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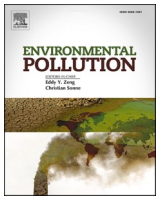

Cellular effects of microplastics are influenced by their dimension: Mechanistic relationships and integrated criteria for particles definition.☆

Alessandro Nardi [a,b,1], Lucia Pittura [a,b,1], Giuseppe d'Errico [a], Deborah Cesaroni [a], Federica Mongera [a], Stefania Gorbi [a,b], Maura Benedetti [a,b], Francesco Regoli [a,b,*]

[a] Dipartimento di Scienze della Vita e dell'Ambiente, Università Politecnica delle Marche, Ancona, Italy
[b] NBFC, National Biodiversity Future Center, Palermo 90131, Italy

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ABSTRACT

The definition of microplastics (MPs) is nowadays too generic from a biological perspective, since different characteristics of these particles might influence their effects. To provide experimental evidence that size is an important factor to be considered, Mediterranean mussels *Mytilus galloprovincialis* were exposed to five size classes of polyethylene fragments (PE-MPs, 20–50 μm, 50–100 μm, 100–250 μm, 250–500 μm, 500–1000 μm). After 10 days of exposure, MPs ingestion and mechanistic relationships between particles size and cellular effects were analysed through a wide panel of biological alterations, including immune system responses, cholinergic function, antioxidant system, lipid metabolism and peroxidation. Results were further elaborated through a Weight of Evidence approach, summarizing the overall biological significance of obtained results in a hazard index based on the number and magnitude of variations and their toxicological relevance. PE-MPs 500–1000 μm were identified as the less biologically reactive size class due to the limited ingestion of particles coupled with the lack of biological effects, followed by PE-MPs 250–500 μm, which slightly altered the cholinergic function and lysosomal membranes. Conversely, PE-MPs smaller than 250 μm provoked a more consistent onset of biological alterations in terms of immune system composition and functioning, redox homeostasis, and lipid metabolism. The overall findings of this study highlight the importance of considering the size of particles for monitoring and risk assessment of MPs, introducing a more integrated evaluation of plastic pollution that, beside particles concentration, should adequately weigh those characteristics triggering the onset of biological effects.

1. Introduction

In the last decade, microplastics (MPs) have received much attention due to their ubiquitous presence in aquatic ecosystems, low degradation rates, wide ingestion, and distribution among marine food webs (Pittura et al., 2023). A continuously growing number of studies is also documenting the effects of MPs on different taxa, highlighting the capability of these particles to modulate several biological processes which range from transcriptional to physiological levels (Avio et al., 2015; Prokić et al., 2021). However, the possibility to establish clear relationships between MPs exposure and biological outcomes has been prevented by the complexity of pathways involved, as well as by the marked discrepancies among species and exposure conditions used in laboratory experiments and environmental scenarios.

Several chemical-physical characteristics of MPs such as polymer typology, additives and adsorbed chemicals, shape, density, and size of particles have been suggested to influence their ingestion and excretion rates, their potential translocation between tissues, as well as cellular compartmentalization and effects. These factors may have contributed to the heterogeneous alterations observed on immune system and inflammatory processes, redox metabolism and oxidative damages, energy reserves, neurotoxic effects, reproduction, growth, and behaviour in organisms exposed to MPs (de Sá et al., 2018; Foley et al., 2018; Paul-Pont et al., 2018; Pittura et al., 2023; Rochman et al., 2019). In this respect, the definition of microplastics as particles smaller than 5 mm has become too generic and not anymore adequate from a biological point of view to properly reflect their heterogeneity: it has also been suggested to consider these particles as a mixture of contaminants rather

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* Corresponding authorDipartimento di Scienze della Vita e dell'Ambiente (DiSVA), Università Politecnica delle Marche, Via Brecce Bianche 60131, Ancona, Italy.
E-mail address: f.regoli@univpm.it (F. Regoli).

[1] Alessandro Nardi and Lucia Pittura equally contributed to the authorship of the manuscript.

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than a single typology of stressor (Rochman et al., 2019; Thornton Hampton et al., 2022).

Although environmental and health hazard of plastic polymers has been linked to monomers' toxicity (Lithner et al., 2011), recent meta-analyses on the effects of MPs on terrestrial, freshwater, and marine biota did not extrapolate any difference based on polymer typologies (Adam et al., 2019; Bucci et al., 2020; Doyle et al., 2022).

On the other hand, the shape of MPs was shown to influence their interaction with aquatic biota in terms of ingestion, retention, and elimination dynamics, as well as targeted biological systems and magnitude of provoked effects. Results from laboratory studies and meta-analyses indicate that irregularly shaped particles such as fibers or fragments may be preferentially ingested, retained for longer periods, and be more harmful compared to regular morphologies particles such as spheres and granules (Cole et al., 2019; Coppock et al., 2019; Graham & Thompson, 2009; Ma et al., 2020; Thornton Hampton et al., 2022; Xia et al., 2022).

Among the characteristics of MPs influencing their biological fate, the size of particles has been often suggested as a key feature, with smaller items having a higher probability of being ingested by a wider number of taxa (Thornton Hampton et al., 2022). An inverse relationship between PS-particles size (0.5, 5, and 50 μm) and the onset of biological alterations has been measured at molecular, cellular and physiological level in *Perna viridis* (Jong et al., 2022). Conversely, in the same species, a 96-h laboratory exposure to polystyrene (PS), polypropylene (PP) and polybutylene succinate (PBS) particles of three different size ranges (small, <30 μm; medium, 30–300 μm; large, 300–1000 μm), highlighted that the larger ones had the more pronounced acute toxicity effects in terms of mortality (Phothakwanpracha et al., 2021). A similar relationship was observed in *Mytilus galloprovincialis*, with larger particles causing more severe immunotoxic effects on haemocytes exposed *in vitro* to 1 μm, 100 nm, and 50 nm PS-particles (Sendra et al., 2020).

