Microplastics, microfibres and nanoplastics cause variable sub-lethal responses in mussels (*Mytilus* spp.)

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ABSTRACT

We compare the toxicity of microplastics, microfibres and nanoplastics on mussels. Mussels (Mytilus spp.) were exposed to 500 ng mL-1 of 20 μm polystyrene microplastics, 10 × 30 μm polyamide microfibres or 50 nm polystyrene nanoplastics for 24 h or 7 days. Biomarkers of immune response, oxidative stress response, lysosomal destabilisation and genotoxic damage were measured in haemolymph, digestive gland and gills. Microplastics and microfibres were observed in the digestive glands, with significantly higher plastic concentrations after 7-days exposure (ANOVA, $P < 0.05$). Nanoplastics had a significant effect on hyalinocyte-granulocyte ratios (ANOVA, $P < 0.05$), indicative of a heightened immune response. SOD activity was significantly increased followed 24 h exposure to plastics (two-way ANOVA, $P < 0.05$), but returned to normal levels after 7-days exposure. No evidence of lysosomal destabilisation or genotoxic damage was observed from any form of plastic. The study highlights how particle size is a key factor in plastic particulate toxicity.

1. Introduction

Plastic debris is a prolific and persistent marine contaminant that has permeated throughout the global ocean. Microplastic (0.1 μm-1 mm) and nanoplastic (< 0.1 μm) debris (Hartmann et al., 2019) may pose a substantial risk to marine ecosystems (Galloway et al., 2017). Micro- and nanoscopic plastics can be directly manufactured (e.g. medical applications, personal care products), or derive from the physico-chemical degradation of plastic debris. Plastic can enter into marine ecosystems through a myriad of pathways including direct disposal, airborne dispersal, terrestrial run-off and riverine flow (Cole et al., 2011). Microplastics and microfibres have been identified in seawater, sediments, polar ice and biota across the globe (Bergmann et al., 2016; Lusher, 2015), while recent methodological advances have allowed for the identification of waterborne nanoplastics in samples taken from the North Pacific gyre (Ter Halle et al., 2017).

Owing to their small size, micro- and nanoplastics are bioavailable to a wide array of aquatic organisms, with evidence of plastic particulates being directly or indirectly (i.e. via trophic transfer) consumed, or inhaled via the gills (Setälä et al., 2018). Microplastic debris has been

identified in a wide range of marine organisms, with highest concentrations found in animals at the base of the marine food web (e.g. bivalve shellfish) (Walkinshaw et al., 2020). Microplastics have been shown to incite adverse health effects including reduced feeding, fecundity and premature moulting in copepods (Cole et al., 2019; Cole et al., 2015; Coppock et al., 2019), diminished reproductive outputs and offspring performance in Pacific oysters (Sussarellu et al., 2016), and reduced feeding, weight loss and reduced activity in benthic polychaetes (Besseling et al., 2013; Wright et al., 2013; Wright et al., 2015). Toxicological effects stemming from nanoplastic exposure include reduced feeding, body size and reproductive endpoints in *Daphnia* (Besseling et al., 2014; Rist et al., 2017), reduced fertilisation success and increases in larval abnormalities in Pacific oyster (Tallec et al., 2018), and heightened immune response, oxidative damage and inflammation in fish (Brandts et al., 2018b; Greven et al., 2016; Lu et al., 2016).

The blue mussel (*Mytilus edulis*) and Mediterranean mussel (*Mytilus galloprovincialis*) have been widely used in assessing the risks posed by environmental stressors (Farrington et al., 2016; Viarengo and Canesi, 1991). Mussels are omnivorous suspension feeders with proven

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capacity for consuming microplastics (Ward et al., 2019a). Numerous field-studies have demonstrated that mussels can readily consume microplastic debris under environmental conditions (De Witte et al., 2014; Li et al., 2018; Li et al., 2015; Rochman et al., 2015; Santana et al., 2016; Van Cauwenberghe and Janssen, 2014; Zhao et al., 2018). In mussels, waterborne particles are captured via the gills, with capture efficiency and ingestion dependent upon particle size, shape, hydrophobicity, and aggregation within biogenic matrices (e.g. marine snow) (Kach and Ward, 2008; Porter et al., 2018; Rosa et al., 2018; Ward and Targett, 1989; Ward and Shumway, 2004); particles are subsequently sorted via the labial palps (Beninger et al., 1995), and either ingested or ejected within pseudofaeces (Ward et al., 2019b; Zhao et al., 2018). Microplastics have been shown to aggregate in the gills and digestive glands (Avio et al., 2015; Browne et al., 2008; Paul-Pont et al., 2016; Pittura et al., 2018; Revel et al., 2019; von Moos et al., 2012) and adhere on organs not associated with feeding, including the adductor muscles, foot and mantle (Kolandhasamy et al., 2018); there is also some evidence that < 20 μm microplastics can translocate into the haemolymph (Browne et al., 2008; von Moos et al., 2012). To date, toxicity studies considering the impacts of plastics on mussels (and may other organisms) have primarily considered the impact of high-doses of spherical microbeads or irregularly-shaped plastic fragments (Lenz et al., 2016); to the authors' knowledge only one other study has considered the impact of plastic fibres on mussels, in which microfibres were shown to significantly reduce filtration rates in *M. edulis* (Woods et al., 2018).

This study sets-out to better understand the risks plastic particulates, varying in shape and size, pose to marine mussels, and highlight differences in the mechanisms of toxic action, caused by microplastics, microfibres and nanoplastics. Microfibres are among the most commonly observed forms of plastic debris in marine samples (Lindeque et al., 2020; Mishra et al., 2019), but are largely underrepresented in ecotoxicological studies on microplastics (Trestrail et al., 2020), and there are relatively few in vivo studies with nanoplastics. Here, acute (24 h and 7-day) exposure studies using the model organism *M. edulis* were undertaken to compare the effects polystyrene microplastics (spherical beads), polyamide microfibers and polystyrene nanoplastics (spherical beads) can have on mussel health. Biomarkers (indicators of an adverse health response) for immune response, oxidative balance, lysosomal destabilisation and genotoxic damage were selected as relevant endpoints, based on the findings of prior toxicological studies (Détrée and Gallardo-Escárate, 2018; Paul-Pont et al., 2016; Revel et al., 2019; von Moos et al., 2012). It was hypothesised that sub-lethal toxicity would vary with the size and shape of the plastic: (1) given their high-aspect ratio (i.e. particle length is far greater than particle width), it is proposed that microfibers will more readily aggregate and become lodged within the digestive tract than spherical microbeads, increasing their likelihood of causing sub-lethal harm (H_1); and (2) owing to their diminutive size, it was anticipated that nanoplastics will more readily penetrate into tissues and the haemolymph than microplastics and will therefore be more likely to trigger an immune response (H_2). The results of the study provide insight into the mechanisms by which plastic particulates can cause harm to mussels, contributing to our understanding of the risks plastic pollution poses to marine life.

