Chemosphere 287 (2022) 132356

Contents lists available at ScienceDirect

Chemosphere

journal homepage: www.elsevier.com/locate/chemosphere

Chronic toxicity of polystyrene nanoparticles in the marine mussel *Mytilus galloprovincialis*

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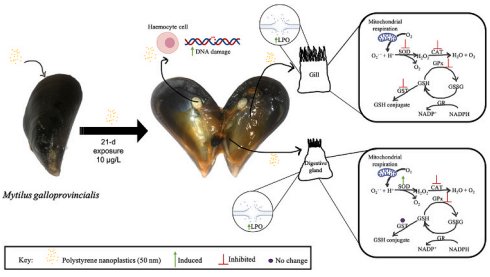
HIGHLIGHTS

- Polystyrene nanoplastics aggregation/ agglomeration is favoured in seawater.
- Genotoxicity occurs in mussel haemolymph after NPs exposure.
- NPs toxicity is tissue and time dependent.
- Gills are the predominate tissue affected by nanoplastic toxicity.

GRAPHICAL ABSTRACT



ARTICLE INFO

Handling Editor: Michael Bank

Keywords:
Polystyrene nanoplastics
Mytilus galloprovincialis
Oxidative stress
Oxidative damage
Genotoxicity

ABSTRACT

Nanoplastics (NP) (1–100 nm) are a growing global concern, and their adverse effects in marine organisms are still scarce. This study evaluated the effects of polystyrene nanoplastics (10 µg/L; 50 nm nPS) in the marine mussel *Mytilus galloprovincialis* after a 21 – day exposure. The hydrodynamic diameter and zeta potential of nPS were analysed, over time, in seawater and ultrapure water. A multibiomarker approach (genotoxicity (the comet assay) was assessed in mussel haemocytes, and the antioxidant enzymes (superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx)), biotransformation enzyme (glutathione – *S* – transferase (GST)), and oxidative damage (LPO)) was assessed in gills and digestive glands to evaluate the toxicity of nPS towards mussels. In seawater, aggregation of nPS is favoured and consequently the hydrodynamic diameter increases. Genotoxicity was highly noticeable in mussels exposed to nPS, presenting a higher % tail DNA when compared to controls. Antioxidant enzymes are overwhelmed after nPS exposure, leading to oxidative damage in both tissues. Results showed that mussel tissues are incapable of dealing with the effects that this emerging stressor pursues towards the organism. The Integrated Biomarker Response index, used to summarise the biomarkers analysed into one index, shows that nPS toxicity towards mussels are both tissue and time dependent, being that gills are the tissue most compromised.

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https://doi.org/10.1016/j.chemosphere.2021.132356
Received 7 June 2021; Received in revised form 16 September 2021; Accepted 23 September 2021
Available online 29 September 2021

1. Introduction

Nanoplastics (NP) (1–100 nm) (Gigault et al., 2018) in the marine environment mostly derive from macro/microplastic degradation (Andrady, 2011; Cole et al., 2011) but can also enter the marine environment in their initial small size, where they have been intentionally manufactured to a fixed size related with specific applications and found in consumer products (e.g., cosmetics, clothing fibres, drug delivery, etc.) (Bessa et al., 2018; Tamminga et al., 2018; Wang et al., 2018). These are stressors of growing global concern, and the time necessary to reach nano-sized plastic particles depends on the scale of the original plastic (Koelmans et al., 2015). Once in the marine environment, plastic particles undergo two main breakdown processes; fragmentation and degradation (Mattsson et al., 2018). After the fragmentation of larger plastic polymer chains into smaller fragments, the polymer itself is unaltered, however, following degradation processes plastic polymers are susceptible to bond-breaking processes, thus changing the polymer properties (Andrady, 2011). Regarding the degradation of plastics, five main processes can occur in the marine environment: hydrolysis, mechanical/physical and thermo oxidative degradation, photodegradation, and biodegradation (Andrady, 2011). Once plastic polymers endure degradation into nano-sized plastic particles, the surface area increases, which in turn increases their potential biological impact (Mattsson et al., 2018; Ferreira et al., 2019). Moreover, at the nanoscale, plastic particles possess novel physical properties which facilitate their entry through biological barriers, accumulating in tissues and consequently impacting organism's metabolism and behaviour (Worm et al., 2017; Mattsson et al., 2018; Ramirez et al., 2019). Recently, an overall greater health change in mussels was associated to NPs exposure compared to microplastics (Capolupo et al., 2021). It is, therefore, crucial to understand the burden of nanoplastic availability and its biological impact on marine biota.

The existence of plastic waste at the nanoscale in the marine environment is known, however, the detection of realistic concentrations of NP remains challenging, as several analytical methods are required to accurately measure the concentration of NP, for various types of NP polymers as well as for several complex matrices (Halle et al., 2017). In the North Atlantic subtropical gyre, Halle et al. (2017) managed to obtain a nanoplastic segment containing nano-scaled plastic polymers such as polyvinyl chloride (PVC), polyethylene terephthalate (PET), polyethylene (PE), and polystyrene (PS). Likewise, in global water shorelines and sand, styrene oligomers have been found, and because of their broad spatial distribution, they have been considered as a global contaminant in sandy beaches (Kwon et al., 2015). PS is known as the 4th most abundant plastic in the world, widely used in building insulation, plastic cutlery and glasses, packaging, toys, and medical equipment (PlasticsEurope, 2019). The breakdown of PS products released in the environment most probably result in styrene oligomers (Ekvall et al., 2019). Moreover, Ekvall et al. (2019) showed that just after 5 min of mechanical breakdown of PS coffee cup lids and polystyrene foams, through simulation of processes that occur along coastlines worldwide, nano-scaled PS particles were generated. Therefore, PS is a significantly important plastic polymer to be evaluated at the nanoscale, as its potential release into the marine environment is increasingly high, especially in coastal shorelines.

When acknowledging NP toxicity towards marine organisms, the plastic particle polymer, size, concentration, and exposure time must be taken into consideration and the fact that their toxicity is also affected by the presence of other contaminants, food availability, species, and species developmental stages (Kögel et al., 2020). Decrease in growth rates, energy, stress, inflammation, and malformations are all associated with NP toxicity and NP effects in marine biota (Kögel et al., 2020), The effects of NP in the aquatic environment have been reviewed recently (Haegerbaeumer et al., 2019; Ferreira et al., 2019; Peng et al., 2020; Gonçalves and Bebianno, 2021; Kihara et al., 2021). In the marine environment, the effects of NP in bacteria, algae, rotifers, nematodes, crustaceans, echinoderms, bivalves, and fish have been evaluated (Ferreira et al., 2019; Gonçalves and Bebianno, 2021; Kihara et al., 2021; Capolupo et al., 2021). Most of the data available are based on PS NPs with functionalized groups (amide or carboxyl groups) rather than virgin PS nanoparticles, being that an amide-group attached are found more toxic than nPS with a carboxyl-group (Gonçalves and Bebianno, 2021). nPS has been shown to affect cell growth, larvae development, malformations of embryos, oxidative stress-induced damage towards lipid membranes, and possible inactivation of the photosystems in algae (Gonçalves and Bebianno, 2021). Ekvall et al. (2019) found nanosized polystyrene particles formed after mechanical breakdown, where nPS had either negative or neutral surface charges, thus incrementing the importance of virgin nPS exposure assessments. However, it is also crucial to understand the toxicity effects of nPS with no functionalized groups namely virgin nPS. Moreover, due to mechanical abrasion from waves and winds, sand and rocks, the nPS produced embrace all various types of nPS (Ekvall et al., 2019).

Bivalves are considered as excellent sentinel organisms for ecotoxicological assessment thanks to their wide geographical distribution and sessile filter-feeding habits, enabling them to accumulate NP from the surrounding environment and therefore they are crucial species to assess the effect of NPs. A chronic exposure of NP in bivalves, acknowledging the potential of ROS generation leading to oxidative stress and damage in tissue – specific evaluations are still rare. Therefore, the present study's main purpose lies within comprehending the effects of PS NP (10 μg/L) in the marine mussel *Mytilus galloprovincialis* based on a multibiomarker approach. In this study, genotoxicity (comet assay) in mussel's haemolymph, effects on the antioxidant enzymes activities (superoxide dismutase – SOD), catalase – CAT, glutathione peroxidase – GPx), biotransformation enzyme activity (glutathione – *S* – transferases – GST), and oxidative damage (lipid peroxidation – LPO) in gills and digestive gland were evaluated after a 21 – day exposure. Environmentally relevant concentrations of NP are still limited, however for microplastics (<5 mm) concentrations range from 0.008 μg/L (Desforges et al., 2014) to 10 μg/L (Ivar Do Sul et al., 2014) for coastal regions, thus a concentration of 10 μg/L of polystyrene nanoplastics was selected.

