Oxidative stress related effects induced by micronized polyethylene terephthalate microparticles in the Manila clam

Marco Parolini[a*], Beatrice De Felice[a], Stefano Gazzotti[b], Luisa Annunziata[b], Michela Sugni[a], Renato Bacchetta[a], Marco Aldo Ortenzi[b]

[a] Department of Environmental Science and Policy, University of Milan, via Celoria 26, I-20133 Milan, Italy

[b] Laboratory of Materials and Polymers (LaMPo), Department of Chemistry, University of Milan, via Golgi 19, I-20133, Milan, Italy

[*] Correspondence should be addressed to Marco Parolini (marco.parolini@unimi.it)

Abstract

Microplastic (MP) contamination represents a serious threat for marine organisms. Several laboratory studies have highlighted the adverse effects caused by the exposure to different MP polymers towards diverse marine species. However, the information of the toxicity of polyethylene terephthalate (PET) MPs is largely unknown. The present study was aimed at investigating the adverse effects induced by 7-days exposure to two concentrations (0.125 and 12.5 μg/mL) of micronized, irregular shaped and variable size PET microparticles (PET-MPs) towards the Manila clam (*Ruditapes philippinarum)*. Histological analyses were performed to assess tissue damage on digestive glands, gonads, gut and gills, whereas oxidative stress related effects, namely the amount of pro-oxidant molecules, the activity of antioxidant (superoxide dismutase - SOD, catalase – CAT and glutathione peroxidase - GPx) and detoxifying (glutathione S-transferase - GST) enzymes, and the levels of lipid peroxidation, were investigated in gills and digestive gland. Our results showed that clams can ingest and egest micronized PET-MPs, but no histological alterations to bivalve tissues occurred. Although PET-MPs did not cause oxidative stress in the digestive gland, they significantly altered the oxidative status of gills, leading to lipid peroxidation. No clear indication of a weakness of bivalve health status was obtained in this study.

Keywords: Manila clam; microplastics; oxidative stress; polyethylene terephthalate (PET)

1. Introduction

In recent years plastics contamination has raised as a worrisome concern for aquatic ecosystems. Almost 10% of the annual plastic production contributes to marine contamination, so that the accumulation of plastic debris has been identified as a global environmental problem (Barnes et al., 2009). Growing interest has raised on microplastics (MPs), which are described as any plastic item < 1 mm (Browne et al., 2008). Microplastics are ubiquitous in marine ecosystems and represents one of the main threat that they have to face (Eriksen et al., 2014). Over 5 trillion microscopic plastic fragments are estimated to float into the oceans worldwide (Eriksen et al., 2014), conferring to them the role of the most numerically abundant oceanic debris (Law and Thompson, 2014). A growing number of studies has found the presence of different MP polymers in both abiotic (water and sediments) and biotic (zooplankton, mussels and fish) matrices (see Cole et al., 2013), from beaches and coastlines, to subtropical oceanic gyres and remote areas, including polar ice caps and the deep ocean (e.g., Wright et al., 2013; Law and Thompson, 2014; Cole et al., 2014). Depending on their size, shape and chemical composition, MPs assume different positions and behaviours along the water column, being involuntary ingested or predated by a vast range of marine species that mistake them for natural food/preys (Galloway et al., 2017). A number of studies has demonstrated that the ingestion of MPs can induce a series of sub-lethal effects towards aquatic organisms though direct and indirect processes, including the decrease of food uptake (Blarer and Burkhardt-Holm, 2016), the onset of oxidative stress (Alomar et al., 2017; Magara et al., 2018; 2019) and inflammation (Lu et al., 2016), as well as developmental alterations (Messinetti et al., 2018) and decrease in growth and reproduction rate (Sussarellu et al., 2016; Lo and Chan, 2018). In contrast, other studies have reported slight or null effects due to MPs ingestion (e.g., Hämer et al., 2014; Kaposi et al., 2014; Imhof et al., 2017; De Felice et al., 2018; Weber et al. 2018). A recent meta-analysis of the effects due to MPs, showed negative effects for consumption, growth, reproduction, and survival of fish and aquatic invertebrates but, simultaneously, many of the effects were neutral, confirming an inconsistency and