2. Methods

2.1. Animal collection and husbandry

Mussels (*Mytilus spp.*) were sampled from the established mussel population at Starcross (Stephenson and Thomas, 2019) in the Exe estuary (UK; $50^0 37' 26.22''$ N $32^0 6' 45.24''$ W) at low-tide in late July 2016 and early August 2017. Mussels with shell length of ~6 cm were hand-picked, placed in coolboxes containing natural seawater and returned to the laboratory within 1 h of sampling. Fouling organisms (e.g. barnacles, algae) were removed from mussel shells using a scalpel. Mussels

were depurated for 24 h in 0.2 μm filtered, 33 ± 0.3 ppt artificial seawater (ASW), prepared using purified water and marine salts (Tropic Marin). Prior to experimental work, mussels were acclimated for between 7 and 14 days in recirculation tanks containing ASW. Mussels were fed daily by pausing the recirculation system for 2 h, and providing 200 μL L^{-1} ASW (approximately 4×10^8 microalgae L^{-1} ASW) of Shellfish Diet 1800® (Reed Mariculture), a commercial formulation of microalgae, comprising *Isochrysis* sp., *Pavlova* sp., *Tetraselmis* sp., *Chaetoceros calcitrans*, *Thalassiosira weissflogii* and *Thalassiosira pseudonana*.

2.2. Microplastics and nanoplastic preparation

Test plastics were selected for their bioavailability to mussels. Polystyrene and polyamide particles have densities similar to that of seawater ($p = 1.05$ g cm^{-3}), and therefore could be expected to remain suspended within experimental beakers; ideally all plastics would have been manufactured from the same polymer, however polystyrene fibres could not be sourced. Particle size ranges overlap with that of the natural prey of mussels, with 50 nm nanoplastics used to represent particulates with capacity for permeating into tissues.

2.2.1. Polystyrene microplastics (PS-MP)

Polystyrene monodisperse beads (20 μm ø; Sigma 87,896) were selected as a representative plastic particulates, similar to those used in other toxicology studies with mussels. To remove any preservatives (e.g. azides) present in the stock solution, we followed the protocol of Cole and Galloway (2015). In brief: stocks were transferred to a Falcon tube, centrifuged and the supernatant removed; the plastic pellet was then re-suspended in ultrapure water, with the process repeated a total of three times.

2.2.2. Polyamide microfibres (PA-MF)

Nylon microfibres (10 ø × 30 μm) were prepared by sectioning polyamide nylon 6,6 polyfilament fibre (Goodfellow AM325705) using a cryogenic microtome and fibre size verified using microscopy per the protocol of Cole (2016). Equivalent spherical diameter of microfibres were calculated as 19.2 μm, per the formulae prescribed by Malvern (2014).

Microplastics and microfibres were suspended in ultrapure water and kept in the dark at 2-4 °C to prevent microbial growth. Microplastic stocks were vortexed for ~10 s to resuspend plastics prior to use, and concentrations were quantified using a Sedgewick-rafter chamber. Microplastic mass (M) was calculated using the formula: M = p x V, with their volume (V) based on the volume of a sphere for microbeads and a cylinder for microfibers, and their density (p) based on their polymer, i.e. 1.05 g cm^{-3} for polystyrene and 1.14 g cm^{-3} for nylon 6,6, with 500 ng particles mL^{-1} equivalent to ~114 PS-MP mL^{-1} and ~186 PA-MF mL^{-1}.

2.2.3. Polystyrene nanoparticles (PS-NP)

Fluorescent polystyrene sulphated-nanobeads (50 nm ø; Ex. 505–545, Em. 560–630; Magsphere, Pasadena, CA, USA) were supplied as suspensions (2.5% w/v; azide free), which was stored at 4 °C as per the manufacturer guidelines. Sulphated PS-NPs were chosen as the surface sulphate groups have been demonstrated to enhance their dispersion in seawater (Wegner et al., 2012) in the same way that eco-corona formation on nanoplastics can facilitate their dispersal under environmental conditions (Saavedra et al., 2019); the stability of the PS-NP was tested using dynamic light scattering (DLS), which confirmed good dispersion within seawater (Fig. S1-S2). Particles were characterised with transmission electron microscopy (TEM) and DLS; test particles demonstrated a zeta potential of −30.9 ± 1.9 mV and a measured hydrodynamic diameter (HDD) of 52.65 ± 2.88 nm (DLS), and mean particle size was 34 ± 8.6 nm (TEM); further characteristics are provided in Table S1. For dosing the test systems, a stock of

1000 mg L^{-1} was used, with 500 ng mL^{-1} equivalent to 7.3×10^9 PS-NP mL^{-1}.

2.3. Experimental set-up (Exposure 1 and Exposure 2)

The study comprised two complimentary experimental set-ups: *Exposure 1* was conducted in August 2016 using cohorts of 6 mussels (allowing for pooling of tissues and haemolymph prior to conducting bio-assays) exposed for either 24 h ($n = 4$ per treatment) or 7 days (n = 4 per treatment) in 5 L ASW; *Exposure 2* was conducted in August 2017, using individual mussels (allowing for greater replication) exposed for 7 days only ($n = 8$ per treatment) in 1 L ASW. Mussels (average ± se: 5.93 ± 0.02 cm; range: 5.06–6.92 cm) were placed carefully at the base of acid-washed glass beakers and then ASW carefully poured in. Filtered air was pumped in via a glass pipette to provide aeration and keep the water sufficiently agitated to maintain particulate dispersion; beakers were covered with cling-film to prevent airborne contamination and evaporative loss. Plastics were added to each beaker via micropipette, with treatments comprising controls (no plastic), or 500 ng mL^{-1} of either PS-MP, PA-MF or PS-NP, chosen with reference to Lenz et al. (2016) and other ecotoxicological studies (see references in results and discussion). Beakers were randomly allocated (Microsoft Excel) a position on a tray within a controlled-temperature room (15 °C; 12 h:12 h light:dark) and maintained for either 24 h or 7 days. For the 7-day exposure, mussels were fed daily with 200 µL L^{-1} ASW of Shellfish Diet 1800® (Reed Mariculture); 1 h after feeding, a complete water change was undertaken, and plastics added. Mussels were observed prior to feeding, after feeding and after dosing with plastics, recording whether they were feeding or not (i.e. valves open, mantle visible, particles visibly drawn into the mussel).

