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Effects of polystyrene microplastics on early stages of two marine invertebrates with different feeding strategies☆

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ABSTRACT

Nowadays, microplastics represent one of the main threats to marine ecosystems, being able to affect organisms at different stages of their life cycle and at different levels of the food web. Although the presence of plastic debris has been reported in different habitats and the ability to ingest it has been confirmed for different taxa, few studies have been performed to elucidate the effects on survival and development of marine animals. Thus, we explored the effects of different environmental concentrations of polystyrene microbeads on the early stages of two invertebrate species widespread in the Mediterranean shallow waters: the pelagic planktotrophic pluteus larvae of the sea urchin *Paracentrotus lividus* and the filter-feeding sessile juveniles of the ascidian *Ciona robusta*. We evaluated the effects on larvae and juvenile development and determined the efficiency of bead ingestion. The feeding stages of both species proved to be extremely efficient in ingesting microplastics. In the presence of microbeads, the metamorphosis of ascidian juveniles was slowed down and development of plutei altered. These results prompted the necessity to monitor the populations of coastal invertebrates since microplastics affect sensitive stages of life cycles and may have consequences on generation recruitment.

Polystyrene microplastics can alter sensitive developmental stages of marine invertebrates, being filter-feeding organisms more effective in ingesting plastic particles.

1. Introduction

Microplastics (MPs) are small plastic particles (1 μm–1 mm; Andrady, 2015) some of which are specifically produced at the micro-level scale, such as sandblasting media, virgin pellet, cosmetics (Fendall and Sewell, 2009), while others originate from the degradation of bigger plastic debris, such as polyester fibers, polyethylene plastic bags and polystyrene particles from buoys and floats (Browne et al., 2011; Davidson, 2012; O'Brine and Thompson, 2010).

Over the past 50 years, an incredible amount of plastic has reached the marine environment and its presence has been recorded in different aquatic habitats at all latitudes (Thompson et al., 2004). The potential negative impact of this big debris on the ecosystems has been taken into consideration since the 1980s (Stefatos et al., 1999) while the MPs have been neglected until recent time, when numerous studies flourished, highlighting the presence of these particles in different environmental compartments. In North American Great Lakes, 43,000 microplastic particles/km^2 have been registered (Eriksen et al., 2013), whereas the sediments of Italian Garda Lake contain more than 1000 particles/m^2 (Imhof et al., 2013). According to Eriksen et al. (2014) there are over 5 trillion microplastics floating in the oceans. More in detail, their amount in the coastal waters has been estimated to vary from 3 to 100,000 item/m^3 (Carpenter et al., 1972; Doyle et al., 2011; Noren and Naustvoll, 2010) and to exceed 67,000 particles/km^2 in the open oceans (Colton et al., 1974). However, this measurement has been determined through the employment of plankton nets that have a mesh size between 80 and 330 μm, generating an important underestimation of the abundance and distribution of the smaller particles, which probably represent the highest threat (Andrady, 2015). Moreover, the influence of tide, wind, wave action and oceans currents determines a high variability of spatial and temporal distribution of particles, making extremely difficult a real quantification of MPs abundance.

Nowadays, the ecological impacts of MPs are of particular interest but our knowledge about their effects on marine organisms is still very limited. MPs ingestion has been demonstrated in different *taxa* including fish (Boerger et al., 2010; Davison and Asch, 2011; Lusher et al., 2012), seabirds (van Franeker et al., 2011), benthic polychaetes (Wright et al., 2013), amphipods, lugworms, barnacles (Thompson et al., 2004), mussels (Browne et al., 2008), decapod crustaceans (Murray and Cowie, 2011) and in different zooplanktonic organisms (Cole et al., 2013). The negative effects of MPs oral uptake vary from damaging and blocking the feeding appendages and digestive system (Derraik, 2002; Laist, 1997; Murray and Cowie, 2011), to limiting the food intake and transferring pollutants in living organisms (Mato et al., 2001; Oehlmann et al., 2009; Talsness et al., 2009; Teuten et al., 2009). In copepods, the co-presence of MPs beads and algae reduces the ingestion rate of the latter (Cole et al., 2013) and ultimately causes fertility reduction, probably due to insufficient nutrition (Lee

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et al., 2013). However, other species, such as polychaete worms, are able to ingest and expel plastic microspheres without any apparent detrimental effects, underlying the variety of responses in the different taxa (Cole et al., 2011).