Previous considerations clearly highlight that monitoring and environmental risk assessment of MPs pollution should not rely only on the number (or mass) of items in abiotic matrices or ingested by organisms: it is necessary that specific characteristics of detected particles are adequately weighed and included in expression of data, based on their capability to modulate the typology and severity of provoked biological effects.

Approaches focused on measuring the onset of cellular alterations may be useful for better understanding the importance of MPs characteristics, highlighting sensitive relationships and early-warning signals of biological disturbance. In this context, the aim of the present study was to provide experimental evidence that size of microplastics can influence the onset of biological effects at cellular level. The Mediterranean mussel *M. galloprovincialis* was chosen as biological model and exposed to five size classes of PE-MPs to compare the onset and the magnitude of alterations on a panel of cellular mechanisms known for their susceptibility and involvement during stress-response phases. Results on immune system composition and functioning, lysosomal integrity, cholinergic responses, oxidative stress, lipid metabolism, and genotoxic damages were elaborated through a Weight of Evidence approach allowing to synthesize the overall biological significance of measured effects in an integrated hazard index. This work was expected to further advance our knowledge on size-mediated effects of microplastics, and possibly suggest the use of criteria which consider this characteristic when expressing data on MPs for monitoring and impact assessment.

2. Materials and methods

2.1. PE-MPs stocks preparation

PE-fragments represent one of the most frequently detected typologies of microplastics, both in terms of shape and polymer, and for this reason they were chosen as model particles for testing the effect of size.

PE-MPs were obtained by a commercial powder of polyethylene fragments supplied by an Italian company (FIMAS Srl). The powder was sieved through a battery of sequential steel sieves (1000 μm, 500 μm, 250 μm, 100 μm, 50 μm and 20 μm mesh size) to obtain stocks of the following classes of size according to a slightly modified categorization proposed by ISO (ISO/DIS, 2021): PE-MPs 500–1000 μm, PE-MPs 250–500 μm, PE-MPs 100–250 μm, PE-MPs 50–100 μm, PE-MPs 20–50 μm. The fractions smaller than 20 μm and larger than 1000 μm, were excluded due to the limited amount of recovered fragments.

For each selected PE-MPs fraction, 100 items were isolated under the stereomicroscope and measured using an image analysis software (Image View) to verify particles sizes and their distribution (Figures and values reported in Supplementary Materials, SM). Then, 1000 particles were isolated under stereomicroscope and weighed (procedure repeated three times per each size class) to calculate the amount of powder (in grams) to be mixed in 0.01% Tween-20 solution (in ultrapure water filtered at 0.22 μm) to obtain experimental stocks of each class of size at a concentration of 5 x 10^6 items/L.

2.2. Experimental design

A total of 96 Mediterranean mussels *M. galloprovincialis* (6.4 ± 0.3 cm, shell length) were obtained from a local shellfish farm in central Adriatic Sea and immediately transferred to laboratory facilities for a 7-days acclimation in 12 beakers (5 L, 8 organisms each) with filtered ASW at 22 °C, salinity 35 and pH 8.10, according to seasonal local seawater characteristics. After acclimation, organisms were randomly divided among six experimental treatments, each performed in duplicate, including control (artificial seawater without PE-MPs, CTL) and the five PE-MPs size classes described before (20–50 μm, 50–100 μm, 100–250 μm, 250–500 μm, 500–1000 μm); for each treatment, particles were dosed at the nominal concentration of 1000 items/L, mixing 1 mL of each stock solution in the respective 5 L exposure beakers. This exposure concentration is greater than levels typically detected in seawater samples (Buckingham et al., 2022; Tanhua et al., 2020), and our results were not expected to be correlated to any environmental scenario. The choice was driven by the general aim to highlight the occurrence of different responses in a short-term exposure, thus reflecting a typical need of toxicological studies exploring possible biological targets and pathways, through the onset of alterations in laboratory experiments (Bringer et al., 2022; Cole et al., 2020; Sui et al., 2022; Xu et al., 2022).

Throughout both acclimation and exposure periods, complete water changes were scheduled every other day and organisms fed after each water renewal with a commercial mixture of phytoplankton (4.4 x 10^7 cells/liter/day, Easy booster prof, easy reefs®; 33% *Isochrysis galbana*, 31% *Nannochloropsis* sp., 18% *Tetraselmis* sp., 18% *Phaeodactylum* sp.): during the experimental phase, beakers were accurately washed twice with filtered ASW after water removal to eliminate possible residual MPs, before re-dosing these at the nominal concentration.

After 10 days of exposure, samples were collected as follows: for biochemical analyses, haemolymph, gills and digestive glands were collected from 8 organisms (4 per replicate beaker) and pooled to obtain 4 samples, each with tissues of 2 individuals, flash frozen in liquid nitrogen and stored at -80 °C until analyses. For immune responses and genotoxic effects on haemocytes, haemolymph was withdrawn from the adductor muscle of 8 organisms (4 per replicate beaker), pooled in 4 samples (two individuals each) and either used immediately for *in-vivo* immune parameters or fixed in Carnoy solution (acetic acid:methanol, 1:3) for the evaluation of micronuclei frequency. From the same 8 organisms, the digestive glands were excised, four of these were individually flash frozen in liquid nitrogen and stored at −80 °C until histological analyses, while the remaining, individually frozen and maintained at −20 °C until processed for MPs extraction and characterization.

2.3. MPs extraction and characterization

For each treatment, 4 digestive gland samples were processed according to validated protocols (Pittura et al., 2022a): briefly, sample were digested at room temperature using 10% potassium hydroxide (KOH) solution prepared in ultrapure water, filtered at 0.45 μm before use. The digestates were then vacuum filtered on mixed cellulose ester (MCE) membranes (Ø 47 mm, pore size 8 μm, ClearLine) and subsequently observed under a stereomicroscope (GZ 808 with IS 4K-8 digital camera, Optech) to isolate extracted fragments. To confirm the match between the particles extracted and those of the original PE-MPs stock, particles were measured using an image analysis software (Image View) and chemically characterized by μFT-IR spectroscopy (Spotlight 200i FT-IR microscopy system, PerkinElmer). Such results are given in SM.

