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# Uptake and transcriptional effects of polystyrene microplastics in larval stages of the Mediterranean mussel *Mytilus galloprovincialis*<sup>☆</sup>

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## ABSTRACT

The widespread occurrence of microplastics (MP) in the marine environment is cause of increasing concerns about the safety of the exposed ecosystems. Although the effects associated to the MP uptake have been studied in most marine taxa, the knowledge about their sub lethal impacts on early life stages of marine species is still limited. Here, we investigated the uptake/retention of 3  $\mu\text{m}$  polystyrene MP by early stages of the Mediterranean mussel *Mytilus galloprovincialis*, and the related effects on gut clearance, feeding efficiency, morphological and transcriptional parameters involved in embryo larval development. Uptake measurements were performed on larvae at 48 h, 3, 6 and 9 days post fertilization (pf) after exposure to a range of 50 10,000 particles  $\text{mL}^{-1}$ . At all tested pf periods, treatments resulted in a significant and linear increase of MP uptake with increasing concentrations, though levels measured at 48 h pf were significantly lower compared to 3 9 d pf. Ingested MP were retained up to 192 h in larvae's gut, suggesting a physical impact on digestive functions. No change was noted between the consumption of microalgae *Nannochloropsis oculata* by larvae when administered alone or in the presence of an identical concentration (2000 items  $\text{mL}^{-1}$ ) of MP. The exposure to 50 10,000 MP  $\text{mL}^{-1}$  did not alter the morphological development of mussel embryos; however, transcriptional alterations were observed at 50 and 500 MP  $\text{mL}^{-1}$ , including the up regulation of genes involved in shell biogenesis (extrapallial protein; carbonic anhydrase; chitin synthase) and immunomodulation (myticin C; mytilin B), and the inhibition of those coding for lysosomal enzymes (hexosaminidase;  $\beta$  glucuronidase; cathepsin L). In conclusion, though not highlighting morphological or feeding abnormalities, data from this study revealed the onset of physical and transcriptional impairments induced by MP in mussel larvae, indicating sub lethal impacts which could increase their vulnerability toward further environmental stressors.

## 1. Introduction

Over the past decades, most of the glass, wooden or metal made items used in the daily life have been progressively replaced by lighter, cheaper and more durable plastic made counterparts, so that the current historical period has been cited as “the Age of Plastic” (Avio et al., 2017). However, besides the multiple benefits

brought by plastic to the human society, increasing concerns are risen as to the impacts associated to its ubiquity within natural systems (UNEP, 2016). Currently, plastics account for the 80–90% of the whole marine litter (Derraik, 2002), and over 5 trillion microscopic plastic fragments are estimated to float into the World Oceans (Eriksen et al., 2014). These particles, referred to as microplastics (MP) based on their < 5 mm size (GESAMP, 2016), can directly enter the marine environment as microscopic items (primary MP), or deriving from the progressive fragmentation of larger plastics due to weathering processes (secondary MP) (Andrady, 2011). Depending on their size, shape and chemical composition, MP assume different positions and behaviours along the water column, being easily mistaken for natural food/preys by a vast

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range of marine species (Galloway et al., 2017).

In recent years, increasing attention has been focused on the potential adverse effects induced by MP on the marine species ontogeny (UNEP, 2016). Given their incomplete neuro-morphological development, early life stages of aquatic species display a higher vulnerability to external challenges compared to adults (Mohammed, 2013). This assumption is particularly critical for marine sessile invertebrates, whose resilience or spatial dispersion depend on recruitment pathways governed by planktonic larvae. Bivalve and echinoderm larvae are widely employed for ecotoxicological investigations, given their high responsiveness to physical and chemical stressors, ease of rearing and adaptability to *in vitro* experimental designs (Fabbri et al., 2014; Passarelli et al., 2018). A study from Kaposi et al. (2014) showed a concentration dependent uptake of polyethylene MP by larvae of the sea urchin *Tripneustes gratilla*, and reduced body width only at the highest dosage of 300 MP mL<sup>-1</sup>.

The exposure to increasing concentrations of polystyrene MP also affected filtration rates in embryos of different asteroid and sea urchin species (Lizarraga et al., 2017). In larvae of the Pacific oyster (*Crassostrea gigas*), the capability to ingest polystyrene MP of different sizes was reported to be a function of the development stage (Cole and Galloway, 2015). Polystyrene MP (2–6 µm) were found to alter the expression of genes involved in gamete differentiation and maturation over a 8 week exposure in adult *C. gigas* (Sussarellu et al., 2016).

Whether the ingestion of plankton sized MP induce morphological and/or transcriptional effects in early stages of marine bivalves is still unclear. Therefore, in the present study, experiments were performed to evaluate the uptake of polystyrene MP by early life stages of the Mediterranean mussel (*Mytilus galloprovincialis*) and the related effects on multiple endpoints. *M. galloprovincialis* is widely distributed in urbanized coastal environments thus likely being exposed to MP sources for its entire life cycle; it is also a species of high ecological and commercial importance, and worldwide utilized as sentinel organism of pollution in coastal marine environments (Viarengo et al., 2007). Establishing the impacts of MP on mussel early life stages is thus important to gain significant insights on the adverse consequences of plastic pollution.

Ingestion and accumulation of MP was microscopically assessed in mussel larvae at different developmental times and increasing MP concentrations. The retention of ingested particles by larvae was evaluated by measuring their egestion over time. Co exposure to MP and microalgae (*Nannochloropsis oculata*) was performed to evaluate potential influences of MP occurrence on food consumption. Finally, in agreement with recent studies (Balbi et al., 2016, 2017), morphological alterations and expression changes of transcripts involved in shell biogenesis, neuroendocrine signaling, detoxification/antioxidant processes, and immune responses were evaluated in mussel embryos grown in the presence of MP to investigate the potential impacts on larval development of mussels.