a high variability of response across taxa (Foley et al., 2018). For these reasons, exploring the effects of diverse MP polymers represent a priority to understand their real risk towards aquatic organisms. Of particular concern is the exposure and the subsequent effects due to MPs in filter-feeder species such as bivalves, whose high filtration activity during their normal breathing and feeding activity confers them a unique capability to ingest high amount of MPs. For this reason, bivalves have been proposed and used as suitable indicator organisms of MP pollution (Van Cauwenberghe et al., 2015; Wesch et al., 2016). The presence of MPs has been predominantly documented in mussels, whereby up to ~ 2 particles/g of diverse µP items have been detected in both farmed and wild mussels from European countries and fishery market of China (Mathalon and Hill, 2014; Van Cauwenberghe and Janssen, 2014; Li et al., 2015; Van Cauwenberghe et al., 2015). However, MPs has been also detected in other bivalve species, including the Manila clam (*Ruditapes philippinarum*), the ark clam (*Scapharca subcrenata*) and oysters (*Alectryonella plicatula*), in concentration ranging between ~3 and ~ 11 particles/g (Li et al., 2015). Considering the amount of MPs found in bivalves tissues, several laboratory studies have investigated the adverse effects caused by the ingestion of MPs, differing in polymeric composition and size. Most of such studies have been performed on mussels, exploring the uptake and the sub-lethal toxicity of uniform, spherical polyethylene (PE) or polystyrene (PS) MPs (e.g., Lusher, 2015), two of the most abundant polymers in the environment (Wagner et al., 2014). However, the ingestion and the toxicity of MPs having different polymeric composition rather than PE and PS have been scarcely investigated because of lack of commercial standards (Paul-Pont et al., 2018). Thus, to mimic real particles in the environment, MPs of various size and shape can be created to reproduce exposure conditions that organisms experience in the wild. The aim of the present study was to assess the ingestion and the potential adverse effects induced by the exposure to micronized polyethylene terephthalate (PET) particles towards *R. philippinarum*. Considering the high density of PET (1.38 g/cm^3), we decided to test the potential toxicity of PET-MPs towards the Manila clam, a bivalve species living sunken in marine sediments that could be exposed to this kind of polymer. Manila clams were exposed for 7 days to two concentrations (0.125 and 12.5 µg/mL) of PET

microparticles (hereafter PET-MPs). The ingestion of PET-MPs and tissue damage were investigated by histological analyses, while oxidative stress-related effects were investigated on the gills and the digestive gland by the measurement of a suite of six different biomarkers. In detail, the amount of pro-oxidant molecules, the activity of antioxidant (superoxide dismutase - SOD, catalase – CAT and glutathione peroxidase - GPx) and detoxifying (glutathione S-transferase - GST) enzymes, and the levels of lipid peroxidation were investigated in both the target organs.

2. Materials and Methods

2.1 Experimental plan

Specimens of *R. philippinarum* were purchased on May 2019 in a fish shop and quickly transferred to aquaria located in the facility of the University of Milan. Aquaria were filled by circulating artificial seawater (Instant Ocean; salinity about 37‰) under constant aeration, temperature (14 °C) and photoperiod (16-hrs light: 8-hrs dark). Clams were left in aquaria without sediment for 1 week to allow their acclimation to laboratory conditions. Clams showed a good health status and a very low mortality occurred during the acclimation period (< 3%). Clam were exposed to PET microplastics because PET is predominantly used as packaging material and accounts for up to 7.1% of the total European plastic consumption (PlasticsEurope, 2014). Studies by Klein et al. (2015) and Gasperi et al. (2014) have highlighted that PET microplastics, though not as dominant as the PE and PP polymers, notably contributes to the overall MP load in large European river systems. However, PET microparticles are one of the least used plastic polymer among the studies on MP effects towards marine organisms (de Sà et al., 2018). As no consistent information concerning the concentration of PET-MPs in marine environments is available, we arbitrarily decided to test two concentrations similar to those administered to other aquatic organisms in previous laboratory studies assessing the toxicity of MPs (Messinetti et al., 2018; De Felice et al., 2018; 2019). Fifteen specimens (~ 4 cm in length) were seeded in 5 L glass beakers filled with 4 L of the same artificial seawater circulating in the acclimation aquaria and exposed for 7 days to two concentrations (0.125 and 12.5 µg/mL) of

119 micronized PET-MPs. Specimens from the control group were maintained in 5 L glass beakers with
120 artificial seawater only. The exposures were performed under semi-static conditions. Specimens of
121 *R. philippinarum* were laid down on a stainless grid placed close to the bottom of the tank. Artificial
122 seawater was renewed every single day and the selected amount of PET-MPs was added to the
123 exposure aquaria. Clams were fed for 1 hour with Algamac2000® (Aquafauna Bio-Marina, USA), an
124 algae replacement-substitute-enrichment medium consisting of spray-dried cells of *Schizochytrium*
125 spp., before the renewal of the exposure conditions. Three independent replicates (= beakers) per
126 experimental group were performed. Moreover, considering the low amount of PET-MPs used in the
127 present study, in order to confirm the MPs uptake by *R. philippinarum*, an additional 1-day exposure
128 to a highest, unrealistic PET-MPs concentration (50 µg/mL) was performed. Because of the lack of a
129 PET-MP analytical standards, particles used in the present study were obtained by mechanically
130 grinding commercial bottle-grade PET chips (Invista 1101 PET) with a blade grinder. This procedure
131 allowed to obtain MPs mimicking a realistic exposure scenario, whereby secondary PET-MPs derive
132 from breakage and erosion of plastic bottles. A commercial bottle-grade PET chips was frozen in
133 liquid nitrogen and after grinding, particles were passed through a 1 mm sieve to select only items
134 included in the MPs range (Browne et al., 2008). Resulting PET-MPs had irregular shape and a size
135 ranging between 8 and 1,054 µm in length (mean length 220 µm; Figure 1a). The relative percentage
136 for each dimensional class composing our PET-MPs standard was measured in a subsample of the
137 resulting grinded MPs (n = 500 particles) and was the following: < 10 µm = 1%, < 50 µm = 28 %; 50
138 < µm < 100 = 24 %; 100 < µm < 1,000 = 47 %. The polymeric composition of micronized PET-MPs
139 was assessed by using a Fourier Transformed Infrared Spectroscope (FT-IR) Perkin Elmer Spectrum
140 100 (Figure 1b).