2.4. Biological analyses

Validated protocols, detailed in SM, were applied to analyse the following classes and typologies of biological endpoints: immunocytes responses (granulocytes on hyalinocytes ratio and phagocytosis rate, haemocytes lysosomal membrane stability, micronuclei frequency); cholinergic function (acetylcholinesterase activity in haemolymph and gills); total oxyradical scavenging capacity toward peroxyl radical (TOSC ROO•), hydroxyl radical (TOSC HO•), and peroxynitrite (TOSC ONOO-) in the digestive gland; lipid metabolism (acyl-CoA oxidase activity and neutral lipids content) and peroxidation (lipofuscin content) in the digestive gland. For each biomarker, 4 samples from each treatment were analysed ($n = 4$).

2.5. statistical analyses and WOE elaboration

Analysis of variance (ANOVA) was applied to evaluate the effects of treatments on each analysed biological parameter, after testing for normal distribution (Shapiro-Wilk test) and homogeneity of variances (Levene's Test). Data were transformed using Box-Cox transformation (R package "MASS") when assumptions were not fulfilled. Tukey HSD *post-hoc* test followed significant ANOVA test to compare the means of interest. Multivariate principal component analysis (PCA) was applied to scale the whole dataset, visualize treatments distribution and evaluate the contribution of analysed parameters.

Since one of the main objectives of this study was to highlight possible differences in terms of hazard of each PE-MPs size class, the whole dataset of biological responses was further elaborated through a quantitative Weight of Evidence (WoE) approach. This approach provides hazard indices for each experimental condition based on toxicological relevance of measured biological endpoints, statistical significance and magnitude of observed variations compared to specific thresholds. The elaborated Hazard Quotients (HQs) are then assigned to one of five classes of hazard, from Absent to Severe. Whole calculations and assumptions are fully detailed in previously published studies (Avio et al., 2015; Gonçalves et al., 2023; Nardi et al., 2022 ; Pittura et al., 2018; Regoli et al., 2019) and provided in SM.

3. Results

3.1. MPs in digestive glands

Extraction of MPs from the digestive gland of experimental mussels confirmed ingestion of all the PE-MPs size classes with a number of items varying from 1 to a maximum of 31 per individual (Table 1). The highest number of fragments was found in mussels exposed to 100–250 μm PE-MPs, significantly higher than in organisms exposed to PE-MPs 500–1000 μm. Statistically comparable levels of MPs were observed in the other treatments, while PE-fragments were not detected in control organisms, as expected. Measurement and characterization of extracted particles confirmed the expected size range and polymer match showing spectra with no change in the position of the characteristic peaks of polyethylene (*i.e.* 2915 cm^{-1}, 2848 cm^{-1}, 1467 cm^{-1}, 718 cm^{-1}), as reported in SM.

Table 1
MPs in digestive gland of exposed mussels. Results are given as mean number of particles extracted from each digestive gland ± standard deviation, n = 4. Different letters indicate statistically significant differences. No PE fragments were detected in control organisms.

TREATMENT	n. items/digestive gland
20–50 μm	11.5 ± 5.41 [a]
50–100 μm	6.5 ± 1.0 [ab]
100–250 μm	21.83 ± 15.02 [a]
250–500 μm	10.0 ± 6.06 [ab]
500–1000 μm	1.83 ± 1.02 [b]

3.2. Immunocytes sub-populations composition, functionality and subcellular damages

Lysosomal membrane stability was significantly reduced in all MPs-exposed mussels (Fig. 1A), except for those exposed to 500–1000 μm PE-MPs. A significant increase of granulocytes on hyalinocytes ratio was observed in mussels exposed to 20–50 μm and 50–100 μm PE-MPs compared to control condition (Fig. 1B). Similarly, phagocytosis significantly increased in the same experimental treatments (Fig. 1C). No significant differences were observed in terms of micronuclei frequency (Fig. 1D).

3.3. Cholinergic function

The activity of AChE in haemolymph showed a significant increase only in organisms exposed to 20–50 μm PE-MPs (Fig. 2A), while in the gills AChE activity was significantly stimulated in organisms exposed to MPs ranging from 100 to 500 μm (Fig. 2B).

3.4. Antioxidant capacity and lipid peroxidation

The total oxyradical scavenging capacity showed a significant increase toward peroxyl radical in 20–50 μm PE-MPs exposed mussels (TOSC ROO•, Fig. 3A), and a slighter increase, not statistically significant, was observed also in 50–100 μm PE-MPs exposed mussels. The total oxyradical scavenging capacity against hydroxyl radical was significantly reduced in mussels exposed to 50–100 and 100–250 μm PE-MPs (TOSC HO•, Fig. 3B), while TOSC toward peroxynitrite significantly increased only in mussels exposed to 20–50 μm PE-MPs (TOSC ONOO-, Fig. 3C). Lastly, lipofuscin accumulation was significantly higher in the digestive gland of mussels exposed to 50–100 μm and 100–250 μm PE-MPs compared to other treatments (Fig. 3D).

3.5. Fatty acids metabolism and neutral lipids accumulation

Acyl-CoA oxidase activity (ACOX) was significantly reduced in the digestive gland of mussels exposed to PE-MPs with a size range between 50 and 500 μm (Fig. 4A); mussels exposed to 50–100 μm and 100–250 μm PE-MPs showed a contemporary increase of neutral lipids content (Fig. 4B).