2.4. Haemolymph and tissue sampling

At the end of each exposure, mussels were removed from experimental vessels, opened-up using sterile, stainless-steel scissors and excess seawater removed (i.e. gently blotting with blue roll). Haemolymph was extracted from the posterior adductor muscle using a hypodermic syringe pre-loaded with 100 µL of cold phosphate buffered saline (PBS); in Exposure 1 haemolymph was pooled and in Exposure 2 haemolymph was collected from a single mussel. The digestive gland and gills were excised using sterile stainless-steel forceps and/or scissors. Haemolymph and tissues were blotted on blue roll, and then placed in Eppendorfs with 50% PBS and either analysed immediately, or snap-frozen in liquid nitrogen and stored at −80 °C prior to analysis.

2.5. Uptake of microplastics and nanoplastic (Exposure 1)

To confirm dietary uptake and assess the accumulation of microplastics in the digestive glands (Exposure 1: 24 h, $n = 4$; 7 days, n = 4), the glands were excised, patted dry, weighed (mg, wet-weight), and then digested for ~16 h in a drying oven (50 °C) using 2 mL of 10% KOH per mg of tissue. Microplastics were quantified using a light microscope (×20 objective), and the concentration of microplastics per mg of digestive gland (wet-weight) calculated.

To demonstrate the uptake of PS-NP into the mussel tissues (Exposure 1: 24 h, n = 4; 7 days, n = 4), the tissues was homogenised with a probe sonicator in 2 mL of ultrapure water. The fluorescence dye was then extracted from the tissue by adding 2 mL of 2-ethoyethyl acetate solvent and agitated on a shaker-plate for 24 h. The phases were then separated via centrifugation (2000 *g*) for 10 min and the supernatant containing the dye was measured using a spectro-fluorometer (Biotek Synergy HT) (Hussain, 2001; Ravnic et al., 2006).

2.6. Immune response: granulocyte-hyalinocyte ratio (Exposure 1)

The proportion of granulocytes (phagocytic haemocytes) and

hyalinocytes (agranular haemocytes that do not display a phagocytic response) present in mussel haemolymph was used as a biomarker of immunological response. Per the protocol of Gorbi et al. (2013) and Avio et al. (2015), haemolymph was spread onto a glass slide, air-dried and fixed with Beker's fixative with 2.5% NaCl, and then washed and stained with 3% May-Grünwald Giemsa. Slides were visualised under a light microscope (×10 objective), and 200 cells classified.

2.7. Oxidative balance: SOD and TBARS (Exposure 1)

The SOD assay provides a measure of antioxidant enzyme levels in tissues, with 1 unit of SOD per mg (U mg^{-1}) corresponding to a 50% reduction of nitroblue tetrazolium (NBT). SOD activation in gills and digestive gland tissues were determined by measuring their ability to inhibit the generation of NBT, as described in Noventa et al. (2018). Briefly, digestive gland and gill tissues were rinsed with phosphate buffered saline, then homogenised on ice and superoxide radicals generated by adding xanthine-xanthine oxidase. Absorbance values were determined at 573 nm for 10 min via spectrophotometer. Absorbance values were compared to a standard curve, plotting inhibition of NBT generation against SOD concentrations (0–20 U/mL; $R^2 = 0.99$), and accounting for the mean protein content of the tissues (Bradford, 1976).

TBARS concentrations in gill and digestive gland tissues were measured using the method of Buege and Aust (1978), adapted to a micro plate reader (Biotek Synergy HT; absorbance: 530 nm). In brief: 150 µL of samples was heated at 60 °C for 60 min in a solution containing 50% trichloroacetic acid, 1% butylated hydroxytoluene, 1 mM phosphate-buffered saline and ethylenediaminetetraacetic acid, and 1.3% thiobarbituric acid (TBA) in 0.3% NaOH. The tubes were then cooled, and after mixing, the TBARS level was quantified at 530 nm by using a micro plate-reading UV-spectrophotometer (Biotek Synergy HT). Results were expressed in nM of TBARS per mg of protein. TBARS production (nM mg^{-1}) was ascertained by comparing the lipid peroxidation activity of tetramethoxypropane standards ($R^2 = 1.00$), accounting for the mean protein content of the tissues (Bradford, 1976).

2.8. Lysosomal destabilisation: neutral red retention (Exposure 2)

The neutral red retention assay provides a quantitative estimation of oxidative damage in cells (Repetto et al., 2008). Healthy cells can rapidly incorporate and store neutral red dye in their lysosomes, while damaged cells are unable to retain the dye; as such, the neutral red retention can be used as a measure of the health of cell populations. Per the protocol of Hu et al. (2015), 100 µL aliquots of fresh haemolymph-PBS solution (50% dilution) were pipetted in triplicate into a 96-well plate (Greiner), with 100 µL of PBS used for blanks. The microplate was incubated on a shaker plate at room temperature for 20 min to facilitate cell adhesion, and then the plate inverted and excess haemolymph/PBS carefully removed. Next, 50 µL of 200 µM neutral red-PBS solution was added to each well, plates incubated for 15 min in the dark and then excess neutral red dye carefully removed. Adhered cells were washed and fixed with 150 µL of 1% glutaraldehyde for 2 min, and then fixative removed. Finally, 200 µL of extraction buffer (1% acetic acid and 50% ethanol) was added to each well, and plates left in the dark for 20 min at room temperature. To allow for calibration of absorbance data, neutral red dye standards (0–30 ng mL^{-1}) were prepared and 200 µL pipetted into each plate prior to analysis. Absorbance of neutral red dye was ascertained using a microplate reader (TECAN, Infinite® 200 PRO) at a wavelength of 540 nm.

2.9. Genotoxicity: micronucleus assay (Exposure 1) and comet assay (Exposure 2)

The micronucleus test was used to quantify micronuclei formation in mussel haemocytes as a biomarker for genotoxicity. Adapting the

protocol described by Avio et al. (2015), haemocytes were fixed in Carnoy's solution (3:1 methanol:acetic acid) and then spread onto glass slides, air dried and stained with SYBRsafe (0.5×, 45 mM Tris-borate, 1 mM EDTA, pH 8.3). Slides were visualised with an optical fluorescence microscope (Axiovert S100; x40 objective, oil immersion), and the number of micronuclei (smaller than one-third of nucleus diameter and on same optical plane) present across 1000 cells recorded.