141 *2.2 Histological analyses*

142 For the histological analyses, five clams from each experimental group were randomly sampled and
143 their whole body was fixed in Bouin's fluid for about one week. Then, the remaining proteinic shell

144 portions were removed under the stereomicroscope, the whole specimens dehydrated in an ascending
145 alcohol series and embedded in ParaPlast Plus tissue embedding medium (Sigma-Aldrich, Italy).
146 Using a Reichert rotary microtome, all fixed clams were cut in 7 μm transverse sections at different
147 levels throughout the whole specimen. Ten serial sections after about every 150 sections, i.e., 1 mm,
148 were placed on microscope slides and left to dry overnight at 37 °C. Slides were stained with Mayer's
149 Haemalum and Eosin, mounted in Eukitt (Kindler GmbH, Freiburg) and observed under a Leica
150 DMRA2 light microscope equipped with a Leica DC300F digital camera. All clams were observed
151 and analyzed microscopically for the occurrence of diseases and inflammatory response.

152 *2.3 Methods of oxidative stress biomarkers*

153 Nine clams per each treatment were dissected under the stereomicroscope, the gills and the digestive
154 glands were isolated, quickly frozen in liquid nitrogen and maintained at -80 °C until the analysis of
155 oxidative stress biomarkers. The suite of biomarkers was applied to homogenates of gills and
156 digestive glands dissected by Manila clams according to the methods described by Parolini et al.
157 (2013; 2017). Gills or digestive glands from three specimens exposed in the same beaker (~ 0.5 g)
158 were pooled and homogenized by an automatic homogenizer in 100 mM phosphate buffer (pH 7.4),
159 with the addition of KCl (100 mM), EDTA (1 mM), protease inhibitors (1:10 v/v) and dithiothreitol
160 (100 mM). Three pools were performed per each treatment, for a total of nine organism per treatment.
161 The amount of reactive oxygen species was measured according to the dichlorofluorescein-diacetate
162 (DCFH-DA, 10 mg/mL in DMSO) method (Deng et al., 2009). The activity of SOD, CAT, GPx and
163 GST was assessed on the supernatant (S9 fraction) obtained by the centrifugation at 16,000 × g for
164 30 min of raw homogenate for both the organs, according to spectrophotometric methods (Parolini et
165 al., 2010). Total protein content was determined according to the Bradford method (1976). In brief,
166 the inhibition of cytochrome c (10 μM) reduction due to the superoxide anion generated by the
167 reaction between xanthine oxidase (1.87 mU/mL) and hypoxanthine (50 μM) was assessed at $\lambda = 550$
168 to measure SOD activity. The CAT activity was assessed by monitoring the consumption of H_2O_2

(50 mM) at λ = 240 nm. The consumption of NADPH (120 μM) in a solution containing 0.2 mM H_2O_2 as a substrate in 50 mM potassium phosphate buffer (pH 7) containing glutathione (2 mM), sodium azide (NaN_3; 1 mM), glutathione reductase (2 U/mL) was monitored at λ = 340 nm to assess GPx activity. The GST activity was evaluated by monitoring the reaction among the sample, reduced glutathione (1 mM) and 1-chloro-2,4 dinitrobenzene for 1 min at λ = 340 nm. Lipid peroxidation was measured according to the thiobarbituric acid reactive substances (TBARS) method (Ohkawa et al., 1979).

2.4 Statistical analysis

Linear mixed models (LMMs) were used to assess the effects induced by the exposure to PET-MPs. The treatment was included in the models as a fixed effect factor, while the identity of the exposure beaker as a random factor. We used biomarker responses obtained by the processing of three pools of gills or digestive glands isolated from three organisms sampled in each of the three experimental replicates (exposure tanks) we prepared for each experimental condition (n = 3 replicates). All the analyses were run by means of SPSS 21.0 statistical package.

3. Results

Histological analysis did not show the presence of PET-MPs in the digestive tract of Manila clams exposed to both the tested concentrations.

No alteration of the histological structure of the digestive tract walls was noted. In fact, samples from both controls and treated groups showed a regular monolayer of high columnar epithelial cells with eosinophilic cytoplasms and elliptical nuclei (Figure 3A-C). The basal portion of these cells laid on a thin basement membrane which identified an underneath layer of connective tissue (lamina propria). On the contrary, the cell membrane along the apical portion of the cells appeared as a thick line, with overlying bristle-like cilia that extended into the gut lumen.