When MPs reach the marine environment, they can interact with a wide range of organisms (Barnes et al., 2009). Animals inhabiting different compartments of the marine environment and displaying different feeding strategies can be differently affected by MPs. Moreover, different stages of the life cycle can have diverse sensitiveness, being the larvae usually the most vulnerable.

Very scant data are available regarding the effects of MPs on the development of aquatic organisms, as most studies have focused on the chemical aspects of MPs. In fact, the toxic effects of leachate from particles have been analyzed in different organisms, such as mussels (Gandara e Silva et al., 2016), fish (Lonnstedt and Eklov, 2016) and sea urchins (Nobre et al., 2015), whereas the physical effects are still scarcely explored (Kaposi et al., 2013).

To fill this important gap of knowledge, we evaluated the effects of environmental MPs concentrations on developmental stages of two different invertebrate species. *Ciona robusta* (Brunetti et al., 2015; Pennati et al., 2015) is a solitary ascidian, inhabiting shallow waters, in particular coastal areas rich of organic material (Satoh, 1994), and proposed as model organism to test water pollution (Zega et al., 2009). The adults develop through tadpole non-feeding larvae which swim for few hours before adhering to a substrate and metamorphosing into sessile juveniles (Chiba et al., 2004; Hotta et al., 2007). Both ascidian adults and juveniles are filter feeding and exploit the pharyngeal gill slits in muco-ciliary plankton feeding (Burighel and Cloney, 1997). *Paracentrotus lividus* is a common echinoid of the Mediterranean and North Atlantic coasts. This herbivorous sea urchin has an important role in coastal ecosystem maintenance as its foraging activity remarkably affects the compositions and the dynamics of algal and rocky littoral pools (Lawrence, 1975). Mature gametes are released directly in sea water and the larvae are pelagic until they become competent and undergo metamorphosis after about three weeks. Feeding activity starts slightly before the pluteus stage is reached, 48 h post fertilization (Giudice, 1986). The larvae feed on plankton employing external ciliary bands (Strathmann, 1971).

The chosen species offered the unique opportunity to evaluate and compare the impact of MPs on the development of two different suspension feeders: the pelagic suspension feeding plutei and the sessile filter-feeding ascidian juveniles. These allowed us to investigate if MPs differently affect animals with different feeding strategies. This information is of key importance to evaluate the existence of a specific sensitive compartment in the marine environment and properly manage its natural resources.

2. Materials and methods

2.1. Microplastics

Polystyrene spherical microparticles with a dark red color and a diameter of 10 μm were used in the experiments; chemical and physical properties of the MPs were provided by the supplier (Sigma, Italy). The red color allowed us to follow the beads track inside the transparent tested animals. The size of microbeads was chosen to be compatible with the plutei mouth opening (about 20 μm) and ascidian juvenile oesophagus (20–30 μm). Polystyrene microbeads were preferred because, unlike other plastics, they have a negligible styrene release in suspension (Cohen et al., 2002), ensuring that the observed effects can be ascribed to the physical presence of plastic particles and not to monomer contamination. The commercial standard was an aqueous suspension with a particle concentration of 50 mg/ml, that was diluted 1:1000 in artificial sea water buffered with 5 mM Hepes pH 8 (ASWH) to produce a stock suspension of 50 μg/ml of beads from which the final exposure suspensions were made. As published protocols suggested (Cole et al., 2013; Kaposi et al., 2013), all the suspensions were freshly prepared every time and sonicated for 10 min before use to ensure a homogenous distribution of the beads in the medium. Based on previous works (Lee et al., 2013), four different microparticles concentrations were tested: 0.125, 1.25, 12.5 and 25 μg/ml.

For both the experimental models, all the experiments were performed in triplicate.

2.2. Ascidians

Adults of the ascidian *C. robusta* were collected from natural populations in Chioggia bay (Venice, Italy) and maintained in aquaria at 18 ± 1 °C. Constant light condition was preferred to promote gamete production and avoid spawning (Lambert and Brandt, 1967). For each experiment, at least three adults were sacrificed. Eggs and sperms were obtained by dissection of gonoducts and cross fertilization was performed *in vitro*. Embryos were cultured at 18 ± 1 °C in ASWH until they reached the desired developmental stages (see below).

