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Exposure to pet-made microplastics: Particle size and pH effects on biomolecular responses in mussels

Francesca Provenza [a], Manuela Piccardo [b,c], Antonio Terlizzi [b,c], Monia Renzi [b,*]

[a] Bioscience Research Center, via Aurelia Vecchia, 32, 58015 Orbetello, Italy
[b] Department of Life Sciences, via L. Giorgieri, 10, University of Trieste, 34127 Trieste, Italy
[c] Stazione Zoologica Anton Dohrn, 80121 Napoli, Italy

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ABSTRACT

This study aims to evaluate the expression of biomarkers of oxidative stress (LPO, GPx, AtCh, SOD) in mussels (*Mytilus galloprovincialis*) following the exposure to suspensions of microparticles irregular shaped fibres of Polyethylene terephthalate of different sizes (small 5–60 μm, S-PET; medium 61–499 μm, M-PET; large 500–3000 μm, L-PET) at a single dose of 0.1 g/L. Mussels were tested under two different starting pH conditions of marine water: standard (8.0) and acidified (7.5). The results obtained from this study show that: *i*) PET microplastics are able to induce biochemical stress in mussels; *ii*) among the biomarkers tested, LPO and GPx were more effective in detecting the stress induced by microplastic in both initial pH conditions; *iii*) the expression of biomarkers was influenced by the size of the microparticle. In particular, greater effects were associated with the largest PET particle tested (0.5–3.0 mm); *iv*) regarding the effect of pH, in experiments starting from 7.5 pH the animals showed a lower biomarker expression than those starting from 8.0 pH.

1. Short note

Plastic litter and microplastic pollution can produce significant direct and indirect impacts in marine ecosystems (Cole et al., 2011; Cole et al., 2014; Avio et al., 2015a; Dehaut et al., 2016; Collard et al., 2017) and the scientific interest towards microplastic is quickly increased in the last few years (Pauna et al., 2019). Plastic litter in marine ecosystems come from inadequate waste management, and from legal or illegal dumps located near coastal areas (AEA, 2006), irrational use and disposal of plastic food packaging (UNEP, 2015). More than 92% of the 12 million tons/year of plastic entering oceans are represented by particles smaller than 5 mm (Jambeck et al., 2015) that are defined by GESAMP (2016) with the umbrella term "microplastics". Fragmentation of larger plastic pieces represents a significant secondary source of microplastics in marine environments. A wide range of marine species from pelagic and benthic fishes to marine mammals resulted able to ingest microplastics (Neves et al., 2015; Van Cauwenberghe et al., 2015; Fossi et al., 2016; Dehaut et al., 2016; Courtene-Jones et al., 2017; Karlsson et al., 2017; Pellini et al., 2018; Renzi et al., 2018a, 2018b).

In 2017, PET represented one of the most produced polymer in Europe (Plastic Europe, 2018); this material was intended to make disposable products for mass consumption (Singh et al., 2018). PET-originated litter can reach the seafloor (Mu et al., 2018) or represent one of the most frequently found macro-litter along beaches (Munari et al., 2016). PET-made tools are affected by photooxidation and hydrolysis processes that producing smallest plastic pieces (Gewert et al., 2015). PET is currently considered a safe material for the human health (Sinha et al., 2010). Nevertheless, recent papers highlighted toxicity from the exposure to PET microplastics (PET-MPs) in both freshwater species such as amphipods, copepods, and fish (Heindler et al., 2017; Rochman et al., 2017; Weber et al., 2018) and marine species (Piccardo et al., 2020). To our knowledge, relationship between the toxicity of PET-MPs on marine organisms and its particle size is less explored by literature (Piccardo et al., 2020). Although fibres, fragments and irregular particles are the most abundant shape of microplastics detected in biota (Bour et al., 2018), most of the laboratory studies on bivalves have been conducted using spherical polystyrene (Browne et al., 2008; Cole et al., 2015; Paul-Pont et al., 2016; González-Soto et al., 2019).