192 The analysis of the suite of oxidative stress biomarkers performed on the gills and the digestive gland
193 showed contrasting results. The exposure to PET-MPs did not induce a significant modulation of the
194 oxidative status of clam digestive gland (Figure 4a-f). PET-MPs treatments did not induce a
195 significant overproduction of reactive oxygen species ($F = 1.555$; $p = 0.316$; Figure 4a). Accordingly,
196 except a significant effect on GPx activity ($F = 39.086$; $p = 0.002$; Figure 4d), with a significant
197 inhibition at the end of the exposure to the highest tested concentration ($p = 0.003$), the activity of
198 SOD ($F = 0.156$; $p = 0.859$; Figure 4a), CAT ($F = 0.160$; $p = 0.587$; Figure 4b) and GST ($F = 4.071$;
199 $p = 0.109$; Figure 4e) was not affected by PET-MPs exposure in comparison with controls. Moreover,
200 no significant alterations of lipid peroxidation levels occurred between treated and control specimens
201 ($F = 1.345$; $p = 0.347$; Figure 4f). Even histological analyses of the digestive glands did not reveal
202 significant differences between controls and exposed groups, both showing intact digestive tubules
203 with regular and well distinguishable digestive and basophilic pyramidal cells. In samples from all
204 the experimental groups the epithelium of the digestive gland did not show any alteration; on the
205 contrary it appeared well comparable to that of controls with well-nourished digestive cells and
206 basophilic pyramidal cells assembled to form crypts.
207 In contrast, the exposure to PET-MPs exposure significantly altered the oxidative status of clam gills
208 (Figure 5a-f). Although the exposure to PET-MPs did not significantly modulate the amount of
209 reactive oxygen species ($F = 3.853$; $p = 0.084$), the activity of antioxidant enzymes was significantly
210 modulated by the exposure to the highest PET-MPs concentrations (Figure 4b-e). Whilst the activity
211 of SOD ($F = 3.356$; $p = 0.119$; Figure 4c) and GST ($F = 1.396$; $p = 0.318$; Figure 5e) measured in
212 PET-MPs exposed clams did not significantly differ with respect to control, a significant increase of
213 CAT activity ($F = 6.005$; $p = 0.037$; Figure 5c) was found. In contrast, a significant inhibition of GPx
214 ($F = 6.712$; $p = 0.029$; Figure 5d) was measured in gills from clams exposed to the highest tested
215 concentration compared to control ($p = 0.013$). A significant increase of lipid peroxidation levels was
216 measured ($F = 8.695$; $p = 0.017$; Figure 5f) in gills from clams treated with the highest PET-MPs
217 concentration compared to controls. Despite these findings, no histological effects on gill structure

and integrity were found. Gills from both controls and treated groups showed the typical structure of the bivalve *ctenidium*, with strictly folded filaments lined by a single layer of ciliated and secretory cells. Inside the filaments, the water channels and the haemal sinuses were evident, these latter only occasionally showing few haemocytes (Figure 3D-F). Anyway, no differences in the number of haemocytes among the experimental groups were recorded. Histological analyses were also performed on gonads of all samples, in order to look for possible effects of PET-MPs on reproduction. Samples from both treated groups showed testes and ovaries well comparable to those observed in controls, with no evident signs of haemocyte infiltration or gonad degeneration. Also looking at the gonadal stages of the samples, no differences were recorded among the groups suggesting that 7-days exposure to PET-MPs did not substantially alter the reproductive biology of *R. philippinarum*.

4. Discussion

The present study showed that micronized PET-MPs having irregular shape and variable length was ingested and induced the imbalance the oxidative status and the onset of oxidative damage in the gills, but not in the digestive gland of the Manila clam. On the contrary, the histopathological analyses of gills, digestive gland, gonads and gut did not reveal differences with respect to controls. The uptake and tissue distribution of MPs have been described in diverse mussel species, mainly in the blue mussel, in both field and laboratory studies (e.g., Mathalon and Hill, 2014; Van Cauwenberghe and Janssen, 2014; Li et al., 2015; Van Cauwenberghe et al., 2015), demonstrating that gills, stomach, intestine and digestive tubules are the main sites of MPs accumulation for high density PE (HDPE) and PS (Browne et al., 2008; Von Moos et al., 2012; Avio et al., 2015). Such studies observed aggregates of MPs within the intestinal lumen and digestive tissues, while a limited occurrence was found in branchial epithelial cells. Moreover, plastic items smaller than 3.0 or 9.6 μm were also found in the haemolymph and inside the haemocytes after translocation from the gut cavity (Browne et al.,

2008). Similarly, PS microplastics were accumulated in the gills and digestive gland of the peppery furrow shell *Scrobicularia plana* (Ribeiro et al., 2017), while PS microbeads (2 and 6 µm in diameter) were found only in the digestive gland of the Pacific oyster *Crassostrea gigas* (Sussarellu et al., 2016). In the present study we did not found PET-MPs on the gills Manila clams treated with the low tested concentrations, while the presence of PET-MPs was shown in the digestive tract of specimens exposed to the highest concentration (50 µg/mL; Figure 2). It must be considered however that the highest concentration was unrealistic, but also that the exposure to such a concentration was very short. The low amount of particles found in clams could be due to the low concentrations of MPs used in our experiments compared to studies performed on other mussel species and/or to the different density of PET (1.38 g/cm^3) with respect to PS (1.04 g/cm^3) and PE (0.88–0.96 g/cm^3). Because of the high density of PET, MPs (mainly the bigger ones) rapidly sinks at the bottom of the exposure tanks, resulting less available for filter-feeder species than PE and PS, which are both prone to float in the water column and do not (PE) or need up 24 hours to sediment (PS; Ribeiro et al., 2018). Alternatively, as bivalves exert a limited control on the types of filtered particles, the limited amount of PET-MPs found in the digestive tract, coupled with the lack of particles on gill surface, might suggest a sorting capacity of clams, allowing them to discriminate prior to ingestion unfavorable particles that are subsequently rejected as pseudofaeces (Gosling, 2003; Ward and Shumway, 2004). According to this hypothesis, we can reasonably suppose that PET-MPs were ingested and egested by the clams, In fact, a number of PET-MPs visible to the naked eye and wrapped in mucus were ejected as pseudofaeces through the inhalant siphon by treated clams. As most of PET-MPs were > 100 µm n length, we cannot exclude that a limited amount of small MPs were ingested by clams. According to this hypothesis,, the additional exposure to a highest, unrealistic concentration (50 µg/mL) pointed out the capability of clams to ingest PET-MPs, as showed by the presence of microparticles within their digestive tract (e.g., the ~ 60 µm PET-MPs showed in Figure 2).