2.2.1. Development and larval survival rate

To test the effects of MPs presence on ascidian embryonic development, 30 embryos at 2-cells stage were exposed to the different bead concentrations in ASWH. Each exposure was performed in triplicate (n = 450). They were reared at 18 ± 1 °C until control larvae (CO), maintained in ASWH, reached the hatching larva stage (−18 h post fertilization (hpf); Hotta et al., 2007). To keep MPs in suspension, embryos were maintained in gently rocking condition. After −18 hpf, the survival rate was evaluated: each experimental group was carefully observed under a stereoscope and the percentage of alive larvae was calculated as: (number of alive larvae/total exposed embryos) x 100. Subsequently, larvae were fixed in 4% paraformaldehyde in standard Phosphate buffer saline (PBS) supplemented with 0.5 M NaCl, for 1.5 h at room temperature. After a few washes in PBT (PBS + 0.01% Tween 20), larvae were mounted on slides and observed under a dissection microscope to evaluate the presence of malformations.

2.2.2. Metamorphosis

Ascidian embryos develop into tadpole swimming larvae that, after few hours, metamorphose into sessile juveniles, in which adult tissues and organs differentiate. To determine the effects of MPs on metamorphosis, embryos were allowed to develop in ASWH until they reached the hatching larva stage. Then, 30 larvae for each treatment were transferred into 5.5 cm Petri dishes and allow to attach to the substrate. After adhesion, ASWH was replaced with the testing suspensions (0.125, 1.25, 12.5 and 25 μg/ml microbeads in ASWH). Control animals (CO) were maintained in fresh ASWH. 100 μl of a concentrated suspension of algae (*Tetraselmis* sp. 4–10 μm of diameter) were added to each treatment. The media were changed every day with freshly prepared ones. Animals were left to develop in the experimental conditions for four days. Then, each individual was observed under a stereoscope to estimate the proceeding of juvenile development. Each treatment was performed in triplicate (n = 90). The metamorphosis in *C. robusta* is a complex series of events (Chiba et al., 2004), mainly consisting in tail reabsorption, organs rotation and development of protostigmata or gill slits. By day 4, juveniles nor-

mally reach stage 4, characterized by completed organs rotation and the presence of two pairs of protostigmata (for a comprehensive description of *Ciona* metamorphosis process see Chiba et al., 2004). To evaluate MPs effects on metamorphosis and juvenile development, we assigned a developmental stage, roughly corresponding to those described in Chiba et al. (2004), to each individuals, mainly evaluating organs rotation and the dimension of the axial complex. Stage 4 juveniles had a small almost negligible axial complex; stage 3 juveniles had completed organ rotation but retained a big axial complex; stage 2 samples did not start the organs rotation. Moreover, we counted animals that had adhered to the dish and died soon after (Fig. 1A–D).

2.2.3. Juvenile survival

Effects on juvenile survival were evaluated exposing −90 metamorphosed individuals (30 for each replica) at stage 3 to the different concentrations of MPs for 8 days. Stage 3 was chosen since it was the stage just before the oral siphon started contracting (Chiba et al., 2004). The suspensions were supplemented with 100 µl of concentrated algae and renewed every day with freshly prepared ones. A control group (CO) was reared in ASWH plus algae. Then, each individual was observed under a stereoscope and the percentage of alive juveniles was recorded.

2.2.4. Feeding behaviour and ingestion rates

To measure the ingestion rate, 30 juveniles at stage 4 (Chiba et al., 2004) were exposed to MPs. As soon as the exposure suspensions were added, the single individuals were observed under a stereoscope and the feeding activity was recorded for 1 min using a Leica DFC-450-C camera. The ingestion rate was calculated as the number of particles ingested in the first minute of exposure. The juveniles were maintained in the exposure media for 12 h then the suspensions were replaced with ASWH plus algae. After 24 h, the percentage of juveniles with MPs in their digestive tract and/or in faecal pellets was calculated to estimate the expelling efficiency.