3.6. Principal components analysis and weighted elaboration

Principal components analysis produced a two-dimensional pattern explaining about 62% of variance (Fig. 5). This pattern revealed a clear separation between control organisms and mussels exposed to PE-MPs smaller than 250 μm along PC1 (35.5% of total variance), with a major contribution of lysosomal membrane stability, granulocytes on hyalinocytes ratio, TOSC HO•, lipofuscin accumulation, phagocytosis,

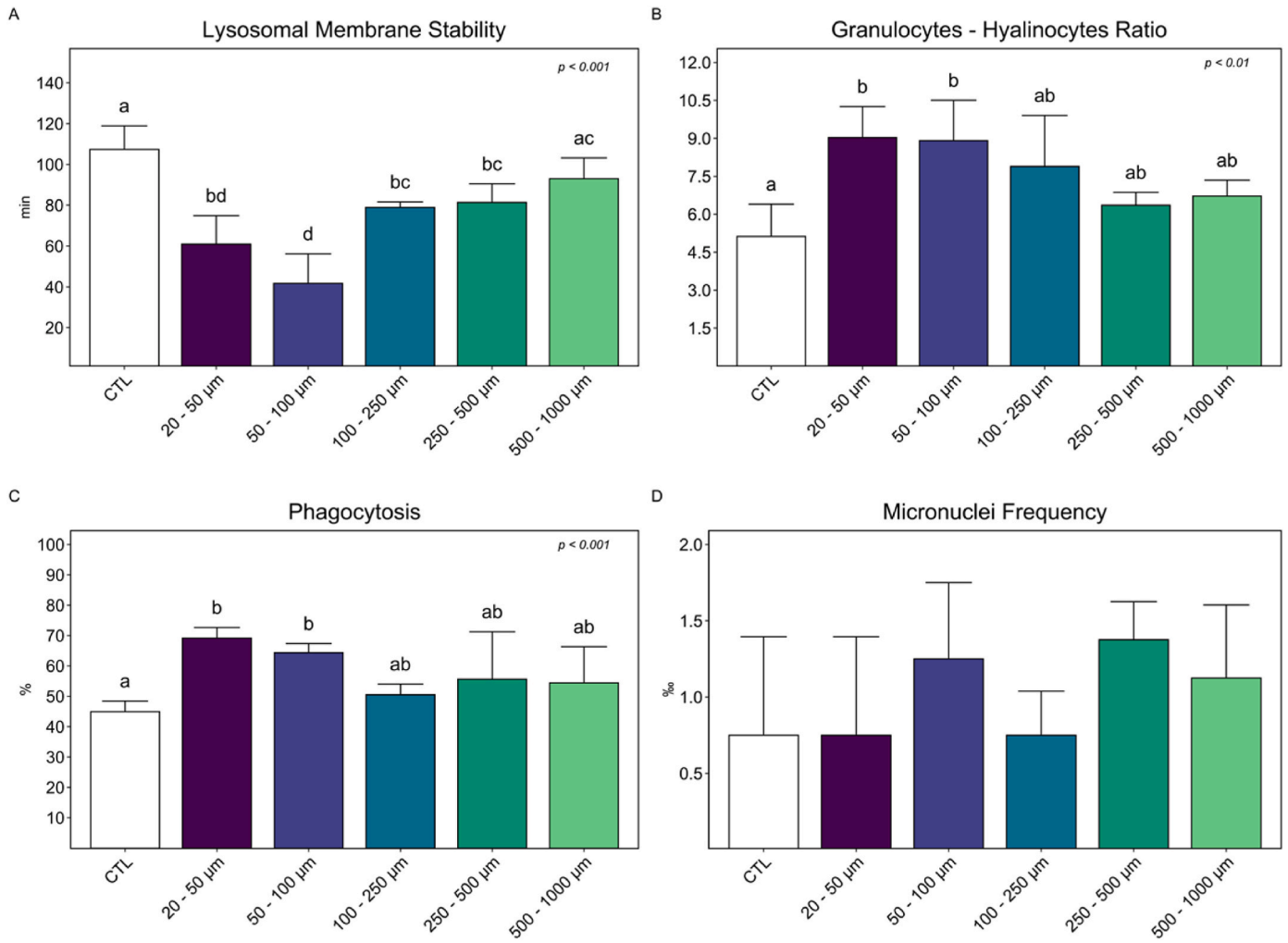

Fig. 1. Immunocytes responses. Haemocytes lysosomal membrane stability (A), granulocytes on hyalinocytes subpopulations ratio (B), granulocytes phagocytosis efficiency (C), and frequency of micronuclei (D) of exposed mussels. Results are given as mean ± standard deviation, n = 4. Different letters indicate statistically significant differences.

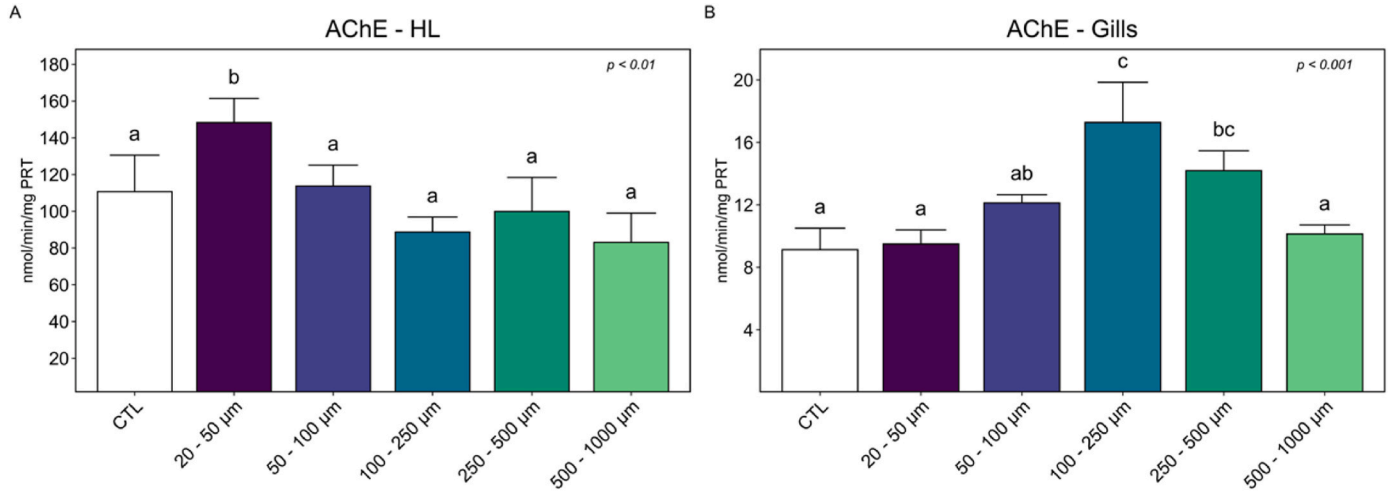

Fig. 2. Cholinergic function. Acetylcholinesterase activity in haemolymph (A) and gills (B) of exposed mussels. Results are given as mean ± standard deviation, n = 4. Different letters indicate statistically significant differences.

