole discussion, not only indicated lines which are just examples). Discussion should be significantly reduced to 3-4 pages with a maximum removal of overstatement or speculation.
R: As suggested discussion was reduced

Answer to reviewer is not accurate. Examples :
L445:
Initial review "According to Fig 2D, there is NO difference between any treatment and Control after 7 and 14 days. Revise letters in Fig 2D, there is no B so no AB at day 7."
R: Letters were corrected
L733:
Initial review "The same contradiction can be seen in discussion about IBR where it is said that "…confirming higher threat of MPs accumulation in S. plana with increasing MPs size." while earlier it is said that "…Therefore, the smaller the particle, the higher the hazardous potential…".
Reply "The contradiction was corrected"
But in revised version of the Ms
L533 "Moreover, the smaller the particles, the higher the hazardous potential of the…"
R: The contradictions were corrected

L733 "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…"

So the same contradiction still exists but in another place not pointed out previously. Again, please check carefully all discrepancies throughout the whole manuscript.
R: The discrepancies were checked and corrected

Reviewer #2: The paper has some interesting data that deserve publication but overall the presented evidence is limited compared to the speculative nature of the interpretation and discussion. The added value of the PCA analysis and true meaning of the IBR remains unclear. See attached file for detailed comments..

R: As suggested by the editor the PCA was deleted. All changes that were included in the file attached and that are listed below were taken into account
The speculative nature of the results and discussion were removed,

Comments of the attached file
Paper MERE_2020_656_R1 has improved after the first revision but several remarks have not been taken into account. The PCA and IBR are still not clearly explained in terms of interpretation and meaning (if needed as such). The discussion remainss confounded with

several speculations that are not or only superficially supported by the data of the study.
Headlines:
Low density polyethylene virgin MPs and with PFOs adsorbed with two different sizes were accumulated in Scrobicularia plana
Not clear to what "were accumulated" refers. It has not been demonstrated that the MP were actually internalized by the clams.
R: The sentence was changed
Text:
Line 18: Microplastics are widespread in the marine environment, whereby the uptake of the these tiny particles by organisms can cause adverse biological responses.
R: The suggested change was made

Line 29: (glutathione-S-transferases) activity ->: (glutathione-S-transferases activity)
R: The suggested change was made

Linhe 47: widely use -> wide use
R: The suggested change was made

Line 56: blown in, blown from landfills -> blown in from where ?
R: The sentence was corrected to include the atmospheric and the land input
Line 71: world oceans
R: We do not agree with this change because the world ocean is only one.

Line 75: pH is rather stable in the oceans, so that effect is expected to be minimal
R: We do agree with the comment and the reference to pH was deleted

Line 81: the gut cavity is the storage for accumulation -> not clear what is meant by this, it is also not clear what is meant by the term "accumulation" as used in the text. No distinction is made between ingestion without internalization and ingestion with internalization.
R: The sentence was clarified

Line 94: Therefore, ingestion, gills and adherence of MPs may be a vector of other contaminants, from where there is a potential source for bioaccumulation -> Poorly phrased sentence, not clear what the role of the gills is. I assume trapping of the MPs during but that is not stated.
R: The sentence was changed
Line 99: The perfluorooctane sulfonic acid (PFOS)-> Perfluorooctane sulfonic acid (PFOS)
R: The suggested change was made

Line 124: A biomarker index was calculated to assess these effects in an integrated manner..
R: the suggested addition was included
Line 134: The use of polypropylene bottles is not exactly avoiding the use of plastic material, why was this not dome in glass bottles ?

R: The polypropylene bottle was only used to prepare the microplastics with the PFOS adsorbed that required strong rotation not compatible with the use of glass material

Line 139: How was the centrifugation done (rpm and g).
R: A rotary shaker was used and the speed was 20 rpm

Line 166: aquaria -> aquarium
R: The suggested change was made

Line 171: The two types of MPs (virgin or with PFOS) were not added to each aquarium as is stated but to different aquaria.
The change was made to indicate that MPs virgin and contaminated to PFOS were added separately to two of the aquaria each.