2.3. Sea urchins

Adults of *Paracentrotus lividus* were collected in the Gulf of Lerici (La Spezia, Italy) and reared in aquaria as previously described (Mercurio et al., 2014). Gametes were obtained by intraoral injection of 1–2 ml of 1 M KCl (Kaposi et al., 2013) from adults of homogenous size (Ø ~ 4 cm). Fertilization and embryos culture were performed at 18 °C with 12 h dark/light cycle in 300 ml of gently aerated ASWH. As sea urchin larvae start eating between 36 and 48 h post fertilization (hpf), Phyto Reef (SHG, Italy), a nutritional supplement based on microalgae (2–8 µm) was added to the media just before feeding activity began.

2.3.1. Development and larval survival rate

Sea urchin embryos were allowed to develop in ASWH for 24 hpf. Then, 25 ml of ASWH with embryos were added to 25 ml of MPs suspensions in order to reach the final testing concentrations (0.125, 1.25, 12.5 and 25 µg/ml microbeads in ASWH + CO). When the larvae reached the 4-armed pluteus stage (72 hpf), 1 ml of each treatment suspension plus plutei was collected and the percentage of alive larvae was evaluated under a microscope as: (number of larvae with ciliary movements/total number of larvae) x 100. To assess the effects on development, a further 1 ml of each suspension was collected and larvae were fixed in 4% paraformaldehyde in PBS supplemented with 0.5 M NaCl. 90 randomly chosen animals for each tested concentration were photographed under an optical microscope and body length, post-oral arm length and body width (Fig. 3A) were measured to verify the presence of developmental delay or malformations as reported by Kaposi and collaborators (2013).

2.4. Comparison between ascidian and sea urchin feeding activity

To compare the ingestion efficiency of ascidian filter-feeding juveniles and sea urchin pelagic plutei, we exposed for an hour stage 4 juveniles and 72 hpf plutei to the different concentrations of MPs. Subsequently, juveniles and plutei were observed under a stereomicroscope and number of individuals displaying MPs in their digestive

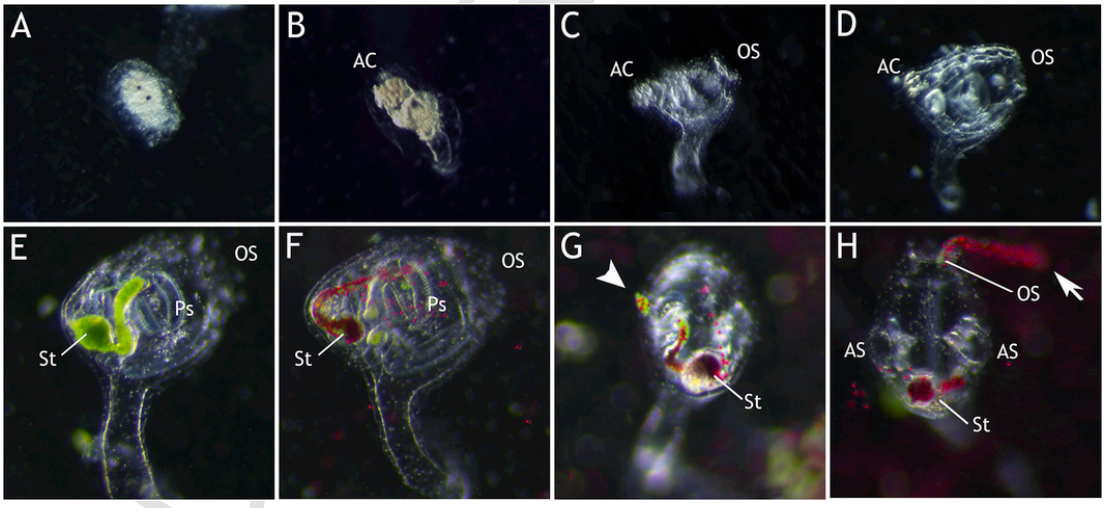

Fig. 1. *C. robusta* developing juveniles. A–C: Different phenotypes observed after exposure to microplastics. A: Dead individual. B: Stage 2. C: Stage 3. D: Stage 4, see text for description. E–H: Feeding juveniles observed 24 h after exposure. E: Lateral view of a control juvenile with algae in the digestive tract. F: Lateral view of a juvenile exposed to 25 µg/ml MPs showing MPs and algae in the digestive tract. G: Dorsal view of a juvenile exposed to 12.5 µg/ml showing MPs and algae in the digestive tract and in a faecal pellet. H: Dorsal view of a juvenile while rejecting MPs from the oral siphon. AC, axial complex; AS, atrial siphon; OS, oral siphon; Ps, protostigmata; St, stomach. Arrowhead = faecal pellet; arrow = rejected MPs.

tract was recorded. Feeding efficiency was calculated as: (number of fed individuals/total number of individuals scored) x 100.