Considering the negligible amount of PET-MPs ingested by clams over the 7-days exposures, we can reasonably exclude that their presence in the digestive tract might impair the digestive system with a

consequent decrease of feeding behavior and growth as showed by previous studies of invertebrates (see review by Foley et al., 2018). However, the ingestion of PET-MPs can result in detrimental sub-lethal effects due to chemical or physical damage, as demonstrated in previous studies on other mussel species (von Moos et al., 2012; Wright et al., 2013; Avio et al., 2015; Nobre et al., 2015). In fact, in marine environments, MPs occur in a wide range of shapes, from fibers to irregular fragments, spheres and rods, which can affect the potential for the physical adverse effects induced by different plastic polymers towards aquatic organisms (Wright et al., 2013). Most of studies that investigated the potential toxicity of MPs towards marine organisms used particles having regular shape and reported null or negligible effects (Foley et al., 2018 and references therein). For instance, a 7-days exposure of *M. galloprovincialis* specimens to 20 g/L of virgin PE and PS microparticles (<100 µm) showed that both polymers were ingested and accumulated in haemolymph, gills and digestive gland, leading to a significant decrease of lysosomal membrane stability and an increase of DNA fragmentation in haemocytes (Avio et al., 2015). Although the destabilization of lysosomal membrane could be related to the over-production of pro-oxidant molecules (Canesi et al., 2002; Jovanovic and Palic, 2012), the antioxidant defenses were not significantly modulated in the mussel digestive gland, except for a significant inhibition of Se-dependent glutathione peroxidases observed at the end of the exposure to virgin PS microparticles (Avio et al., 2015). In the present study, the onset of oxidative stress was investigated on the gills because, being involved in nutrient uptake, digestion and gas exchange, they are continuously brushed by seawater and represent the first organ to be exposed to different waterborne contaminants (de Oliveira David et al., 2008), while the digestive gland is the main organ involved in digestion/assimilation and in phase I and II pathways of xenobiotic metabolism (Livingstone et al., 1994). For these reasons, both the organs can be prone to suffer contaminant-induced oxidative stress. However, the exposure to irregular shaped PET-MPs could return different results. In fact, PET-MPs administered to clams showed a variety of shapes, with serrated or sharp extremities (Figure 1a), which might result in physical damage to clam tissues and, simultaneously, to the onset of inflammatory responses and oxidative stress situation. In contrast to

294 our expectation, the exposure to our PET-MPs did not induce an oxidative stress situation in the
295 digestive gland of Manila clams, as neither the overproduction of pro-oxidants nor a modulation of
296 antioxidant responses and GST, with the exception of a significant inhibition of GPx, nor a change in
297 the levels of lipid peroxidation were observed (Figure 4). However, opposite responses were noted in
298 the gills, whereby the exposure to the highest PET-MPs concentration induced a modulation of the
299 oxidative status, as observed by the significant increase of CAT and the significant inhibition of GPx
300 activity (Figure 5). Changes in the activity of these enzymes, which play a concurrent role in the
301 removal of hydrogen peroxide (Regoli and Giuliani, 2014), might suggest a production of this pro-
302 oxidant molecule. The lack of activation of SOD might suggest an inhibitory effect and/or negative
303 feed-back caused by hydrogen peroxide, suggesting that SOD reaction already produced cytosolic
304 H_2O_2 (Vlahogianni and Valavanidis, 2007). Alternatively, H_2O_2 might be originated by the
305 spontaneous dismutation of superoxide radical through non-enzymatic pathways (Gwoździński et al.,
306 2010) or by other cellular enzymes like those included in peroxisomes (Khessiba and Roméo, 2005).
307 Whilst GPx is mainly responsible for eliminating the H_2O_2 produced by metabolic processes, CAT
308 acts also toward the exogenous source of this molecule (Avio et al., 2015). The contemporary,
309 opposite modulation of these enzymes in the gills suggests that PET-MPs induced an overproduction
310 of H_2O_2. We might suppose that the activation of CAT was able to efficiently counteract the onset of
311 pro-oxidants induced by PET-MPs, explaining the lack of changes in ROS levels observed in treated
312 groups compared to controls. The inhibition of GPx activity may be due to the competition for the
313 same substrate with CAT (Kappus, 1985). Alternatively, as CAT is activated only at high H_2O_2
314 concentrations (Pereira et al., 2013), while GPx acts also at lower substrate levels, the significant
315 decrease of GPx activity suggest a possible excess of hydrogen peroxide that this enzyme cannot
316 offset. This hypothesis was supported by similar trends of CAT and GPx observed in the gills of *M.*
317 *galloprovincialis* exposed to 250 ng/L of ibuprofen (Gonzalez-Rey and Bebianno, 2011), as well as
318 in the freshwater zebra mussel (*Dreissena polymorpha)* after the exposure to 8,000 ng/L of the same
319 pharmaceutical (Parolini et al., 2011). The imbalance of the oxidative status induced by a short-term

