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(Article begins on next page)

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Are mixtures of micro/nanoplastics more toxic than individual micro or nanoplastic contamination in the clam *Ruditapes decussatus*?

Emma Ventura[a,b], Joanna M. Gonçalves[a], Juliano M. Vilke[a], Giuseppe d'Errico[b], Maura Benedetti[b,c], Francesco Regoli[b,c], and Maria João Bebianno[a]

[a]CIMA, Centre of Marine and Environmental Research\ARNET - Infrastructure Network in Aquatic Research, University of Algarve, Campus de Gambelas, 8000-139 Faro, Portugal.
[b]Dipartimento di Scienze della Vita e dell'Ambiente, Università Politecnica delle Marche, Via Brecce Bianche, 60131 Ancona, Italy
[c]National Future Biodiversity Centre (NFBC), Palermo, Italy

*Corresponding author: mbebian@ualg.pt / +351 289 244 434

Abstract

It is quite evident/impressive that there are now more micro (MPs) and nano (NPs) sized plastic particles than ever before, being extremely harmful once they reach the ocean. Toxicology and ingestion routes in marine biota may vary because of potential interactions between MPs and NPs in the marine environment, however, this information is yet not fully understood. The clam *Ruditapes decussatus* was used to test the cytotoxicity (*in vitro;* 24h) and the genotoxicity, neurotoxicity, oxidative stress, oxidative damage as a result of ingestion (*in vivo;* 10 days) of polystyrene NPs (PSNPs; 50nm, 10 μg/L), polyethylene MPs (PEMPs; 4-6 μm, 10 g/L), and their mixture. (Mix; 10 μg/L of PEMPs + 10 μg/L of PSNPs) in the gills and digestive glands. At all treatment exposures, MPs and NPs were ingested by clams,. Results obtained in *in vitro* assay indicate that PSNPs are more harmful to clam's hemolymph than other treatments. Genotoxicity was not detected in any exposure treatment, while clams treated with MPs and Mix showed a significant time-dependent increase in AChE enzymatic activity. In addition, the activity of the enzymes superoxide dismutase (SOD) and catalase (CAT) showed a exposures are tissue- and time-dependent increase statistically significant in response to all treatments; the lack of oxidative damage support the neutralization efficiency of reactive oxygen species generated during exposure by the antioxidant defence system. The overall hazard assessment, according to the weighted evidence criterion revealed the greatest impact in clam's digestive glands and the tenth day of exposure. An antagonistic interaction between MP/NPs was also highlighted.

Keywords: Microplastics; Nanoplastics; Mixtures of Plastics, Bivalves, *Ruditapes decussatus*, Polystyrene, Polyethylene

1. Introduction

As a result of the high applicability of plastic materials, global plastic production (GPP) has grown rapidly over time reaching 390.7 million tons in 2022 (PlasticsEurope, 2022). Polyethylene (PE), polypropylene (PP), polyvinyl chloride (PVC), and polystyrene (PS) are among the most requested polymers by the global plastic industry. Particularly PE and PS are highly used in the packaging (e.g., coffee cups, takeaway lids, detergent bottles, milk cartoons), building and construction (e.g., rigid pipes, park benches, air conditioners, roofing, building walls) end markets contributing the most to the GPP (44% and 18%, respectively) (PlasticsEurope, 2022). The number of plastic materials that are currently entering the ocean is strictly associated with the increasing trend in plastic production and human population density (Jambeck et al., 2015). In fact, despite a rise in plastic recycling from 2006 until today (+117%), a considerable portion of plastic waste still ends up in landfills (37%) and finally into the marine environment due to inadequate waste management systems (Alimi et al., 2018; PlasticsEurope, 2022). This has a significant negative impact on marine life as well as consequences on goods (e.g., food, pharmaceutical components, mineral sources) and services (e.g., climate regulation, carbon sequestration, oxygen production) that the ocean provides to our society. Adverse effects are aggravated by the increasing concentration of smaller plastic fragments (Kiran et al., 2022), originated from a variety of degradation and fragmentation processes of plastic materials including UV-induced photodegradation, thermo-oxidation, hydrolysis, and microbial degradation. These processes eventually cause the plastic material to shrink in size, reaching a measurement scale between micro (μ) and nano (n) meters. Microplastics (MPs) and nanoplastics (NPs), despite the trim level of consensus reached among the scientific community, are commonly defined as particles with a size range of 1 μm to 5 mm and 1 nm to 1000 nm, respectively (Gigault et al., 2018; Guzzetti et al., 2018). The formation and the persistence of MPs and NPs in water can alter their physical and chemical properties (Li et al., 2019; Zhang et al., 2021). For instance, free ions present in seawater can be absorbed by MP particles changing their surface charges (Rahman et al., 2023), thus allowing them to possibly form aggregates that influence their fate and mobility (Gonçalves & Bebianno, 2021; Li et al., 2019). At the same time, due to their small size, MPs and NPs are easily ingested by marine organisms (Rodrigues et al., 2022) either accidentally or because of their similarity to the prey (Fossi et al., 2018). The smaller the particle size, the easier it is for them to cross biological membranes, thus causing bioaccumulation and transfer along the trophic food web. MPs and NPs can induce mechanical and physical damages (e.g., blockage of feeding structures, inflammation, abrasion) along with the alteration of critical cellular structures and biological functions (e.g., genotoxicity, neurotoxicity damage) in many marine organisms (Fossi et al., 2018; Gonçalves et al., 2022; Guzzetti et al., 2018;

2

Zaki & Aris, 2022). Even though it is widely recognized that MPs and NPs can negatively affect marine environments and organisms, many gaps still remain, especially in relation to NP sources, distribution, and toxicity pathways, due to the lack of analytical methodologies to quantify their ingestion and assess their effects (Baudrimont et al., 2020; Gonçalves & Bebianno, 2021; Hsieh et al., 2023; Zaki & Aris, 2022). Marine bivalves are important sentinel organisms that can therefore be used to better understand the effects caused by emerging contaminants in the marine environment, such as plastic particles (Bebianno et al., 2004; Sendra et al., 2021; Silva et al., 2021). It has been already demonstrated the capacity of marine bivalves to ingest small-sized plastic particles that can then be translocated and accumulated in many tissues (e.g., gills and digestive gland) (Bendell et al., 2020; Ribeiro et al., 2017; Magara et al., 2018; Sendra et al., 2021; Thomas et al., 2020). Genotoxicity, neurotoxicity, oxidative stress, and oxidative damage have also been assessed in filter-feeding organisms such as mussels (*Mytilus galloprovincialis, Mytilus coruscus, Mytilus edulis)* and clams *(Scrobicularia plana, Ruditapes decussatus)* (Bebianno et al., 2004; de Alkimin et al., 2022; Huang et al., 2021; Islam et al., 2021), with increasing effects caused by smaller particles sizes (Capolupo et al., 2021).

In this study, to assess the biological effects of polyethylene MPs (PEMPs), polystyrene NPs (PSNPs) and their interaction, an *in vitro* (24 h) and an *in vivo* (10 d) exposure assays to PSNPs (50nm, 10 µg/L), PEMPs (4-6µm, 10 µg/L) and a mixture of the two (Mix: 10 µg/L of PEMPs +10 µg/L of PSNPs) were performed using the clam *Ruditapes decussatus*. A multi-biomarker approach was used to assess changes in genotoxicity, neurotoxicity, oxidative stress, damage, and the ingestion of MPs/NPs in clams' gills and digestive glands.

2. Materials and Methods

2.1. Polystyrene nanoparticles (PSNPs)

Fluorescent ® Plain YG 50 nm polystyrene nanoparticles (9003-53-6) were purchased from Polysciences, Inc. (Germany). Characterization details of PSNPs are found in Gonçalves et al. (2022). Briefly, when distributed in FSW (filtered sea water), the hydrodynamic of the particle increases (852 ± 103 nm) suggesting an aggregation/agglomeration due to the high salt content of seawater, whereby the higher the concentration of PSNPs, the higher the aggregation. For experimental purposes, a concentration of 10 µg/L was used.

2.2. Polyethylene microparticles (PEMPs)

Polyethylene microparticles of 4-6 μm in size (MPP-635XF; density: 0.96) were purchased from Micro Powders Inc. (NY-USA). A 10 mg/L stock solution of PEMPs was prepared for the experiment by mixing 10 mg of PEMPs with 1 L of distilled water and sonicated to prevent floating particles at the surface. A final concentration of 10 μg/L was used for experimental purposes.

2.2.1 PEMP characterization.

A Malvern MATERSIZER 3000 (MALVERN INSTRUMENTS Ltd, UK) was used to analyze plastic suspensions (PEMPs and a mixture of PEMPs with PSNPs) made in separate 600 mL beakers. The first suspension contained 0.1 g of PEMPs (4-6 μm) in 500 ml of ultrapure water, whilst the second one had 0.1 g of PEMPs and 1 ml of PSNPs (50 nm) also in 500 ml of ultrapure water. In the specific case of the mixture, analyses were conducted twice. The first time, polyethylene was selected as the polymer, and the second time, polystyrene was chosen as the polymer to analyze. Six consecutive measurements were carried out by the Malvern matersizer, and the particles were maintained suspended by a submergible stirrer. For all measurements, results are expressed both in terms of percentage of volume (% volume) and particle size (μm).