DNA damage was evaluated using the alkaline version of the single cell gel electrophoresis assay (i.e. comet assay), following the protocol of Lewis and Galloway (2009). The Comet assay can be used to assess induced genetic damage, both as a direct and indirect impact of a contaminant, through identifying single-strand DNA breakages in individual cells (Jha, 2008). In brief: the haemolymph of each mussel was centrifuged at 1000 rpm for 3 min at 4 °C and the supernatant discarded. The pellets with the isolated cells were resuspended in low melting point agarose containing 1% Kenny's salt solution and spread onto microscope slides pre-coated with high melting point agarose with 1% Tris–acetate EDTA. The embedded cells were lysed by immersion in a cold lysis buffer (2.5 M NaCl, 10 mM Tris, 0.1 mM EDTA, 10% DMSO, 1% Triton X-100, pH 10, in dark conditions at 4 °C) for 1 h. The slides were placed in a horizontal electrophoresis unit and incubated in an alkaline electrophoresis solution (300 mM NaOH, 1 mM EDTA, deionized water, pH > 13, in dark conditions at 4 °C) for 40 min. Electrophoresis was conducted for 30 min at 25 V, 300 mA. One slide with two gels was prepared per mussel, coloured with SYBRsafe Green (0.5×, 45 Mm Tris-borate, 1 Mm EDTA, pH 8.3) and observed with an optical fluorescence microscope (Axiovert S100, x20 objective with 420-490 nm excitation and 520 nm emission filters) coupled with a digital camera (Sony). An image analysis programme (Komet 5.5, Kinetic Imaging Ltd) was used to analyse the comet images. For each gel, 50 nuclei were analysed blindly of treatment, making a total of 100 cells examined in each slide. % Tail DNA was used to grade DNA damage.

2.10. Statistical analysis

Data was tabulated, and figures prepared using Microsoft Excel. Statistical analyses were undertaken using R (R, 2019). Data was tested for normality using a Shapiro-Wilk test, transformed where applicable, and homogeneity of variance was visually inspected to satisfy *apriori* parametric requisites. An ANOVA with post hoc Tukey tests were used to compare parametric data, and a Kruskal-Wallis with post hoc Wilcox test used to compare non-parametric data; post-hoc analysis was used to compare data with the controls. A binomial General Linear Model (GLM) was used to compare DNA damage (Comet tail %). Data is presented as mean ± standard error, with statistical significance assigned to $P < 0.05$. For comparisons of SOD and TBARS data by exposure length (24 h and 7-day) the data was normalised to the control for the respective experiment to prevent a shift in the baseline of the experiment creating false positives, using the formula: (sample concentration / average (sample concentration)) * 100, where 'sample concentration' refers to the measured value of substance within the sample, and 'average (sample concentration)' refers to the mean value of the replicates within the sample; two-way ANOVAS were then conducted on the percentage data with Tukey post-hoc analysis.

3. Results and discussion

3.1. Microplastic and nanoplastic uptake

Mussels were observed to be actively feeding following addition of microalgae and exposure to PS-MP, PA-MF and PS-NP. Food ingested by mussels is physically homogenised by the rotating crystalline style, digested extracellularly in the stomach, and then shunted into the digestive gland to undergo intracellular digestion (Gosling, 2003; Koukouzika et al., 2009); the glands comprise terminal epithelial
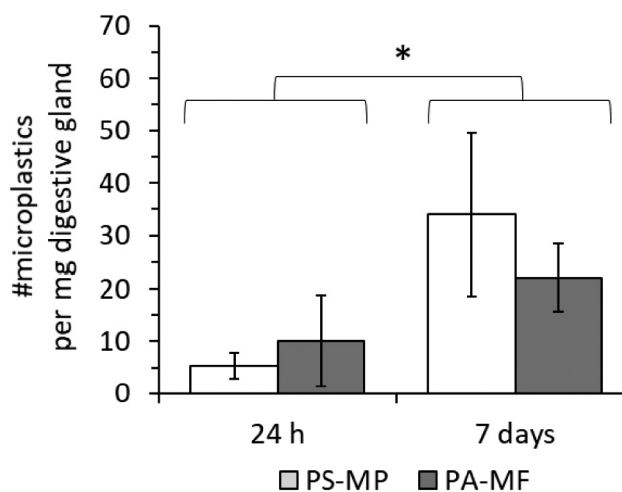

Fig. 1. The number of polystyrene microbeads (PS-MP; $n = 3$) and nylon microfibres (PA-MF; $n = 4$) identified per mg of digestive gland tissue of individual mussels, following 24 h or 7-day exposure to 500 ng mL^{-1} microplastics. * denotes statistical difference between microplastic concentrations in digestive glands of mussels following 24 h and 7-day exposures (two-way ANOVA, Time: $P < 0.05$).

tubules, with undigested food returned to the stomach prior to egestion, and as such may be considered a "dead-end" in which particulates might be retained (Faggio et al., 2018). Both PS-MP and PA-MF were identified in the digestive glands of mussels, with a significantly greater amount of microplastics identified in digestive glands following the 7-day exposure (two-way ANOVA, Time: P < 0.05; Fig. 1). Our results demonstrate microfibres aggregate in the digestive gland of mussels, corroborating the findings of experimental studies using PS-MP (2–16 μm), and irregularly-shaped, high-density polyethylene (HDPE, < 80 μm), low-density polyethylene (LDPE, 20–25 μm), polyethylene and polystyrene (< 100 μm), and a polyethylene-polypropylene mix (< 720 μm) (Avio et al., 2015; Browne et al., 2008; Paul-Pont et al., 2016; Pittura et al., 2018; Revel et al., 2019; von Moos et al., 2012).

We observed no significant difference in the concentrations of PS-MP and PA-MF in the digestive glands after either 24 h or 7-days exposure (two-way ANOVA, Treatment: $P = 0.57$): PS-MP concentrations averaged 5.3 ± 2.4 microplastics mg^{-1} after 24 h exposure and 34.0 ± 15.5 microplastics mg^{-1} following the 7-day exposure (Tukey, Time: $P = 0.30$), while PA-MF concentrations averaged 10.0 ± 8.7 microplastics mg^{-1} after 24 h exposure and 22.0 ± 6.4 microplastics mg^{-1} following the 7-day exposure (Tukey, Time: $P = 0.28$). Based on this data, we conclude microfibres do not aggregate to any greater extent than spherical microbeads. As we explore below, there was also no difference in the levels of sub-lethal toxicity caused by 500 ng particles mL^{-1} of microfibres and microplastic. In combination, these results lead us to reject hypothesis H$_1$. Conversely, Gray and Weinstein (2017) identified that polypropylene microfibres (50 × 10^6 particles mL^{-1}) resulted in significantly greater mortality in adult daggerblade grass shrimp when compared with exposure to microplastic beads and granules; further, Au et al. (2015) demonstrated that polypropylene microfibres cause mortality in the amphipod *Hyalella azteca* at far lower concentrations than polyethylene microplastic particles. Further experimental work using microfibres of varying length and aspect ratio are recommended to explore differences in toxicity.