exposure to PET-MPs in Manila clam gills might lead to an oxidative stress situation, with detrimental consequences to cellular macromolecules, including lipids, proteins and DNA. The significant increase of lipid peroxidation levels observed in clam gills at the end of the exposure to the highest tested concentration (Figure 5) suggest that the exposure to PET-MPs can induce oxidative damage to lipids and lead to cellular alteration, including disruption of the cell membrane (Yajima et al., 2009). Thus, we may speculate that the contact or rubbing of PET-MPs with the gill surface induces an inflammatory, and consequently, an oxidative stress response in this organ, even though no histological effects were noted.

5. Conclusions

Our results suggest that short-term exposure to low concentrations of micronized, irregular shaped and variable size PET-MPs might represent a threat for the health status of Manila clams. In fact, although these MPs did not modulate the oxidative status of clams' digestive gland, an oxidative stress situation occurred in the gills, which did not show histological alterations. Considering that organisms are exposed to MPs for their whole lifespan, long-term exposure to PET-MPs should be necessary to confirm the hazard of these particles towards filter-feeders organisms. Moreover, because of the high density of PET, PET-MPs tend to deposit on marine sediments, representing a potential threat for benthic organism with different feeding strategy, such as grazers. For these reasons, further investigations on the potential adverse effects caused by the exposure to irregularly shaped PET MPs towards marine organisms represent a priority in marine ecotoxicology. Indeed, irregular shaped with different size MPs are predominant in marine ecosystems and might represent a major threat for free-living organism compared to regular shaped MPs, whose toxicity was commonly tested under controlled laboratory conditions.

Author contribution

Marco Parolini: Conceptualization, Writing - Original Draft, Supervision; Beatrice De Felice: Investigation; Stefano Gazzotti, Luisa Annunziata: Investigation; Michela Sugni: Investigation, Renato Bacchetta: Investigation; Marco Aldo Ortenzi: Investigation.

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Figures and Figure captions

Figure 1: Scanning electron microscopy (SEM) image (a) and Fourier Transformed Infrared Spectroscope (FT-IR) spectrum (b) of micronized polyethylene terephthalate microplastics (PET-MPs).

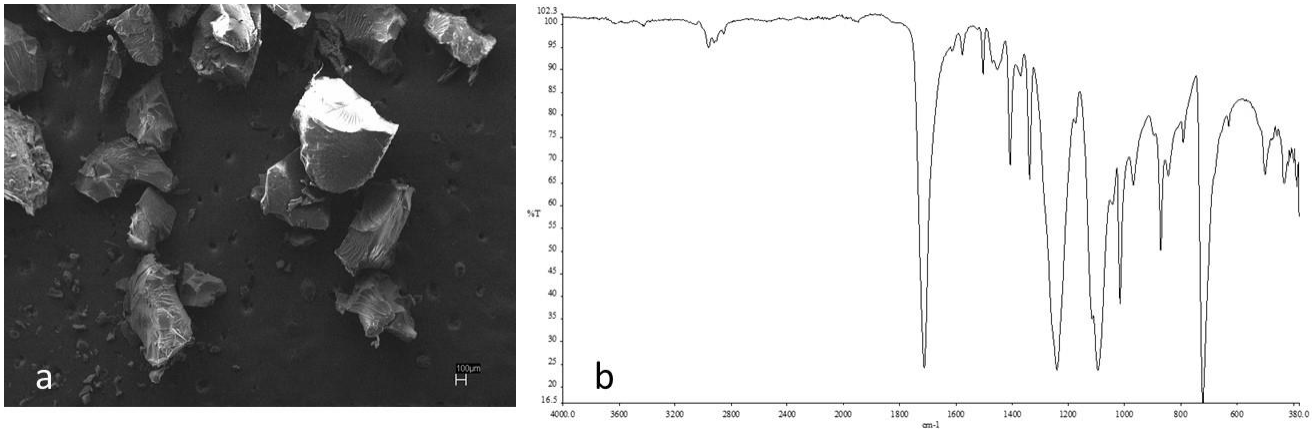

Figure 2: Histological cross sections of the digestive system from control (A) and micronized 50 µg/mL PET-MPs exposed *Ruditapes philippinarum* specimen (B). A PET-µP fragment is visible in panel (B) inside the gut lumen (arrowhead). T = typhosole; t = testis; ov = ovary.