Line 176: How can it be that salinity showed so much variation, does not seem to be under control.
R: We checked the numbers and there was a typing error that was corrected

Line 181: What was measured, the length of the shell ?
R: The shell length was measured and information included

Line 182: Not clear whether the gills, digestive gland and whole soft tissues were from the same individuals.
R: No the individuals were not the same. The whole soft tissues were stored at -20ºC for MPs and PFOS analysis and for the enzymes other clams were collected and tissues were dissected intro gills and digestive gland

Line 196: …each individual clam was placed into a glass beaker…
R: The suggested change was made

Line 197: Chlorela->Chlorella. What was the Chlorella concentration in the beakers and what is meant by Chlorella suspension. What is the origin of the algae and why was a freshwater algae used and not a marine algae.
R: We do agree with the reviewer and there was a mistake on the name of the species used. The algae used was Tetraselmis chuii and the concentration of the suspension was 2.2×10^{6} cells/mL

Line 207: delete “kept”
R: The suggested change was made

Line 211: Then a filtered NaCl solution…What was the concentration of NaCl used.
R: The concentration of the solution was included

Line 215: …for observation and quantification of the MPs under…

R: The suggested change was made

Line 240: Not clear where “both” refers to
R: The sentence was corrected
Line 247: A DA dwell time…
R: The correction was made

Line 262: How was the protein concentration determined.
R: The reference to total protein concentrations were deleted here because the determination of total protein concentrations were included in point 2.7

Line 270: Brand of the UV spectrophotometer ?
R: the brand of the UV spectrophotometer was included
Line 303: What is meant by 15.4 M ?

: 15.4 M
R: M means molar

Line 310: Why only in gills and not digestive gland ?
R: Because normally AChE is only determined in the brain or in the gills. It was clarified in the sentence that the determination was only made in the gills.

Line 322: …were included in each 96 well microplate…
R: The suggested change was made

Line 334: Which type of reader used ?
R: The information was included

Line 347: A blank reaction was included…
R: The suggested change was made

Line 362: Why were the other data not included in the PCA ?
R: As suggested by the editor and by the other reviewer the PCS information was deleted

Table 2: Filtration rate for P4 has an unlikely low SD value. These data need verification.
R: There was a typing mistake and the SD value of the filtration rate was corrected on the Table

Line 402: reference is made to accumulation in tissues, this is misleading since the accumulation may be limited to ingestion and accumulation in the gut lumen.
R: Change was made to indicate that MPs were ingested

Line 410: How does the accumulation of PFOS compare with the expected concentration of PFOS on the basis of the MPs measured in the tissues (Figure 1)? The PFOS analysis only done after 14 days ?

R: PFOS concentration was only analysed after 14 days and this is 100 less than expected

Line 423: What about V4 ?
R: Information regarding V4 was included.

Line 483: …than at day zero and day 7
R: The change was made as suggested

Line 491: What is the Caspase activity in the control at day zero ?
R: Caspase activity was only measured at the end of the exposure period because there were not tissue available at time zero.

Line 494: What about P20 ?
R: P20 was added in the sentence

Line 509: What is meant by “are jointly with”. Do you mean cluster together ?
R: As suggested by the edidor the results of PCA analysis were deleted.

Line 520: The IBR values are presented without variance bars. Since the values are based on the analysis of individuals it should be possible to provide variance bars. As it is presented it seems that there are no differences. It is stated that the IBR pattern was similar among tissues but in the next sentences it is indicated that the levels are not the same among treatments. The star plots are not explained and their meaning is unclear. In general the added value of the PCR and IBR approaches is poorly presented and explained.

R: As suggested the IBR values are presented with variance bars. Start plots are explained and the added value of the IBR included.Thr

Line 526: What evidence exists that this is a real concern for human exposure given other exposure routes.
R: this information related to the impact of human exposure was deleted

Line 546: Again how relevant is 1 mg/l.
R: this information was already included and the sentence was rephrased

Line 548: The meaning of the term “accumulation” is still not explained. Very important to make clear that this does not necessarily refers to internalization.
R: The use of the term accumulation was replaced by ingestion

Line 547: The results are presented in a complex manner. Focus on the evidence presented in this study.
R: Changes were made to focus on the evidence of the results obtained.