The principal aim of this study is to verify if PET-MPs are able to significantly affect, and related to the size of microparticles, the expressions of stress biomarkers in exposed mussels. Secondly, this study evaluates the occurrence of significant differences in biochemi-

* Corresponding author.
E-mail address: monia.renzi@bsrc.it (M. Renzi)

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cal responses based on the initial pH condition by comparing the results obtained following exposure to pH 8.0 with those obtained at 7.5 pH, the latter being levels which could be likened to a temporary impact from a water acidification hotspot. The occurrence of such temporary sources of pH acidification in coastal marine ecosystems does not represent a rare phenomenon. In fact temporary water acidification is reported to originate from effluents of municipal or industrial wastewater treatment plants (Aniyikaiye et al., 2019), from acidification of estuarine inputs (Sammut et al., 1995) or from intense activities by primary producers (Hinga, 2002). Coastal transitional ecosystems are naturally pH pulsating environments due to their fluctuating overall balance between surplus of respiration or of primary production (Specchiulli et al., 2008; Basset et al., 2013). The ocean pH decreased by about 30% compared to pre-industrial levels (Doney et al., 2009) and is expected to decrease further, following the climate change scenario, to a level of 7.7 pH units within 2100 (Feeley et al., 2009). Under this global scenario the occurrence of local and temporary conditions of 7.5 pH could be not an infrequent occurrence in coastal marine areas. For the exposed reasons, effects recorded in this study under starting acidified condition could be of significant help in future researches to focus the matter of PET-MPs pollution and to weight their potential toxicity for marine species. Recent papers suggested that ocean acidification is able to determine significant effects in marine species (Knutzen, 1981; Roleda et al., 2015). For example, it can increase physiological stress for marine shellfish and, consequently, affecting fertilization (Shi et al., 2017), metabolism (Zhao et al., 2017), behaviour (Peng et al., 2017), and immune function (Su et al., 2018). In addition, it is well know that microplastics are able to adsorb and leach hydrophobic compounds (Engler, 2012; Bergmann et al., 2015; Chen et al., 2019) and that such chemical equilibrium can be influenced by a wide range of variables; including pH fluctuations (Tourinho et al., 2019). Accordingly, PET toxicity could potentially change under different environmental conditions. Furthermore, it is supposed by the literature that particle size could represent a key factor modulating microplastic bioactivity and associated toxicity (Lee et al., 2013; Jeong et al., 2016; Kinjo et al., 2019). Therefore, the coexistence of acid stress and plastic pollution may produce a synergistic effect impacting severely the marine mussels as reported by literature (Shang et al., 2020).

This study aims to fill some of the exposed knowledge gaps testing the interactive biochemical stress induced in mussels (*Mytilus galloprovincialis*) by the exposure to three different sizes of environmentally realistic flakes (with jagged edges and surface irregularity) of PET-MPs under two different starting conditions of pH of water (8.0 and 7.5 pH). The water pH was corrected to 7.5 pH units at the beginning of the exposure and then it was allowed to the system to follow its natural evolution without water renewal or additional pH corrections in order to evaluate the resilience of the system after the initial hotspot perturbation. To evaluate stress induced, lipid peroxidation (LPO), enzymes involved in the oxidative stress responses (glutathione peroxidase GPx, superoxide dismutase SOD), and in neuro-status (cholinesterase activity analysing Acetylthiocholine, AtCh) were used as rapid and recognized biomarkers tools to detect the exposure to xenobiotics (Regoli et al., 2014; Tim-Tim et al., 2009).