## 2. Materials and methods

### 2.1. Polystyrene microplastics (MP)

Fluorescently labeled polystyrene microspheres (Fluoresbrite Plain YG, 441/486 nm excitation/emission;  $3 \pm 0.15$  µm diameter) were purchased from Polyscience Inc. (Washington, PA, USA). Such particles were chosen as a proxy of MP exposure since their density (1.05 g/cm<sup>3</sup>) is almost identical to that of seawater (1.025–1.035 g/cm<sup>3</sup> according to the UNESCO, 1981), so that they should not sink within 24–48 h from suspension in water. The MP diameter was chosen based on the size of phytoplanktonic

species commonly preyed by bivalve larvae (Southgate et al., 2017). Surfactants were not present or added to the original MP suspension ( $1.69 \times 10^9$  MP mL<sup>-1</sup>), whose medium was ultrapure H<sub>2</sub>O (Polyscience, inc.). A stock solution of  $10^7$  MP mL<sup>-1</sup> was prepared in milli Q H<sub>2</sub>O, aliquoted and stored at 4 °C according to the manufacturer's specification. At each MP administration, 1 aliquot of the stock solution was serially diluted in filtered (0.22 µm) seawater (FSW) to achieve the suitable volume to be added to each experimental treatment. Before spiking, the lack of aggregation was verified by epifluorescence microscopy (Zeiss Observer, Axiovert S100, 20–40 × magnification) at  $\lambda_{\text{excitation}}$  365 nm and  $\lambda_{\text{emission}}$  397 nm.

### 2.2. Animal holding and oocyte fertilization

Sexually mature specimens of Mediterranean mussel (*M. galloprovincialis*) were collected from a government certified mussel farm (COPRALMO, Cesenatico, Italy), immediately transported to the laboratory, and acclimated for 3 days in static tanks containing filtered seawater (FSW) at 16 °C and at a density of 5 mussels L<sup>-1</sup>. Gamete collection, oocyte fertilization and larvae handling were performed following the procedure described by Fabbri et al. (2014). Briefly, when mussels begun to spontaneously spawn, each individual was placed in a 250 mL beaker containing 200 mL of aerated FSW until complete gamete emission. After spawning, mussels were removed from beakers and sperms and eggs sieved through 50 µm and 100 µm nylon meshes, respectively, to remove impurities. Egg quality (shape, size) and sperm motility were checked using an inverted light microscope (OPTECH, IB series; Munchen, Germany; 40 × magnification). Pools of gametes from at least three specimens per sex were employed for experimental purpose. Eggs were fertilized with a 1:10 egg to sperm ratio in the suited experimental systems (see detailed description below). In any case, embryos were grown at  $16 \pm 1$  °C with a 16 h: 8 h (light: dark) photoperiod and continuous aeration.

### 2.3. MP uptake and retention by mussel larvae and effects on food consumption

#### 2.3.1. Experimental treatment

Oocyte fertilization was performed as previously described in 2 L glass beakers containing 1.5 L FSW (35 eggs mL<sup>-1</sup>) (S1 stock culture). Starting from 48 h post fertilization (pf), larvae were fed daily with a suspension of microalgae (*N. oculata*; 1200 cell mL<sup>-1</sup>). Every 3 days, larvae were collected by sieving the S1 culture on a nylon mesh (20 µm pore filter) and employed for experimental treatments as described below. At each time interval, unemployed larvae were re suspended in FSW and the S1 rearing conditions restored.

#### 2.3.2. Uptake of polystyrene MP

Larvae were collected at 48 h, 3, 6 and 9 days pf, from the S1 stock and aliquoted in 12 well plates at a density of 50 larvae mL<sup>-1</sup> in a total volume of 3 mL FSW. MP were added to each well to obtain final nominal concentrations from 50 MP mL<sup>-1</sup>, which is the lowest concentration allowing all exposed larvae (50 organisms mL<sup>-1</sup>) to feed on suspended particles (MP to larvae ratio 1:1), to 10,000 MP mL<sup>-1</sup>, which is comparable to the microalgae concentration at which the maximum ingestion rate was measured in mussel larvae (Sprung, 1984). Treatments were performed in quadruplicate. After 24 h of exposure, samples were fixed with buffered formalin (4%). MP uptake by larvae was assessed using an inverted epifluorescence microscope (Zeiss Observer, Axiovert S100; 20–100 × magnification) equipped with a 365/397 nm ( $\lambda_{\text{excitation}}/\lambda_{\text{emission}}$ ) filter. Data were expressed as the

mean  $\pm$  SEM of the percentage of larvae showing MP ingestion or the number of ingested MP larva<sup>-1</sup> (N = 4).

#### 2.3.3. Retention of ingested MP

Larvae were collected at 6 d pf from the S1 stock and added to 100 mL glass beakers containing 50 mL of FSW (50 larvae mL<sup>-1</sup>). MP stock solution was diluted into each beaker to obtain a final nominal concentration of 1000 MP mL<sup>-1</sup>. After 24 h of exposure, the beakers content was pooled in a 100 mL PVC container having a sieved bottom (20  $\mu$ m nylon filter mesh) to isolate larvae from eventually non ingested MP. Rapidly, the container was half submerged in 6 L tanks containing 5 L FSW, and thereby blocked by mechanical support to allow egested MP to pass through the sieved bottom and avoid re ingestion. At 2 h intervals for the first 12 h, and every 12 h thereafter, aliquots of about 150 larvae were collected from each treatment condition and fixed with 4% buffered formalin. At each time interval, the number of larvae showing ingested MP was examined by an inverted fluorescence microscope (Zeiss Observer, Axiovert S100; 20–100  $\times$  magnification) and the FSW volume contained in each 6 L tank renewed. The assessment was stopped when two consecutive sampling showed no D veligers with ingested MP. Data were expressed as mean  $\pm$  SEM (N = 4) of the percentage of larvae showing MP over time.

#### 2.3.4. Effects of MP on food consumption

The effects of MP on food consumption were evaluated by measuring the uptake of microalgae *N. oculata* by larvae either in the presence or absence of the selected MP. Six d pf larvae were collected from the S1 stock and placed into 100 mL beakers containing 50 mL FSW at a density of 50 larvae mL<sup>-1</sup>. All treatments were performed using total item concentration (i.e. MP, algae, or MP + algae) far below levels at which the maximum ingestion rate is expectable in mussel larvae (around 10,000 items mL<sup>-1</sup> according to Sprung, 1984), to avoid satiation or gut saturation. MP and/or microalgae were added to each beaker to obtain a mixture containing each item (MP and algae) at a concentration of 2000 particle mL<sup>-1</sup>. Single exposures to algae (2000 cell mL<sup>-1</sup>) or MP (2000 particles mL<sup>-1</sup>) were also performed. For each treatment, a control condition containing no larvae was maintained in parallel. All treatments were performed in quadruplicate (N = 4). After 24 h, samples were filtered on a 20  $\mu$ m nylon filter mesh to eliminate larvae and the solution was centrifuged at 1100 $\times$ g for 10 min. The obtained pellet was re suspended in 500  $\mu$ L FSW and the concentration of MP and/or microalgae assessed microscopically (Axioskop 40, Carl Zeiss, Milan, Italy; 40  $\times$  magnification). The consumption of both administered items was assessed by measuring the percentage of concentration variations in the exposure medium with respect to control.