Efforts to quantify PS-NP within mussel tissues was unsuccessful, as we were unable to distinguish the red fluorescence signal of the particles from autofluorescence. However, we remain confident that uptake occurred given our observations of active feeding, and evidence from other research papers that PS-NP (either free-floating or aggregated with microalgae) can be filtered-out by mussels; in these studies, PS-NP
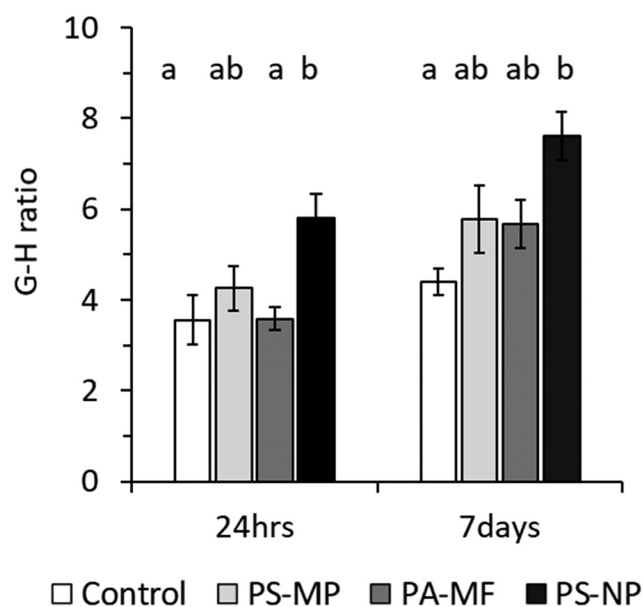
Fig. 2. The Granulocyte-hyalinocyte ratio in mussel haemolymph; a biomarker of immune response (two-way ANOVA, treatment: $P < 0.05$; $n = 4$) following a 24 h and 7-day exposure to: controls (no plastic), 500 ng mL^{-1} polystyrene microbeads (PS-MP), 500 ng mL^{-1} polyamide microfibers (PA-MF) and 500 ng mL^{-1} polystyrene nanoparticles (PS-NP). Treatments that don't share letters are significantly different (Tukey post-hoc analysis).

uptake was ascertained by measuring reduction of nanoparticle concentrations in the surrounding water and/or visualising uptake of PS-NP within tissues via fluorescence imaging (Brandts et al., 2018a; Sendra et al., 2019; Wegner et al., 2012), although such imaging studies have been criticised as leaching of fluorophores may give false positives (Catarino et al., 2019).

3.2. Immune response: granulocyte-hyalinocyte ratio

Haemocytes comprise spherical, eosinophilic 'granulocytes', containing hydrolytic enzymes, that play an active role in phagocytosis, and basophilic 'hyalinocytes' (Lowe and Moore, 1979); as such, an increase in the proportion of granulocytes present in the haemolymph is indicative of a heightened immune response. Our data shows the haemocyte ratio was significantly different between treatments for the 24 h and the 7-day exposures (two-way ANOVA, Treatment: P < 0.05; Fig. 2), with a significant increase in the granulocyte-hyalinocyte ratio of mussels exposed to PS-NP (Tukey: P < 0.05 (24 h); P < 0.01 (7-day)). There was no difference in haemolymph profiles after 24 h and 7-day exposures (two way-ANOVA, Time: P = 0.18). We recommend that chronic exposures could be designed to elucidate whether this immune response is sustained longer-term, following both continued plastic exposure and after mussels are depurated and kept in clean water.

The significant increase in the proportion of phagocytic haemocytes observed in the PS-NP treatments may have resulted from the 50 nm diameter nanoparticles translocating from the intestinal tract into the mussel's haemolymph triggering the innate immune response and a proliferation of the granulocytes, or localised inflammation resulting in granulocytic proliferation. In our previous studies with oysters, nanoparticles were taken up across the bivalve gut epithelium through an endocytic pathway and could be identified within the lysosomes and phagocytic cells (Noventa et al., 2018). Nanoparticles engulfed by phagocytes have been shown to trigger autophagy, fuse with lysosomes (potentially affecting lysosomal stability as the polymers are resistant to the hydrolytic processes) and cause toxicity through reactive oxygen species (ROS) formation, mitochondrial damage and localised inflammation; ultimately nanoparticles tend to accumulate in excretory

organs (Gustafson et al., 2015).

In contrast to PS-NPs, exposure to PS-MP and PA-MF resulted in a non-significant increase in granulocyte-hyalinocyte ratio after 7-days exposure (Fig. 2), leading us to accept hypothesis H$_2$. In other studies, over longer exposure periods, it has been observed that polyethylene (< 100 µm; 10-day exposure) and LDPE microplastics (20–25 µm; 28-day exposure) can significantly reduce granulocyte-hyalinocyte ratio in *Mytilus* sp. (Avio et al., 2015; Pittura et al., 2018). While not statistically significant, the marked increase in the proportion of granulocytes in response to microplastics observed here is curious. While it is thought that microplastics < 20 µm can be translocated into the haemolymph of *Mytilus* sp. (Avio et al., 2015; Browne et al., 2008), it is not anticipated that the 20 µm PS-MP or 10 × 30 µm PA-MF used in these experiments would be able to enter the circulatory fluid. However, nanoparticles stemming from these microplastics certainly could translocate and incite an immune response. In Antarctic krill (*Euphausia superba*), amphipods (*Gammarus duebeni*) and shore crabs (*Carcinus maenas*), the gastric mill has been shown to physically damage microplastics resulting in the formation of nanoplastics (Dawson et al., 2018; Mateos-Cárdenas et al., 2020; Watts et al., 2015). We consider it plausible that mechanical grinding of PS-MP and PA-MF in the gastro-intestinal tract of mussels could also result in the formation of nanoplastics; we recommend this as an area for future consideration. Alternatively, inflammation resulting from abrasion or microplastic incursion into tissues could result in recruitment of granulocytes at a localised level.

3.3. Oxidative balance: SOD and TBARS

Superoxide dismutase (SOD) is an antioxidant enzyme that protects cells from toxic reactions with reactive oxygen species (ROS) and free radicals (e.g. O_2^-, OH*, HO_2*); prolonged increases in antioxidant activity can require energy that takes away from growth and reproduction (Trestrail et al., 2020). Here, SOD activity was significantly influenced by treatment in the gills (two-way ANOVA, treatment: $P < 0.05$; Fig. 3A) being significantly increased in response to PS-NP in the 24 h exposure (Tukey, P < 0.05). SOD activity was also significantly influenced by treatment in the digestive gland (two -way ANOVA, treatment: P < 0.05; Fig. 3B), with significantly higher SOD concentrations in PS-MP and PA-MF treatments in the 24 h exposure (Tukey, P < 0.05). After 7-days exposure, SOD concentrations were markedly reduced and not significantly different from the controls in either the gills (Tukey: P = 0.2–1.0; Fig. 3A) or digestive glands (Tukey: P = 0.4–0.9; Fig. 3B).