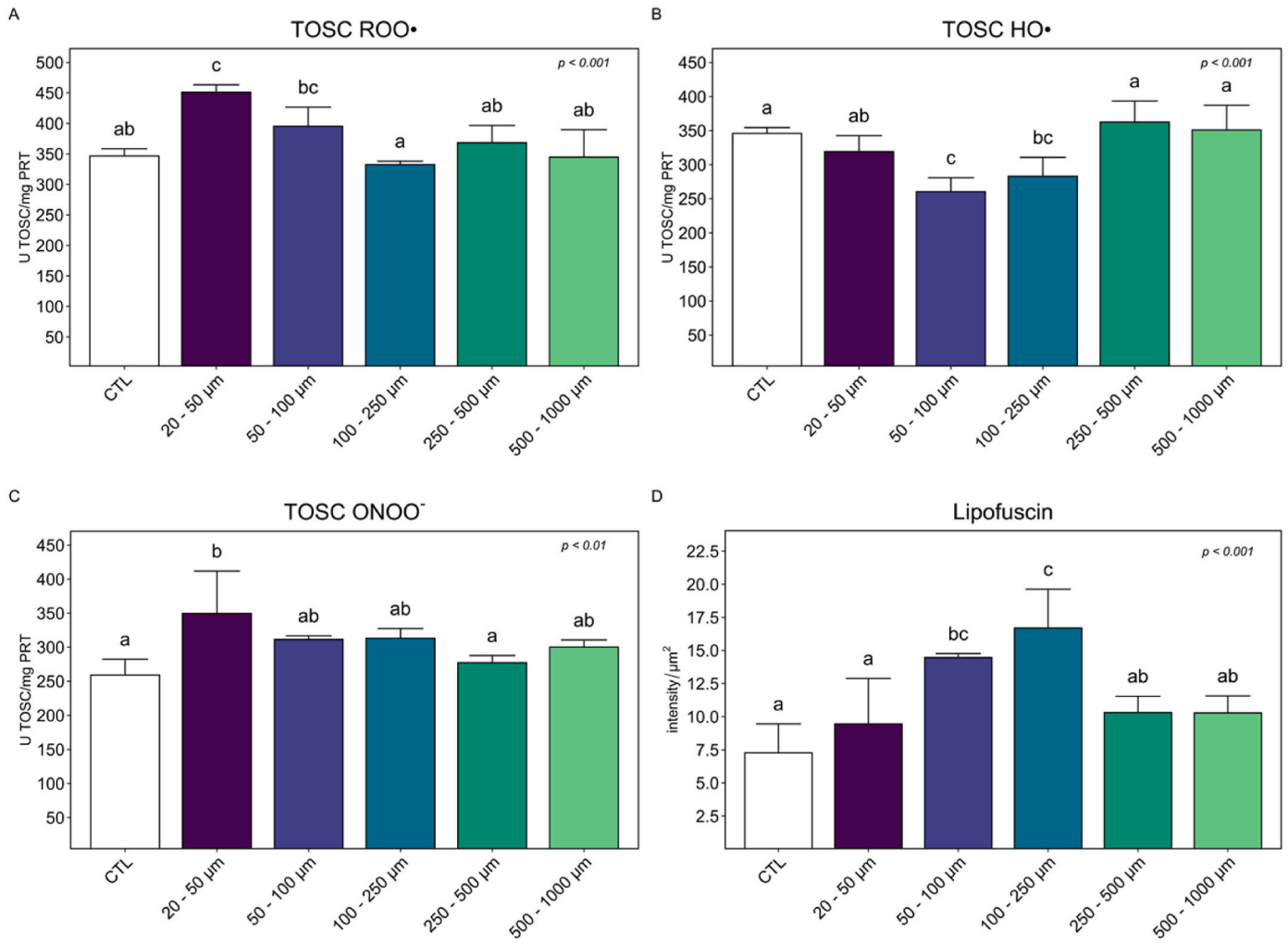

Fig. 3. Antioxidant capacity and lipid peroxidation. Total oxyradical scavenging capacity toward peroxyl radical (A), hydroxyl radical (B) and peroxynitrite (C), lipofuscin accumulation in 8 µm thick digestive gland sections (D) of exposed mussels. Results are given as mean ± standard deviation, n = 4. Different letters indicate statistically significant differences.