### 2.4. Embryotoxicity and transcriptional effects of MP on mussel embryos

#### 2.4.1. Experimental treatments

Oocyte fertilization was performed in 6 well (mRNA expression) or 12 well (embryotoxicity test) plates as previously reported (Fabbri et al., 2014; Balbi et al., 2016). After 30 min, fertilization success (n. fertilized eggs/n. total eggs  $\times$  100) was verified microscopically (>85%). The same concentration range tested for uptake assessment (50, 100, 500, 1,000, 5000 and 10,000 MP mL<sup>-1</sup>) was used for the embryotoxicity test. For transcriptional analyses two concentrations in the low range tested for embryotoxicity (50 MP mL<sup>-1</sup> and a 10X concentration, 500 MP mL<sup>-1</sup>) were chosen, based on the general assumption that functions at lower biological hierarchies, such as transcriptional pathways, are more sensitive and respond much faster to stress exposure than biological

endpoints at higher biological hierarchies (Franzellitti et al., 2010). MP were added to each well at the selected nominal MP concentrations (for the embryotoxicity test; 50 and 500 MP mL<sup>-1</sup> for mRNA expression analyses). A control treatment containing only FSW was simultaneously performed.

#### 2.4.2. Embryotoxicity test

The embryotoxicity test was performed as described by Fabbri et al. (2014). After a 48 h incubation, samples were fixed with buffered formalin (4%). All larvae in each well were examined by optical microscopy using an inverted microscope (OPTTECH, IB series; Munchen, Germany; 40  $\times$  magnification). Observations were carried out by an operator blind to the experimental conditions. A larva was considered normal when the shell was D shaped (straight hinge) and the mantle did not protrude out of the shell, and malformed if had not reached the stage typical for 48 h pf (trochophore or earlier stages) or in the presence of developmental defects (concave, malformed or damaged shell, protruding mantle). The recorded endpoint was the mean  $\pm$  SEM (N = 4) of the percentage of normal larvae (D veligers). The acceptability of test results was based on controls for a percentage of normal D shell stage larvae >70% (ASTM, 2004).

#### 2.4.3. mRNA expression by quantitative real time PCR (qPCR) analysis

Embryo handling and total RNA extraction was performed as described in detail by Balbi et al. (2016). RNA concentration and quality were verified using the Qubit RNA assay through the Qubit 2.0 system (Thermo Fisher, Milan, Italy) and electrophoresis using a 1.2% agarose gel under denaturing conditions. First strand cDNA for each sample was synthesized from 1  $\mu$ g total RNA using the iScript supermix (Bio Rad Laboratories, Milan, Italy) following manufacturer's instructions.

Gene transcription was evaluated in 4 independent RNA samples (about 6000 embryos/replicate). Primers pairs employed for qPCR analyses were as reported in previous studies or were designed with Primer Express (Thermo Fisher, Milan, Italy) using nucleotide sequences retrieved from the GeneBank database for *M. galloprovincialis* (Table S1). Reactions were performed in duplicate in a final volume of 10  $\mu$ L containing 5  $\mu$ L iTaq Universal master mix with ROX (BioRad Laboratories, Milan, Italy), 2  $\mu$ L diluted cDNA, and 0.2  $\mu$ M specific primers. A control lacking cDNA template (no template) and a minus reverse transcriptase (no RT) control were included in the qPCR analysis to determine the specificity of target cDNA amplifications. Amplifications were performed in a StepOne real time PCR system apparatus (Thermo Fisher, Milan, Italy) using a standard "fast mode" thermal protocol. For each target mRNA, melting curves and agarose gel electrophoresis were utilized to verify the specificity of the amplified products and the absence of artifacts. The amplification efficiency of each primer pair was calculated using a dilution series of cDNA (Table S1). Helicase and elongation factor 1 $\alpha$  were selected as the best performing combination of reference gene products for qPCR data normalization through a preliminary stability analysis amongst 6 candidates (Balbi et al., 2016). Calculations of relative expression of target mRNAs was performed by a comparative C<sub>T</sub> method (Schmittgen and Livak, 2008) using the StepOne software tool (Thermo Fisher, Milan, Italy). Data were reported as relative expression (log<sub>2</sub> transformed fold change) compared to control samples.

#### 2.5. Data analysis

After being tested for normality (Shapiro Wilk test) and variance homogeneity (Levene's mean test) data from uptake parameters, feeding behavior and embryotoxicity test were subjected to a

one way ANOVA followed by Bonferroni multiple *post hoc* comparisons to test for significance amongst different treatments or between single treatments and control condition (SigmaPlot 13, Systat Software Inc. San Jose, CA, USA). Data from qPCR analyses were evaluated with the REST software (Pfaffl et al., 2002) that uses a randomisation test with a pairwise reallocation to assess the statistical significance of the differences in expression between each treatment group and the controls.

The putative occurrence of concentration dependent variations of MP uptake parameters, including the percentage of larvae showing ingestion of MP and the average number of ingested MP, was analysed by means of the Pearson correlation test. In addition, for all tested MP concentrations and larval age, the Pearson correlation coefficient was calculated to evaluate relationships between the percentage of larvae showing ingestion and the average number of ingested MP larva<sup>-1</sup>. In all approaches,  $p < 0.05$  was set as the threshold level of statistical significance.

Additional permutation statistical approaches were used to evaluate:

- i) Influences of MP concentrations on MP uptake at different post fertilization times. Data obtained from the analysis of the two uptake variables (i.e. the percentage of larvae showing ingestion and the average number of ingested MP larva<sup>-1</sup>) were submitted to a permutation multivariate analysis of variance (PERMANOVA) using the PERMANOVA + add on in PRIMER v6 (Anderson et al., 2008). Standardized data were used to calculate similarity matrices based on the Euclidean distance (999 permutations). Factors were “larval development”, represented by the tested post fertilization times (4 levels: 48 h, 3, 6 and 9d pf), and “treatment”, referring to the tested MP concentrations (6 levels: 50, 100, 500, 1,000, 5,000, and 10,000 MP mL<sup>-1</sup>). Pseudo F values in the PERMANOVA main tests were evaluated in terms of significance ( $P < 0.05$ ). When the main test revealed statistical differences, PERMANOVA pairwise comparisons were carried out (using the Euclidean distance matrix and 999 permutations).
- ii) Interactive effects of MP treatment on transcript expressions across embryo development. Relative expression data from MP treatments at different post fertilization periods were submitted to PERMANOVA analysis using the PERMANOVA + add on in PRIMER v6. Log<sub>2</sub> transformed fold change variations of the target transcripts were used to calculate similarity matrices based on the Euclidean distance (999 permutations). Factors were “developmental stage” and “treatment”. Numerical metric for life stage progression was indicated by the post fertilization time (0, 24, 48 h). Treatment was indicated by the nominal MP concentrations of exposure (0, 50, and 500 particles mL<sup>-1</sup>). Pseudo F values in the PERMANOVA main tests were evaluated in terms of significance ( $P < 0.05$ ). Distance based redundancy linear modeling (DISTLM) followed by a redundancy analysis (dbRDA) in PRIMER was also performed to examine the relationship between the multivariate dataset (i.e. the suite of transcripts assayed and their expression levels) and the predictor variables (developmental stage and MP treatment). DISTLM used the BEST selection procedure and adjusted R<sup>2</sup> selection criteria.