2. Materials and methods

2.1. Polystyrene nanoplastics (nPS)

Fluoresbrite® Plain YG 0.05 μm Microspheres (9003-53-6) was acquired from Polysciences, Inc. (Germany). 0.05 μm particles packed as 2.5% aqueous suspension, 3.64×10^{14} particles/mL in water (7732-18-5), CV = 15%, Excitation max. = 441 nm, Emission max. = 486 nm. A concentration of 10 μg/L was used, achieved by adding 3 μL of nPS to 10 L of seawater.

2.2. Characterization of nPS

Commercially available nPS (from Polysciences, Inc., LOT#A780141) was submitted to a DLS particle sizer (ZetaSizer Nano ZS90, Malvern Inc.) to measure the hydrodynamic diameter of nPS in both ultrapure water [7732−18−5] and filtered seawater (FSW). Zeta potential values of NP were determined by electrophoresis mobility measurements at 25 °C, using the same equipment, in a disposable 1 × 1 × 1 polycarbonate capillary cell (DTS1061, Malvern Inc.). Time-resolved DLS measurements were used to evaluate aggregation kinetics. Samples were measured over a period between 2 and 12 h. The estimated time gap between the beginning of aggregation and data collection was 50 s.

2.3. Experimental design

Mussels *M. galloprovincialis* Lam. (50 ± 5 mm shell length) were

handpicked from the Ria Formosa Lagoon (Portugal) (37°00′30.6″N 7°59′39.6″W) and transported alive to the laboratory. All mussels were scrap – cleaned and placed into 10 L glass tanks (2 mussels/L) containing natural seawater (S: 35 ± 1) with a 12 h/12 h light/dark cycle and kept under constant aeration. Mussels were acclimated for four days. After acclimation, mussels were exposed to 10 μg/L of nPS jointly with a control group kept in clean seawater in a duplicate design (2 tanks per treatment) for 21 days. Seawater was changed every other day with redosing of nPS concentration. Seawater quality was analysed daily by measuring salinity (36.5 ± 0.75), temperature (24.5 ± 1.5 °C), pH (7.9 ± 0.2) and oxygen saturation (100 ± 1.7%). Mussels were only fed with the plankton existing in the natural seawater. Mortality of mussels was observed in mussels subjected to nPS exposure at day 1 (one dead mussel) and 3 (one dead mussel) of exposure. No mortality was observed between unexposed mussels. For this experiment, five mussels from both control and nPS tanks were removed at the beginning of the experiment and after 3, 7, 14 and 21 days of exposure, where the volume of water, at water exchanging days and reposition of nPS contamination, was adjusted to the number of mussels in each tank to maintain the ratio of 2 mussels/L. At each sampling time, mussels from each experimental condition were collected and dissected into gills and digestive glands, and immediately frozen in liquid nitrogen and stored at −80 °C until further use. Moreover, at days 0, 3 and 14 of exposure, five mussel's haemolymphs were collected for genotoxicity (comet assay). For condition index purposes, mussels were sampled on days 0, 7 and 14. A battery of biomarkers consisting of antioxidant enzymes (SOD, CAT, GPx), biotransformation enzyme (GST) and oxidative damage (LPO) was used to analyse nPS effects in gills and digestive glands of *M. galloprovincialis*.

2.4. Quality control quality assessment

To eliminate plastic contamination, aeration was supported by glass pipettes and glass tanks were enclosed, eliminating aerial plastic contamination. Additionally, during tissue dissection, no gloves nor plastic material/equipment were used to eliminate any further plastic contamination.

2.5. Condition index

The condition index (CI) was assessed in mussels (5 per treatment and time of exposure) to determine their physiological status at the beginning (day 0) and after 7 and 21 days of exposure. The CI was calculated as the percentage (%) of the ratio between the whole mussel weight (tissue and shell) (g) and the wet weight (g) of the soft tissues (Gomes et al., 2013).

2.6. Genotoxicity assay

DNA damage was assessed by the alkaline comet assay, adapted from Singh et al. (1988) and Gomes et al. (2013). Haemolymph of five mussels collected after 0, 3 and 14 days of exposure to nPS and from five unexposed mussels was extracted from the posterior adductor muscle with a sterile hypodermic syringe (1 mL) (25 G needle). From each experimental condition, 100 μL of sub-sample was stained with 100 μL trypan blue to assess cell viability, where the % of live cells was measured by randomly counting 100 cells. Briefly, for DNA damage assessment, microscopic slides were previously cleaned in ethanol/ether (1:1) and coated with 0.65% normal melting point agarose (NMA) in Tris-acetate EDTA. Upon collection, mussel haemolymph cells were centrifuged at 3000 rpm for 3 min (4 °C), and pellets with isolated cells suspended in 0.65% low melting point agarose (LMA, in Kenny's salt solution) and casted on the microscopic slides. Slides with embedded cells were subsequently immersed in a lysis buffer (2.5 M NaOH, 100 mM EDTA, 10 mM Tris, 1% Triton X-100, 10% dimethylsulfoxide, 1% sarcosil, pH 10, 4 °C) for 1 h for the diffusion in agarose of cellular components and DNA immobilization. Slides were gently placed in an electrophoresis containing electrophoresis buffer (300 mM NaOH, 1 mM EDTA, adjusted pH 13, 4 °C) after the lysis stage. Electrophoresis was then carried out for 5 min at 25 V and 300 mA. Once concluded, the slides were removed and soaked in a neutralizing solution (0.4 mM Tris, pH 7.5), rinsed with bi-distilled water and left to dry overnight. Afterwards, slides were stained with 4,6-diamidino-2-phenylindole (DAPI, 1 μg/mL) and the presence of comets was analysed using an optical fluorescence microscope (Axiovert S100) coupled to a camera (Sony). With a total magnification of × 400, the Komet 5.5 image analysis system was used to score 50 randomly chosen cells for each slide (total of 200 cells scored per group), following the determination of the amount of DNA in tail. Results are expressed as mean ± standard deviation.

2.7. Tissue preparation for enzymes activities analysis

Mussel gills and digestive gland were homogenized individually in 5 mL of 20 mM Tris-Sucrose buffer (0.5 M Sucrose, 0.075 M KCl, 1 mM DTT, 1 mM EDTA, pH 7.6) in an ice bath for 2 min, according to the protocol described by Geret et al. (2002). Homogenates were then centrifuged (500 *g*, 15 min, 4 °C) and the resulting supernatant re-centrifuged (12 000 *g*, 45 min, 4 °C) to obtain the cytosolic fraction. Aliquots (150 μL) of the cytosolic fraction were separated for the determination of the activities of each antioxidant enzyme activity (SOD, CAT, GPx) and biotransformation (GST) enzymes.

In addition, total protein concentrations (mg protein g^{-1} tissue), adapted for microplate reader using bovine serum albumin (BSA) as standard, were determined using the method defined by Bradford (1976).

2.8. Superoxide dismutase (SOD) activity

SOD activity was assessed in gills and the digestive gland by calculation of the decrease in absorption by the xanthine oxidase/hypoxanthine system of the substrate cytochrome *c* at 550 nm, and the results are expressed as U mg^{-1} protein (McCord and Fridovich, 1969).

2.9. Catalase (CAT) activity

The quantitative determination of CAT activity in the mussel gills and digestive gland, followed the procedure defined by Greenwald (1985) and is based on the measurement of consumption of hydrogen peroxide (H_2O_2) at 240 nm spectrophotometrically. Results are expressed as mmol min^{-1}mg $protein^{-1}$.

2.10. Glutathione peroxidase (GPx) activity

GPx activity in the gills and digestive gland was measured at 340 nm, with cumene hydroperoxide as substrate at 28 °C, using a microplate reader (Infinite® 200, Pro-Tecan), based on the method adapted from McFarland et al. (1999). Outcomes are represented as mmol min^{-1}mg $protein^{-1}$.