2.5. Statistical analysis

All data were analyzed using the software R (R-Core-Team, 2013). A single way Analysis of Variance (ANOVA) was performed, after a Cochran Test to test the homogeneity of variance. If H_0 of homoscedasticity was rejected, the data were square root transformed to meet the assumptions of the analysis. If the results of ANOVA were significant ($P < 0.05$), a Tukey's Post-hoc Test was performed to disentangle differences among groups.

3. Results

3.1. Effects on ascidians

3.1.1. Development and larval survival rate

Larval survival rate was not affected by MPs exposure at all the tested concentrations ($F_{4,10} = 2.0433$; $p = 0.1639$; Fig. S1). Moreover, exposed larvae showed a normal phenotype, perfectly comparable to control samples (Fig. S2).

3.1.2. Metamorphosis

We recorded in all the experimental groups the presence of dead individuals and juveniles at different developmental stages (Fig. 1A–D). Dead animals were observed in all the experimental groups and no statistically significant difference was reported. The percentage of individuals that after 4 day reached stage 4 (Fig. 1D) was significantly lower in all the exposed groups compared to control group ($F_{4,10} = 11.388$, $p = 0.0009$) (Fig. 2A). Moreover, the percentage of juveniles scored as stage 3 (Fig. 1C) was significantly higher at 12.5 and 25 μg/ml concentrations than in control group ($F_{4,10} = 4.6175$, $p = 0.0226$) (Fig. 2A). Metamorphosis was slowed down but manifest malformations were not observed at all the tested concentrations.

3.1.3. Juvenile survival

No statistically significant difference in juvenile survival was observed between control and treated samples: survival rate ranged from 83.7%, observed in control group and 67.5% registered at the highest MPs concentration ($F_{4,10} = 1.147$; $p = 0.3894$; Fig. S3).

3.1.4. Feeding behaviour and ingestion rates

After 1 min of exposure at the three highest concentrations, juveniles had already MPs in their digestive tract (Fig. 2B) (Video 1). The number of styrene MPs ingested increased with particle concentration ($F_{4,10} = 40.804$, $p = 3.66*10^{-6}$). In particular, the number of MPs ingested in juveniles exposed to 12.5 and 25 μg/ml was significantly higher than that ingested by juveniles exposed to 1.25 μg/ml (Fig. 2B). After 24 h (Fig. 1E–G, 2C), very few individuals had MPs only in the digestive and the differences were not significant for all the concentrations ($F_{4,10} = 0.7388$, $p = 0.5865$). Most of the juveniles exposed to 0.125 and 1.25 μg/ml had no MPs or had expelled them in the faecal pellets. At the two highest concentrations (12.5 and 25 μg/ml), a significant higher percentage of individuals displayed MPs both in the pellets and in the digestive tract (12.5 and 25 μg/ml vs the other three treatments: $F_{4,10} = 32.217$, $p = 1.092*10^{-5}$). Some juveniles were observed while rejecting MPs and algae from oral siphon (Fig. 1H).

Supplementary video related to this article can be found at https://doi.org/10.1016/j.envpol.2017.11.030.