### 3. Results

#### 3.1. Uptake assessment

According to the PERMANOVA (Table S2), factors “MP concentration” and “time post fertilization”, as well as their interaction, had a significant effect on the overall MP uptake levels. The

PERMANOVA pairwise comparisons (Table S3) revealed significant differences between MP uptake levels at 48 h pf and those recorded at 3, 6, 9 d pf for all performed treatments. Larvae exposed at 3 d pf showed significant differences compared to 6 d pf at 500–10,000 MP mL<sup>-1</sup>, as well as to 100–5000 MP mL<sup>-1</sup> when compared to 9 d pf. Levels measured at 6 and 9 d pf significantly differed each other in the presence of 100 and 10,000 MP mL<sup>-1</sup>.

Levels of MP uptake by mussel larvae tended to increase with increasing concentrations (Fig. S1 A–D). At all tested post fertilization periods, the exposure to 50–10,000 MP mL<sup>-1</sup> resulted in significant increases of both the percentage of larvae showing MP ingestion (Fig. 1A–D) and the number of ingested particles (Fig. 1E–H). However, only at 48 h, 3 and 6 d pf the levels of both parameters were significantly and positively correlated to the nominal MP concentrations, while no significant relationship was noted at 9 d pf. The lowest uptake levels and variations among treatments were showed by larvae at 48 h pf, in which significant increases of both parameters were detected only at the highest tested concentration (Fig. 1A, E). Major changes in the number of ingested MP were detected at 6 d pf, with levels reaching up to 14.2 MP larva<sup>-1</sup> at 10,000 MP mL<sup>-1</sup> (Fig. 1G). The only significant decrease in the number of ingested MP with increasing concentration was detected in larvae at 9 d pf exposed to 10,000 MP mL<sup>-1</sup> compared to 5000 MP mL<sup>-1</sup> (Fig. 1H). At all post fertilization periods, the percentage of larvae showing MP ingestion was significantly and positively correlated with the average number of ingested MP larva<sup>-1</sup> (Fig. S2). The analysis in epifluorescence microscopy did not highlight MP aggregation or plate wall adherence after 24 h of suspension.

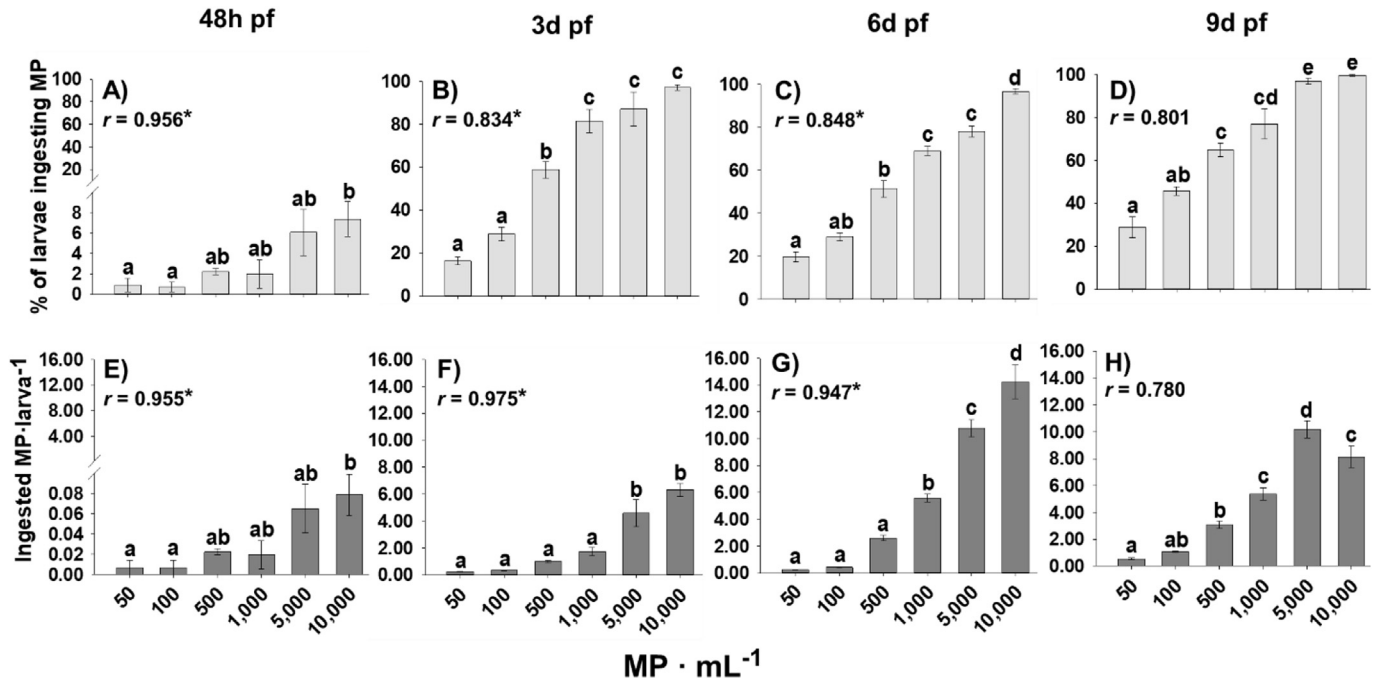
#### 3.2. Evaluation of MP retention time and effects on food consumption

Results of the MP retention assessment performed on larvae exposed to MP for 24 h are shown in Fig. 2. Larvae were exposed to a concentration of 1000 MP mL<sup>-1</sup> leading to an average accumulation lower than 10 MP larva<sup>-1</sup> (based on uptake information reported in Fig. 1E–H), thus avoiding the possible effect of a high gut load on the subsequent elimination of ingested particles. At the end of the exposure period, 92% of exposed larvae showed ingested MP. A remarkable egestion was observed at 24 h from exposure, when only 23% of D veligers showed ingested MP. A further drop to 9.1% was recorded after the following 24 h, while fluctuations in the 3–10% range were noted in subsequent observations. The experiment was stopped at 204 h (8.5 days) from exposure, time at which all examined larvae showed no MP for two consecutive intervals. No mortality of larvae was registered during the test.