2.11. Glutathione-S-transferases (GST) activity

The biotransformation enzyme GST was measured in the gills and the digestive gland by the conjugation of 0.2 mM reduced glutathione (GSH) with 0.2 mM 1-chloro-2,4-dinitrobenzene (CDNB) in a reaction mixture of 0.2 M KH_2PO_4/K_2HPO_4 buffer (pH 7.9), at 340 nm, in a microplate reader (Infinite® 200, Pro-Tecan) following the method adapted from Habig et al. (1974). Results are expressed as μmol CDNB $min^{-1}mg^{-1}$protein.

2.12. Lipid peroxidation (LPO)

Mussel gills and digestive gland were homogenized individually on

ice with 5 mL of 0.02 M Tris-HCl buffer (pH 8.6) and butylated hydroxytoluene (BHT) in a 1:10 ratio. Homogenates (3 mL) were centrifuged at 30 000 *g* for 45 min (4 °C) and the resulting supernatant was used to determine total protein concentrations (Bradford, 1976) and LPO levels. LPO levels were determined by the absorbance of malondialdehyde (MDA) and (2 E)-4-hydroxy-2-nonenal (HNE), at 540 nm, following the method adapted from Erdelmeier et al. (1998). Results are presented as nmol/mg prot.

2.13. Integrated biomarker response index

The integrated biomarker response index (IBR) is an effective tool to visualize biological effects of pollutants and simplify the interpretation of the relationship between the battery of biomarkers and contamination levels (Devin et al., 2014). Thus, the biomarker data from gills and digestive gland of *M. galloprovincialis* exposed to polystyrene NPs were integrated using a biomarker response index version 2 (IBR) proposed by Sanchez et al. (2013), being this version modified from the IBR index defined by Beliaeff and Burgeot (2002) and described in Serafim et al. (2012). IBR allows the integration of the different biomarker responses into a numeric value. A reference deviation concept is based on a disturbed and undisturbed state, where the IBR index was developed with the aim of removing the IBR result dependency on arrangement of the biomarkers as well as the induction and inhibition of each biomarker (Sanchez et al., 2013). IBR represents a sum of the deviation between unexposed and nPS at each sampling day.

Briefly, combined data of each individual biomarker (X_i) was compared to the data (X_0) of each biomarker from the control group and log transformed (Y_i) to reduce variance [$Y_i = \log (X_i/X_0)$]. The mean (μ) and standard deviation (σ) for Y_i was calculated and the data of each parameter were further standardized according to the following equation:

$$Z_i = \frac{(Y_i - \mu)}{\sigma}$$

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

$$A = Z_i - Z_0$$

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

$$IBRv2 = \sum |A_i|$$

2.14. Statistical analysis

The significant differences between treatments and time were evaluated by using parametric tests (ANOVA, followed by Tukey's Post-hoc test), or non-parametric equivalent test (Kruskal-Wallis and a two-tailed multiple comparisons test), according to data distribution and variances homogeneity (Shapiro-Wilk test). Results were significant when $p < 0.05$. A Principal Component Analysis (PCA) was also used to evaluate the relationship between treatments (unexposed and exposed to nPS) and the analysed parameters [antioxidant and biotransformation enzymes (SOD, CAT, GPx, GST) activities and oxidative damage (LPO)] in gills and digestive gland of mussels along the exposure period (21 days). These statistical analyses were performed on R software (R. Core Team, 2017) and Statistica 7.0 software (Statsoft Inc., 2005; USA). For IBR, statistical differences were evaluated using a *t*-test on Microsoft Excel (Microsoft Corporation, 2018. *Microsoft Excel*, Available at: https://office.microsoft.com/excel), and results were considered significant when $p < 0.05$.

3. Results

3.1. nPS characterization

The hydrodynamic diameter and zeta potential (ζ-potential) of polystyrene nanoplastics (nPS; 50 nm) were studied between a period of 2 and 12 h with measurements taken every 50 s, in ultrapure water (Fig. 1 A) and filtered seawater (FSW; Fig. 1 B). Results showed that in ultrapure water the size of nPS did not vary with time, meaning that there is no aggregation of NPs under these conditions. According to Fig. 1 A, the average size of nPS is approximately 25 nm, which is less than the size reported by the manufacturer. This difference is common, as they are usually related to the techniques used to measure the size of these particles. The hydrodynamic diameter of nPS in ultrapure water agrees with the ζ-potential, which indicates that in these conditions ζ-potential is approximately −68.8 ± 0.66 mV, very far from zero, thus NPs are stable, and no aggregation takes place. When nPS are dispersed in FSW, the size increases (852 ± 103 nm), indicating that aggregation/agglomeration occurs due to the high concentration of salts in seawater (Fig. 1 B). The ζ-potential, under these conditions, also demonstrates that aggregation/agglomeration is favoured as ζ-potential is practically zero (−0.068 ± 0.23 mV).

3.2. Condition index

There are no significant variations in the condition index of *M. galloprovincialis* between treatments nor between the times of exposure, whereby values ranged between 37.30 ± 2.81 and 42.93 ± 1.90% ($p > 0.05$) (Table 1).

3.3. Genotoxicity

DNA damage was assessed in haemocytes of unexposed and nPS exposed mussels, using the comet parameter of % tail DNA, at the beginning of the experiment, and after 3 and 14 days of exposure (Fig. 2). No major differences were observed at each exposure period in the haemocytes of unexposed mussels ($p > 0.05$). However, in mussels

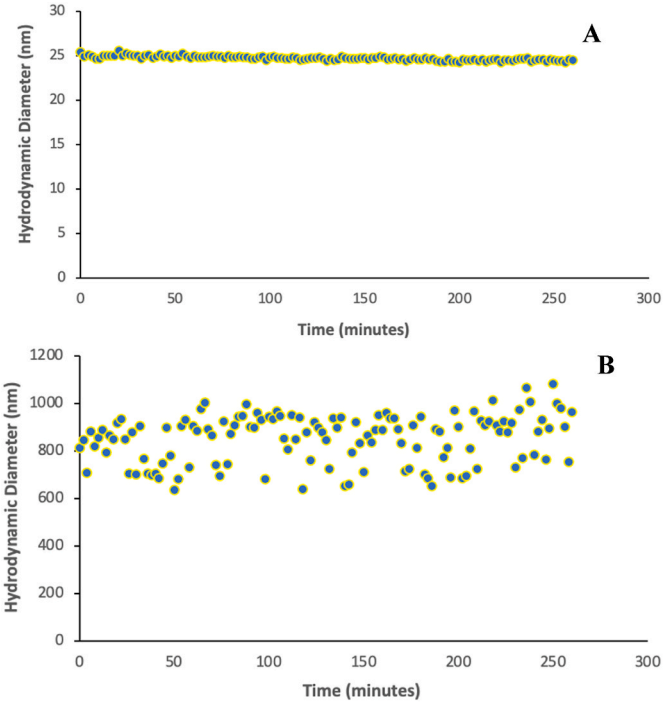

Fig. 1. Hydrodynamic diameter of polystyrene nanoplastics (50 nm) in **(A)** ultrapure water and **(B)** seawater over time (minutes).

Table 1
Condition index (mean ± S.D.) (%) of *M. galloprovincialis* exposed to nPS at the beginning and end of exposure.

Time (day)	CT	nPS
0	37.30 ± 2.81	
7	40.67 ± 8.16	41.66 ± 5.54
21	37.22 ± 3.03	42.93 ± 1.90

exposed to nPS, DNA damage was significantly higher (4 – fold) than in unexposed mussels at both exposure days ($p < 0.05$). A few examples of comets in mussel haemocytes from control and exposed to nPS are documented in Fig. 3. Haemocytes of *M. galloprovincialis* from controls showed a nucleoid core with no DNA migrating into the tail region at 0, 3 and 14 days, whilst in nPS exposed mussels, the nucleoid core presents broken DNA fragments or damaged DNA migrating into the tail region after 3 and 14 days of exposure. Therefore, nPS exposure leads to genotoxicity in haemolymph of exposed mussels (see Fig. 2A).

3.4. Enzymatic activity

Antioxidant enzymes activities in tissues of unexposed mussels did not change over time ($p > 0.05$; Fig. 3 A – H), with exceptions in CAT (gills, $p < 0.05$; Fig. 3 C), GPx (gills, $p < 0.05$; Fig. 3 E), and GST activities (gills, $p < 0.05$; Fig. 3 G).