Line 615: Seems rather speculative given te limited evidence presented.

R: The sentence was deleted to avoid speculation

Line 654: What is the ecological meaning of these unrealistic exposure scenario's.
R: As suggested by the other reviewer the sentence was deleted to avoid speculation.

Line 715: If so poorly explained in the results section.
As mentioned above the PCA data was removed from the results and the discussion

Line 728: This sentence seems to contradict itself
R: The sentence was change to avoid contradiction

Line 735: No real evidence that this could be a "an important indicator" as claimed.
R: The reference to the expression an "important indicator" was removed

Line 742: Why PFOS in particular, no evidence presented.
R: the information was deleted

Highlights

- Low density polyethylene virgin MPs and with PFOs adsorbed of two different sizes were ingested in *Scrobicularia plana*
- MPs act as a vector of PFOS in *S. plana* tissues and PFOS accumulation was independent of MPs size
- Higher MPs accumulation was in the clams exposed to the bigger size of virgin LDPE MPs
- Oxidative stress was MPs size and tissue dependent.

Manuscript File Click here to view linked References

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

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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.

Keywords: Microplastics, *Scrobicularia plana*, perfluorooctane sulfonic acid, biomarkers, toxicity

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 *et al.,* 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.,* 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 20-30 % (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 'sandblasting' 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, Lozano and Mouat, 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 235–1,770 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℃ for 48h (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 hours. 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 ºC) 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 hours, 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 ºC ± 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 -80ºC until further analysis. Whole soft tissues collected for determination of MPs and PFOS concentrations were collected under a fume hood, weighed and frozen at -20ºC 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 hours 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 (T0) and after 14-day exposure (T14). 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:

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

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 ºC 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 minutes 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 minutes and subsequently centrifuged at 1690 *g*, for 10 minutes. 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 (150×4.6mm) 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 0.5M, Tris 20 mM, KCL 0.5 M, DTT 1M, EDTA 1 mM, and Milli-Q water at pH 7.6), according to the protocol of Geret et al. (2012). The homogenate was centrifuged at 500 *g*, at 4ºC for 15 minutes. The cytosolic

fraction was separated and centrifuged a second time at 12,000 *g*, at 4ºC for 45 minutes. Supernatant was divided into aliquots for the determination of the antioxidant enzymes (SOD, CAT and GPx) and GST activities. Aliquots were frozen at -80 ºC 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 & 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 minute 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 & Burk (1978) 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 and Jakoby (1981) 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, 0.02M Tris, pH 8.6) and 50 µL of butylated hydroxytoluene solution (BHT). The homogenate was centrifuged at 30,000g, at 4 ºC for 45 minutes 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 ºC 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 ºC, for 60 minutes, 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 ºC for 10 minutes. 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 ε = 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 ºC for 30 minutes. The supernatant was subdivided into two aliquots and stored at -80 ºC, 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 minutes. 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 minutes, with an interval of 30 seconds. 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), acording to the method described by Fernandes et al. (2020). Tissues were homogenized 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 minutes, 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 one hour 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 ($p < 0.05$).

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 (Figure 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 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 the Figures 2A-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 (Figure 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 (V20) ($p < 0.05$).

SOD activity in digestive glands was significantly lower than in gills ($p < 0.05$) (Figure 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 Figures 2C-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 ($p > 0.05$) (Figure 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 (Figure 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 ($p > 0.05$) (Figures 2E-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, (Figure 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 ($p < 0.05$) (Figure 2F).

3.6. Glutathione-S-transferases

GST activity in gills and digestive glands of *S. plana* are presented in Figures 2G-H. Results showed no significant variation in GST activity in gills from unexposed clams throughout the bioassay ($p > 0.05$) (Figure 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 (Figure 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 ($p > 0.05$) (Figures 2I-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 ($p < 0.05$) (Figure 3B).