Other studies have also shown SOD activity to increase in the digestive glands and gills of *Mytilus* spp. exposed to 10–100 µg L^{-1} PE-PP for 10 days (Revel et al., 2019), as well as the clam *Scrobicularia plana* following exposure to 1 mg L^{-1} PS-MP for 14 days (Ribeiro et al., 2017). Transcriptomic analyses of *M. galloprovincialis* exposed to HDPE for 18 days also showed an increased expression of genes related to immune and stress response in the digestive gland and mantle (Détrée and Gallardo-Escárate, 2018). However, Paul-Pont et al. (2016) observed no difference in SOD activity in *Mytilus* spp. following a 7-day exposure to PS-MP, and Magara et al. (2018) showed no effect on SOD activity in the gills or digestive glands of *M. edulis* exposed to 100–1000 PE-MP mL^{-1} for 4 days. Wang et al. (2020) identified a non-significant increase in SOD activity in the digestive glands of the mussel *Mytilus coruscus* following 24 h exposure to microplastics, and no changes in SOD activity with longer exposure periods.

Thiobarbituric acid reactive substances (TBARS) form as a by-product of lipid peroxidation, stemming from the oxidative degradation of lipids by reactive oxygen species. TBARS concentrations in gill tissues were significantly affected by treatment (two-way ANOVA, treatment: $P < 0.01$; Fig. 4A), with significantly lower TBARS in the PS-NP exposure after 24 h exposure (Tukey, P < 0.05). We observed no difference in TBARS concentrations in the gills after 7-days exposure, nor the digestive glands after 24 h or 7-days exposure (two-way
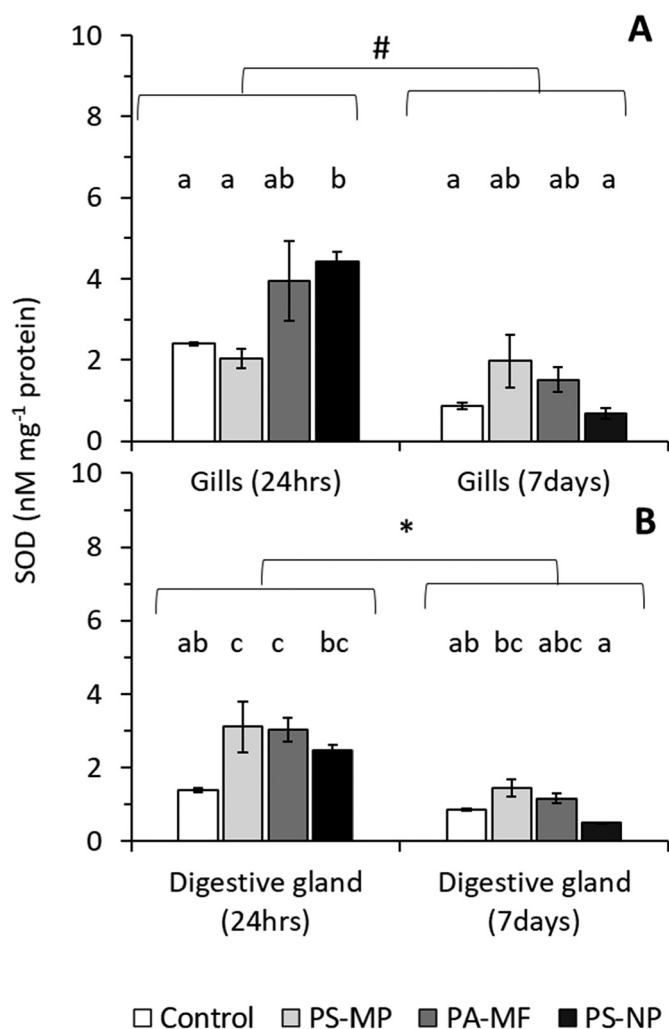
Fig. 3. Superoxidase (SOD; mg^{-1} protein) activation in mussel tissues following 24 h and 7-day exposure to: controls (no plastic), 500 ng mL^{-1} polystyrene microbeads (PS-MP), 500 ng mL^{-1} polyamide microfibers (PA-MF) and 500 ng mL^{-1} polystyrene nanoparticles (PS-NP). **(A)** SOD activation in gills (two way-ANOVA, treatment: $P < 0.05$, $n = 4$). **(B)** SOD activation in digestive glands (two way-ANOVA, treatment: P < 0.05, n = 4). Treatments that don't share letters are significantly different (Tukey post-hoc analysis); # denotes a statistical difference in the SOD response across treatments in gill tissue following 24 h and 7-day exposures (two-way ANOVA, treatment*time: P < 0.05); * denotes statistical difference between SOD concentrations in digestive glands of mussels following 24 h and 7-day exposures (two-way ANOVA, time: P < 0.05).

Fig. 4. The response of Thiobarbituric acid reactive substances (TBARS, nM mg^{-1} protein) in mussel tissues in mussels following 24 h and 7-day exposure to: controls (no plastic), 500 ng mL^{-1} polystyrene microbeads (PS-MP), 500 ng mL^{-1} polyamide microfibers (PA-MF) and 500 ng mL^{-1} polystyrene nanoparticles (PS-NP). **(A)** TBARS in gills (two-way ANOVA, Treatment: P < 0.05; n = 4) and **(B)** digestive glands (two-way ANOVA, Treatment: $P = 0.66$, n = 4). Treatments that don't share letters are significantly different (Tukey post-hoc analysis).

ANOVA, treatment: $P = 0.53$; Fig. 4B). Further, there was no significant difference found between the 24 h and 7-day exposure data (two way-ANOVA, time: $P = 0.09$ (gills) and $P = 0.34$ (digestive glands)). Given no significant increases in TBARS concentrations were observed, our data would suggest no substitutive lipid peroxidation has occurred within our test models.