PET microparticles tested in this study were obtained by the trituration of 1 mm pellets to a final shape of heterogeneous flakes, with jagged edges and surface irregularity that well simulate MPs particles present in environmental matrices (Lambert et al., 2017). Three desired particle-sizes: small (5–60 μm; S-PET), medium (61–499 μm; M-PET), and large (500–3000 μm; L-PET) were tested. Microparticles were preliminary rinsed to remove external contamination and chemically analysed by microscopy associated to Fourier Transform Infrared Spectroscopy (μFT-IR, Nicolet iN10, Thermo®) technique as extensively described by Piccardo et al. (2020) for the same PET-MPs tested in this study. Adult animals (n = 18 for treatment; 144 in total) were collected from natural unpolluted marine area (Talamone, Natural Park of Maremma, Tuscany), put in stabulating aquarium for acclimation before starting the experiment (Avio et al., 2015b). Then, their length were determined using a calibre to calculate the mean value of the maximum length of the valves. Selected animals, in order to reach a standardized mean valves length ranging within 3.2–3.5 cm, were distributed in glass jars with 1 L of natural marine water (9 mussels/jar; 2 jars for treatment; 111.1 mL water/mussel ratio). As reported in Table 1, mean length were controlled to keep homogeneous the distribution of specimen among jars with respect to body size. In fact, organisms with similar length have similar age and dimensions of body-structures (i.e. syphons, etc.), and the same possibilities to interact with PET-MPs. Following a previous study on microplastics in mussels, exposure time was fixed to 7 days (Avio et al., 2015b), while concentrations of microplastic were fixed to 0.1 g/L for each of the three tested particle-sizes of PET-MPs. Exposure doses are higher than plastic levels recorded in natural ecosystems (Lenz et al., 2016). Nevertheless, PET is considered a non-toxic plastic material (Singh et al., 2018) and we decided to use doses higher than natural ones to detect a clear signal after 7 days of exposure according to the

Table 1
Water parameters expressed as mean ± standard deviation. After 7 days of exposure, pH (t = 7) shows similar values regardless initial pH conditions. Mussels maximum shell length (mean ± standard deviation) were measured just before the starting of the exposure. Qualitative description of microplastic macroscopic behaviour in different testing jars: Starting time (t = 0), initially due to cohesion effect, PET-MPs are floating on the water; nevertheless, during the next day microparticles progressively settled on the bottom due to density and bio-secretion production by mussels. These phenomena occurred under both starting conditions; only recorded differences were related to particle size that favoured the settlement of L-PET earlier than others.

		Mussel size (mm)	Water parameters				
			pH t = 0 (pH unit)	pH t = 7 (pH unit)	Temperature (°C)	Oxygen (% sat.)	Salinity (PSU)
Standard	Control	33.6 ± 10.5	8.00 ± 0.01	**7.86** ± 0.04	17.40 ± 0.11	79.64 ± 0.09	39.29 ± 1.03
	S-PET	34.7 ± 8.0	8.01 ± 0.00	**7.54** ± 0.04	17.30 ± 0.07	80.52 ± 2.16	39.19 ± 0.82
	M-PET	33.4 ± 10.3	8.01 ± 0.00	**7.39** ± 0.00	17.40 ± 0.07	80.63 ± 9.44	39.33 ± 1.14
	L-PET	33.2 ± 10.3	8.00 ± 0.01	**7.50** ± 0.04	17.40 ± 0.11	77.31 ± 2.96	39.26 ± 1.16
Acidified	Control	33.8 ± 10.9	7.49 ± 0.00	**7.51** ± 0.04	17.33 ± 0.33	84.33 ± 13.61	39.59 ± 1.60
	S-PET	33.1 ± 10.8	7.51 ± 0.00	**7.45** ± 0.01	17.40 ± 0.14	87.57 ± 5.55	39.49 ± 1.50
	M-PET	33.9 ± 10.9	7.50 ± 0.01	**7.53** ± 0.04	17.33 ± 0.11	85.50 ± 4.67	39.69 ± 1.74
	L-PET	33.5 ± 10.5	7.49 ± 0.00	**7.48** ± 0.04	17.40 ± 0.07	85.35 ± 1.70	39.02 ± 1.60