In general, results from digestive glands show a concentration of LPO by-products 3-fold lower than those observed in gills (Figure 3C), with only significant differences between 4-6 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 Figure 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).

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 ($p < 0.05$) (Figure 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 Figure 6. 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 ($p < 0.05$) (Figure 6A) Similarly, the start plots showed that changes in biomarkers were treatment dependent (Figure 6B).

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 et al., 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., 2018; 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; Farrel 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., 2019), 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 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., 2018; 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) (Figure 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 Gonzalez-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; Wegner et al., 2012).. The accumulation of PFOS in *S. plana* soft tissues supports the hypothesis that MPs

543 are potential vector of hazardous substances, particularly of persistent organic pollutants
544 (Ferreira et al. 2016; Paul-Pont et al., 2016; O'Donovan et al., 2018; Pittura et al., 2018; Qu et
545 al., 2018; González-Soto et al., 2019; Sikdokur et al., 2020). It is important to bear in mind
546 that longer residence times and ageing of plastics in the natural environment will favour the
547 transference of adsorbed or absorbed chemicals from MPs to the surrounding environment,
548 increasing the threats to marine biota (Wang et al., 2018). It is known that PFOS alone induces
549 a vast array of biological effects, including metabolic disturbance, developmental and
550 reproductive impairments, oxidative stress, and hepatomegaly (Lau, 2012; Lankadurai et al.,
551 2013; Chen et al., 2016; Balbi et al., 2017; Du et al., 2017; Kariuki et al., 2017; Sant et al.,
552 2017, 2018). Therefore, in order to assess to what extent uptake and accumulation of the
553 chemical in the water column is altered when binding to MPs (Koelmans et al., 2016; Paul-
554 Pont et al., 2016; Tourinho et al., 2019), it is recognized that the pattern of accumulation of
555 PFOS via MPs sorption should be compared to the single-PFOS exposure scenario.
556
557
558 **4.1 Biochemical biomarkers**
559 MPs are known to modulate oxidative stress (Ribeiro et al., 2017; O'Donovan et al., 2018,
560 2020; Pittura et al., 2018) and therefore a battery of biomarkers of oxidative stress,
561 neurotoxicity, oxidative damage and apoptosis was used to assess the ecotoxicological effects
562 of both types and sizes of MPs.
563 The enzyme SOD is the first line of defence in protecting tissues against oxidative stress, by
564 means of catalysing the dismutation of superoxide anion radical ($O_2^{\bullet-}$) into hydrogen peroxide
565 to reduce oxidative damage (Jo *et al.*, 2008, Ribeiro *et al.*, 2017). In clams exposed to V20,
566 SOD activity was first induced in the gills and later in the digestive gland indicating an efficient
567 response of this antioxidant enzyme to counteract the toxic by-products generated by physical
568 stress provoked by the accumulation of this MPs size (Figures 2A-B). Ribeiro *et al.* (2017) also
569 found a significant increase in SOD activity in the same biological model exposed to a similar
570 concentration of PS MPs (20 μm). Moreover, consistent with the superoxide scavenging, CAT
571 activity was unable to counter act the removal of the excess of hydrogen peroxide and defence
572 mechanism toward the exogenous source of H_2O_2 (Regoli & Giuliani, 2014) in the digestive
573 gland of V20-exposed clams in the end of the exposure period while in V4-exposed clams the
574 behaviour of CAT activity was the opposite (Figure 2D) (Catalano et al., 2014; Gomes et al.,
575 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 & 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 (Figures 2E-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 (Figures 2G-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 ug 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 ug 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 (Figures 2G-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., 1999; Prabhu et al., 2004). Accordingly, LPO levels increased in clam gills exposed for two weeks to virgin LDPE 20-25 µm MPs 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 (Figures 2I-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 MPs (P20), LPO also increased in gills of *S. plana* (Figure 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., 2018); 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