2.3. *in vitro* assay

In vitro analyses were conducted following the adapted Gómez-Mendikute & Cajaraville (2003) and Katsumiti et al. (2014) protocols. Twenty individuals of *Ruditapes decussatus* (4.2 ± 0.21 cm) were purchased from Formosa-Cooperativa de Viveiristas of the Ria Formosa lagoon (37.007117, -7.834466), and acclimatized for four days (12 h/12 h light/dark cycle) in a 10 L glass tank (2 clams/L) filled with FSW. After acclimatization, the hemolymph was extracted from the clams' abductor muscle using a sterile hypodermic syringe (1 mL; 25 G needle) under aseptic conditions in a vertical laminar airflow cabinet. In ice, five pools, containing four clams each, were prepared for each treatment (CT, PSNPs, PEMPs and Mix). From each pool, 10 μl of hemolymph were taken and placed in an anti-aggregation solution (0.2 M Tris; 171 mM NaCl; 24 mM EDTA; 0.15% (v/v) HCl 1 N) (Katsumiti et al., 2014) to avoid cell aggregation. Firstly, cell viability was assessed by staining 10 μl of hemolymph with 10 μl of Trypan Blue Stain (0.4 %) and then the percentage of live cells in a group of 100 randomly counted cells was calculated according to the formula:

$$Concentration \ (cells \ mL^{-1}) = \frac{n^{\underline{o}} \ cells \ \times \ 10\ 000}{n^{\underline{o}} \ squares} \times dilution \ factor$$

A Neubauer chamber, a hemocytometer, and light microscopy (Compound Light Microscopy; 400x) were used. Once this was calculated, a hemolymph volume containing 2×10^5 cells/mL was suspended in an anti-aggregation solution.

In 96-well microplates, 100 µl of hemolymph were placed in the cell culture media Dulbecco's Modified Eagle Medium (DMEM, pH 7.4) (6 replicates per treatment), and 10 µg/L of PSNPs (50 nm), 10 µg/L of PEMPs (4-6µm) and 10+10 µg/L of PSNPs and PEMPs added respectively for PSNPs, PEMPs and Mix treatments. Microplates were then incubated for 24 hours at 18°C.

The DMEM medium was removed from the microplate wells and the cell status was checked using light microscopy (Compound Light Microscopy; 400x). Neutral red working solution (0.4%, pH 7.3-7.4) was then added to each well, and empty wells to use as a negative control, and incubated for 1 hour in the dark. Microplates were then centrifuged at 270 g (4°C; 10 mins) to separate the supernatant. The latter was then removed and carefully washed with PBS to remove all the dye not attached to the cells. Once ready, samples were added to a U-bottom 96-well microplates and incubated for an additional 20 mins at 18°C with 100 µl of extraction solution (acetic acid and ethanol 50%) to extract the dye from the cells. Then, samples were transferred to V-bottom 96-well microplates and centrifuged at 270 g (4°C; 10 mins) and the supernatant extract and placed into another V-bottom 96-well microplate for absorbance determination. The latter was measured (Infinite M200 Pro, TECAN®, CH) at 550 nm to detect the amount of Neutral Red dye retained by the cell's lysosomes.

2.4. *in vivo* Experimental design

Clams *R. decussatus* (4.2 ± 0.21 cm) were purchased from an aquaculture facility in the Ria Formosa lagoon, Portugal (Formosa-Cooperativa de Viveiristas of the Ria Formosa lagoon; 37.007117, -7.834466). Clams were then placed into 30 L tanks (20 L of FSW) with a ratio of 2.5 clams/L, in a duplicate design. Clams were acclimatized for 7 days, the SW exchanged every other day, and the condition index was evaluated in five organisms, from randomly selected tanks, to ensure that the physiological status of organisms was not compromised. Then, clams were exposed for 10 days to 10 µg/L of PSNPs, 10 µg/L of PEMPs, and a mixture of the two plastic particles (Mix = 10 µg/L of PSNPs + 10 µg/L of PEMPs). Every other day, the seawater and contaminants were renewed, and abiotic parameters of seawater remained unchanged throughout the exposure period and were assessed using a multiparametric probe (Odeon, PONSEL, FR) (S: 37.8 ± 2.2; T: 19.0 ± 0.8ºC; pH: 7.8 ± 0.1; O_2 sat: 100 ± 1.7%). During the experiment clams were fed only with the plankton contained in natural seawater and were maintained under constant aeration and at a 12 h/12 h light/dark cycle.

Clams were sampled at 0, 7 and 10 days of exposure for a multi-biomarker analysis and at days 0 and 10 for ingestion analyses. On sampling days, for genotoxicity assay, the hemolymph of the clams was collected, and the gills and digestive glands of clams were dissected and immediately frozen in liquid nitrogen and stored at -80ºC until further analysis.

2.5 Ingestion of PSNPs

According to Gagné (2019), a fluorescence-based technique was used to assess the ingestion of PSNPs utilizing the molecular rotor probe 9-(dicyanovinyl)-julolidine (DCVJ). First, individual tissues (gills and digestive glands; n=6 per each treatment and time) of clams, from days 0 and 10, were homogenized at 20% (w/v) in an ice-cold buffer solution (50 mM NaCl, 10 mM Hepes - NaOH [pH 7.4], 1 mM EDTA, and 1 mM DTT), using a VWR Star-Beater (5 min, 20/s shaking, with grinding balls). The cytosolic fraction was obtained by samples centrifugation for 20 min at 2°C and 15000 g. The supernatant was then promptly frozen at 80°C for further analysis. Then, samples were examined using a spectrofluorometric microplate reader (Tristar 5, BERTHOLD, DE), with 450 nm excitation and 400–800 nm emission spectra. PSNPs have the same wavelength of 510 nm. In comparison to controls, results are given as PSNPs µg/g wet weight.

2.6 Ingestion of PEMPs

The ingestion of PEMPs in clams (gills and digestive glands; n=6 per each treatment and time) at days 0 and 10 were evaluated using the density separation method (Bebianno et al., 2022). The gills and digestive glands of clams were weighed and then digested over 2 days at 50°C using a 10% potassium hydroxide (KOH) solution with a ratio of 5:1 mL per gram of tissue. Pre-filtered sodium chloride (NaCl) solution with a density of 1.2 was added separately to each digested sample to allow plastic particles to float. After an incubation period of 30 minutes, the superficial layer of the solution was collected in a glass beaker and then filtered to collect the PEMPs. A Vacuum pump (Pall®) and cellulose filters with a mesh size of 0.45 µm were used. Once dry, the filters were colored with Nile Red. A working solution of 5 µg/mL of the latter was prepared from a 1 mg/mL stock solution. 2 mL of working solution was then added to the filters, which, once dried, were observed under a light microscope (Compound Light Microscopy; 400x) to measure the size of the PEMPs. Pictures of the filters were taken using a camera (Moticam 1080, MOTIC EUROPE, ES), and the images were analyzed for plastic detection using the program Motic Image Plus 3.1.

2.7. Condition index (CI)

The CI was assessed in five clams, from random tanks, after the acclimatization period and calculated as a percentage (%) of the ratio between the whole clam weight (tissue and shell) (g) and both wet and dry weight of the whole soft tissue (g) (Gomes et al., 2013).

2.8. Antioxidant enzymes

For the analysis of antioxidant enzymes activity, firstly the gills (n=6) and digestive glands (n=6) (per each treatment and period of exposure) of clams were homogenized in 5 mL of Tris-Sucrose buffer (0.5 M Sucrose, 0.075 M KCl, 1 mM DTT, 1 mM EDTA, pH 7.6) for five minutes in a TissueLyser Star-Beater (VWR, USA) (20/s shaking, with grinding balls) (Geret et al., 2002). Following two centrifugations (1st at 1500 g, 15 minutes, at 4°C, and the 2nd at 12000 g, 45 minutes at 4°C), the cytosolic fraction was collected from the resulting supernatant. Then, the activities of superoxide dismutase (SOD) and catalase (CAT) were evaluated.

All enzymatic activities were standardized by the determination of total protein concentration (TP; mg protein g^{-1} tissue) following the method described by Bradford (1976). The bovine serum albumin (BSA) was used as a reference to quantify TP and calibrated for the 96-well microplate reader (Infinite M200 Pro, TECAN®, CH)

The activity of SOD was assessed following the method described by McCord & Fridovich (1969), by the reduction of cytochrome c by the xanthine oxidase/hypoxanthine system at 550 nm (U mg^{-1} protein).

The activity of CAT (mmol min^{-1} mg protein^{-1}) was measured spectrophotometrically by the consumption of hydrogen peroxide (H_2O_2) at 240 nm (Greenwald, 1985).

2.9 Acetylcholinesterase activity (AChE)

Following a modification of Ellman's colorimetric approach (Ellman et al., 1961), AChE activity was exclusively measured in the gills of *R. decussatus* (n = 5 per each treatment and period of exposure). This process is based on the formation of a yellow product, 5-thio-2-nitrobenzoic acid (TNB) ion, by the interaction of acetylthiocholine (ATC) with 5, 5'-dithiol-bis (2-nitrobenzoic acid) (DTNB). First, 3 mL of Tris-HCl buffer (100 mM, pH 8) and 30 µL of Triton-X 100 (0.1%) were used to individually homogenize each gill, using the TissueLyser Star-Beater (VWR, USA) (20/s shaking, with grinding balls). The supernatant was removed after centrifugation (12 000 g, 4 °C, 30 min), and kept at -80°C until further analysis. For analysis, ATC solution (3 mM) was used to start the reaction after adding DTNB (0.75 mM) to the samples and allowing them to sit at room temperature for 5 minutes to evaluate the AChE activity. Infinite 200 Pro (TECAN®, CH) microplate reader was used to measure the absorbance at 412 nm for 5 minutes at 30-second intervals. AChE activity is expressed as nmol ATC min^{-1} mg protein^{-1}.