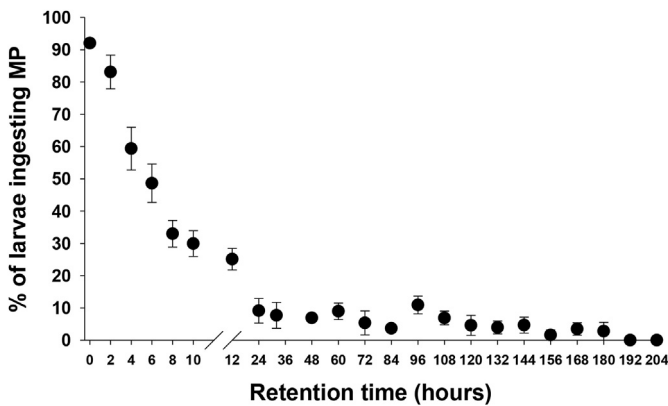
The co exposure to 2000 MP mL<sup>-1</sup> and 2000 cell mL<sup>-1</sup> of the microalga *N. oculata* revealed a significant preference for nutritious items (algae) by mussel larvae, which consumed the 80% of administered cells vs the 20% of MP (Fig. 3). Similar results were obtained in single exposures, in which algae consumption was significantly higher (87%) compared to MP (about 40%) after 24 h of exposure. The consumption of both MP and *N. oculata* did not change significantly between single and co exposure treatments.

#### 3.3. Embryotoxicity test

Results of the embryotoxicity test performed on *M. galloprovincialis* embryos exposed to MP (50–10,000 particle mL<sup>-1</sup>) are shown in Fig. 4. At 48 h pf the  $86.2 \pm 4.7\%$  of normally developed D shape veligers was noted in control samples, thus achieving requirements for test acceptance ( $\geq 75\%$ ; Fabbri et al., 2014). Compared to control, no significant decreases in the fraction of normally developed D shape larvae were observed in



**Fig. 1. MP uptake by mussel larvae.** Graphs report the percentage of D-shaped veligers showing uptake of MP (A–D) and average number of MP ingested per single larva (E–H) as a function of larvae age (i.e. post fertilization time) and MP concentration (mean  $\pm$  SEM; N = 4). Different letters indicate statistically significant variations between groups (p < 0.05). Pearson's correlation coefficients (r) between assessed parameters and MP concentrations are also reported (\*p < 0.05).

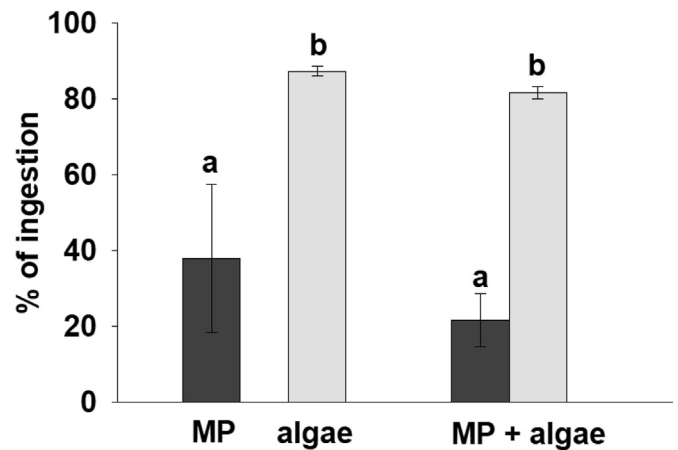


**Fig. 2. Time employed for MP egestion by mussel larvae.** Experiments were performed on 6 d pf larvae exposed for 24 h to 1000 MP mL<sup>-1</sup> and transferred to clean FSW for different post-exposure periods. Observations were performed until samples at two consecutive time points showed no larvae with ingested MP. Data are reported as mean  $\pm$  SEM (N = 4) of the percentage of larvae showing MP uptake.

samples grown in the presence of increasing MP concentrations.

#### 3.4. Effects of MP on gene transcription

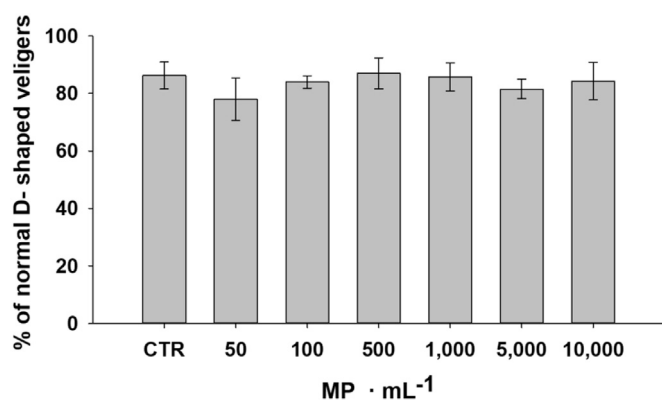
Treatments with polystyrene MP induced overall up regulation of immune related gene products in D veligers (48 h pf), in particular of *LYS* at 500 MP mL<sup>-1</sup>, and of *MYTC* and *MYTLB* at 50 and 500 MP mL<sup>-1</sup> (Fig. 5A). Transcripts encoding lysosomal enzymes were differently regulated at the tested nominal MP concentrations, with *GUSB* being significantly down regulated at both tested MP concentrations, *HEX* being down regulated at 50 MP mL<sup>-1</sup>, and *CTSL* being significantly up regulated at 500 MP mL<sup>-1</sup> (Fig. 5B). As to neuroendocrine related transcripts, significant up regulation of the 5 *HT1* gene product was observed in D veligers at 500 MP mL<sup>-1</sup>,



**Fig. 3. Effects of polystyrene MP (3  $\mu$ m) on the consumption of microalgae (*N. oculata*, 2.5  $\mu$ m) by mussel larvae.** Single exposure treatments consisted of a 24-h exposure to 2000 MP mL<sup>-1</sup> or 2000 algae mL<sup>-1</sup>. Co-exposure treatment consisted of a 24-h exposure to a mixture of 2000 cell mL<sup>-1</sup> and 2000 MP mL<sup>-1</sup>. Data represent the percentage of MP or algae consumed by mussel larvae (mean  $\pm$  SEM; N = 4). Different letters indicate statistically significant differences (p < 0.05).