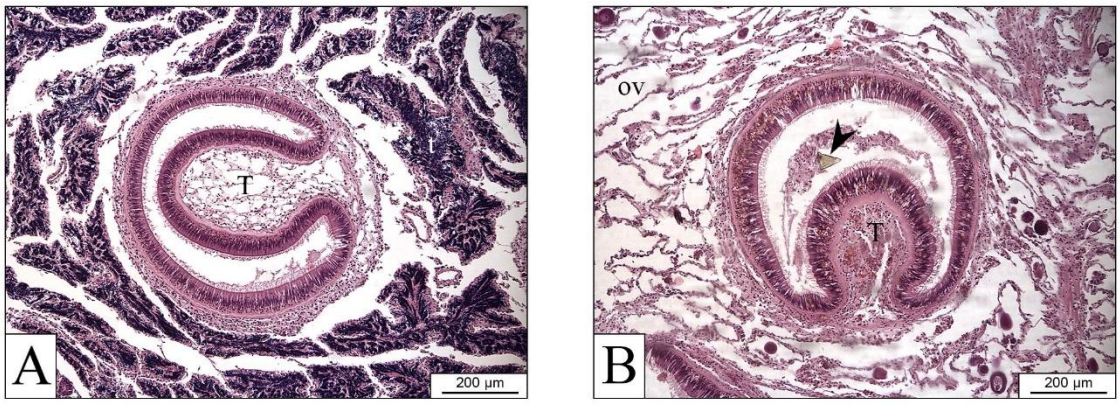

Figure 3: Details of the type 1 epithelium at the crystalline style sac level (A-C), and the gill filaments (D-F) of Ruditapes philippinarum. The digestive epithelium shows the characteristic columnar cells with their bristle-like cilia, and gills their internal structure. * = lumen; → = bristle-like cilia; cep = columnar epithelium; o = oocyte; GF = gill filament; wc = water channel; hs = haemal sinus.