2.10 Lipid peroxidation (LPO)

The gills (n=6) and digestive glands (n=6) (per each treatment and period of exposure) of *R. decussatus* were individually homogenized in 5 mL of a Tris-HCl buffer (0.02 M; pH 8.6) and butylated

hydroxytoluene (BHT) in a 1:10 ratio. Then, 3 mL of the homogenate was centrifuged at 30 000 g (4°C; 45 mins) and the resulting supernatant was used to calculate the total protein concentration (Bradford, 1976) and the levels of LPO (nmol/mg prot). According to a technique adopted by Erdelmeier et al. (1998), LPO levels were evaluated by measuring the absorbance of malondialdehyde (MDA) and (2 E)-4-hydroxy-2-nonenal (HNE) at 540 nm.

2.11 Genotoxicity

The alkaline comet test, which was modified by Singh et al. (1988) and Gomes et al. (2013), was used to measure DNA damage. Using a sterile hypodermic syringe (1 mL) and a 25 G needle, hemolymph from six clams collected at 0- and 7-days following exposure to PSNPs, PEMPs and Mix, as well as from six clams that had not been exposed the hemolymph was removed from the posterior adductor muscle. To determine cell viability, 100 μL of a sub-sample from each experimental condition was stained with 100 μL of trypan blue. The percentage of living cells was calculated by counting 100 cells randomly. Briefly, microscopic slides were washed in ethanol/ether (1:1) before being coated with 0.65% normal melting point agarose (NMA) in Tris-acetate EDTA to assess DNA damage. Clam hemolymph cells were collected and centrifuged at 3000 rpm for 3 min (4°C), and the pellets with separated cells were then suspended in 0.65% low melting point agarose (LMA, in Kenny's salt solution), and cast on the microscope slides. Following the diffusion of cellular components in agarose and DNA immobilization, slides containing embedded cells were submerged in a lysis buffer (2.5 M NaOH, 100 mM EDTA, 10 mM Tris, 1% Triton X-100, 10% dimethyl sulfoxide, 1% sarcosil, pH 10, 4°C) for 1 h. After the lysis phase, slides were carefully added to an electrophoresis solution containing an electrophoresis buffer (300 mM NaOH, 1 mM EDTA, adjusted pH 13, 4 C). Following that, electrophoresis was performed for 5 min at 25 V and 300 mA. After the experiment was finished, the slides were taken out, neutralized (0.4 mM Tris, pH 7.5), washed with bi-distilled water, and then allowed to dry overnight. The existence of comets was evaluated using an optical fluorescence microscope (Axiovert S100, ZEISS, DE) connected to a camera (Sony) after slides were stained with 4,6-diamidino-2-phenylindole (DAPI, 1 μg/mL). Following the assessment of the quantity of DNA in the tail, the Komet 7.1 image analysis system was used to score 50 randomly selected cells for each slide (a total of 300 cells scored per group). This was done at a total magnification of 400x. The results are presented as the mean ± standard deviation.

2.12 Synergism and antagonism model

The single-dose factorial design method reported by Ritz et al. (2021) was used to evaluate the presence of synergism or antagonism between PSNPs and PEMPs in gills and digestive glands. With

this approach, four treatments are produced by combining two factors (the two plastic types) with two levels: control, PSNPs, PEMPs and a mixture (Mix) of the two. For all sampling days (0, 7 and 10 days) all biomarkers (SOD, CAT, LPO, AChE and DNA damage) levels were evaluated using the following dose addition and independent action models. Dose addition defines the reference effect (when neither synergism nor antagonism occurs) as the sum of the variance of treatments compared to controls:

$$E_{add}= (E_{PSNPs}-E_{CT}) + (E_{PEMPs}-E_{CT})$$

The difference between the observed effect of the mixture of the two contaminants ($E_{Mix}-E_{CT}$) and the predicted response (E_{add}) defines the presence of either antagonistic or synergistic effect:

$$D_{da}= E_{Mix}-E_{CT}- E_{add}= E_{Mix}-E_{PSNPs}-E_{PEMPs}+E_{CT.}$$

Independent action defines the reference effect as the product between the variance of treatments compared to controls:

$$E_{ind}=E_{CT} \left(1 - \frac{E_{CT}-E_{PSNPs}}{E_{CT}}\right)\left(1 - \frac{E_{CT}-E_{PEMPs}}{E_{CT}}\right)= \frac{E_{PSNPs}\times E_{PEMPs}}{E_{CT}}$$

Alike dose addition, any antagonistic or synergistic effect can be defined as the difference between the observed (E_{Mix}) and the predicted response (E_{add}):

$$D_{ia}= E_{Mix}-\left(\frac{E_{PSNPs}\times E_{PEMPs}}{E_{CT}}\right)$$

A synergistic effect is observed when D_{da} and D_{ia} are higher than zero; otherwise (<0), an antagonistic effect exists. Results of synergistic and antagonistic impact are valuable only for the concentrations used during the experiment (10 µg/L of PSNPs, 10 µg/L of PEMPs, 10+10 µg/L of PSNPs + PEMPs).

2.13 Weight of Evidence (WOE)

The Weight of Evidence (WOE) quantitative model was used to integrate the set of data, and line of Evidence (LOE) obtained from biomarkers analyses (SOD, CAT, LPO, AChE and DNA damage) analyzed on gills and/or digestive glands. This approach is used to simplify further results interpretations and obtain more robust and weighted conclusions (Regoli et al., 2019). Briefly, a percentage of variation is calculated for each biomarker individually. Single values are then normalized/corrected by comparing them with their specific threshold (level of variation above which there is biological relevance), weight (toxicological relevance), and statistical differences with controls, with the final creation of classes of effects (Regoli et al., 2019). Whole calculations, detailed flow charts, the rationale for weights, thresholds, and expert judgments have been previously described in detail (Regoli et al., 2019).

2.14 Quality control and assessment

No plastic equipment was used during the exposure time, all tanks had glass lids and glass pipettes for aeration to avoid additional plastic contamination, including airborne pollution. Moreover, no plastics were used during tissue dissection, biochemical assays, and the evaluation of ingestion of plastic particles in gills and digestive glands in all treatments.

2.15 Statistical analyses

Statistically significant differences between times and treatments were evaluated according to data distribution and variance homogeneity (Shapiro-Wilk test) using parametric tests (2-way ANOVA followed by Tukey's post-hoc test) or non-parametric equivalent tests (Kruskal-Wallis and a two-tailed multiple comparison test) at a 95% confidence interval. All the statistical analyses were performed using GraphPad Prism 9. A Principal Component Analysis (PCA) was used both for gills and digestive glands to study the relationship between treatments (CT, PSNPs, PEMPs, and Mix) and between the oxidative stress, oxidative damage, and neurotoxicity biomarkers (SOD, CAT, LPO, AChE). In this case, statistical evaluations were conducted using the Statistica 7.0 program.

3. Results

3.1. PSNPs and PEMPs characterization

PSNPs were analyzed in a mixture with PEMPs (4-6 µm) through laser diffraction analyses. The average results on particle size distribution showed a high level of aggregation, in fact, no particles smaller than 3 µm were found (Fig. 1A). This is probably due to an aggregation of the PSNPs with the PEMPs in the solution and demonstrated by the highest presence of particles with a size between 8 and 9 µm, size of the PEMPs used. On the other hand, laser diffraction analyses on PEMPs (4-6 µm) show a certain level of aggregation even when PEMP particles are tested alone. In fact, the highest percentage was found for particles between 4 and 6 µm (PEMPs fabrication size) but also for particles up to 118 µm (Fig. 1C). However, as for PSNPs, when particles are texted in the mixture (PSNPs + PEMPs suspension) a higher level of aggregation even for PEMPs is observed. This is demonstrated by the presence of a high percentage for particle sizes between 100 µm and 625 µm (Fig. 1B).

3.2. *In vitro* assay

Results show a significant increase in the number of living hemocytes after a 24-hour exposure to PSNPs treatment compared to PEMPs and the mixture ($p<0.05$) (Fig. 2).

3.3. Condition Index

Before the experimental exposure, the CI measured in organisms of *Ruditapes decussatus* collected in the Ria Formosa lagoon during summertime showed a mean value of 31.6±1.7.

3.4. Ingestion of NPS and MPs

Clams exposed to PSNPs and Mix treatments show a significant increase in the amount of ingested PSNPs in both gills and digestive glands ($p<0.05$) with similar levels of nanoparticles in the two tissues (Fig 3A-B).

Concerning PEMPs, a similar level of ingestion was observed for both the tissues of PEMPs and Mix exposed organisms (Fig. 4A-B and supplementary data 1).

3.5. Enzymatic activities

3.5.1. Superoxide dismutase (SOD)

In the gills of *Ruditapes decussatus* exposed to PEMP particles an increase in SOD activity is seen at day 10 compared to the control and to day 7 ($p<0.05$) (Fig. 5A), whilst no significant variation is observed within treatments and times for organisms exposed to either PSNPs or Mix *($p>0.05$)* (Fig. 5A).

In digestive glands, a significant increase in SOD activity is present at day 10 for clams exposed to PSNPs, PEMPs, and to their mixture ($p<0.05$) compared to control and to day 7 (Fig. 5B).

3.5.2. Catalase (CAT)

Variations of CAT activity in the gills are presented in Figure 5C. No significant changes are observed between treatments on days 0 and 7 of exposure ($p>0.05$) (Fig. 5C); nevertheless, at day 10, a major increase in enzymatic activity was observed for PSNPs and, particularly, for Mix treatments compared to controls ($p>0.05$) (Fig. 5C).