In the gills, a significant decrease trend in SOD activity is shown after 3, 7 and 14 days in mussels exposed to nPS when compared to unexposed ($p < 0.05$; Fig. 3 A). SOD activity in mussels after 14 days of exposure to nPS present significant differences when compared to nPS exposed mussels after 21 days and to unexposed mussels ($p < 0.05$). On the other hand, in the digestive gland, SOD activity increased after 3 and 7 days in nPS exposed mussels ($p < 0.05$; Fig. 3 B), whilst in nPS exposed mussels a significant decrease at 14 days is noticeable when compared to unexposed and exposed mussels at 21 days ($p < 0.05$).

CAT activity in the gills of mussels exposed to nPS were significantly different from unexposed mussels throughout exposure period ($p < 0.05$; Fig. 3 C). A decrease in CAT activity is noteworthy in nPS exposed mussels after 7 days of the bioassay, and a significant difference was detected between mussels after 14 days compared to mussels at 3 and 21 days of exposure ($p < 0.05$). In the digestive glands of nPS exposed mussels, a general decrease in CAT activity was observed until the end of the exposure period, being this decrease in activity significant between unexposed and exposed mussels at both 3 and 21 days of exposure ($p < 0.05$; Fig. 3 D). Also, after 14 and 21 days of exposure to nPS, mussels' digestive glands present a significant decrease when compared to the

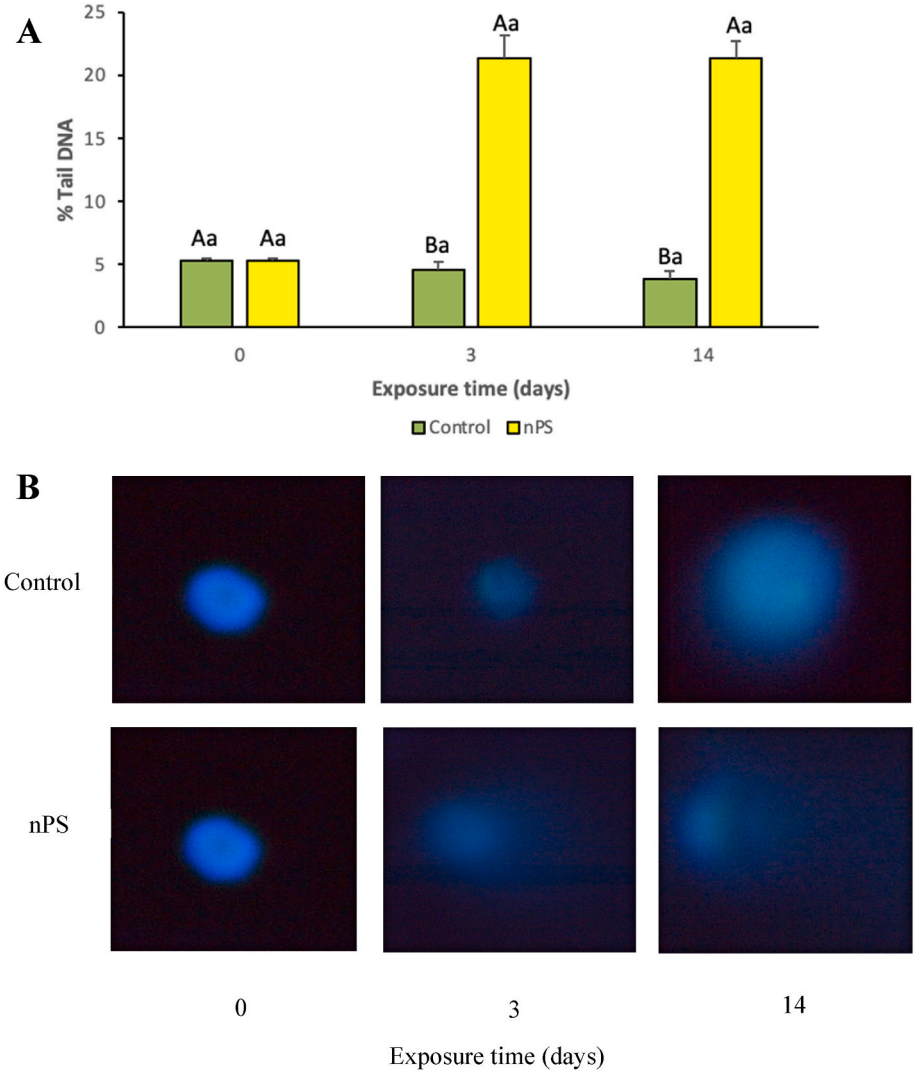

Fig. 2. **(A)** Genotoxicity effects of *in vivo* exposure of polystyrene NPs in the haemolymph of *M. galloprovincialis* and **(B)** examples of comet assay images of unexposed *M. galloprovincialis* haemocytes and those exposed to polystyrene NPs. Different upper and lower case letters indicate significant differences between treatments for the same time, and between times for the same treatment, respectively ($p < 0.05$).

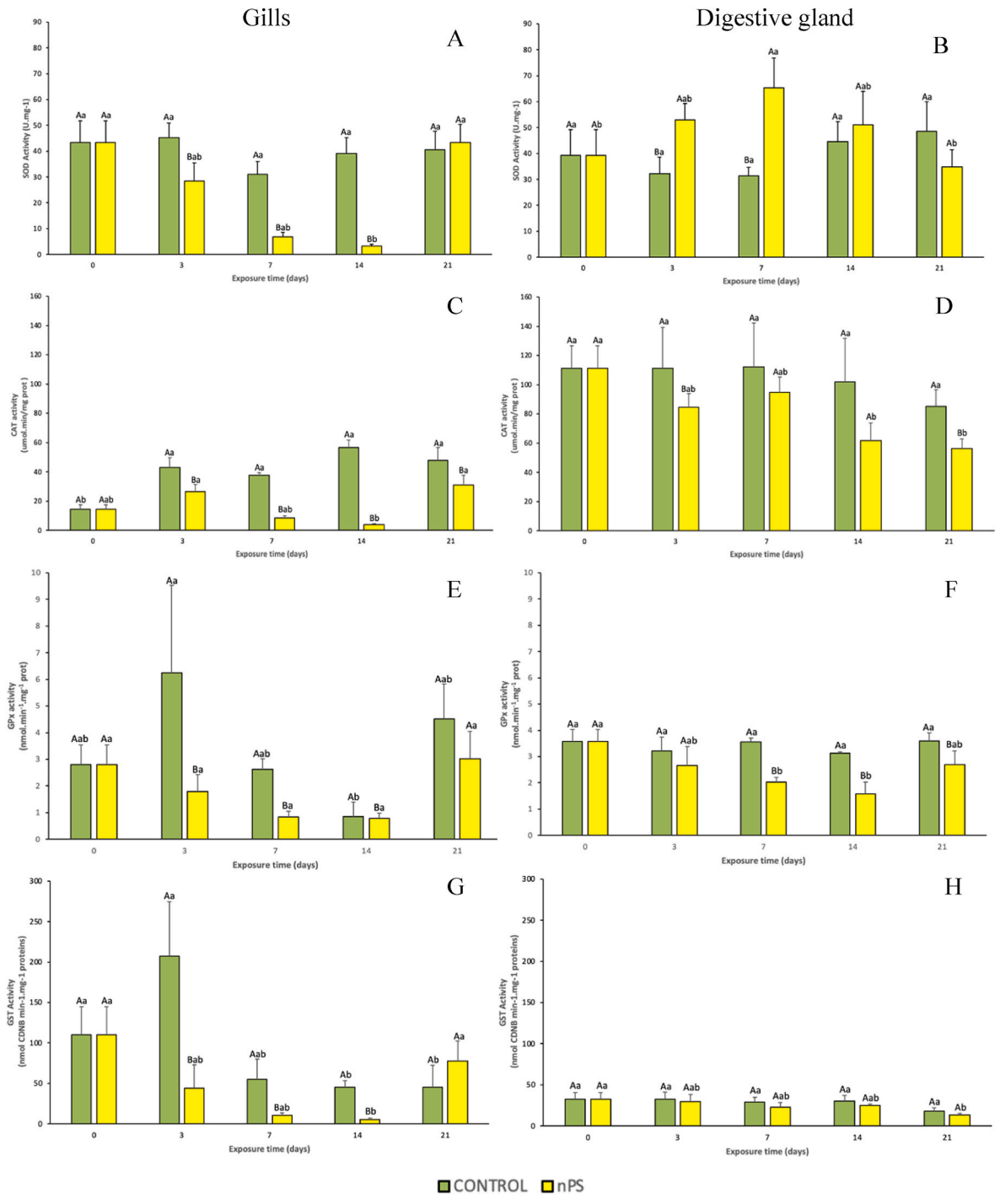

Fig. 3. Superoxide dismutase (SOD) (A–B), catalase (CAT) (C–D), glutathione peroxidase (GPx) (E–F) and glutathione-S-transferase (GST) (G–H) activities (mean ± std) in the gills and digestive gland of mussels *M. galloprovincialis* from controls and exposed to 10 μg/L nPS for 21 days. Different upper and lower case letters indicate significant differences between treatments for the same time, and between time for the same treatments, respectively ($p < 0.05$).

beginning of exposure ($p < 0.05$).