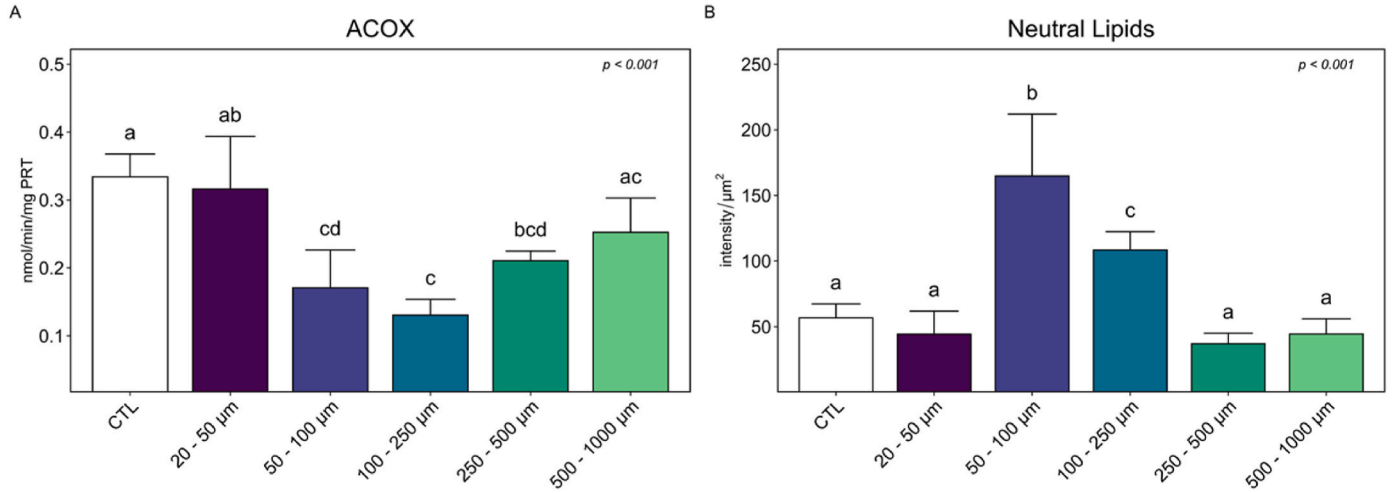

Fig. 4. Fatty acids metabolism and neutral lipids storage. Activity of acyl-CoA oxidase in the digestive gland (A) and content of neutral lipids in 8 µm thick digestive gland sections (B) of exposed mussels. Results are given as mean ± standard deviation, n = 4. Different letters indicate statistically significant differences.

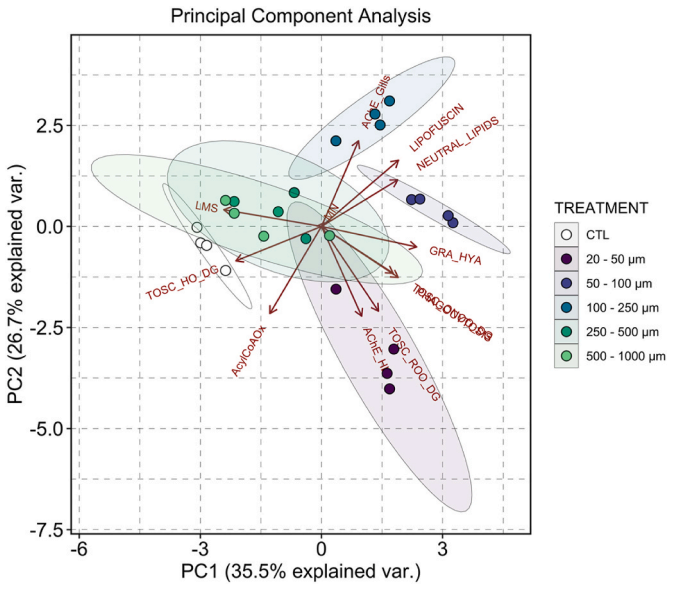

Fig. 5. Two-dimensional visualization of principal components analysis. Dots represent experimental observations, ellipses represent the 95% confidence interval of each experimental condition grouping, arrows indicate variables and their length express their relative contribution.

neutral lipids content, and TOSC ONOO^{-}, listed in descending order of percentage contribution. At the same time, a less pronounced but still relevant separation was observed along PC2 (26.7% of total variance) among 20–50 µm, 50–100 µm and 100–250 µm PE-MPs experimental treatments, driven by activity of acetylcholinesterase in haemolymph and gills, acyl-CoA oxidase and TOSC ROO•, listed with the same rationale used for PC1.

The elaboration of the whole dataset through weighted criteria provided the hazard classification for each class of size (shown in Fig. 6) considering the number and magnitude of measured variations and the toxicological relevance of each biological endpoint. The hazard was classified as "SLIGHT" in organisms exposed to PE-MPs larger than 250 µm, with slight to moderate variations observed for neutral lipids and ACOX in PE-MPs 250–500 µm treatment, and ACOX for PE-MPs 500–1000 µm. On the other hand, a "MODERATE" class of hazard was assigned to experimental conditions with PE-MPs smaller than 250 µm, driven by immunocytes alterations (for PE-MPs 20–50 µm and 50–100 µm), antioxidant defences (TOSC ROO• and ONOO- for PE-MPs 20–50 µm), lipid metabolism and oxidative damages (lipofuscin, neutral lipids and ACOX for PE-MPs 50–100 µm, lipofuscin and ACOX for PE-MPs 100–250 µm).

4. Discussions

This study was aimed to demonstrate whether PE-MP fragments can provoke size-dependent effects on biological responses measured in *M. galloprovincialis*. Considering the biological reactivity of MPs, short-term toxicological studies exploring the diversity of possible alterations and biological targets, typically require experimental concentrations higher than those represented in environmental scenarios. Concentrations of MPs similar to the one used in the present study (1000 items/L) were recently used to investigate the sensitivity of mussels *Mytilus coruscus* to concomitant MPs and seawater acidification (Sui et al., 2022), as well as the trophic transfer, intake and elimination of MPs in the gastropod *Reishia clavigera* (Xu et al., 2022); a comparative study among PS-MPs, polyamide (PA)microfibers and PS-nanoplastics (Cole et al., 2020) tested the effects of roughly 100,000 up to 7.3 x 10^{12} items/L on *Mytilus* spp. immune response and redox balance, while even greater MPs concentrations (from low dose: 0.1 mg/L