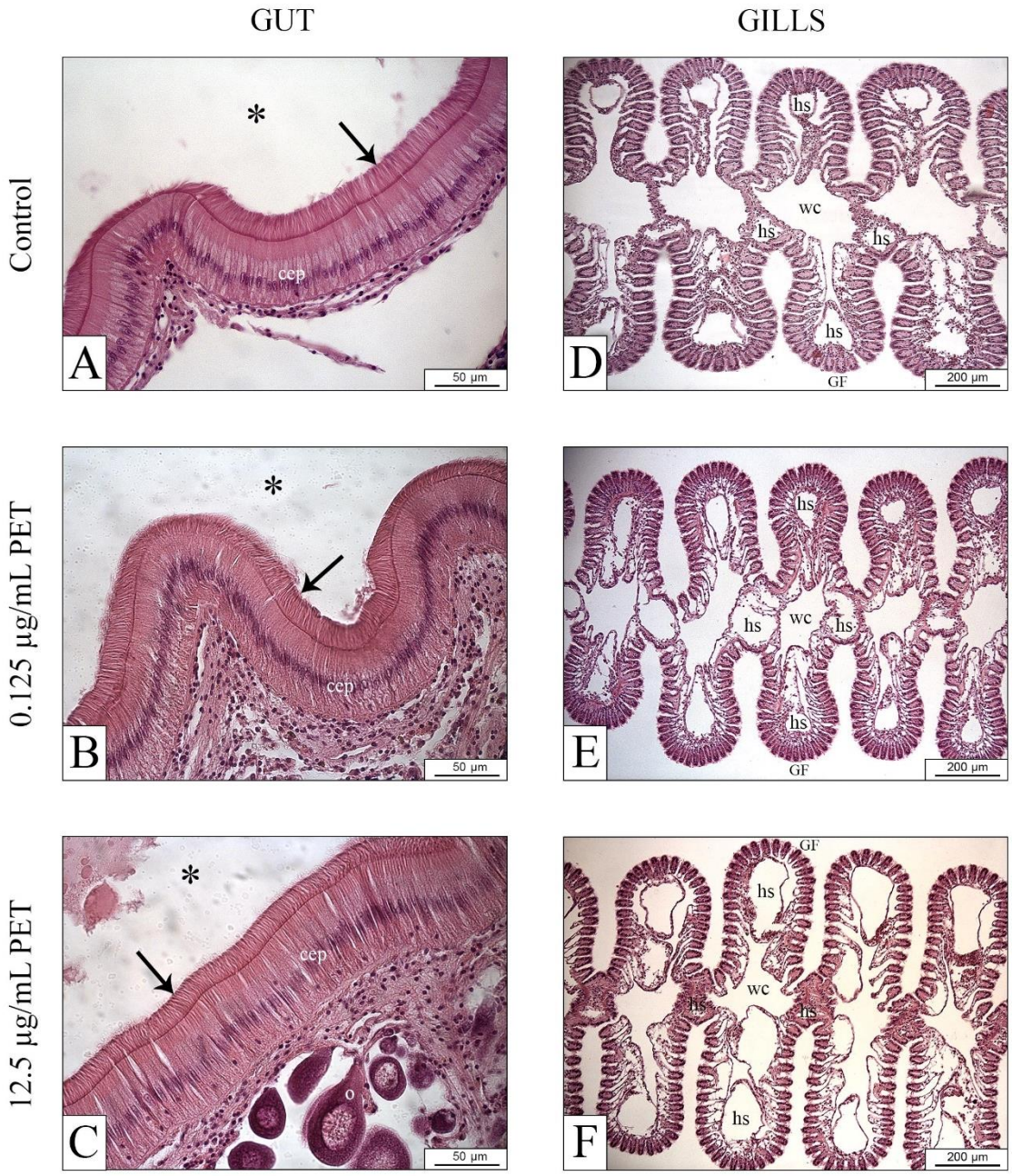

Figure 4: Box and whiskers plot of the total amount of reactive oxygen species (a), activity of superoxide dismutase (b), catalase (c), glutathione peroxidase (d), glutathione S-transferase (e) and lipid peroxidation levels (f) measured in homogenates of digestive gland dissected from clams exposed to two concentrations of micronized PET-MPs. The '×' symbol within the box-plots represents the mean of values, while asterisks above the box-plots show significant differences in the biomarker response between treated and control group (** $p < 0.01$).

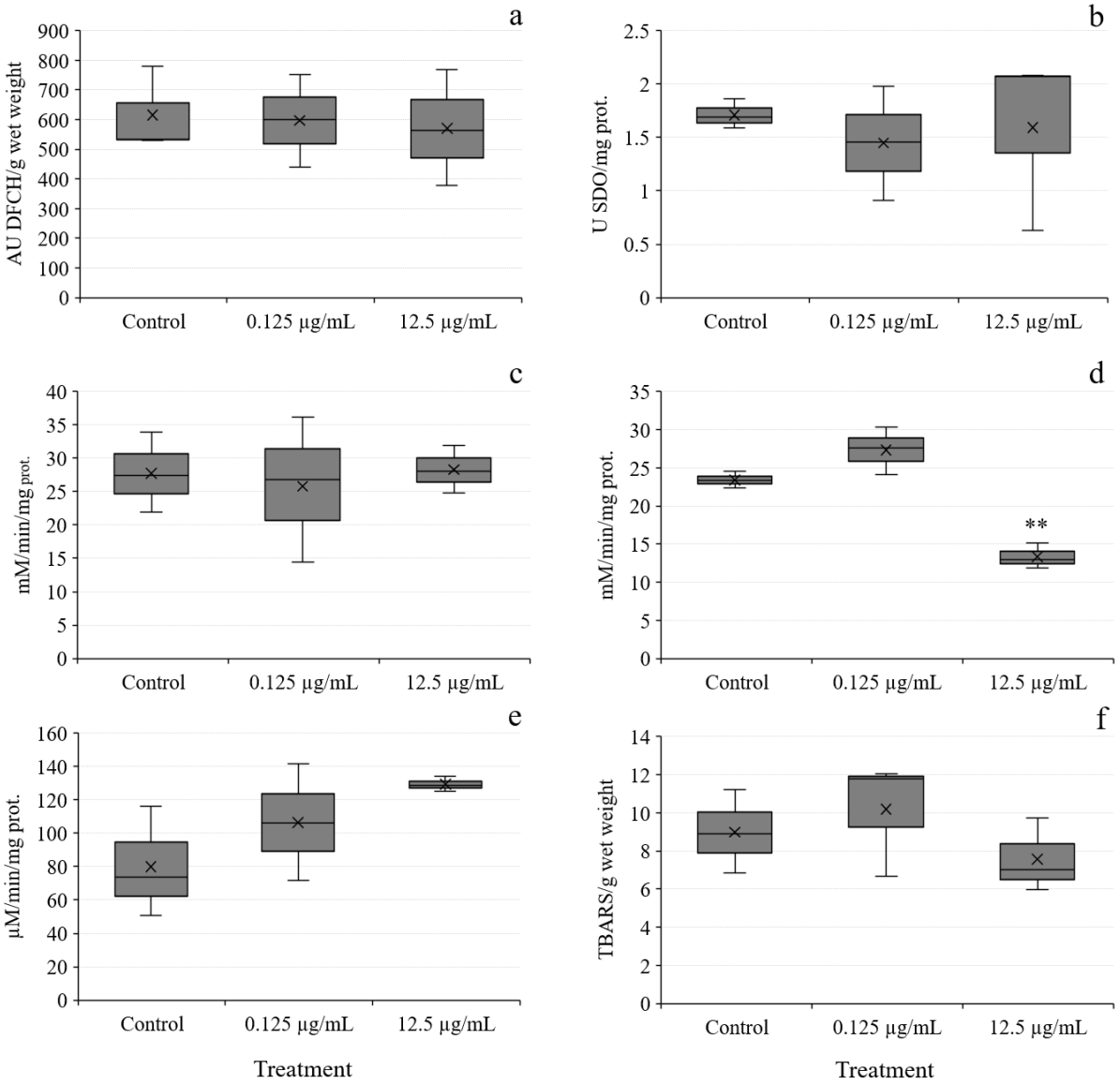

Figure 5: Box and whiskers plot of the total amount of reactive oxygen species (a), activity of superoxide dismutase (b), catalase (c), glutathione peroxidase (d), glutathione S-transferase (e) and lipid peroxidation levels (f) measured in homogenates of gills dissected from clams exposed to two concentrations of micronized PET-MPs. The '×' symbol within the box-plots represents the mean of values, while asterisks above the box-plots show significant differences in the biomarker response between treated and control group (** $p < 0.01$).