while levels of *MeER1* and *MeER2* transcripts resulted unchanged (*MeER1*) or differently regulated (*MeER2*: significant up regulation at 50 MP mL<sup>-1</sup> and down regulation at 500 MP mL<sup>-1</sup>) (Fig. 5C). *CA*, *CS*, and *EP* transcripts involved in shell biogenesis were significantly up regulated at 500 MP mL<sup>-1</sup> (Fig. 5D). Finally, mRNA levels of *MT10* and *MT20* methallotionein gene products (here considered as involved in ROS scavenging/antioxidant processes) were unchanged (*MT10*) or significantly up regulated (*MT20*) at 500 MP mL<sup>-1</sup> (Fig. 5E).

A multivariate permutation statistical approach was used to infer the putative interactive effects of MP treatment on the observed transcript expressions across embryo development. The



**Fig. 4. Effects of 3 µm polystyrene MP on *M. galloprovincialis* normal embryo-larval development in the 48-h embryotoxicity assay.** Data represent the mean ± SEM (N 4) of the percentage of normal D-shaped larvae at each treatment. No statistically significant differences ( $p < 0.05$ ) were observed between treatments and control.

employed dataset comprised levels for the target transcripts in unfertilized eggs and their variations at 24 h pf (Fig. S3), other than those at 48 h pf reported in Fig. 5A–E. PERMANOVA analyses demonstrated a significant effect of MP treatment on gene transcription and a significant interaction with embryo development (Table S4). Indeed, distance based linear model (DISTLM) analysis revealed that though expression profiles were strongly dependent on embryo development (explaining about 84% total variation), MP treatment accounted for about 8% of total variation, which mostly explained the observed changes in transcript expressions at 48 h pf (Fig. 5F). DISTLM ordination analysis by functional group revealed that expression patterns of transcripts involved in shell biogenesis, lysosomal response, and immune responses mostly explained the observed MP effects at 48 h pf, while effects on antioxidant responses and neuroendocrine signaling peaked at earlier developmental stages (Fig. 5G).

#### 4. Discussion

The emerging paradigm about plastic pollution of marine environments is that micro to nano size particles may have far more subtle effects compared to bigger litter fragments, since their size range overlaps with the preferred particle size ingested by marine zooplankton (reviewed by Galloway and Lewis, 2016). In marine and freshwater copepods, nanoplastics and microplastics have been associated to reduced feeding and sub lethal health outcomes (Cole et al., 2015; Lee et al., 2013; Besseling et al., 2014). Aimed at providing further insights, the present study evaluated the potential for 3 µm polystyrene MP to be ingested by larvae of *M. galloprovincialis*, measured the dynamics of MP uptake as a function of larvae development and particle concentration, and assessed the potential physiological adverse outcomes of MP ingestion at the morphological (48 h embryotoxicity assay) and molecular (transcriptional effects) levels. Employed MP were selected to closely match size range (2–6 µm) and shape (spherical) of phytoplanktonic species known as food sources for mussel larvae (Widdows, 1991).

Previous investigations were performed using the same typology of polystyrene microspheres to assess the impact of MP or nanoplastics on marine taxa, including phytoplankton (Long et al., 2017), crustaceans (Jeong et al., 2017) and molluscs (Paul Pont et al., 2016). Data obtained in this study showed that MP were readily ingested by mussel larvae, with uptake rates positively correlated with MP concentration and larvae development. Sprung (1984)

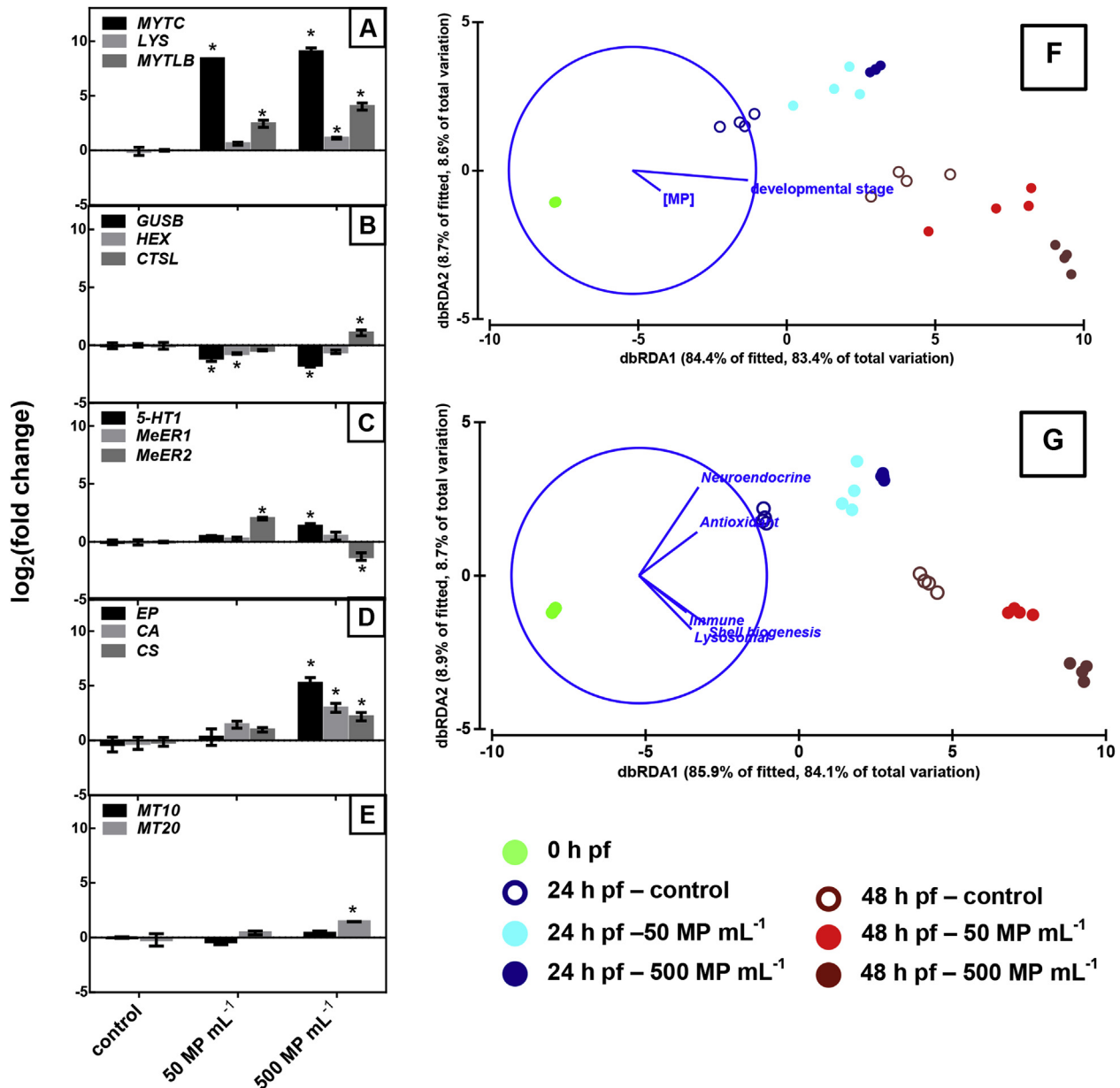
found that within a range of 0–10,000 cells mL<sup>-1</sup> of the alga *Isochrysis galbana*, mussel larvae adapt ingestion rate to the external food availability, suggesting that a similar feed back regulation may occur in response to increasing MP concentrations.