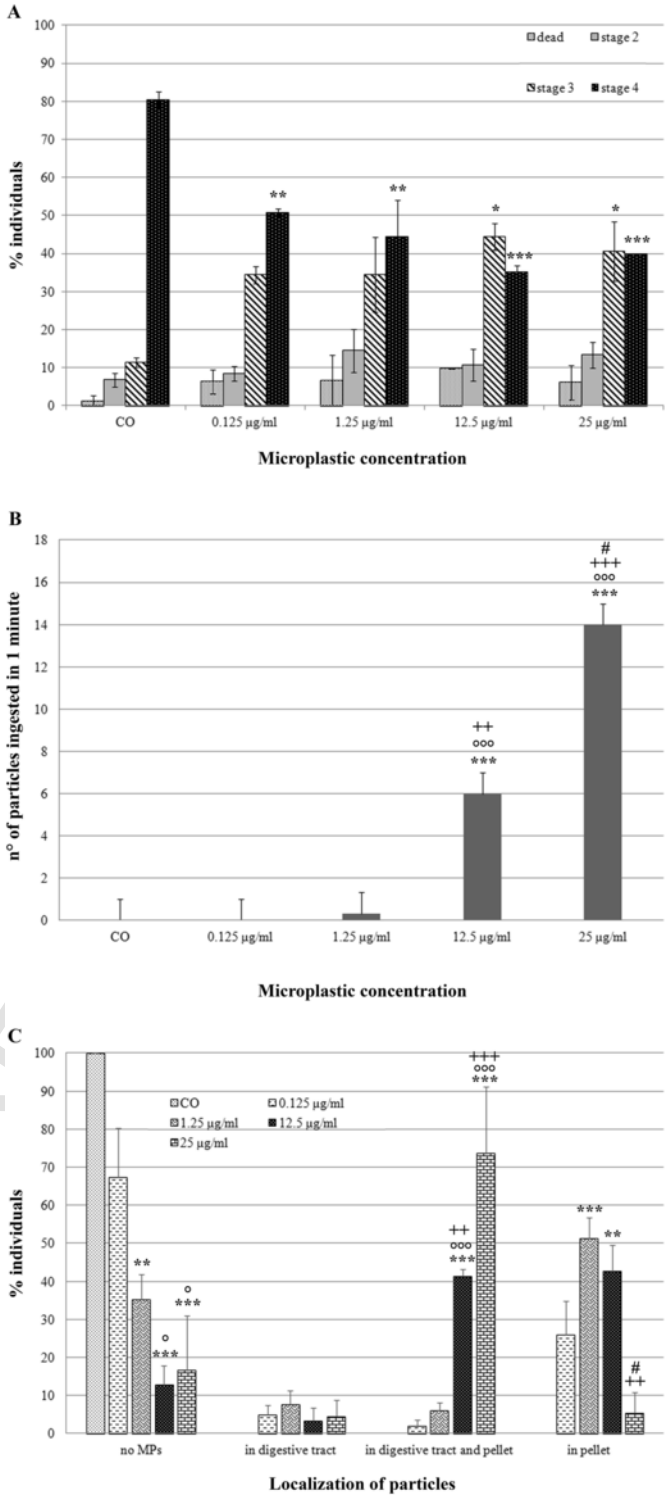

Fig. 2. Effects of polystyrene microplastics on *C. robusta*. Data are means of 3 replicates ± standard error (SE). A: Percentages of juveniles at different developmental stages observed after 4-day exposure to different concentrations of MPs. B: Number of MPs ingested after 1 min of exposure at different concentrations. C: Localization of MPs after 24 h from exposure. Legend of symbols: * = differences from control;° = differences from 0.125 μg/ml; + = differences from 1.25; # = differences from 12.5 μg/ml. The repetition of each symbol indicate the level of significance according to R significance codes: $p < 0.001$ ***; $p < 0.01$ **; $p < 0.05$ *.

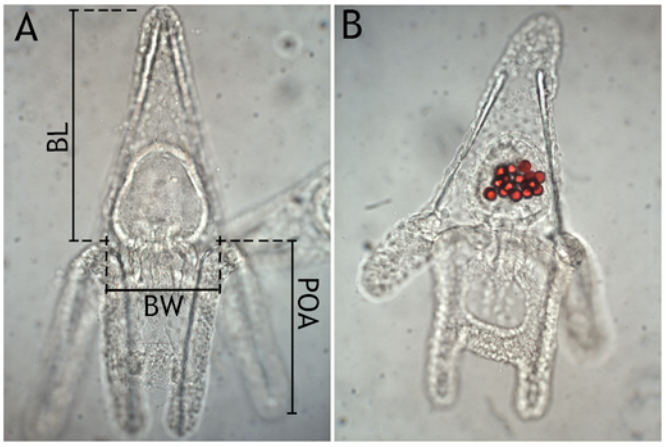

Fig. 3. *Paracentrotus lividus* plutei. A: Control pluteus 72 h post fertilization. B: Pluteus developed from an embryo exposed to 25 µg/ml MPs. BL: body length; BW body width POA: post oral arm.