Our results differ from studies using plastic at concentrations a magnitude greater than used here; for example, Brandts et al. (2018a) demonstrated that exposure to PS-NP (0.05 mg L^{-1}) induced lipid peroxidation in the digestive glands of *Mytilus galloprovincialis*, and Oliveira et al. (2018) observed significant increases in TBARS in the freshwater bivalve *Corbicula fluminea* following exposure to 0.13 mg L^{-1} of 1–5 μm microplastics. Notably Lei et al. (2018) observed substantial physical damage to intestinal epithelia, including removal of villi, in zebrafish (*Danio rerio*) exposed to microplastics (0.1–5 μm; 1 mg L^{-1}). We posit that higher plastic concentrations may
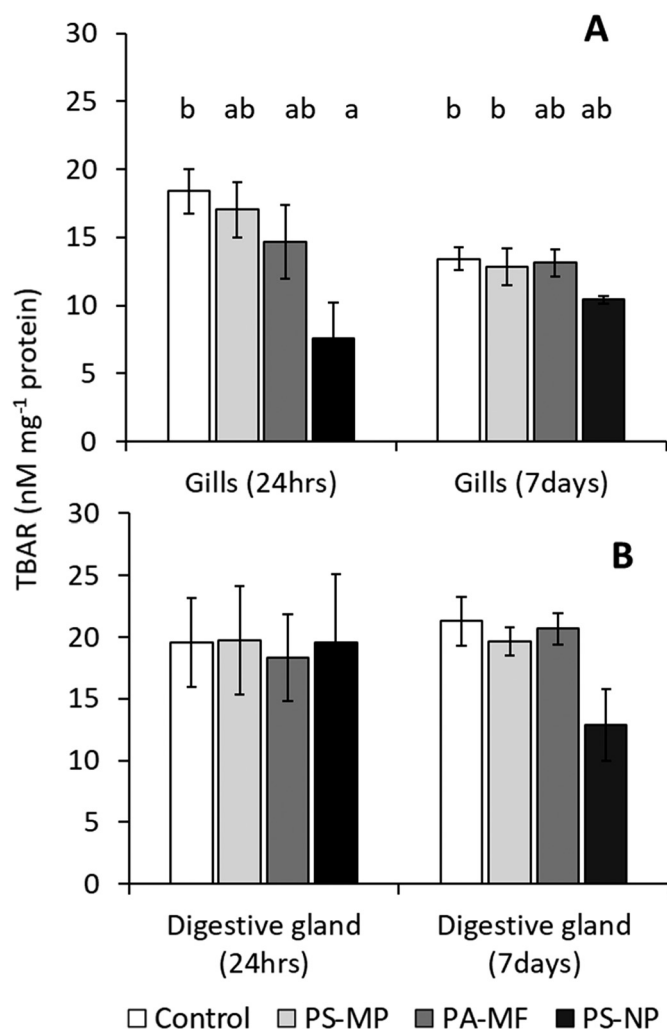
be sufficient to cause attrition within the gastro-intestinal tract resulting in the abrasion of tissues, leading to cellular damage and lipid peroxidation.

It is recognised that oxidative stress responses can be variable, differing with genotype, age, reproductive status, feeding and temperature, and that a broader suite of biomarkers and wider literature should be considered in inferring biological pathways (Magara et al., 2018; Trestrail et al., 2020). Histological studies have shown that microplastics can permeate into the epithelial cells of the digestive gland resulting in epithelial thickening, the formation of granulocytomas and reduced epithelial cilia densities (Bråte et al., 2018; Paul-Pont et al., 2016; von Moos et al., 2012). This localised inflammation can stem from release of ROS and impaired intracellular homeostasis, and has been widely evidenced in tissues exposed to nanoparticles in vivo and in vitro (Chaudhari et al., 2014; Khanna et al., 2015; Manke et al., 2013; Rosen et al., 1995). In assessing the response of the copepod *Paracyclopina nana* to 0.05 and 0.5 μm PS-MP, Jeong et al. (2017) drew similar conclusions, hypothesising prolonged cellular exposure to microplastics elevated intracellular ROS levels, triggering the activation of antioxidant genes. In this study, the increase in SOD activity after 24 h
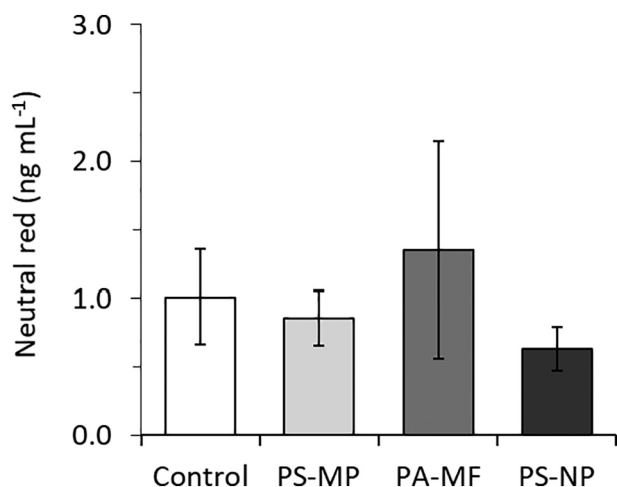
Fig. 5. The response of Neutral red retention (ng neutral red retained per mL of haemolymph); a biomarker of lysosomal stability in mussel haemocytes (Kruskal-Wallis, P = 0.63; *n* = 3), following a 7-day exposure to: controls (no plastic), 500 ng mL^{-1} polystyrene microbeads (PS-MP), 500 ng mL^{-1} polyamide microfibers (PA-MF) and 500 ng mL^{-1} polystyrene nanoparticles (PS-NP). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

would indicate micro- and nanoplastics triggered an oxidative challenge; but given SOD activity was normal after 7-days exposure, we can infer this oxidative challenge was short-lived and would therefore not have been energetically taxing (Trestrail et al., 2020).

3.4. Lysosomal destabilisation: neutral red retention

Lysosomes are membrane bound organelles, containing hydrolytic enzymes maintained at a low pH, with the function of removing endogenous and extraneous waste from the cell (Xu and Ren, 2015). Lysosomal membrane stability can be disrupted by environmental stressors (e.g. pollution, hypoxia), reducing their capacity to retain the tracer neutral red (Petrović et al., 2001). Our neutral red retention data showed that a 7-day exposure to plastic particulates at 500 ng mL^{-1} had no impact on lysosomal stability (Kruskal-Wallis, *P* = 0.63; Fig. 5).

This concurs with other recent studies. For example, in the Eastern oyster (*Crassostrea virginica*) 4 h in vitro and 48 h in vivo exposures to 3 μm PS-MP and 50 nm PS-NP (100 ppb) had no effect on lysosome stability, despite microscopic evidence for PS-NP accumulation within lysosomes via endocytic pathways (Gaspar et al., 2018). Further, no impact on lysosomal stability was observed in *M. galloprovincialis* exposed to LDPE microplastics for 7–28 days, as measured using the NRRT assay (Pittura et al., 2018). Conversely, in *M. edulis*, epithelial uptake of HDPE was linked with reduced lysosomal membrane stability (von Moos et al., 2012), and a 10-day exposure to PS-MP resulted in decreased lysosome membrane stability of *M. galloprovincialis* haemocytes (Avio et al., 2015).