642 *R. phillipinarum,* at 6.74 mg L^{-1} (Zhang et al., 2020) but this need to be confirmed in MPs
643 contaminated with PFOS.
644
645 Regarding neurotoxicity, AChE activity increased in the gills of the clams exposed to the
646 smaller size of virgin MPs (V4) while there was no alteration in the gills of clams exposed to
647 the bigger size. Similarly, an increase was also detected for the same species exposed to the
648 same virgin MPs with BaP or oxybenzone (PB-3) adsorbed MPs (11-13 μm) (O´Donovan et
649 al., 2018, 2020). The present results seem to indicate that LDPE virgin MPs do not induce
650 neurotoxicity (O'Donovan et al., 2018, 2020). In contrast, AChE activity decreased in the gills
651 of the clams exposed to PFOS contaminated MPs with increasing size indicating that MPs
652 contaminated with PFOS induce neurotoxicity. Polystyrene microplastics (20 μm) also induce
653 a reduction in AChE activity in gill tissues of *S. plana* following two weeks exposure at similar
654 concentrations (Ribeiro *et al.*, 2017) and in mussels *M. galloprovincialis* gills exposed to PS
655 MPs (<100 μm) with and without pyrene adsorbed (Avio et al., 2015) indicating that PS MPs
656 along with size might have an effect on neurotransmission.
657
658 The overwhelming prooxidant intracellular status upon MPs exposure could also affect
659 metabolic pathways and cell survival via an apoptotic cascade of events through which the
660 intrinsic pathway is activated (Grutter, 2000). Extensive research on aquatic pollution has
661 reported the induction of caspase activity and upregulation of pro-apoptotic and caspase genes
662 in aquatic organisms exposed to xenobiotics, such as MPs (Liu et al., 2007, 2019; Détrée and
663 Gallardo-Escárate, 2017; Sant et al., 2018; Zhao et al., 2019; Shi et al., 2020). Surprisingly, the
664 present results indicated that the effect on caspases 3/7 was significantly inhibited in the
665 digestive gland of *S. plana* under exposure to any of the MPs treatments (Figure 4), thus
666 signifying an interruption in apoptotic mechanism. Tang et al. (2020) demonstrated the
667 significant up- and down-regulation of Bcl-2 and caspase-3, respectively, in cockles *Tegillarca*
668 *granosa*, as a response to single and combined exposures to MPs and organic pollutants.

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

PFOS in tissues (Figure 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.

4. 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.

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Figure
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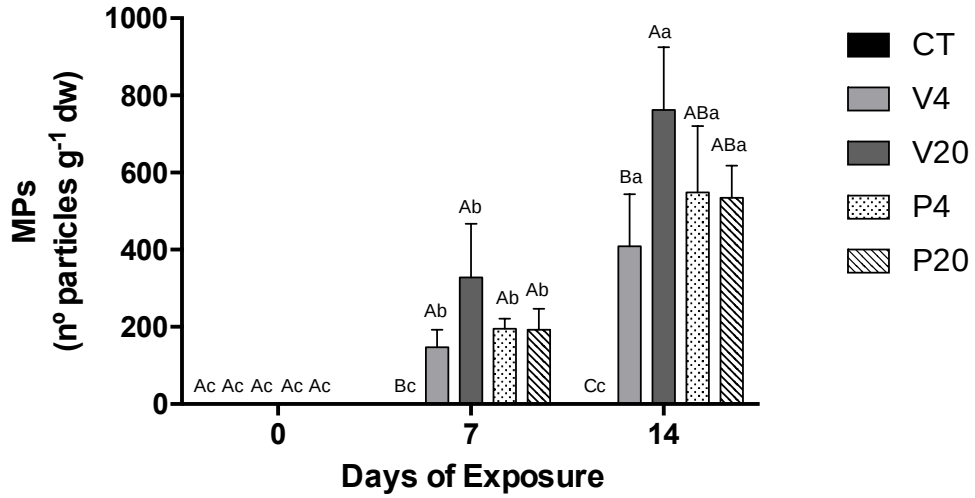

Figure 1: MPs (mean ± s.d.) in the whole soft tissues of *S. plana* unexposed (CT) and exposed to two sizes of virgin (V4 and V20) and PFOS adsorbed MPs *(*P4 and P20*) for* 14 days. Different capital letters indicate significant differences between treatments within the same time. Different lowercase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