principal aim of this study that was to relate biochemical responses to the particle size of PET and not to assess the environmental significance of the response. Natural marine 0.45 μm-filtered water collected from an unpolluted sampling site (Talamone, Natural Park of Maremma, Tuscany) was used as testing medium. Animals were fed with dry food before the beginning of the exposure. Acidified water (pH = 7.5) was obtained from the natural marine water by pH correction with HCl (100 μL of 0.1 M, ultrapure reagent, Sigma Aldrich); this correction was performed before adding the animals. To evaluate the natural evolution driven by animals' metabolic activities, initial pH characteristics of water was not corrected, and water was not renewed during the experiment. Principal parameters were recorded at time zero ($t = 0$), and at the end of the experiment ($t = 7$) and the natural evolution over time of pH, after the induced stress of initial acidification, was monitored. Observed fluctuations (mean; standard deviations) are reported in Table 1. In the same Table, a qualitative description of microplastic macroscopic behaviour is reported. At the end of the experiment, mussels from each jar were sacrificed by rapid excision, pooled together and stored in liquid N_2. Pools of animals were then homogenized keeping sample temperature under control and extracted using phosphate buffer and EDTA (Vidal-Linan et al., 2013) in order to collect cellular fraction (S9). Tissues were stored under liquid N_2 until analysis. On collected samples, the superoxide dismutase activity (SOD; Gao et al., 1998), the lipid peroxidation level (LPO; Mihara and Uchiyama, 1978), the glutathione peroxidase activity (GPx; Bocchetti and Regoli, 2006), and the cholinesterase activity (AtCh; Vidal-Linan and Bellas, 2013; Mennillo et al., 2017) were determined. LPO, GPx, and AtCh were expressed referred to the total protein contents fraction S9 according to the Lowry colorimetric method (Lowry et al., 1951). Control samples (negative control) were analysed in order to determine natural expression of targeted biomarkers both under 8.0 and 7.5 pH levels. Results reported in this study are expressed as percentage of variation of each measured biomarker compared to levels recorded in the correspondent negative control. Statistical analyses (ANOVA two-ways test) were performed by the software Prism® (GraphPad, v.4.0).

Concerning data reported in Table 1, the comparison among water pH levels measured at the beginning and at the end of the experiment, highlights that in controls animals' metabolic activities tend to weakly reduce water pH in samples that they were non-acidified at the beginning of the experiment (−0.14 pH units). On the contrary, in initial acidified samples, pH varied not significantly at the end of the experiment (+0.02 pH units). This could be considered a natural trend to acidification of water due to the physiological accumulation of waste metabolites produced by animals during the 7 days of exposure. Animals exposed to PET-MPs, showed a larger effect of acidification of water compared to controls. Concerning animals exposed to initial 8.0 pH, this effect ranged within −0.62 pH units (M-PET) and −0.47 (S-PET). This could be due to an increase of metabolites production in animals exposed to microplastics compared to negative controls. Concerning animals exposed to initial 7.5 pH, this effect was less evident. In our opinion, the less evident effect could be due to an increased buffering action driven by the mussel involving reactions affecting $CaCO_3$ balance to counteract water acidification. This effect is stimulated by the initial acidified condition as yet evidenced in a previous research on freshwater mussels (Heming et al., 1988). In fact, under acidified conditions, Heming et al. (1988) showed that the regulation of pH in acid water did not involve any valve closure rather the excess of protons was buffered using $CaCO_3$ reserves. Further and opportunely focused studies will be performed on this specific aspect to confirm that the exposure to microplastics could be associated to an increase in metabolite production by stressed mussels and that resulting acidification is counterbalanced using animal's $CaCO_3$ reserves keeping water pH closed to 7.5 units.

The recorded expression of biomarkers of stress in mussels exposed to PET-MPs of different size both under 8.0- and 7.5-units initial pH conditions is reported in Fig. 1; data are also associated to the significance recorded by the ANOVA two-ways test. Results on negative controls, highlighted a different sensitivity of considered biomarkers to the diverse initial pH conditions. In fact, only a very slight increase (not significant) of SOD and AtCh activity have been measured in specimen at pH 7.5. An opposite behaviour characterized the LPO and GPx activities in controls which showed an increase of 59.5% of lipid peroxidation and a reduction of 22.6% of glutathione peroxidase in the initial acidified condition (data not shown). For this reasons data have been normalized as percentage variations respect to each control. These results were partially agreed with recent literature recording no significant effects of acidification on GPx and SOD (Wang et al., 2020). These differences may be due to multiple factors. First of all, the initial exposure pH in the study of Wang et al. (2020) was 7.7 units while, in our study, it was 7.5. Furthermore, the exposure time used by the cited study was 14 days followed by 7 days of recovery; on the contrary, the exposure time used in this study was 7 days followed by immediate animals sacrifice at the end of the experiment according to Avio et al. (2015b). Furthermore, in the case of the aforementioned study, the tests were conducted only on the digestive glands while, in this research, the whole animal was used. The foregoing may have determined the differences found between the two studies with respect to GPx.