Also, in the digestive gland, no significant differences are found between days 0 and 7 for all treatments ($p>0.05$) (Fig. 5D), while on day 10, a significant increase in CAT activity is observed for clams exposed to PEMPs ($p<0.05$) (Fig. 5D).

3.5.3. Acetylcholinesterase activity (AChE)

Differences in AChE activity are found in clams' gills between different treatments at days 7 and 10 (Fig. 5E). More in detail, at day 7, there is a significant increase of AChE activity ($p<0.05$), in clams exposed only to PEMPs whilst the lowest values are noticeable in organisms exposed only to PSNPs (Fig. 5E). On the other hand, at day 10, AChE is significantly higher in the Mix treatment($p<0.05$) (Fig. 5E).

3.6. Lipid peroxidation

No variations in MDA levels is observed for all treatments in gills at days 0 and 7, while a significant decrease appears at day 10 in organisms exposed to PEMPs ($p > 0.05$) (Fig. 5F).

As shown in figure 5G, no variation in MDA levels is detected in the digestive glands of clams.

3.7. Genotoxicity assay (Comet assay)

As shown in Fig. 6A, there are no significant differences between days 0 and 7 for all treatments ($p < 0.05$), indicating that no DNA damage occurred. However, a significant decrease in the % of tail DNA was found at day 7 for PSNPs and Mix treatment when compared to unexposed and PEMPs-exposed clams ($p < 0.05$).

3.8. Synergism and Antagonism

3.8.1. Dose addition results

In the gills of *Ruditapes decussatus,* an increasing antagonistic interaction ($D_{da} < 0$) is observed concerning SOD activity between days 7 and 10 (Table 2). Considering CAT, synergism ($D_{da} > 0$) at day 7 and antagonism ($D_{da} < 0$) at day 10 were observed (Table 2). No interaction ($D_{da} \approx 0$) was detected for LPO, neurotoxicity, and genotoxicity (Table 2). On the other hand, in digestive glands, a decreasing synergistic interaction ($D_{da} > 0$) for SOD activity and an increasing antagonistic interaction ($D_{da} < 0$) for CAT activity was observed between days 7 and 10 (Table 3). Neither synergism nor antagonism was observed for LPO (Table 3).

3.8.2. Independent action results

When considering D_{ia} as the reference value, gills show the presence of antagonistic interactions ($D_{ia} < 0$) in relation to SOD activity on day 10 and to CAT activity on day 7 (Table 4). Synergistic interactions ($D_{ia} > 0$) occur for CAT activity on day 10 (Table 4). No interactions ($D_{ia} \approx 0$) are observed for LPO, neurotoxicity, and genotoxicity.

Also, in digestive glands, an antagonistic interaction ($D_{ia} < 0$) is observed for SOD activity at day 10 (Table 5). In relation to CAT activity, an increasing antagonistic interaction was observed between days 7 and 10 (Table 5). No interactions ($D_{ia} \approx 0$) was present for oxidative damage (Table 5).

3.9. Weight of Evidence

The overall WOE elaboration of biomarker results (AChE, LPO, CAT, SOD) shows a time-related response, whereby for PSNPs, PEMPs, and Mix treatments, the highest level of hazard is observed at day 10 (Table 6). A Slight level of hazard at day 7 that increases to Moderate at day 10 is seen only in relation to the PSNPs treatment while in other treatments the increase is sharper, passing from Absent to Moderate from day 0-7 to day 10 (Table 6). CAT activity in gills and SOD activity in digestive glands are the biomarkers that mostly contribute to the overall increase in the hazardous levels at sampling day 10.

3.10. PCA

To further understand the effects of PSNPs, PEMPs, and their mixture on biomarker responses, a principal component analysis (PCA) was applied. The two principal components represent 84.7% in the gills (PC1 = 48.5%, PC2 = 36.2%) and 87.3% in the digestive glands of clams (PC1 = 61.5, PC2 = 35.8%) of the total variance (Fig. 7A – B). Overall results confirm a time-specific effect in both clam tissues, whereby day 10 is most influential (Fig. 7A – B). In the clam's gills, SOD, CAT, and AChE activities are the most influential biomarkers relative to the observed effects, as is the exposure to the Mix and PEMPs (Fig. 7A). In the digestive gland of clams, PEMP particles are the most prominent treatment, with LPO levels, SOD and CAT activities having a powerful effect on the results observed (Fig. 7B). These PCA descriptive analyses suggest that the clam's digestive gland is the most compromised tissue compared to the gills and that the Mix and PEMPs are most influential on biomarker responses compared to PSNPs (Fig. 7A – B).

4. Discussion

Repercussions of plastic pollution on the entire ocean ecosystem are of great concern nowadays. Indeed, with the increasing world population and GPP, the amount of plastic entering the ocean is growing along with the detection of smaller plastic fragments (MPs and PSNPs). Combining both primary and secondary sources of plastic particles, it was estimated that 5.25 trillion fragments are currently polluting the global sea surface most of which are less than 10 mm in size (Alimi et al., 2018). There is evidence that once MPs and PSNPs enter the marine environment, they can form aggregates due to their interaction with seawater components (e.g., colloids, algae, ions) and other dispersed plastic particles of equal or similar size, that change their chemical/physical properties, structure, and size, potentially influencing their level of toxicity, bioavailability, fate, and transport (Abdul Rahman et al., 2023; Gonçalves et al., 2022; Gonçalves & Bebianno, 2021; Li et al., 2019). Taking this into account, this study aims to assess how the occurrence of possible interactions between PSNPs (50 nm) and PEMPs (4-6 μm) when in a mixture with each other, can positively or negatively influence

13

the particles' toxicological hazard towards the clam *Ruditapes decussatus*. According to Alimi et al. (2018), the phenomenon of particle attachment after a collision is known as "aggregation". The process is controlled by Van der Waals and electrical double-layer forces and can be furthermore influenced by diffusion and particles' surface charge properties. When compared to data on PSNPs found in Gonçalves et al. (2022) and Shams et al. (2020), the PEMPs characterization in ultrapure water performed here reveals a higher tendency of MPs to form aggregates (Fig. 1B). Because of the repulsive interactions between their negatively charged surfaces, NPs do not aggregate in ultrapure water (Gonçalves et al., 2022; Shams et al. 2020). PE is a non-polar polymer, while PS was seen to enhance its negativity due to the dissociation of functional groups (Shams et al., 2020); in this respect, the increased MPs tendency to aggregate (Fig. 1B) identified in this study may be attributable to differences in the polymers used. Interestingly, when analyzed in the mixture, both PSNPs and MPs showed, in ultrapure water, a higher level of aggregation (Fig. 1A-B): in seawater, aggregation of NPs and MPs was found to be even higher compared to ultrapure water (Gonçalves et al., 2022; Sun et al., 2021). It is possible that this effect is connected to the breakdown of energy barriers between particles caused by an increase in ionic strength (IS), which is strictly dependent on the rising concentration of sodium chloride (NaCl) (Alimi et al., 2018; Shams et al., 2020). In fact, free ions present in seawater can be absorbed by plastic particles changing their surface charges properties and allowing them to form aggregates (Abdul Rahman et al., 2023; Gonçalves & Bebianno, 2021; Li et al., 2019). When PSNPs and PEMPs are suspended together, they were seen to form aggregates of a size up to 10 μm and between 100 and 1000 μm, respectively (Fig. 1A-B). This may be explained by variations in surface charge energy, higher for MPs compared to NPs (Sun et al., 2021), which reduce repulsive attractions and favour higher attachment probabilities between NPs, and MPs compared to particles of equal sizes. When taking this into account, it is possible to forecast that, in a more environmentally realistic condition, when NPs and MPs are dispersed together in seawater, they might present an even higher level of aggregation. This could significantly affect their mobility, persistency, and bioavailability in the environment (Alimi et al., 2018; Gonçalves & Bebianno, 2021).

Up to date, available data indicate that smaller plastic fragments might be more easily ingested by marine organisms, potentially causing a higher toxicological hazard (Baudrimont et al., 2020; Gonçalves & Bebianno, 2021; Rodrigues et al., 2022; Wang et al., 2021; Ward et al., 2019). In accordance, a high level of ingestion was obtained for both gills and digestive gland of *Ruditapes decussatus* after 10 days of exposure to individual PSNPs and PEMPs (Fig. 3A-B and Fig. 4A-B). Recently, also Gonçalves et al. (2023) found a high level of ingestion in *Mytilus galloprovincialis* exposed for 28 days to PSNPs (50 nm), and smaller plastic fragments prevailed in wild bivalves' species (Abidli et al., 2019; Cozzolino et al., 2021). Interestingly, elevated ingestion of both PSNPs and PEMPs

was also observed here for organisms exposed to the Mix treatment (Fig. 3A-B and Fig. 4A-B), where particles were seen to form bigger aggregates (Fig. 1A-B). The initial step in bivalves feeding processes is particle capture, which results from contacting and retaining gills filaments (Rosa et al., 2018). Pre-ingestion capture efficiency (CE) was found to rise asymptotically as particle size increases (Ward et al., 2019). Therefore, the higher efficiency in trapping larger particles in the gills may account for the high ingestion found here in the gills of *R. decussatus* exposed to the Mix treatment (Fig. 3A and Fig. 4A). Despite evidence that post-capture selection mechanisms, such as gills' muscular contraction, can allow bivalves to finally ingest smaller particles and reject bigger ones (Ward et al., 2019), foraging theories suggest that for suspension-feeding organisms, such as *R. decussatus,* it might be more advantageous in terms of food value, to ingest also larger phytoplanktonic cells (Ward & Shumway, 2004). Indeed, several data showed how pre-ingestion selection of filtered material is generally not based on particles sizes but on other characteristics, such as the physiochemical properties (e.g., particle's charge and hydrophobicity) of the particles that interact with the feeding organs (Rosa et al., 2017, 2018; Ward et al., 2019). When considering this, along with the higher CE of bigger particles (Ward et al., 2019) and the higher filtration rate observed for *R. decussatus* compared to other suspension feeders (Abidli et al., 2019; Cozzolino et al., 2021; Sobral & Widdows, 2000), it can be concluded that the likelihood of ingesting bigger aggregates is high for this organism. This might explain the high level of ingestion observed here for both PSNPs and PEMPs in the digestive gland of *R. decussatus* when in a mixture (Fig. 3B and Fig. 4B). Abidli et al. (2019) and Cozzolino et al. (2021) found that the majority of particles ingested by wild specimens of *R. decussatus* were in the size range of mm. Nonetheless, further analyses should be conducted on ζ-potential values of the aggregates formed when NPs and MPs are suspended together to better understand results.