3.2. Effects on sea urchins

3.2.1. Development and larval survival rate

Plutei can efficiently ingest MPs that can be observed in their digestive tract (Fig. 3B). After 72 h exposure, no difference in the survival rate was observed between control larvae and those developed in presence of MPs beads at all tested concentrations ($F_{4,10} = 0.6298$; $p = 0.6524$) (Fig. S4).

Significant differences were found in body length and arm length of plutei reared at different bead concentrations (Fig. 4A–C). Plutei exposed to 12.5 µg/ml were significantly longer than control ones ($F_{4,445} = 20.24$, $p < 0.0001$; Tukey's post hoc test $p = 0.0001$), while plutei exposed to 25 µg/ml were shorter ($F_{4,445} = 20.24$, $p < 0.0001$; Tukey's post hoc test $p = 0.0002$). Differences in body width were found between control group and 0.125 µg/ml, between 0.125 µg/ml and all the other treatments and between 12.5 µg/ml and 25 µg/ml ($F_{4,445} = 18.29$, $p < 0.005$). At last, treated plutei had longer arms than control group when developed at 0.125 and 12.5 µg/ml ($F_{4,445} = 39.67$, $p < 0.0001$; Tukey's post hoc test $p < 0.0001$). Plutei developed at 1.25 µg/ml had shorter arms compared to 0.125 µg/ml (Tukey's post hoc test $p = 0.0336$) and those developed at 25 µg/ml had shorter arms compared to all the other treatments (Tukey's post hoc test $p < 0.0001$).

3.3. Comparison between ascidian and sea urchin feeding efficiency

After 1 h exposure to MPs, both ascidian juveniles and plutei ingested beads at all tested concentrations but they displayed different efficiencies. In both species, the percentage of individuals with beads in their digestive tract increased with MP concentration. At 1.25, 12.5 and 25 µg/ml, more than 87% of ascidian juveniles had MPs in their digestive tract. These values were significantly higher than the percentage of juveniles that ingested MPs at 0.125 µg/ml concentration and in control group ($F_{4,10} = 106.1$, $p = 3.08*10^{-8}$) (Fig. 5). Only 20% of plutei had ingested MPs at 0.125 µg/ml, while 100% of plutei at 25 µg/ml had them in the digestive tract ($F_{4,10} = 283.55$, $p < 0.0001$). At highest concentrations, there is not a significant difference in the efficiency of ingestion between the two species, as almost all individuals fed on MPs (ANOVA: 12.5 µg/ml: $F_{1,4} = 0.4895$ $p = 0.5227$; 25 µg/ml $F_{1,4} = 1$ $p = 0.3739$). On the other hand, at the lowest concentrations, the efficiency of ingestion of planktonic plutei resulted significantly lower than that of filter-feeding benthic juveniles of *C. robusta* (ANOVA: 0.125 µg/ml: $F_{1,4} = 7.9014$ $p = 0.04827$; 1.25 µg/ml: $F_{1,4} = 27.888$ $p = 0.006166$) (Fig. 5).