TREATMENT	Number and ID of biologial responses in each class					Hazard Classification
	ABSENT	SLIGHT	MODERATE	MAJOR	SEVERE	
PE-MPs 20 - 50 µm	7	2 TOSC ONOO-; TOSC ROO•	2 LMS; PHAGOCYTOSIS	1 GRA/HYA	0	MODERATE
PE-MPs 50 - 100 µm	6	1 PHAGOCYTOSIS	5 ACOX; GRA/HYA; LIPOFUSCIN; LMS; NEUTRAL LIPIDS;	0	0	MODERATE
PE-MPs 100 - 250 µm	8	2 AChE (Gills); LMS	0	2 ACOX; LIPOFUSCIN	0	MODERATE
PE-MPs 250 - 500 µm	10	1 NEUTRAL LIPIDS	1 ACOX	0	0	SLIGHT
PE-MPs 500 - 1000 µm	11	1 ACOX	0	0	0	SLIGHT

Fig. 6. Results of weighted elaboration. For each PE-MPs class of size, number and identification (ID) of biological responses in each class are given, together with the overall hazard classification.

corresponding to 667,000–943,000 items/L, to high dose: 10 mg/L) were used to address the intergenerational effects of environmentally-aged microplastics in *Crassostrea gigas* (Bringer et al., 2022). While the levels of MPs used in such exposures are much higher than environmental ones, these studies contributed to identify the biological targets of MPs and the pathways that these particles could hamper, also elucidating possible mechanisms of action.

Microplastics typically enter the mussels via the inhalant siphons during water filtration, being then captured by the gills, transported through the mouth towards the stomach and the digestive gland, and finally eliminated in faeces or translocated to the circulatory system and other tissues (Li J. et al., 2021).

Our results confirmed that ingestion occurred for all the size classes tested, from 20 μm to 1000 μm: the lowest number of particles was identified in the digestive gland of mussels exposed to the largest ones (500–1000 μm), supporting the hypothesis that, above a certain dimension, these particles are not ingested but efficiently retained by gills and eliminated through pseudofaeces (Ward et al., 2019). The limited ingestion of PE-MPs 500–1000 μm was associated with the lack of remarkable cellular alterations, allowing to quantitatively identify this dimensional size as the less biologically reactive. According to the typical route of ingestion, all MPs initially interact with the gills during filtration; interestingly, those ranging from 100 to 500 μm exhibited the most pronounced effects in terms of cholinergic function in this tissue, probably because particles of these dimensions are likely to be temporarily entrapped between gills lamellae. This entanglement would cause physical disturbance to the normal organ functioning, demanding an increased movement of cilia and consequent performance of the cholinergic system (Bülbring et al., 1953). A similar effect on gills might accelerate the transfer of these MPs to digestive gland where particles of 100–250 μm were detected with the highest values.

Once ingested, MPs can be retained within digestive gland for a time strongly dependent on many factors, including dimension of particles. Mussels, *M. galloprovincialis*, excreted the majority of PS-microspheres of 1 and 10 μm faster than those of 90 μm (Kinjo et al., 2019); the same study, however, demonstrated that among the few retained particles, smaller items remained in the digestive tract for longer periods (up to 40 days) compared to larger ones (28 days). Similarly, Fernández and Albentosa (2019a, b) highlighted that the time of retention of irregularly shaped HDPE-MPs increased with decreasing size in the digestive glands of mussels.

In our study, despite limited statistical differences among tested classes of size, the highest number of particles in the digestive gland was observed for PE-MPs 100–250 μm: comparable retention rates among small (<30 μm), medium (30–300 μm) and large (300–1000 μm) MPs were recently observed in *P. viridis* exposed to 1333 items/L of PS-, PP- and PBS-fragments for 96 h (Phothakwanpracha et al., 2021).

Overall, in the digestive gland we identified a size-threshold for the onset of cellular effects below 250 μm, with specific differences according to size for the affected biological processes and the magnitude of measured variations. A decreased efficiency of the antioxidant defence towards hydroxyl radical was observed in organisms exposed to MPs ranging from 50 to 250 μm, coupled to an increase of oxidative damage and lipofuscin content in the tertiary lysosomes. These results support the hypothesis that MPs may alter redox homeostasis during their retention in the digestive tissue, by promoting ROS formation, through either clogging and inflammatory processes, or by decreasing the efficiency of antioxidant defences (Benedetti et al., 2022; Chen et al., 2022; Hu and Palić, 2020; Li B. et al., 2021; Li et al., 2022). MPs in the range between 50 and 250 μm also negatively affected lipid homeostasis, in terms of decreased acyl-CoA oxidase activity and increased neutral lipids in digestive gland. Acyl-CoA oxidase is the first, rate-limiting enzyme involved in the peroxisomal β-oxidation system, and its deficiency has been previously linked to accumulation of neutral lipids droplets in *Acox1* gene deficient murine microglial BV-2 cell line (Raas et al., 2019), as well as with the onset of steatosis in mice (Fan et al., 1996; Vluggens et al., 2010). Commercial PS-spheres of 4.5 μm have been reported to inhibit ACOX activity in *M. galloprovincialis* (von Hellfeld et al., 2022), and mRNA levels of acyl-CoA oxidase were downregulated in zebrafish embryos following a 7-days exposure to 5 μm and 50 μm PS-MPs (Wan et al., 2019); the coupling of decreased ACOX activity and increased neutral lipids content was also observed in *M. galloprovincialis* exposed to PA-microfibers (Pittura et al., 2022b). Whether ACOX inhibition is the cause of neutral lipids accumulation, or the consequence of steatosis caused by MPs in the digestive tissue, remains to be clarified. However, the involvement of this energy reserve storage mechanism may be prognostic of further metabolic disorders under longer lasting MPs retention scenarios. The increased total oxyradical scavenging capacity toward peroxyl radical (ROO•) and peroxynitrite (ONOO-) in mussels exposed to PE-MPs 20–50 μm, allowed to address pro-oxidative challenge even to this size class and to hypothesize that the presence of such small-sized particles may trigger inflammatory processes mediated by immunocytes that involve the production of those ROS; the lack of peroxidation products accumulation confirms the efficiency of such enhanced capability to counteract oxidative insult.