Once ingested, plastic particles can either accumulate in the digestive tract or can be translocated in hemolymph or other tissues (Fossi et al., 2018; Sendra et al., 2021; Sıkdokur et al., 2020; Wang et al., 2021). The neutral red uptake assay was quantitatively used to assess changes in cell viability of hemocytes subjected to a 24 h *in vitro* exposure to PSNPs, PEMPs, and Mix. PSNPs was the only treatment to cause significant changes, increasing hemocytes cell viability (Fig. 2). Such an effect might be In accordance with the higher localized toxicity of PSNPs towards lysosomes, as stated by Repetto et al. (2008). Ingested PSNPs, due to their smaller size, are able to easily cross biological membranes (Gonçalves & Bebianno, 2021; Kiran et al., 2022), possibly accumulating in lysosomes (Zhou et al., 2023) and reducing their membrane stability (Capolupo et al., 2021). A higher level of PSNPs accumulation in lysosomes compared to MPs was also observed by Gaspar et al. (2018), in accordance with the absence of effects observed here for hemocytes exposed to PEMPs and Mix treatments (Fig. 2).

Exposure to emerging environmental contaminants, such as PSNPs and MPs, was seen to potentially increase ROS production in marine organisms, thus altering antioxidant defense mechanisms and inducing, in many cases, oxidative damage (lipid peroxidation, protein oxidation, DNA damage, and unbalance of intracellular redox status) (Benedetti et al., 2022). Taking this into account, a multi-biomarker approach was used to assess genotoxic (% of DNA tail), neurotoxic (AChE), oxidative stress (SOD, CAT), and oxidative damage (LPO) responses in relation to different plastic particles types and sizes. Different times and tissues were also considered to further understand possible time and tissue-dependent effects.

The first line of the enzymatic antioxidant defense system in bivalves is represented by the superoxide dismutase enzyme (SOD), which allows the conversion of the highly reactive ROS, anion superoxide ($O_2^{\cdot-}$), in the less reactive one, hydrogen peroxide (H_2O_2) (Gonçalves et al., 2022; Guo et al., 2021). Moreover, SOD activity was also seen to be strictly correlated to catalase (CAT) enzymatic activity that, in fact, can finally convert H_2O_2 in water (H_2O), preventing ROS from binding to other molecules (Guo et al., 2021). Alteration of both SOD and CAT catalytic action was assessed in *R. decussatus* exposed to both NPs and MPs particles of different sizes and polymers, inducing changes in total oxidant status (TOS) and total antioxidant capacity (Sendra et al., 2021). Gonçalves et al. (2022) found significant inhibition of SOD and CAT activities for *Mytilus galloprovincialis* specimens exposed for 21 days to PSNPs (50 nm). Time-dependent and tissue-dependent responses were also observed, whereby the gills seem to be the most affected tissue in the short term (3 and 7 days) with significant inhibition of both enzymes, whilst digestive gland effects were less severe and visible after a longer exposure time (14 and 21 days) (Gonçalves et al., 2022). Guo et al. (2021) provided evidence on how, in the freshwater clam *Corbicula fluminea*, a very high increase in SOD activity after PSNPs (80 nm) and MPs (6 μm) exposure, caused an overproduction of H_2O_2, therefore overwhelming CAT enzyme activity, that was indeed inhibited. This means that the organism was not capable of counteracting ROS production, thus leading to an increased level of membrane lipid peroxidation (LPO) that indirectly reflects cell damage (Guo et al., 2021). Results obtained here also show a time and tissue-dependent oxidative stress, whereby an increased enzymatic activity (SOD and CAT) was observed at the end of the exposure time. The digestive gland appears to be the most impacted tissue (Fig. 5B & D). Different uptake, translocation, and elimination processes associated with different tissues could explain those differences (Sıkdokur et al., 2020). Indeed, once plastic ingestion occurs through feeding strategies from the gills, they can be redirected to the digestive gland, where they accumulate due to the absence of enzymatic pathways that allow plastic breakdown (Faggio et al., 2018; Wright et al., 2013). Moreover, the digestive gland in bivalves is involved in important digestive, food transfer/assimilation, and detoxification functions, potentially increasing its direct level of exposure to environmental

16

pollutants and the overall effect on the organism (Détrée & Gallardo-Escárate, 2017; Guo et al., 2021). Ribeiro et al. (2017) observed an important level of oxidative damage (increase LPO levels) in the digestive gland already after 7 days of exposure to *Scrobicularia plana* to PS MPs (18 μm), whilst in the gills lipid peroxidation decreased after 3 days meaning a higher antioxidant system efficiency in this tissue (Li et al., 2019; Ribeiro et al., 2017). In both gills and digestive gland, the highest increase of enzyme catalytic activity is observed in relation to both PEMPs and Mix treatments (Fig. 5A-B & C-D). Nonetheless, also PSNPs showed a time-dependent enzymatic induction that, although less important, affects only CAT in gills and SOD in the digestive gland (Fig. 5B-C).

Overall, the enzymatic antioxidant defense system of *R. decussatus* appears able to efficiently counteract the effects of plastic-induced ROS in terms of LPO production (Fig. 5F-G), which was even decreased at day 10 in the gills of PEMPs-exposed clams (Fig. 5F). Ribeiro et al. (2017) also observed a similar effect which might be related to the increased activity of antioxidant defenses. Indeed, ROS are normally produced in aerobic organisms because of mitochondrial respiration (Bounous & Molson., 2003; Abele & Puntarulo, 2004), thus, even though their production is counteracted by the antioxidant defense system, a slight level of LPO can be present in unexposed clams. When environmental pollutants, as observed here, increase the activity of antioxidant enzymes, this might allow the organism to eliminate all reactive oxygen species more efficiently, thus causing a slight decrease of LPO in exposed organisms compared to controls.

Clam's haemocytes did not show signs of DNA damage after 7 days of exposure for all treatments (Fig. 6A-B). Since ingested plastic particles are firstly translocated to the digestive gland and only subsequently enter the circulatory system (Fossi et al., 2018; Sendra et al., 2021; Sıkdokur et al., 2020; Wang et al., 2021), a 7 days exposure period might not be long enough for the plastic particles to induce toxicity in hemolymph cells (Marisa et al., 2016). In accordance with the present results, Cole et al. (2020) also observed no DNA damage in mussel *M. edulis* exposed to PS MPs (20 μm) for 7 days. Furthermore, Ribeiro et al. (2017) did not find increase of % DNA tail in the clam *S. plana* after 14 days of exposure to PS MPs (18 μm) while this effect was observed after 7 days of post-exposure depuration, meaning that not egested MPs had a delayed effect on DNA (Ribeiro et al., 2017). Genotoxicity could either be promoted directly by plastics-DNA interactions within the nucleus or indirectly through reactive oxygen species (ROS) production (Auguste et al., 2020; Marisa et al., 2016; Ribeiro et al., 2017). Therefore, the absence of effects could also be related to the efficient removal of ROS by the antioxidant system of the organism, as observed here in relation also to LPO (Fig. 5F-G). Also, no signs of AChE inhibition were seen in the gills of *R. decussatus* exposed for 10 days to the different treatments (Fig. 5E), indicating that no neurotoxicity occurred (Capolupo et al., 2021). An increase in its enzymatic activity was observed in this study for specimens exposed to PEMPs and Mix

treatments (Fig. 5E). AChE plays an important role in voluntary muscle movement by catalysing the breakdown of acetylcholine (ACh) after its interaction with neuromuscular synapsis for signal transmission, preventing continuous and uncontrolled muscular contractions (Guo et al., 2021). An increase of AChE activity when organisms were exposed to bigger particles, as observed here (Fig. 5E), might be related to the need for increasing muscular movement of gills filaments to avoid their constraint (Ward et al., 2019). Consequently, higher production of acetylcholine (Ach) is needed, potentially increasing AChE enzymatic activity for its degradation. This is in accordance with previous ingestion results, as the higher gills' capture efficiency for bigger aggregates might allow them to accumulate here before ingestion (Rosa et al., 2018; Ward et al., 2019).

Finally, the biomarker data elaboration using a Principal Component Analysis (PCA) and the Weight of Evidence (WOE) quantitative model show how day 10 is the most influential time in relation to all tissues and treatments considered, indicating once again a time-specific response to plastic particles exposure. (Fig. 7A-B and Table 6). NPs and MPs can induce intracellular ROS production due to their ability to enter inside cells (Hu & Palić, 2020). This process is regulated either by endocytosis (MPs) or pinocytosis (PSNPs), where particles incorporated in vesicles are brought inside the cells (Hu & Palić, 2020). Here they are treated as foreign material, thus triggering the innate immune system of the organism that, consequently, increases ROS production to neutralize possible damaging effects (Hu & Palić, 2020). Considering this, we can understand how a time lag between particle ingestion and the set-off of an effect might need to be considered. Moreover, it is also important to consider tissue translocation, which indeed might provoke tissue and time-dependent effects (Capolupo et al., 2021; Gonçalves et al., 2022; Marisa et al., 2016).