In gills of mussels, GPx decreased significantly in activity after 3, 7 and 14 days of exposure to nPS compared to unexposed mussels ($p < 0.05$; Fig. 3 E), however no significant differences were encountered in nPS exposed mussels between times of exposure. A decrease in GPx activity was also found in the digestive gland of mussels exposed to nPS after 3 days of exposure, being significantly different to control mussels at 7, 14 and 21 days of exposure ($p < 0.05$; Fig. 3 E). Regarding time of exposure, nPS exposed mussels GPx activity is significantly different at 7 and 14 days of exposure compared to the beginning of the experiment ($p < 0.05$).

Activities of the biotransformation enzyme (GST) also decreased in gills of nPS exposed mussels after 3, 7 and 14 days of exposure compared to unexposed ($p < 0.05$; Fig. 3 G). After 14 days of nPS exposure, gills activity is significantly inferior to nPS exposed mussels at the beginning and end of the exposure period ($p < 0.05$). On the other hand, in the digestive gland of nPS exposed mussels, no significant differences were found compared to unexposed ($p > 0.05$; Fig. 3 H), however significant differences were found within nPS exposed mussels at 21 days of exposure compared to the beginning of the experiment ($p < 0.05$).

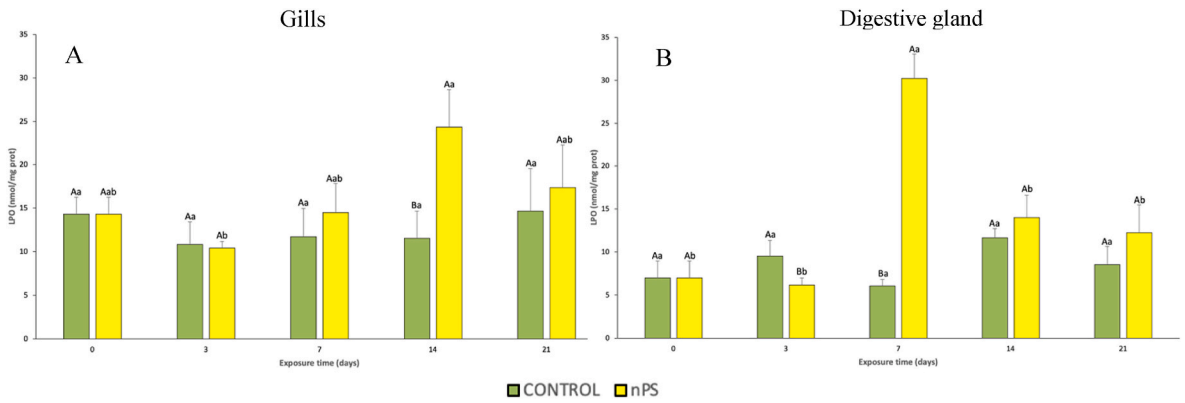

Fig. 4. Lipid Peroxidation (LPO) in gills (A) and digestive gland (B) of mussels *M. galloprovincialis* from control and exposed to 10 μg/L nPS for 21 days (mean ± S.D.). Different upper and lower case letters indicate significant differences between treatments for the same time, and between time for the same treatments, respectively ($p < 0.05$).

Overall, enzymatic activities decreased in gills after 3 days of exposure to nPS, whilst in digestive glands the decrease in enzymatic activity is noteworthy after 7 days of exposure with exception in SOD and GST activities. Time of exposure to nPS is critical for mussel gills at 7 and 14 days of exposure, whilst in digestive glands, longer exposure to nPS has more adverse effects (14 and 21 days).

3.5. Oxidative damage

Oxidative damage in both tissues of unexposed mussels did not change throughout time of exposure ($p > 0.05$; Fig. 5 A – B). In gills of nPS exposed mussels, LPO levels increased significantly at 14 days of exposure compared to controls ($p < 0.05$; Fig. 5 A), being LPO levels in nPS exposed mussels at 3 days significantly lower than LPO levels found after 14 days of exposure ($p < 0.05$). In the digestive glands, a significant increase in LPO levels was encountered earlier, at 7 days of exposure to nPS compared to gills ($p < 0.05$; Fig. 5 B) being also significantly different from all other exposure times of nPS exposed mussels and unexposed mussels from the same exposure time ($p < 0.05$). After 3 days of exposure to nPS, digestive glands of mussels presented lower LPO levels in comparison to unexposed ($p < 0.05$).

Predominately, LPO occurred earlier in digestive glands of nPS exposed mussels compared to gills.

3.6. Principal component analysis (PCA)

PCA was applied to all the data obtained for both tissues (gills and digestive gland) of mussels to describe the impact of nPS on the response of biomarkers (SOD, CAT, GPx, GST activities and LPO; Fig. 5 A – B). The two principal components represent 82.5% and 83.5% of total variance in gills (PC1 = 62.9%, PC2 = 19.5%; Fig. 5 A) and digestive glands (PC1 = 59.0%, PC2 = 24.4%; Fig. 5 B) of mussels, respectively.

In mussels, gills and digestive glands exhibited different responses regarding exposure to nPS, thus nPS toxicity seems to be tissue specific. This may be due to the ability these nano-sized plastic particles may have passing through cellular boundaries. To conclude, in both tissues, LPO was shown to be positively related to the exposure of nPS, whilst remaining enzymatic activities (SOD, CAT, GPx and GST) activities, except for SOD activity in the digestive gland, are negatively related to nPS exposed mussels and positively related to controls. Also, of importance, PCA results of both tissues show that time of exposure is also influential, presenting nPS toxicity as chronic (+7 days).

3.7. Integrated biomarker response

The Integrated Biomarker Response (version 2) (IBR) was calculated for the data on gills and digestive gland biomarkers from all exposure times to nPS. Graphical representation of IBR and star plots for both mussel tissues are in Fig. 6. IBR pattern was tissue dependent and time dependent. No significant differences were observed in the digestive gland ($p > 0.05$) (Fig. 6A), though a linear increase is observed. In the gills, significant differences between exposure times are encountered. The IBR value is significantly higher, in gills, after 14 days of exposure to nPS when compared to other sampling days ($p < 0.05$) (Fig. 6A). IBR

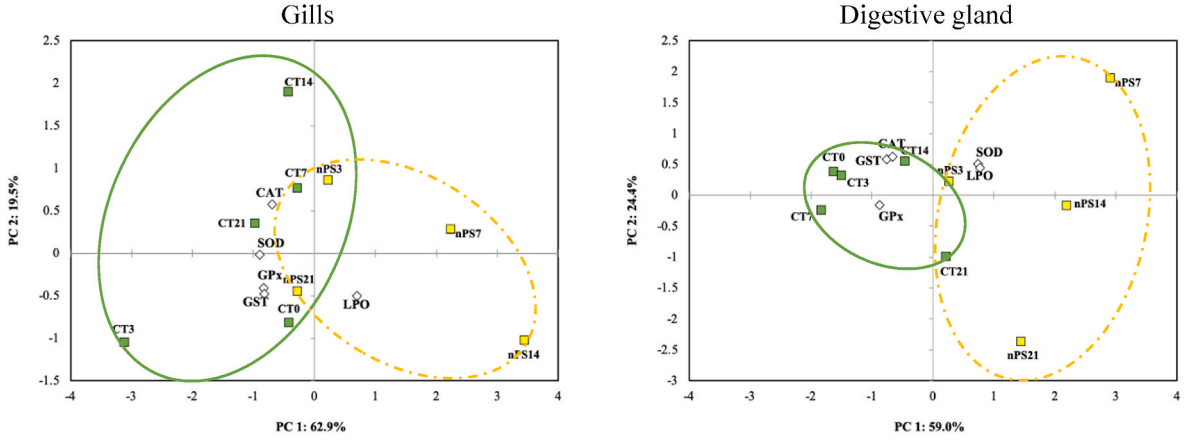

Fig. 5. Principal component analysis (PCA) of a battery of biomarkers (SOD, CAT, GPx, GST activities and LPO) in gills and digestive glands of mussels *M. galloprovincialis* from controls (CT), exposed to nPS for 21 days ($p < 0.05$).