The same sensitivity is confirmed by animals exposed to PET: no significant effects were detectable in SOD and AtCh activity regardless of standard or acidified starting conditions (Fig. 1). Even if our results agree with Wang et al. (2020), the lack in significant alteration of AtCh activity could be due to the selection of tissue for the analysis. We evaluated the AtCh activity in the whole animal rather than in adductor muscle, as done by Guilhermino et al. (2018); our choice could have decrease the responses. Counter wise a general increase of LPO expression (up to 210%) was recorded in specimens tested under initial 8.0 pH conditions. In particular, higher expressions were recorded after the exposure to L-PET. Smaller effect was recorded for initial pH 7.5. The expression of GPx (8.0 pH conditions) was very variable and PET size-depending: animals exposed to S-PET showed a reduction of the enzyme activity (53.4%) while specimen exposed to L-PET highlighted an increase of 25.6%. A similar behaviour was recorded for initial 7.5 pH. Our results confirmed the elevated sensitivity of certain enzymes: among these, GPx and LPO. Similar results have been reported by other experiments performed with different polymers (Magni et al., 2018; Paul-Pont et al., 2016). LPO and GPx levels of plastic-exposed specimen were lower under acidified conditions than under natural pH for marine water. These results could be explained by a larger production of mucous proteins recorded at the end of the experiment in animals exposed under acidified conditions that could trap microplastic preventing their ingestion and reducing animals' intake and, consequently, the activation of biological responses. Results reported in this paper highlighting a PET-induced stress (LPO, GPx) under the tested exposure doses (0.1 g/L, 7 days) are partially agreeing with results reported by Wang et al. (2020). These last cited authors evidenced effects related only to catalase and glutathione during the experiment, that were associated to a quick recovery once MPs stressors were removed; furthermore, no significant effects on GPx and SOD were recorded. Even in this case differences among the exposure time, microplastics sizes, and their chemical types could be responsible of recorded differences. Furthermore, biomarkers pathways have a different time-course of activation and antioxidants levels