This work contributes to a further understanding of how NPs and MPs behave in water and how their change in size can influence ingestion and toxic effects. NP's ability to more easily cross biological barriers can allow them to potentially interfere, in the long term, with important molecular and cellular processes(Capolupo et al., 2021; Gonçalves et al., 2023; Gonçalves et al., 2022; Gonçalves & Bebianno, 2021). NP and MPs tendency to form aggregates once dispersed in seawater (Li et al., 2019; Sun et al., 2021) is considered to increase their sizes, thus reducing the level of danger (Alimi et al., 2018; Gonçalves & Bebianno, 2021). This study assessed, for the first time, how NPs, and MPs when suspended together, tend to increase their level of aggregation (Fig. 3B). This might also induce bigger changes in surface charge properties that, as previously mentioned, can be strictly related to organisms' selective feeding strategies. Moreover, both PCA and WOE results show that the most influential treatments on biomarker responses are PEMPs and Mix compared to PSNPs (Fig. 7A-B and Table 6). Analyses of synergisms and antagonisms were also conducted to further understand how the interaction between NPs and MPs might pose a bigger threat to organisms (Table 2, 3, 4 and 5)

indicating a tissue-specific effect, whereby in gills, a synergistic effect was observed for CAT (Table 4), which according to PCA and WOE, is one of the most influential biomarkers on the overall effect (Fig. 7A-B and Table 6). This is potentially related to the fact that gills capture efficiency asymptotically increases in relation to bigger aggregates (Ward et al., 2019) thus allowing them to be the first ones to enter in touch with them. On the other hand, antagonistic effects were observed in the digestive gland (Tables 3 and 5), where probably the biggest aggregates formed might be selectively egested either through pseudofeces or fecal pellets production (Ward et al., 2019). Different ingestion processes might occur depending on the particle sizes; indeed, PSNPs can be ingested through body adhesion and subsequential internal translocation (Gonçalves., 2023), while bigger particles are most likely captured by gills, ingested, and transported to the digestive gland (Fossi et al., 2018; Sendra et al., 2021; Sıkdokur et al., 2020; Wang et al., 2021; Ward et al., 2019). Thus, higher effects observed here in relation to bigger particles might be because particles ingested through water filtration potentially enter faster in touch with gills and digestive glands. Therefore, a longer time of exposure is needed to better comprehend alteration in the long term, especially in relation to PSNPs. In accordance with this, this work shows a higher level of toxicity when cells were exposed *in vitro* to PSNPs. As mentioned before, this possibly means that PSNPs's smaller size can induce more severe effects at the cellular level (Gonçalves & Bebianno, 2021). Nonetheless, this study clearly shows the possibility of bigger particles to induce intracellular alterations potentially more rapidly due to their higher capture efficiency also correlated to important levels of ingestion. Even though no effective signs of oxidative damage were observed, a possible co-exposure of organisms to smaller and bigger plastic fragments, at environmentally realistic condition, might induce a longer stimulation of the antioxidant defense system reducing the amount of energy available for important biological processes such as growth and reproduction (Trestrall et al., 2020). Furthermore, the formation of bigger aggregates because of higher combining potential, can also induce important post-ingestion mechanical damage (Fossi et al., 2018; Capolupo et at., 2021; Teng et al., 2021). Therefore, in future studies, it would be also useful to assess particle rejection mechanisms to better define particle size limits related to bivalves' ingestion.

5. Conclusion

This dataset provides evidence of the interaction of two different polymers (PS and PE) of plastic at different sizes (NPs) and MPs and their toxicity towards the clam *R. decussatus.* Ingestion of these particles was seen to be independent of the particle size as PSNPs, PEMPs, and the bigger aggregates formed when in a mixture with each other are ingested by the clam. Individual PSNPs induced changes in cellular viability and antioxidant enzymes activities. Nonetheless, PEMPs and Mix's

19

treatments were seen to be the most influential treatments causing essential changes in the short term in the antioxidant enzymes' (SOD, CAT) activity. Moreover, no signs of oxidative damage, genotoxicity, and neurotoxicity were seen for all treatments during this exposure period. In gills and digestive glands of clams exposed to the mixture, antagonistic interactions were calculated between NPs and MPs. However, a longer exposure time and particles' egestion mechanisms should be further evaluated to better understand results. Furthermore, there is also the need to assess the ability of NPs and MPs of the same polymer to form aggregates and evaluate the level of toxicity of other polymers present in the marine environment.