At our experimental conditions, a lower ingestion was observed in 48 h pf larvae compared to elder specimens (3–9 d pf). Conversely, no significant changes in MP uptake propensity were found in larvae of the sea urchin *T. gratilla* at 1–5 d pf (Kaposi et al., 2014). This points out the possible influence of different developmental timing of the filter feeding behavior between the larval stages of the two species. In fact, relevant neuro myogenic events occur in mussel larvae between 48 and 60 h pf, resulting in the functional differentiation of the velum retractor muscles involved in particle catching and ingestion (Dyachuck and Odintsova, 2009). According to the PERMANOVA, MP concentrations higher than 50 particles mL<sup>-1</sup> induced significantly different trends of uptake between larvae at 3 d pf and those at 6–9 d pf. All larvae showed similar propensities to the ingestion (>95% larvae ingesting MP at 10,000 MP mL<sup>-1</sup>), while increasing concentrations caused a higher MP accumulation in 6–9 d pf larvae compared to younger specimens, in line with trends previously observed in oyster larvae (3–24 d pf) exposed to increasing MP size (Cole and Galloway, 2015).

Interestingly, a lower MP accumulation was noted at 9 d pf in larvae exposed to the highest tested concentration (10,000 MP mL<sup>-1</sup>). Such response may be the consequence of growth related variations of swimming/feeding behavior in relation to the particles availability. According to Cragg (1980), as the shell weight/size increase, bivalve larvae alternate active to passive periods passed on the substrate, resulting in a prolonged static filtration and a lower uptake rate at higher concentrations of microalgae. Therefore, the interaction between MP and planktonic larvae might be influenced by their progressive morpho functional development (e.g. from free swimming to sessile behavior), ultimately leading to changes in uptake trends. It is important to consider that MP concentrations employed in this far exceeded those detected in the marine environment, which range from 0.01 particles per m<sup>3</sup> (GESAMP, 2016) to 102,000 particles per m<sup>3</sup> seawater (Norén, 2007). However, since levels of marine contamination by plankton sized MP are thought to increase for effect of weathering (Anderson et al., 2016; Lusher, 2015), obtained findings provide reliable clues on the associated enhancement of uptake by marine larvae.

MP retention experiments showed that about 80% of analysed larvae egested MP within 24 h from exposure. However, 192 h (8 days) were necessary to achieve gut clearance in 100% individuals, suggesting that, although in the same size range of microalgae, MP do not easily pass the larvae digestive tract. Differently, pluteus larvae from the sea urchin *T. gratilla* showed a fast elimination of polyethylene MP, with the 100% egestion achieved after 480 min from exposure (Kaposi et al., 2014). Such discrepancy could reflect the distinct morpho structural features characterizing the gastrointestinal tract of mussel veligers and sea urchin plutei (Aranda Burgos et al., 2014; Rahman et al., 2012), likely resulting in a more efficient elimination route displayed by sea urchins. Intestinal stasis of MP is a hazardous condition for the organism fitness, with effects spanning from reduced nutritional status and energy storage to severe digestive blockage (Grigorakis et al., 2017). A further concern associated to a prolonged gut retention relies on the finding that MP may act as a vehicle for the ingestion of chemical or microbiological agents. Modeling studies demonstrated that the transfer of organic pollutants sorbed onto plastics is of limited importance compared to other routes of exposures (Bakir et al., 2016; Koelmans et al., 2016). However, the role of MP as vectors of pathogens has recently been confirmed (Lamb et al., 2018) and





**Fig. 5. Effects of exposure of mussel embryos to 3 µm polystyrene MP on gene transcription.** Graphs report relative expressions of transcripts involved in (A) immune responses, (B) lysosomal responses, (C) neuroendocrine signaling, (D) shell biogenesis, (E) antioxidant responses at the D-veliger stage (48 h pf). Gene acronym explanations are reported in Table S1. Data represent the mean  $\pm$  SEM of the relative variations ( $\log_2$  fold change) with respect to untreated samples (N 4). \* $p < 0.05$ . (F) Distance-based redundancy modeling (DISTLM) with distance-based redundancy analysis (dbRDA) to explore the amount of the variation in gene transcription to be attributed to MP treatment across the 48-h developmental period (Euclidean Distance resemblance matrix, 999 permutations). (G) DISTLM ordination with projected average transcript profiles by functional group. To account for time-course variations of transcriptional profiles, the dataset employed in both DISTLM analyses comprises qPCR data from control and polystyrene MP treated embryos at 24 h pf and 48 h pf compared to unfertilized oocytes (0 h pf).

growing concerns are expressed on the risks posed by the leakage of additives from ingested MP (Revel et al., 2018).

Impairment of feeding efficiency is one of the most concerning issues related to the occurrence of MP in marine environments (Galloway et al., 2017). In this study we showed that the presence of MP did not affect the average consumption of *N. oculata* algae by mussel larvae. This may indicate that, though apparently equipped with a rudimentary feeding apparatus, mussel D veligers may express food preferences by selectively preying on planktonic items of relatively high nutritional value, as it occurs in adults (Ward and Shumway, 2004). This finding also suggests that polystyrene MP may have a low chemotactile attractiveness for larvae compared to

nutritious particles. Kaposi et al. (2014) reported that presence of biofouling on polyethylene microspheres reduced their ingestion by *T. gratilla* larvae. Although a specific investigation on this issue was out of the scope of our work, it has to be acknowledged that in the framework of environmentally realistic scenarios, MP properties can be modified due to desorption of organic compounds or adsorption of biomolecules from biological fluids (e.g. proteins and/or polysaccharides), thus forming an eco/bio corona (Canesi and Corsi, 2016). As discussed by Galloway et al. (2017), this could strongly influence the interaction of MP with cells and tissues, and ultimately their bioavailability, persistence and toxicity.