3.5. Genotoxicity: micronucleus and comet assays

The results of the micronucleus and comet assays showed that the micro- and nanoplastics used in these exposures did not cause significant genotoxic damage to *M. edulis* following 24 h or 7-day exposures. However, we did observe a significant increase in the number of micronuclei per one thousand cells between exposures (two-way ANOVA, time: *P* > 0.05). Following exposures, an average of 1–1.5 micronuclei per 1000 cells were identified in the control treatment, with notably greater micronuclei abundance following plastic exposure, although such increases were not significant (ANOVA, treatment: *P* = 0.33; Fig. 6A). Similarly others found, a 21-day exposure to virgin
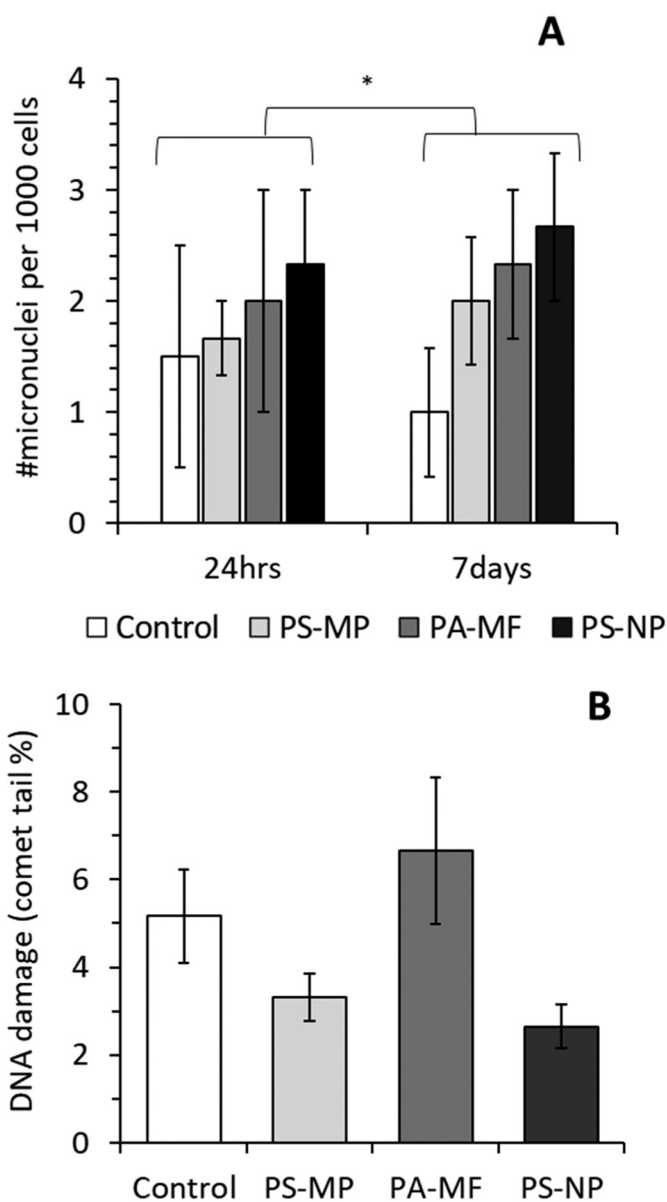

Fig. 6. The response of genotoxicity biomarkers in mussels, following exposure to: controls (no plastic), 500 ng mL^{-1} polystyrene microbeads (PS-MP), 500 ng mL^{-1} polyamide microfibers (PA-MF) and 500 ng mL^{-1} polystyrene nanoparticles (PS-NP). **(A)** Number of micronuclei per 1000 haemocytes from 24 h and 7-day exposures (two-way ANOVA, treat = *P* < 0.05, *n* = 4). **(B)** DNA damage (measured as percentage of DNA in comet tail) (ANOVA, treat = P < 0.05, *n* = 8). Treatments that don't share letters are significantly different (Tukey post-hoc analysis). *denotes statistical difference between micronuclei per 1000 haemocytes in mussels between 24-h and 7-day exposures (two-way ANOVA, time = P < 0.05).

and weathered polyethylene granules (50–600 μm), a 7–28 day exposure to LDPE microplastics (20–25 μm) and a 10-day exposure to PE or PS microplastics (< 100 μm) had no effect on micronuclei formation in the haemocytes of *M. galloprovincialis* (Avio et al., 2015; Bråte et al., 2018; Pittura et al., 2018).

In the control treatment, cells showed a baseline average of 5.2% DNA damage (i.e. proportion of fragmented DNA in the "comet tail": single strand breaks), which is well within normal parameters (Mitchelmore et al., 1998). It was observed that DNA damage did not differ significantly between controls and other treatments (Binomial GLM; PS-MP, *P* = 0.86; PA-MF, *P* = 0.90; PS-NP, *P* = 0.82; Fig. 6B). However, Revel et al. (2019) did observe a significant increase in

haemocytic DNA damage in mussels exposed to > 10 μg L^{-1} of PE-PP microplastics after a 10-day exposure (potentially stemming from nanoplastics in the sample), while Brandts et al. (2018a) showed significantly increased DNA damage in the haemocytes of *M. galloprovincialis* exposed to PS-NP for 96 h. Genotoxic damage could stem from physical-interactions between cell nuclei and nanoparticles, the leaching of toxic chemicals present within different plastics or high concentrations of reactive oxygen species, but in these two studies the underlying mechanisms could not be isolated.

4. Conclusions

In this study we explored how the shape and size of plastic particulates might affect their toxicity in the model organism *Mytilus* spp. Exposure to microplastics, microfibres and nanoplastics (500 ng mL^{-1}) resulted in significantly higher SOD activity after 24 h exposure; after 7-days exposure, SOD activity has returned to normal values, indicating the oxidative challenge was short-lived and therefore would be unlikely to result in adverse health effects. We did not observe any significant difference in the sub-lethal toxicity of microfibres and microplastic beads of similar equivalent spherical diameter, leading to a rejection of our hypothesis that particle aspect-ratio would influence toxicity. However, our results did indicate that particle size influences sub-lethal toxicity, as nanoplastic exposure increased the proportion of phagocytic haemocytes (indicative of a heightened immune response) and resulted in a marked increase in micronuclei formation. We postulate nanoscopic plastics can more readily permeate into the circulatory fluid and tissues inciting an immune response and causing cytotoxicity. We recommend further research is required to consider if: (1) prolonged exposure to plastic results in a sustained heightened immune response; (2) passage through the intestinal tract causes microplastics to abrade into nanoplastics; (3) changes in ecophysiology and apical endpoints (growth, reproduction, mortality) can be observed from chronic exposures using both juvenile and adult life stages.

CRediT authorship contribution statement

Matthew Cole: Conceptualization, Methodology, Investigation, Writing - original draft, Writing - review & editing, Funding acquisition.**Corin Liddle:**Conceptualization, Methodology, Investigation, Writing - original draft.**Giulia Consolandi:**Investigation, Writing - original draft.**Claudia Drago:**Investigation, Writing - original draft.**Cameron Hird:**Investigation, Writing - original draft.**Penelope K. Lindeque:**Writing - review & editing, Supervision.**Tamara S. Galloway:**Funding acquisition, Project administration, Supervision.

Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.marpolbul.2020.111552.

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