6. Acknowledgements

7. References

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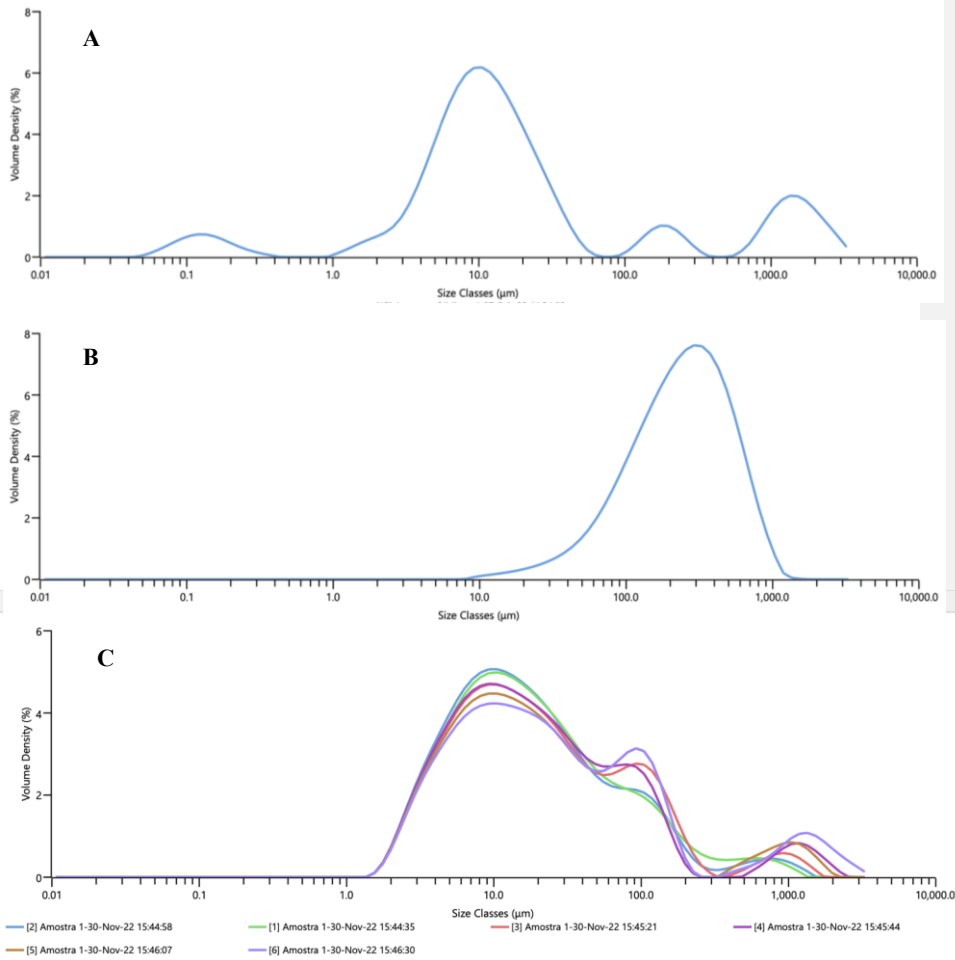
Figure 1. **(A and B)** Average particle size distribution obtained from laser diffraction analyses on a mixture of PSNPs (50 nm) and PEMPs (4-6 μm) and **(C)** of only PEMPs (4-6 μm). **(A)** The graph refers only to polystyrene, **(B)** the graph refer only to polyethylene.
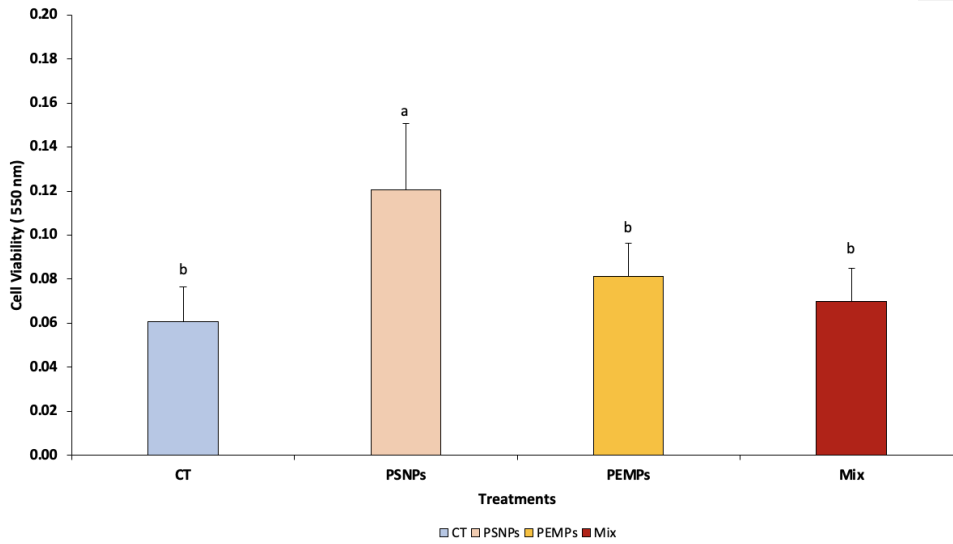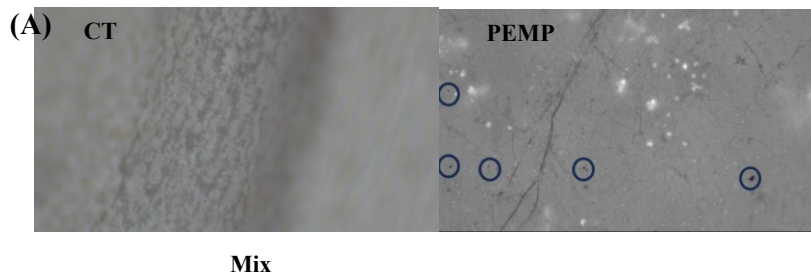
Figure 2. Cell viability in *Ruditapes decussatus* unexposed hemocytes (CT), those exposed to PSNPs (10 µg/L), PEMPs (10 µg/L) and a Mix (10 µg/L PSNPs + 10 µg/L PEMPs) for 24 hours. Different letters indicate significant differences between treatments (*p*<0.05).
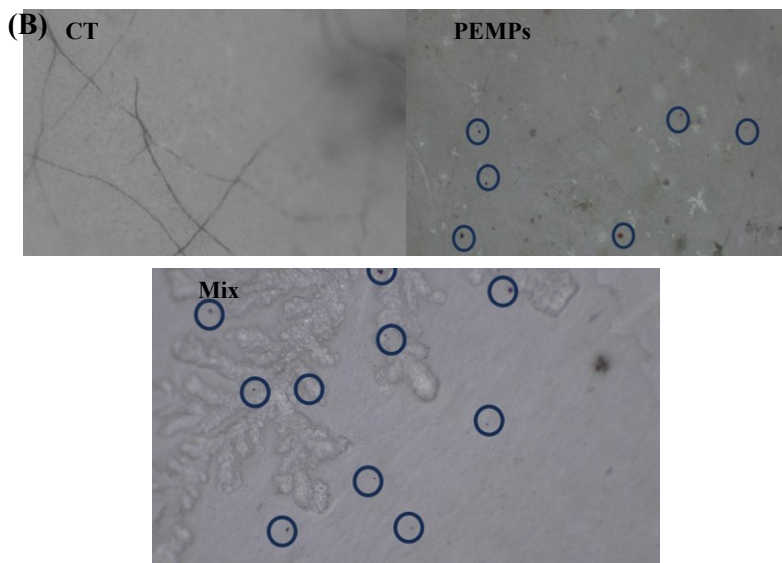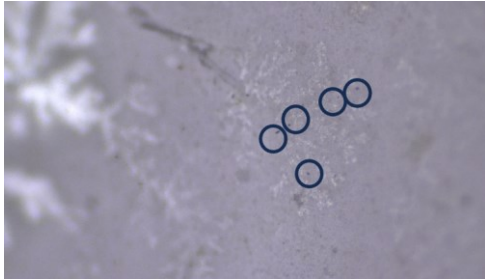
Figure 4. PEMPs ingested in gills of unexposed (CT), PEMPs and Mix exposed *R. decussatus* after 10 days **(A)**; PEMPs ingested in digestive glands of unexposed (CT), PEMPs and Mix exposed *R. decussatus* after 10 days **(B)**. Blue circles highlight PEMP particles detected. Compound Light Microscopy; 400x.
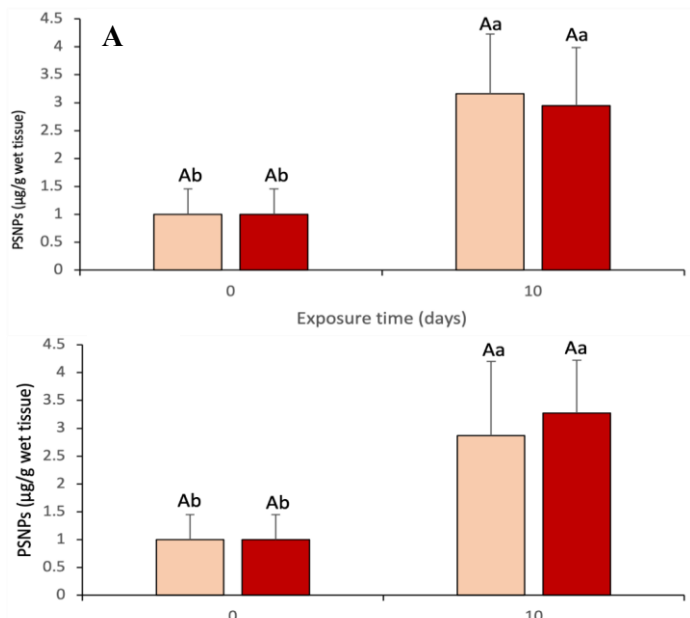
Figure 3. Amount of PSNPs detected in the gills (**A**) and the digestive gland **(B)** of clams and expressed as μg of nanoplastic/μg of wet tissue relative to controls. Significant variations between treatments at the same time and between times for the same treatment are indicated, respectively, by different upper- and lower-case letters (p <0.05).