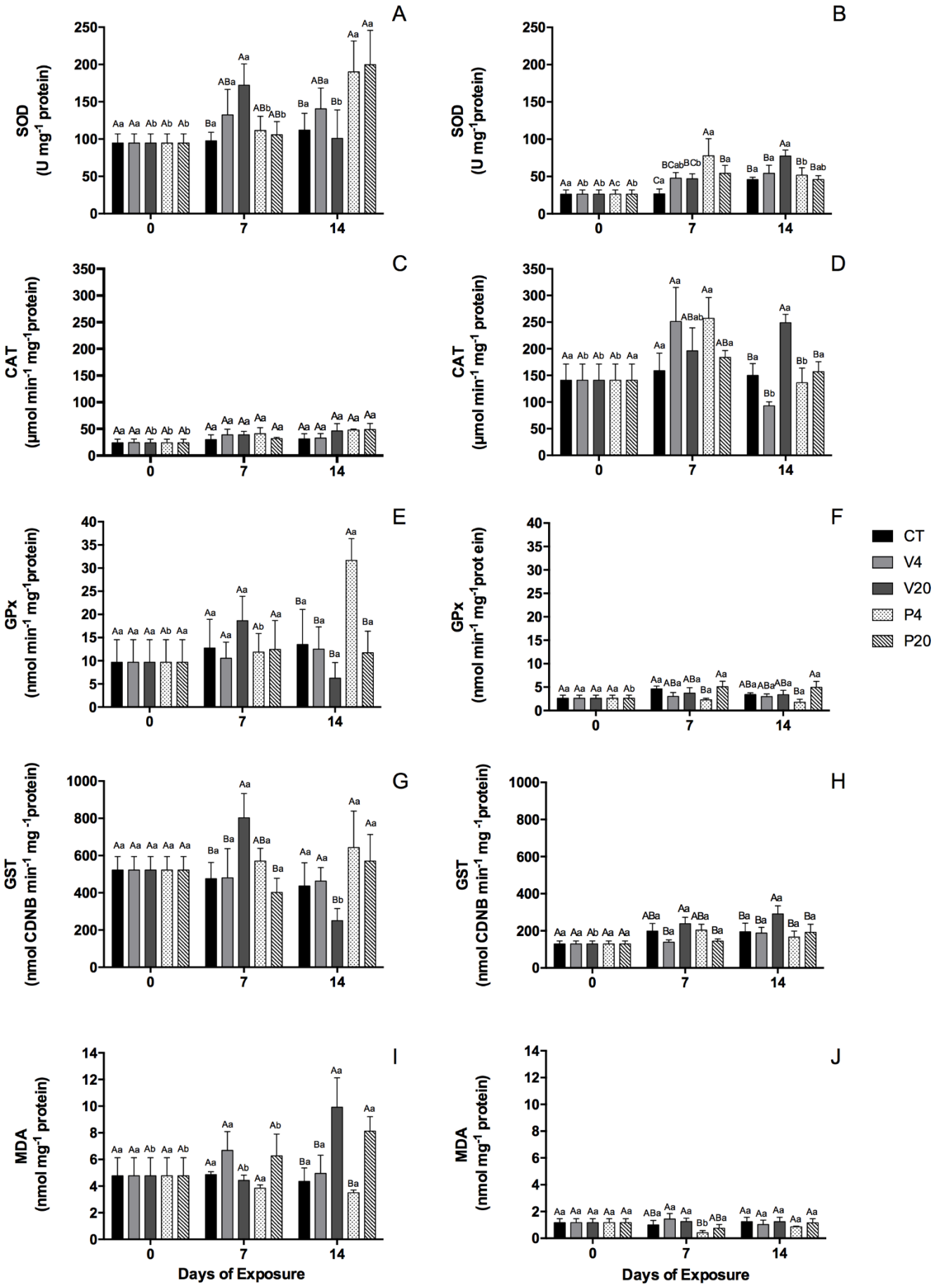
A
B
C
D
E
F
G
H
I
J
SOD (U mg-1 protein)
CAT (µmol min-1 mg-1protein)
GPx (nmol min-1 mg-1protein)
GST (nmol CDNB min-1 mg -1protein)
MDA (nmol mg-1 protein)
Days of Exposure
CT
V4
V20
P4
P20

Figure 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; $p < 0.05$).