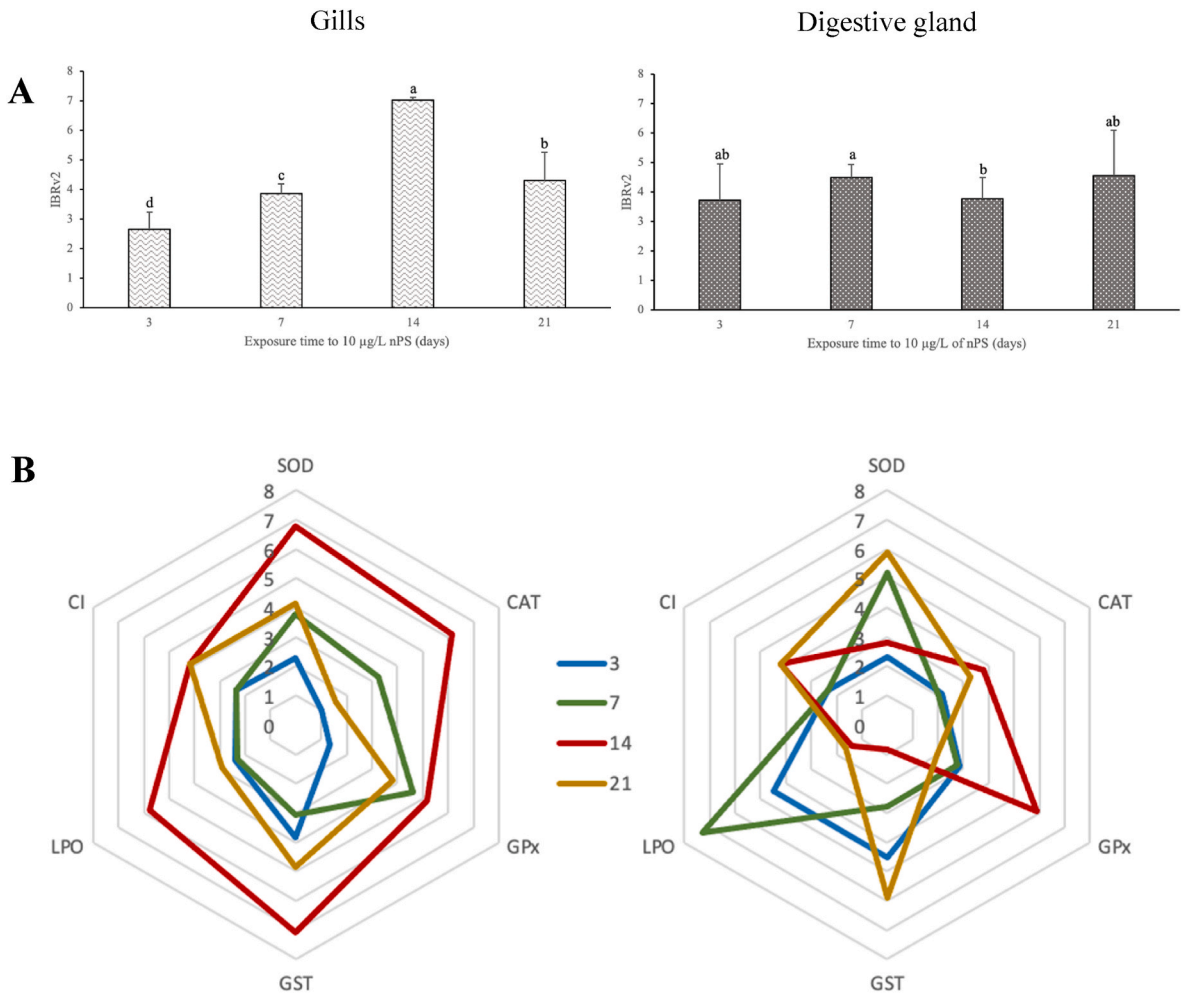

Fig. 6. **(A)** Integrated biomarker response index version 2 (IBR) (mean ± S.D) and **(B)** star plots of the gills and digestive glands of *M. galloprovincialis* exposed to polystyrene nanoplastics for 21 days. Lower case letters indicate significant differences between times of exposure ($p < 0.05$).

value for the gills after 3 days of exposure is significantly lower than other exposure times ($p < 0.05$) (Fig. 6A). No significant differences were found between 7 days and 21 days of exposure in mussel gills ($p > 0.05$) (Fig. 6A). Star – plots showed that changes in biomarkers were also tissue and time dependent (Fig. 6B). IBR plot show that the 7th day of exposure was the most critical for digestive glands of mussels, being LPO the main contributor to overall IBR score. For gills, the 14th day stands as the most critical, with SOD and GST, followed by CAT and LPO, all contribute to overall calculated IBR score.

4. Discussion

Plastic pollution, in the marine environment, at a nanoscale is prominent, and the toxicity of these particles in bivalves is highly worrying. Data has shown that NPs are recognised and consumed by these filter feeders as low nutritional food and that they compromise the integrity of bivalve's immune system, causing fertilization and embryo-larvae development to be impaired (Wegner et al., 2012; Canesi et al., 2015; Balbi et al., 2017; Brandts et al., 2018; González-Fernández et al., 2018; Tallec et al., 2018; Rist et al., 2019; Auguste et al., 2020; Baudrimont et al., 2020; Cole et al., 2020; Li et al., 2020; Sendra et al., 2020a,b; Capolupo et al., 2021). Taking this into account, this study aimed to assess genotoxic, antioxidant and, oxidative damage effects after chronic exposure to nPS in the Mediterranean mussel *M. galloprovincialis*.

Results demonstrate that nPS at a low concentration (10 µg/L), compared to other studies that have evaluated nPS effects in mussels (Brandts et al., 2018; Auclair et al., 2020; Capolupo et al., 2021), significantly affect both gills and digestive gland of *M. galloprovincialis*, in which nPS mediated toxicity is found to be tissue-specific as well as time-dependent.

Regarding nPS characterization, in FSW aggregation/agglomeration was favoured, possibly due to the high salt concentrations, which is supported by an increase in hydrodynamic diameter and with zeta – potential being close to zero. Natural organic matter (NOM), inorganic colloids, ultraviolet (UV) radiation are recognised as factors that alter the nano-specific properties, structure, cohesion, and aggregation of particles (Andrady, 2011; Oriekhova and Stoll, 2018; Shen et al., 2019). Therefore, nPS characterization suggests that NPs behaviour under FSW conditions differs completely from nPS in ultrapure water, and consequently leads to high aggregation/agglomeration kinetics of NPs. Also, in comparison to polystyrene microplastics (PS-MPs; 20 µm) the zeta-potential within seawater (SW) differs greatly from nPS (50 nm). Ribeiro et al. (2017) observed a zeta-potential value of -12.4 ± 2.36 mV for PS-MPs (20 µm) whereas for nPS values are almost zero (-0.068 ± 0.23 mV), showing that nPS have a higher tendency for aggregation/agglomeration when compared to PS-MPs. Although the initial size of plastic particles was unknown, El Hadri et al. (2020) showed that for nPS the zeta-potential was -44 ± 2 mV (25 °C, pH = 6.7 ± 0.3), and the size distribution was of 306 ± 15 nm. Comparatively, PE and PE + PP at the nanoscale presented zeta-potentials of -36 ± 3 and -30 ± 2 mV, and size distributions of 129 ± 7 and 460 ± 23 nm, respectively (El Hadri

et al., 2020). Elsewhere, the difference in behaviour of the NP particles (50 and 100 nm), with and without fluorescence, was observed in different media in Sendra et al. (2020b). Here, zeta-potential presented negative values in all media, whereby differences between nPS (50 nm) with and without fluorescence presented non-fluorescent particles to have zeta-potential values closer to zero in FSW, whereas in ultrapure water the opposite was observed (Sendra et al., 2020b). In Sendra et al. (2020b), the NP (50 nm) evaluated is like the present data, whereby the fluorescence dye has been incorporated internally, being this dye highly hydrophobic and remaining trapped within the particle in aqueous environments (Polyscience Inc., 2018). However, there are minor differences in particles zeta-potential between fluorescent nPS and nPS without fluorescence in FSW (−24.8 and −13.3 mV, respectively) (Sendra et al., 2020b). So, we must consider the chemical composition, and size, of each nanoparticle, and how they interact with NOM, inorganic colloids, and UV radiation as well as salinity, pH and O_2 levels within SW.