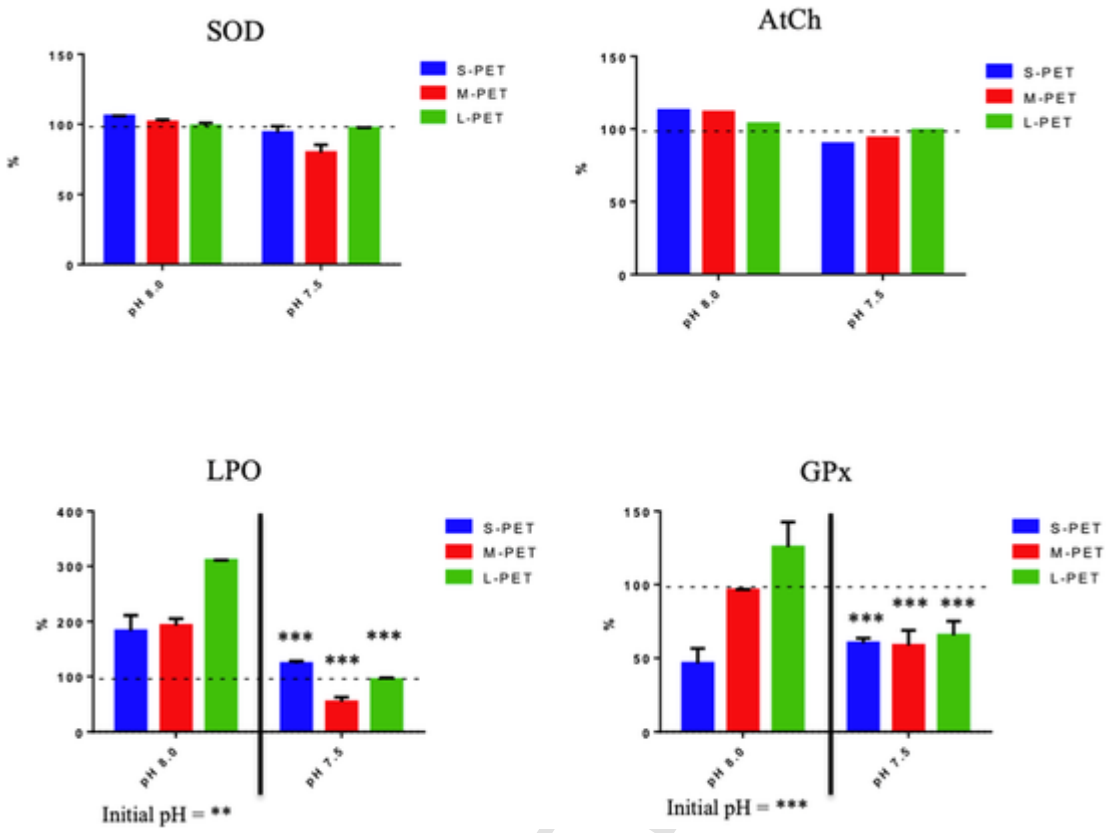

Fig. 1. Biomarkers in mussels. Results are grouped according to different size of PET-MPs (S-PET; M-PET; L-PET) and initial pH of water (8.0 vs 7.5 units). Results are given as percentage respect to negative controls (represented by dashed lines; mean values ± CV%). CV% in AtCh is not visible because it resulted very small. SOD = superoxide dismutase; AtCh = cholinesterase; LPO = lipid peroxidation; GPx = glutathione peroxidase. ANOVA two-ways significance are reported in figure.

measured in animals could vary in a not synchronous way; in particular, as reported by literature, some defences can increase, while other decrease or do not vary at all (Regoli et al., 2014). Even if the reduction of particle size is associated by literature on nanoparticles to an increasing of toxicity (Jeong et al., 2016; Lu et al., 2016; Karami et al., 2017; Cui et al., 2017), our study showed a different effect highlighted that the highest LPO and GPx expression were associated to the exposure to L-PET (larger particle size). A previous study performed on mussels selected within population from different geographical areas, highlighted that fibres of a mean of 1150–2290 μm (range 750–6000 μm of maximum length) represent the particle size class trapped in mussels' tissues (Renzi et al., 2018b); the retained particle size is comparable to L-PET (500–3000 μm) recorded in this study. Results obtained in this study showed a significant interaction among PET-MPs dimensions and initial pH (LPO, $p < 0.0001$; GPx, $p = 0.0002$) suggesting the occurrence of specific and case-by-case interactions that cannot be a priori predicted and that need of more specific researches. Further researches, sized to evaluate both retention time of mussel according to different MPs particle-size and associated biochemical stress, will be performed. Furthermore, according to the literature, further studies will be performed to evaluate biochemical responses recorded when animals are exposed to PET-MPs levels similar to levels measured in marine environments. A larger biomarkers pathways than those tested will be explored to evaluate statistical significance of reported associations and responses (Regoli et al., 2002) and to better understand recorded differences among literature and our results.

Summarizing, even if further researches are needed, results obtained in our study show that: i) PET microplastic are able to induce after 7 days of exposure biochemical stress in mussels; ii) among tested biomarkers, LPO and GPx resulted to be sensitive enzymes to both pH initial hotspot stress and plastic contamination; iii) biomarkers expression resulted affected by microparticle size and, in particular, higher expressions resulted associated to L-PET; iv) animals tested under initial 7.5 acidified pH conditions resulted less stressed than animal tested under natural pH condition; v) results opposite to other studies highlight the case-by-case occurrence of responses and the impossibility to generalize attended effects.

CRediT authorship contribution statement

Francesca Provenza: Investigation, Data curation, Writing - original draft.**Manuela Piccardo:**Methodology, Investigation, Data curation, Writing - original draft.**Antonio Terlizzi:**Supervision, Writing - review & editing.**Monia Renzi:**Conceptualization, Writing - review & editing, Funding acquisition.

Uncited references

Berit et al., 2015
De Marchi et al., 2017
Dubois et al., 1956
Raven, 2005
Regoli and Pellegrini, 2014

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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