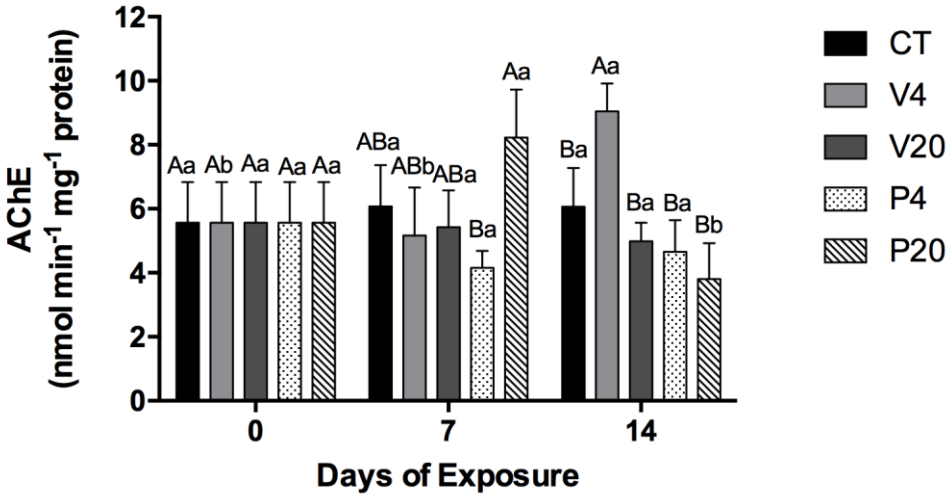

Figure 3: AChE activity (mean ± standard deviation) in the gills of *S. plana.* of unexposed *S. plana* (CT), exposed to two sizes of virgin (V4 and V20) and PFOS adsorbed MPs (P4 and P20) for 14 days. Different capital letters indicate a significant difference between treatments within the same time.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

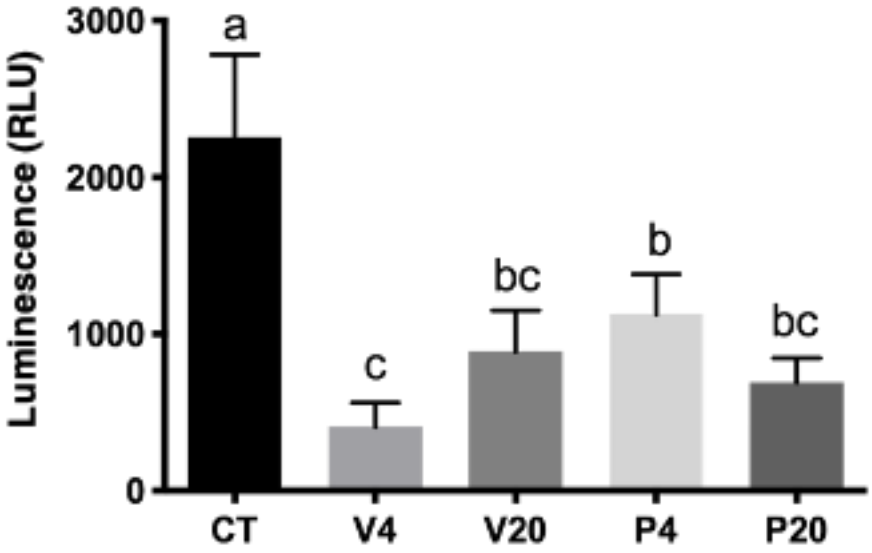

Figure 4: Caspase activity in digestive glands of *S. plana* unexposed and exposed to virgin (V4 and V20) and to PFOS adsorbed MPs (P4 and P20) after 14 days (mean luminescence ± SD). Different letters indicate significant differences among treatments (ANOVA, $p < 0.05$)