No significant increase in the occurrence of macroscopical

abnormalities was noted in mussel embryos grown for 48 h pf in the presence of 50–10,000 MP mL<sup>-1</sup>, thus apparently suggesting no relevant interactions of tested MP with normal larval development. When converted to particle weight volume<sup>-1</sup> values, the tested MP concentrations were 0.8–154 µg L<sup>-1</sup>, a range of values partially overlapping that employed by Balbi et al. (2017), where significant embryotoxicity effects were found in 48 h pf mussel D veligers in the presence of 50 nm amino polystyrene MP (0.001–20 mg/L). The differences in MP size may explain such discrepancy. Indeed, particles smaller than 100 nm were showed to be more efficiently accumulated by marine zooplankton, to have longer retention times, and to display unique properties that may enhance their toxicity (Canesi and Corsi, 2016; Cole and Galloway, 2015; Bouwmeester et al., 2015). Moreover, as discussed by Mazurais et al. (2015) and Sussarellu et al. (2016), spherical microparticles may display a lower impact on larvae with respect to plastic fibers or rough fragments, which are the most frequently detected MP in the marine environment. In fact, Ziajahromi et al. (2017) recently reported that polyester fibers induce higher adverse effects than polyethylene microbeads on the survival, growth and reproduction of the waterflea *Ceriodaphnia dubia*.

Despite the lack of relevant morphological effects, qPCR data reported in this study did show interaction of MP exposure with transcriptional regulation of several genes involved in different physiological processes across the 48 h pf period. Among these, transcripts related to immune responses, shell biogenesis, and lysosomal responses were the most affected by MP exposure of embryos. The development of the immune system is inextricably linked with the formation of the digestive system in bivalves, and at the early larval stages the immune cells are believed to act as digestive system cells (Dyachuk, 2016; Balseiro et al., 2013). Furthermore, in adult bivalves, a molecular evolution of immune related proteins from defense to digestive functions has been postulated (Jollès et al., 1996), leading these proteins to be also expressed in the digestive gland and other tissues (Wang et al., 2012; Balbi et al., 2014). Therefore, the over expression observed for the immune related transcripts *LYS*, *MYTC* and *MYTLB* may result from the uptake activity displayed by D veligers towards MP, and the consequent stimulation of the digestive/immune apparatus.

Transcripts involved in shell biogenesis were significantly up regulated at 500 MP mL<sup>-1</sup>. Previous evaluations across the 48 h embryo development under normal growing conditions showed the selected transcript expressions to be highest at 48 h pf, confirming their key role in initial shell deposition (Balbi et al., 2016, 2017). In general, carbonic anhydrase (CA) regulates matrix mineralization by generating an acidic environment at the calcification sites (Clark et al., 2010); the mussel extrapallial protein (EP) regulates the production of the different polymorphs of calcium carbonate (Yin et al., 2009), and chitin synthase (CS) catalyzes the synthesis of chitin, a key structural component of the shell matrix that drives initial calcium carbonate deposition (Schonitzer and Weiss, 2007; Weiss and Schonitzer, 2006). Though the down regulation of these transcripts were previously reported in D veligers showing gross shell malformations and reduced calcification (Balbi et al., 2016, 2017), their up regulations observed in this study may boost the molecular machinery responsible for calcium carbonate fixation (CA, EP) and organic matrix functions (CS), thus explaining the apparent lack of effects of MP in the embryotoxicity test.

A significant down regulation of transcripts encoding the lysosomal enzymes hexosaminidase (*HEX*),  $\beta$  glucuronidase (*GUSB*), and cathepsin L (*CTSL*) was observed at both tested MP concentrations in 48 h D veligers. Dysfunctional effects on lysosomes were previously observed in digestive glands and haemocytes of adult

mussels exposed *in vivo* to polyethylene and polystyrene MP (Avio et al., 2015; von Moos et al., 2012). These investigations showed significant reduction of lysosomal enzyme stability in both immune and digestive tissues as related to MP accumulation within the organelles and the induction of an inflammatory response (Avio et al., 2015; von Moos et al., 2012). To the best of our knowledge, though based on transcriptional data, the present study is the first reporting the onset of a lysosomal dysfunctional response in larval stages of mussels. It must be observed that Izagirre et al. (2014) reported concomitant *HEX*, *GUSB*, *CTSL* down regulation, and decreased lysosomal membrane stability in digestive glands of adult mussels exposed to thermal stress and Cd<sup>2+</sup>. The authors suggested that such an apparent down regulation might result from genotoxic effects caused by Cd but also from molecular degeneration and loss of lysosomal functionality.

## 5. Conclusions

Data reported in this study showed that mussel larvae can effectively ingest and accumulate polystyrene MP, suggesting their potential role as plastic primary consumers in marine ecosystems. MP ingestion took place from the very early larval stage, and uptake/accumulation levels showed a linear relationship with the particle concentrations and the larvae morphological development up to 6 d pf. The consumption of microalgae by mussel larvae was not affected by the co occurrence of MP, suggesting that their impacts on exposed organisms could be limited by their ability to discriminate among nutritious and non food items. On the other hand, larvae showed a prolonged MP gut retention. The long lasting presence of non nutritious particles in the gut is *per se* associated to adverse biological impacts for the individuals, thus leading to consider mussel early life stages as particularly subjected to the effects of MP pollution; further, retained particles will more likely be accessible to higher trophic levels. Although no consistent effects on the morphological development were observed, transcriptional data obtained in this study outline the potential impacts of MP on shell biogenesis, immune and lysosomal systems.

The present investigations were performed using spherical polystyrene MP in controlled laboratory conditions; thus, observed responses might not necessarily reproduce those occurring in natural systems, where organisms are simultaneously exposed to a broad range of structurally heterogeneous MP of irregular shape/size and further associated stressors. Nevertheless, data highlight the vulnerability of bivalve early life stages towards MP, providing baseline information for future investigation addressing their impact on marine ecosystems.

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at

<https://doi.org/10.1016/j.envpol.2018.06.035>.

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