The antioxidant defence system of mussels exposed to nPS suffered alterations, presenting time-dependent and tissue-specific toxicity. A continuous increase in the activity of antioxidant enzymes can require energy that can compromise energy directed to growth and reproduction (Trestrail et al., 2020).

The antioxidant defence system, regulated by the enzymes studied here, aid in minimizing the effects of ROS within the cell. However, when there is an overproduction of ROS, the antioxidant defence system can be overwhelmed and not counteract the effects, leaving mussels' innate immune system to be compromised and lead to oxidative damage. This is observed in the gills, where the first line of defence, SOD, was unable to counteract ROS production mediated by nPS exposure. On the other hand, in the digestive gland, the increase in SOD activity suggests that superoxide radicals generated by nPS exposure were counteracted in this tissue. As filter-feeding organisms, mussel's gills are a primary route for mussels to uptake xenobiotics (Jørgensen, 1996), and this observation can be a consequence of this factor. On the contrary, in mussels *M. edulis* (nPS - 50 nm; 500 μg/L; 24h) (Cole et al., 2020), and *Mytilus* spp. (polyethylene (PE) and polypropylene (PP) microplastics - < 400 μm; 10–100 μg/L; 10 days) (Revel et al., 2019) and in the clam *Scrobicularia plana* (PS microplastics - 20 μm; 1000 μg/L; 14 days) (Ribeiro et al., 2017), SOD activity increased in both tissues, wherein Cole et al. (2020) SOD activity in the digestive gland returned to control values after 7 days of exposure to nPS, and a similar pattern was observed in this study. This was also observed in the digestive gland of *M. coruscus* after exposure to PS microspheres (2 μm; 10, 10^4 and, 10^6 particles/L; 14 days) (Wang et al., 2020). Alternatively, after exposure to nPS (70 nm; 3.64×10^3 particles/L; 14 days), no significant alterations were observed in SOD activity in the digestive gland of *M. coruscus* (Wang et al., 2021). Thus, as the first line of defence, SOD presents to counteract ROS mediated by nPS toxicity in the digestive gland, meanwhile in the gills, this protective enzyme is overwhelmed.

Comparatively, CAT activity was also overwhelmed, but in both tissues of *M. galloprovincialis*, where the inhibition observed represents the incapability to eliminate hydrogen peroxide generated as a result of nPS exposure. Conversely, data from other species revealed either no alterations or an increase of CAT activity. In *M. coruscus* (nPS; 70 nm; 3.64×10^3 particles/L; 14 days) no significant changes were encountered in the digestive gland (Wang et al., 2021), and Ribeiro et al. (2017) had similar findings in the clam *S. plana* after exposure to microplastics (PS; 20 μm; 1000 μg/L; 14 days), whereas PS microspheres (2 μm; 10, 10^4 and 10^6 particles/L; 7 days) and PE and PP microplastics (<400 μm; 10 days) led to an increase of CAT activity in *M. coruscus* (Wang et al., 2020) and in gills and digestive gland of *Mytilus* spp. (Revel et al., 2019). These comparisons substantiate the differences in toxicity of nano and micro-sized plastic particles, whereby results suggest that the smaller the plastic particle size, the more toxic it is towards bivalves. Also, the concentrations used may have an influential role in the toxicity that plastic particles pursue in bivalves, as the higher the concentration the higher the probability of aggregation/agglomeration within seawater, increasing the size of aggregates and possibly decreasing the capacity of these aggregates to be ingested by the organism.

GPx activity is exceptionally vulnerable, even at low levels of environmental disruption, to the early onset of the pro-oxidant crisis (Regoli and Giuliani, 2014). In the present data, GPx activity was unable to cope with excess hydrogen peroxide present by nPS mediated toxicity, and this was observed in both tissues. The inhibition of GPx activity occurs after 3 days of exposure, being the lowest observed activity at 7 and 14 days. Interestingly, a slight increase in GPx activity is noteworthy in both tissues after 21 days, whereby a possible adaptative response may be taking place. Studies that have evaluated plastic particles effects in bivalves have noticed either no significant alterations in either tissue of *M. edulis* (PS microspheres; 2 μm; 10, 10^4 and 10^6 particles/L; 7 days) (Wang et al., 2020), or an increase in GPx activity prior to 3 days of exposure to PS microplastics (20 μm; 1000 μg/L; 14 days) in both gills and digestive glands of the clam *S. plana* (Ribeiro et al., 2017). In comparison, nPS exposure is leading to excess ROS being produced which is overwhelming GPx ability to catalyse the reaction transforming hydrogen peroxide in water, and consequently, mussel tissues are incapable of dealing with the negative effect of this stressor. However, before 21 days, an activation of an adaptative response may explain the slight increase observed. As shown by Wegner et al. (2012), these filter-feeding organisms recognise and ingest plastic nanoparticles as a low nutritional food. Considering this, an adaptative response of the mussel towards nPS toxicity may be the upregulation of autophagy, normally associated with the lack of nutrients as well as other stressors (Moore et al., 2006). Autophagy plays a role in sequestering and recycling oxidatively damaged proteins and organelles, offering a selective advantage for cell survival (Moore et al., 2006). The adaptative response observed in the gills for all enzymatic activity assessed and in the digestive gland for GPx activity after 21-days of exposure to nPS suggests that mechanisms such as autophagy can be providing recovery of the mussels' tissues.

The biotransformation enzyme GST promotes the binding of glutathione and mercapto-transferase to form glutathione peroxidase with high degradation of hydrogen peroxide (Yu et al., 2018). After an inhibition was observed in GPx activity, it is expected that GST will step in defending the organism from ROS generated after nPS exposure, though here, that is not the case. GST activity, in the gills, had a similar pattern to the other enzymes assessed, whilst in the digestive gland, GST activity maintained control values. On the other hand, GST activity increased in the gills of *S. plana* and decreased in the digestive gland after PS microplastic exposure (20 μm; 1000 μg/L; 14 days) (Ribeiro et al., 2017). Nonetheless, this increments the importance to evaluate plastic particles at the nanoscale, as their behaviour and mediated toxicity differ from larger-sized particles. Another reason behind these differences can be related to that preferably, mussels ingest smaller particles (Wang et al., 2021). Furthermore, the ingestion of these NPs was found to be favourably in the digestive tract, though over time particles translocate to the mantle, being that the gills are not a typical tissue to accumulate (Wang et al., 2021). So, from ingestion through filter-feeding habits, nPS enter the gills and translocate to the digestive tract of the mussel, thus explaining the tissue-dependent and time-dependent toxicity observed. Overall, in both tissues, results suggest that these enzymes were incapable of dealing with the negative effect of this stressor, leading to oxidative damage, and results of LPO confirm this.