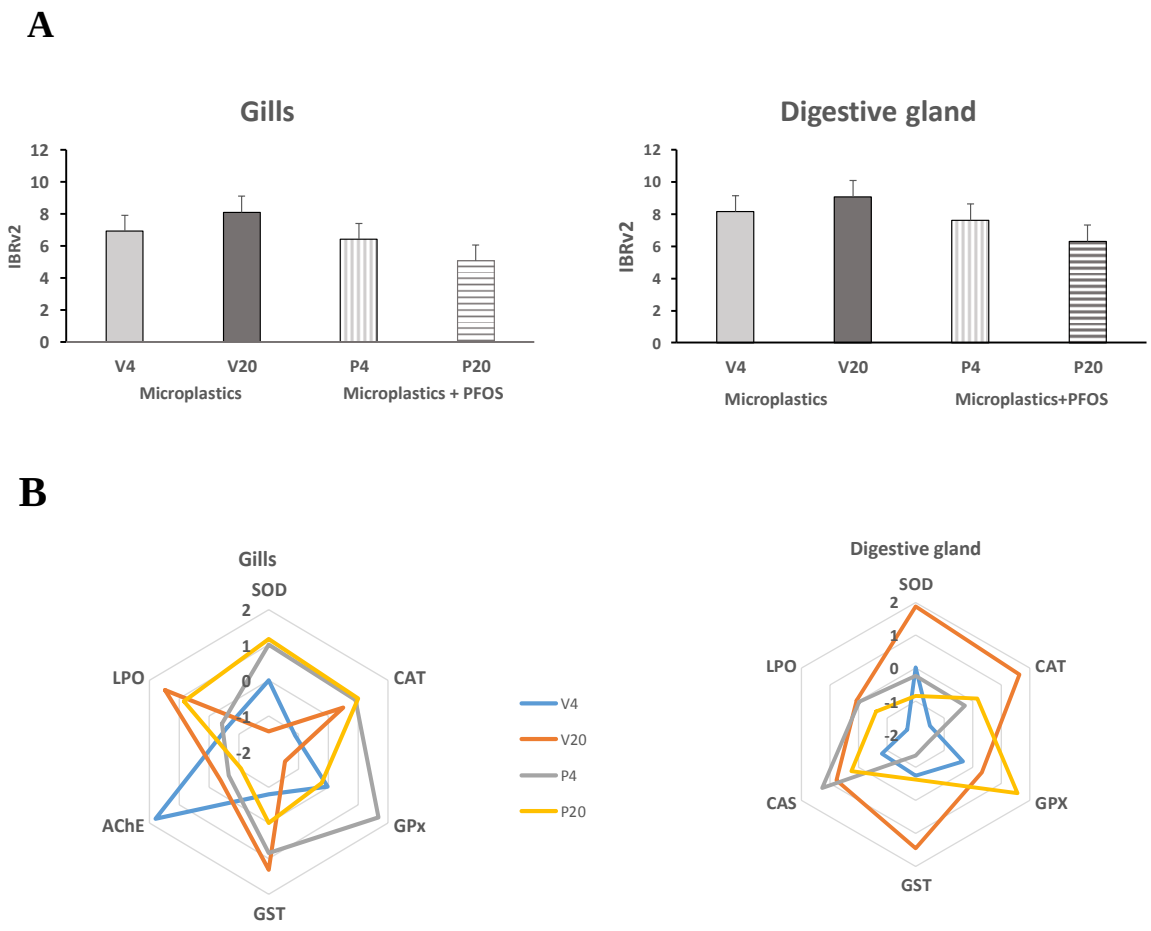

Figure 5 – (A) Integrated biomarker response index version 2 (IBRv2) (mean ± s.d.) and (B) star plots of the gills and digestive gland tissues of *S. plana* exposed to two sizes of virgin (V4 and V20) and PFOS adsorbed MPs (P4 and P20) for 14 days.

Table
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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)

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Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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

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 newrotoxic and oxidative damage, Paulo Pedro ‾ investigation of microplastic accumlation 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