The onset and persistence of an inflammatory state may be further enhanced by translocation processes: indeed, following the route described by Li J. et al., (2021), once in the digestive tract, MPs may be translocated to the circulatory system if not promptly eliminated. This process has been previously demonstrated to be size-dependent (Browne et al., 2008; Li B. et al., 2021; Li J. et al., 2021; Pyl et al., 2022) and to involve different mechanisms, either cell-mediated endocytosis and transcytosis for small-sized MPs and NPs (Browne et al., 2008; Scanes et al., 2019), or through the movement of particles in spaces between the epithelial cells (Powell et al., 2010). The mechanisms of translocation are yet to be elucidated, and a clear relationship with particle size is still under debate: MPs larger than 100 μm have never been detected in circulatory system of different model species (Pyl et al., 2022), and translocation from the digestive tracts toward other tissues was estimated to occur for particles smaller than 83 μm (Mehinto et al., 2022). Laboratory studies have detected MP fragments and spheres up to 50 μm in the haemolymph of mussels (Franzellitti et al., 2019; Pavičić-Hamer et al., 2022; Pittura et al., 2018), and 20–100 μm microfibers were observed in the coelomic fluid of *Holothuria cinerascens* and *Apostichopus japonicus* (Iwalaye et al., 2020; Mohsen et al., 2019). Although in our study we did not analyse the presence of MPs in haemolymph, the onset of harsher immunocytes alterations in organisms exposed to 20–50 μm and 50–100 μm PE-MPs does not allow to exclude gut-circulatory system translocation of particles smaller than 100 μm. Indeed, mussels exposed to these size classes showed an increase of granulocytes on hyalinocytes ratio, as well as of the phagocyting capacity and of lysosomal destabilization; these alterations allowed to identify 100 μm as size-threshold for the onset of immunotoxic effects. Granulocytes proliferation was previously observed in *Mytilus* spp. exposed to 50 nm PS-NP for 7 days, explained by particles translocation leading to immune response and onset of inflammation (Cole et al., 2020). As a consequence of the activation of the cell-mediated immune responses and the shift of haemocytes sub-populations, the increased number of circulating granulocytes observed in our study provided mussels an enhanced phagocytic capability.

The activation of immune response following the presence of exogenous particles in the circulatory system can also trigger ROS production and oxidative damages to membranes (Benedetti et al., 2022). Although we observed a generalized reduction of lysosomal membranes stability, the onset of immunological alterations in mussels exposed to PE-MPs smaller than 100 μm could be the cause of the more pronounced lysosomal destabilization measured below this size-threshold. Despite several studies reported lysosomal alterations after MPs exposure, the mechanisms of such adverse effects are not yet completely understood. Reduced lysosomal membrane stability was observed in mussels exposed to PA-microfibers 11 ± 1 μm diameter (Pittura et al., 2022b), HDPE-MPs smaller than 80 μm and PS-MPs smaller than 100 μm (Avio

et al., 2015; Pittura et al., 2018; Von Moos et al., 2012) and in *Ruditapes decussatus* exposed to 40–48 μm PE-MPs (Abidli et al., 2023). These studies used particles larger than the few micrometers of lysosomes, corroborating the hypothesis that MPs toxicity to lysosomes may not be directly driven by particle-lysosome interactions, but rather involves indirect mechanisms, as ROS production and oxidative unbalance following immune system activation.

The induction of AChE activity in haemolymph of mussels exposed to PE-MPs 20–50 μm further narrows the size-threshold for onset of biological effects, linking immunotoxicity to the neuro-endocrine system. Similarly to gills, we hypothesize the induction of AChE as a compensatory mechanism, rather than a direct effect of MPs, aiming to boost phagocytic capacity through a positive regulatory feedback on neurotransmitters homeostasis: in this respect, it is now widely recognized the role of ACh accumulation on NF-κB signalling pathway as immune response suppressor (Du et al., 2020; Shi et al., 2014, 2015).

The analysis of principal components on the whole dataset of responses, confirmed a clear separation of the cellular health status among mussels exposed to MPs smaller than 250 μm and control organisms, while those exposed to MPs ranging from 250 to 1000 μm showed a mild onset of cellular disturbance. This 2-dimensional pattern gained even more relevance when the results on biological responses were elaborated through weighted criteria which consider not only the magnitude of variations but also the toxicological relevance of these biological endpoints. The algorithm assigned indeed a "MODERATE" class of hazard to MPs smaller than 250 μm, while this was classified as "SLIGHT" in organisms exposed to MPs ranging from 250 to 1000 μm.

5. Conclusions

The overall results of this study and their elaborations pose the attention on the importance of considering MPs size when assessing the biological and ecological risk of plastic particles.

The identification of size-related thresholds for the onset of effects reflecting ingestion, translocation and various typologies of cellular alterations, will contribute to define appropriate "weights" to be assigned to MPs as a function of their size. A similar approach is fundamental for monitoring and risk assessment of MPs providing comprehensive hazard indices of MPs pollution based not only on the number of particles but also on the characteristics relevant to their biological and ecological fate. Beside deepening our knowledge on size-dependent effects towards the nano-size, future studies should aim to address weights for other typical features of plastic particles, like shape and polymer typology.

CRediT authorship contribution statement

Alessandro Nardi: Conceptualization, Data curation, Formal analysis, Investigation, Supervision, Visualization, Writing – original draft, Writing – review & editing. **Lucia Pittura:** Conceptualization, Data curation, Formal analysis, Investigation, Supervision, Visualization, Writing – original draft, Writing – review & editing. **Giuseppe d'Errico:** Formal analysis, Software. **Deborah Cesaroni:** Data curation, Investigation. **Federica Mongera:** Data curation, Investigation. **Stefania Gorbi:** Conceptualization, Resources, Supervision. **Maura Benedetti:** Project administration, Resources, Supervision. **Francesco Regoli:** Conceptualization, Funding acquisition, Project administration, Writing – original draft, Writing – review & editing.

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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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