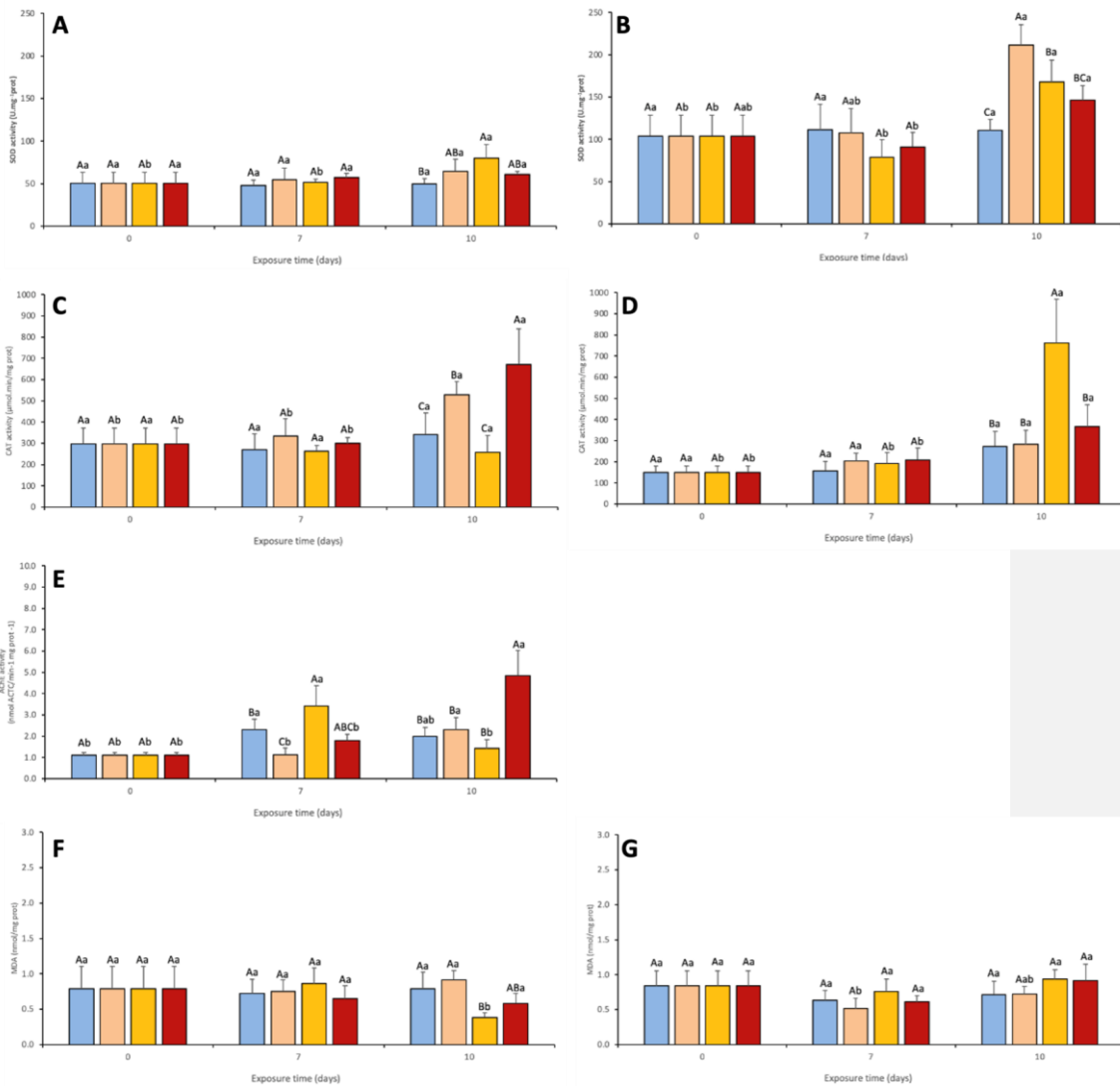
Figure 5. SOD, CAT, AChE activity and LPO levels in the gills (**A, C, E, F**) and digestive gland (except for AChE) (**B, D, G**) of *Ruditapes decussatus* between different treatments (CT, PSNPs, PEMPs, Mix) and different times of exposure. Significant variations between treatments at the same time and between times for the same treatment are indicated, respectively, by different upper- and lower-case letters (*p* <0.05).
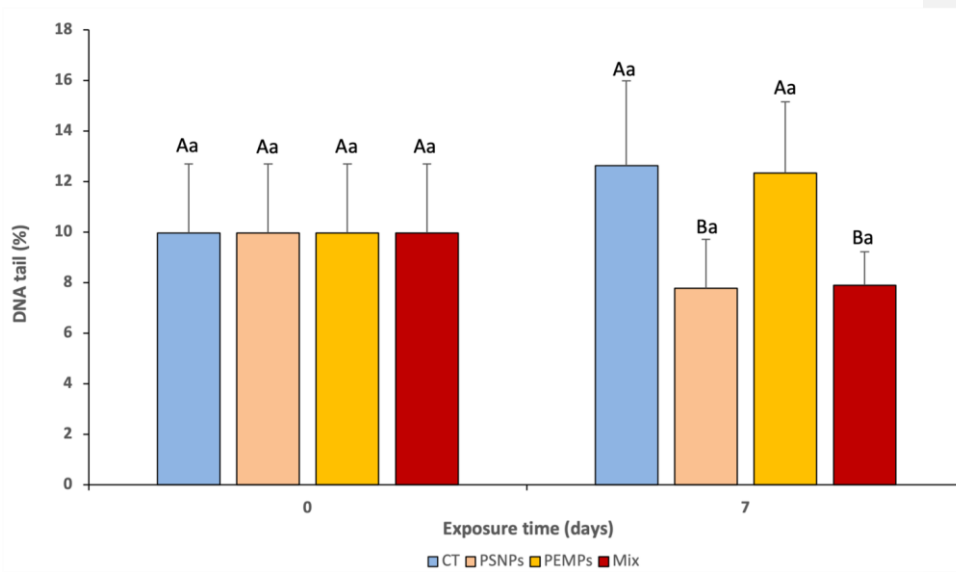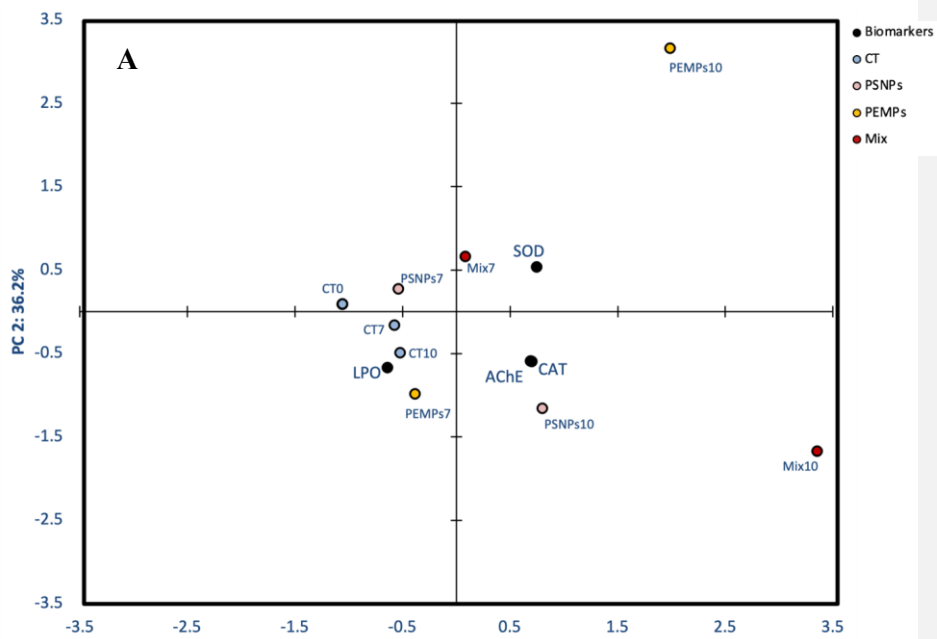
Figure 6. DNA damage (% Tail DNA) (mean ± s.d.) after different treatments (CT, PSNPs, PEMPs, Mix) and different days of exposure in *Ruditapes decussatus.* Significant variations between treatments at the same time and between times for the same treatment are indicated, respectively, by different upper- and lower-case letters (p <0.05).
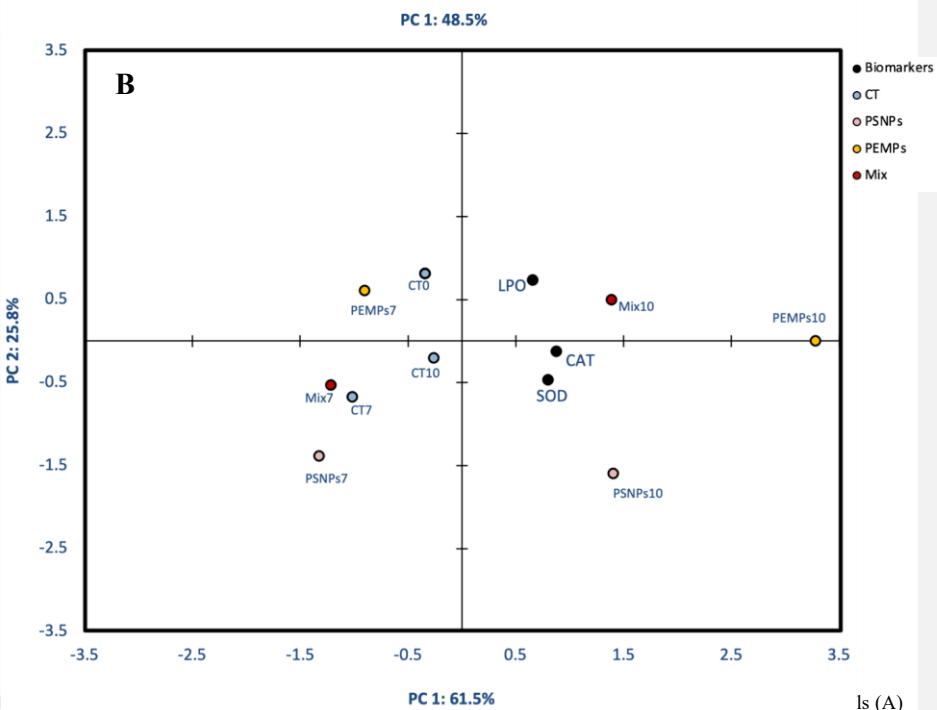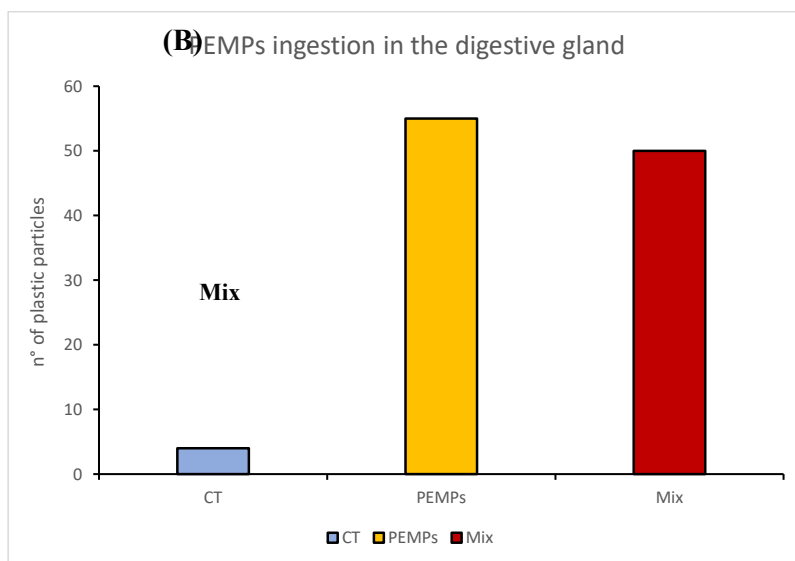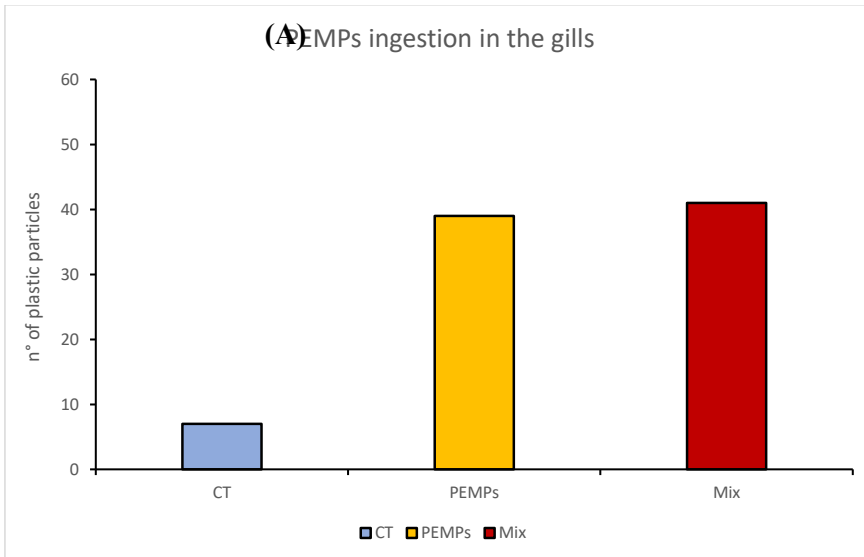
ls (A) and digestive glands (B) of *Ruditapes decussatus* for all treatments (CT, PEMPs, PSNPs, Mix) and times of exposure.

SUPPLEMENTARY DATA

(A) PEMPs ingestion in the gills

(B) PEMPs ingestion in the digestive gland

1. Quantitative graph bar for PEMPs ingested in gills **(A)** and digestive gland **(B)** of unexposed (CT), PEMPs and Mix exposed *R. decussatus* after 10 days.