When the antioxidant defence system is overwhelmed, the membrane lipids are not salvaged from attacks by residual ROS, and therefore lead to lipid peroxidation (LPO). Oxidative damage occurred in both tissues of the marine mussel *M. galloprovincialis*, whereby the highest significant levels of LPO registered in the gills and in the digestive gland are encountered at different times of exposure. NPs induce oxidative stress after exposure to 0.05 mg/L of nPS with attached amide group

(100 ± 6.9 nm; 96 h) in *M. galloprovincialis*, whereby LPO levels increased significantly in the digestive gland, though no significant alterations were found in the gills (Brandts et al., 2018). Wang et al. (2021) showed that LPO was induced in the digestive gland of *M. coruscus* after exposure to nPS (70 nm; 3.64 × 10^3 particles/L; 14 days). In gills and digestive gland of *M. edulis* exposed to 500 μg/L of nPS (50 nm; 24 h and 7 – days), no significant differences in oxidative damage were observed, except an initial decrease found in gills after 24 h exposure, being that no substitutive LPO occurred (Cole et al., 2020). In the clam *S. plana* after exposure to PS microplastics (20 μm; 1000 μg/L; 14 days), LPO levels decreased in gills and, an increased tendency was observed in the digestive gland at 7 – days of exposure though not significant (Ribeiro et al., 2017). Furthermore, no sign of LPO was observed in *Mytilus* spp. before 7 – days of exposure to PS microplastics (2 and 6 μm; 2000 microbeads mL^{-1} day^{-1}; 7 days) although a significant enhancement of ROS was found in haemocytes of the digestive gland of mussels (Paul-Pont et al., 2016). Our results disagree with others, as the gills and digestive glands do suffer oxidative damage (Fig. 4) contradicting the suggestion made by Li et al. (2020) that the gills of *M. galloprovincialis*, do not seem to have a stronger antioxidant capacity when compared to the digestive gland. Therefore, LPO results indicate that the production of ROS overwhelmed the efficiency of the antioxidant enzymes of cells in gills and digestive gland to maintain redox balance, resulting in oxidative damage of membrane lipids. The PCA and IBR results (Figs. 5–6) further confirm that both mussel tissues are susceptible to alterations in their antioxidant defence mechanism after exposure to nPS, being gills the tissue mostly compromised by the presence of nPS throughout the time of exposure. IBR demonstrates that the crucial time of exposure for gills is at 14 days, which is confirmed by the lowest enzymatic activities and highest oxidative damage being observed at 14 days. The PCA also agrees with IBR. Moreover, results for digestive glands are also agree with the PCA and IBR, being that 7 days is the most crucial time of exposure to nPS, further confirmed by oxidative damage observed at this time point.

In unexposed mussels, cells showed an average of DNA damage (5.26% fragmented DNA) which is well within normal levels for *Mytilus* (Mitchelmore et al., 1998). Findings showed that polystyrene NPs are genotoxic on mussel's immune system since DNA damage increased after 3 days of exposure maintaining similar levels until 14 days (Fig. 2). Therefore, in the haemolymph of mussels, the DNA damage observed is independent of the time of exposure. However, this needs to be confirmed with a longer time of exposure. This genotoxic damage may be caused by physical interactions between cell nuclei and NPs, as well as the production of a high concentration of reactive oxygen species (ROS), but the underlying mechanism has yet to be established. The DNA integrity decreased in *M. galloprovincialis*, observed by an increase in DNA damage after a 96-h exposure to nPS (106 ± 10 nm; 0.05–50 mg/L) (Brandts et al., 2018). Similarly, an *in vivo* acute toxicity exposure (96-h; 50 nm; 10 μg/L) of nPS with an amide group attached show a negative impact on the *M. galloprovincialis* immune defence capacity (Auguste et al., 2020). After exposure to microplastic, microfibre and NP, Cole et al. (2020) observed that in *M. edulis,* exposed to a similar NPs size, a higher concentration, and a shorter period (50 nm; 24-h and 7-d; 500 μg/L) had no significant DNA damage. In Cole et al. (2020) nPS exposed mussels presented 3% of DNA damage whereas, in this study, 21.4% of DNA damage was observed. Comparing concentrations used in Cole et al. (2020) and used in this study, there is more available NP particles at 500 μg/L than at 10 μg/L, which in turn form larger aggregates, thus decreasing the probability of occurring physical interactions between cell nuclei and NP particles as they are unlikely to cross cellular boundaries due to size. Moreover, although no significant alterations in DNA damage were encountered, a significant increase, between times of exposure was found, concerning the number of micronuclei per one thousand cells (Cole et al., 2020). Therefore, an increase in micronuclei formation, as well as a decrease in haemocyte cells circulating the haemolymph, are possible genotoxic consequences of NP exposure. Results indicate that the DNA integrity of mussels is compromised after acute and chronic exposure to nPS, and this may be a consequence of the incapacity of the mussel's antioxidant defence mechanism to reduce any ROS generated by exposure to nPS, leaving the remaining ROS to attack the DNA bases by oxidizing the molecules, and consequently causing oxidative damage. The % of tail DNA observed (Fig. 2) may be explained by the continuous attacks of free radicals formed, making DNA non-viable, and therefore interfering with the flow of information, thus compromising the mussel's immune system. Furthermore, an immediate translocation of nPS in haemocytes was confirmed, as well as the main uptake route in *M. galloprovincialis* (nPS; 50 nm; 10 mg/L; 3h) (Sendra et al., 2020b). This internalization of nPS in haemocyte cells also prompted changes in mussels' immune response (Sendra et al., 2020b). Although parameters analysed by Auguste et al. (2020) differ from those analysed here, NPs led to an increase in extracellular ROS production and consequently may have contributed to the oxidative stress and DNA damage observed here in the present results. Enhanced oxyradical generation through the interaction of NP particles with cellular boundaries may be a primary effect of genotoxicity, however, further investigation is necessary to acquire a deeper understanding. Related to the characterization of NP particles in the marine environment, results suggest that the smaller the concentration, the lower the formation of aggregates, the higher the probability of crossing cellular boundaries, and in turn increasing genotoxicity and oxidative damage these particles.

It is understood that NPs enter the internal structure of mussels by ingestion through feeding processes and are predominantly sorted in the gills, whilst larger NPs and aggregates are redirected to the digestive gland where they accumulate and/or partly translocate to the haemolymph, is then distributed to other tissues (Faggio et al., 2018; Sendra et al., 2020b). Moreover, the uptake of NPs can disrupt the internal organization of cells, interfering with the enzymatic activity involved in energy metabolism (Auclair et al., 2020). To conclude, it can be hypothesized that after nPS reach the digestive gland cells and cause LPO after a week of exposure, NPs are redistributed via haemolymph to other tissues, such as gills, overwhelming any possible defence managed by antioxidant enzymes, leading to oxidative damage observed after two weeks of exposure, as well as explain the genotoxicity observed in the haemolymph of mussels.

5. Conclusion

The present study provides evidence that NPs toxicity is tissue-specific as well as time-dependent. Abiotic and biotic characteristics of seawater lead to an increase in hydrodynamic diameter and aggregation of polystyrene nanoplastics. In *M. galloprovincialis*, 10 μg/L of 50 nm polystyrene NPs caused genotoxicity, overwhelmed antioxidant defences, and led to oxidative damage in both tissues. The inhibition and induction of antioxidant defences may be a result of reactive oxygen species generated by NP toxicity and may be attributed to the ingestion, translocation, and accumulation of nanoplastics. In the gills of mussels, an adaptive response seems to lead to an activation of repair mechanisms, such as autophagy though further studies are necessary to fully comprehend this activation. In the future, it is important to comprehend further the behaviour and toxicity of NPs towards marine biota, and their mode of action, as particles interactions differ substantially when compared to ultrapure water. Also, further analysis on longer exposure periods is essential to comprehend how mussels respond to nanoplastic exposure post 21 – days.

Author statement

All authors should have made substantial contributions to all of the following: Joanna M. Gonçalves, Vânia Serrão Sousa, Margarida Ribau-Teixeira, Maria João Bebianno: the conception and design of the study, or acquisition of data, or analysis and interpretation of data, Joanna M.

Gonçalves, Maria João Bebianno: drafting the article or revising it critically for important intellectual content, Maria João Bebianno: final approval of the version to be submitted.

Declaration of competing interest

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

Acknowledgments

This work was conducted under the framework of the PhD grant (UI/BD/150758/2020) and the EMERGEMIX (PTDC/BIA-BMA/30922/2017) and RESPONSE (FCT JPIOCEANS/0005/2020) JPI Oceans projects, funded by Fundação para a Ciência e Tecnologia, I.P., by the European Regional Development Fund (ERDF) through the Portugal 2020 and Portuguese national funds via FCT. We further thank FCT for the funds attributed to CIMA, University of Algarve UID/00350/2020.

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