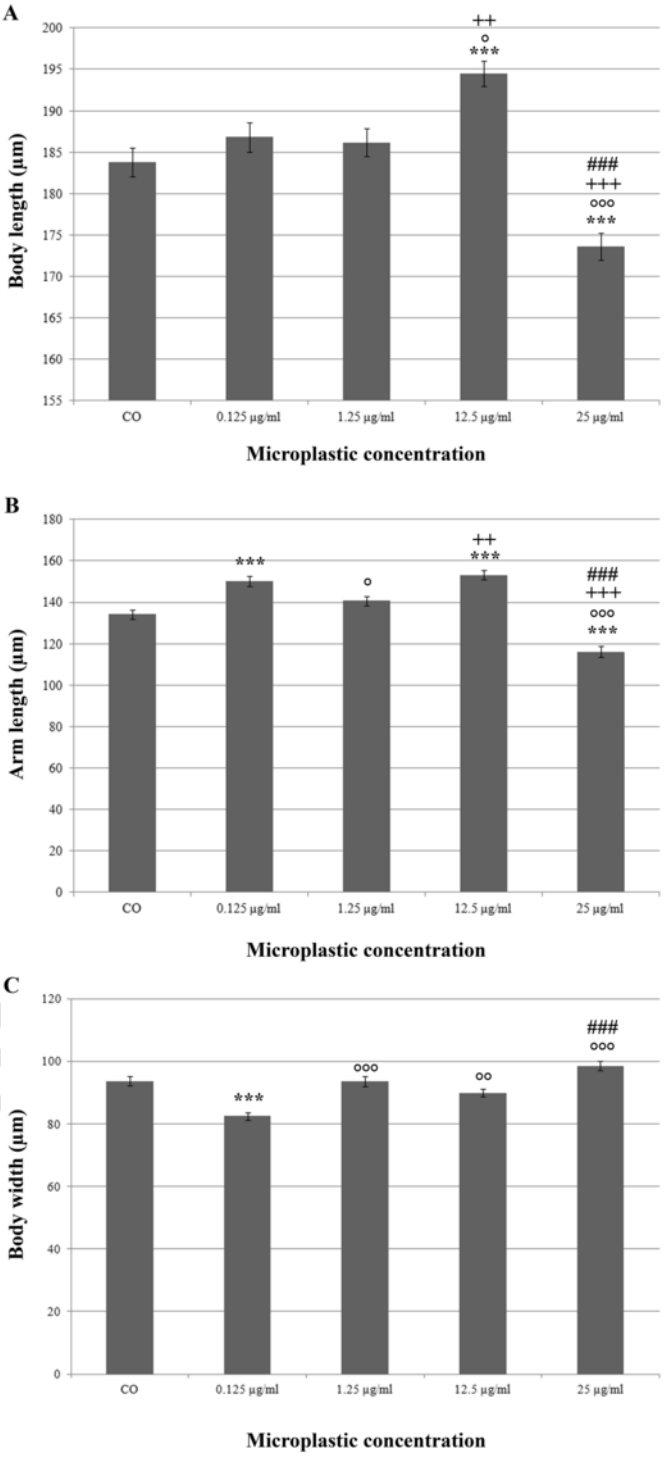

Fig. 4. Morphometric analysis of plutei exposed to MPs. Legend of symbols: * = differences from control;° = differences from 0.125 µg/ml; + = differences from 1.25; # = differences from 12.5 µg/ml. The repetition of each symbol indicates the level of significance according to R significance codes: $p < 0.001$ ***; $p < 0.01$ **; $p < 0.05$ *.

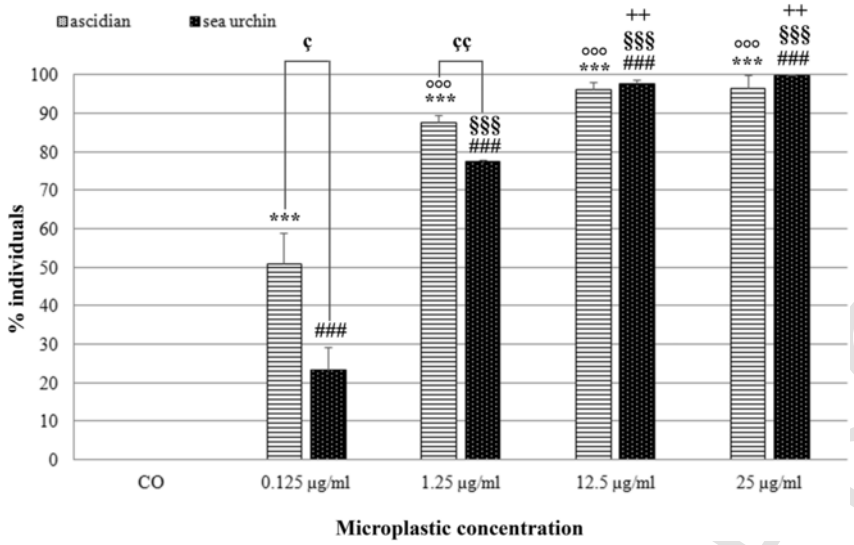

Fig. 5. Percentage of *C. robusta* juveniles and *P. lividus* plutei with MPs in their digestive tracts, after 1 h of exposure to particles. Data are means of 3 replicates with ±SE. Legend of symbols: * = differences of *C. robusta* from control;° = differences of *C. robusta* from 0.125 µg/ml; # = differences of *P. lividus* from control; § = differences of *P. lividus* from 0.125 µg/ml; # = differences of *P. lividus* from 1.25 µg/ml ç = differences between the two species at the same concentration. The repetition of each symbol indicates the level of significance according to R significance codes: $p < 0.001$ ***; $p < 0.01$ **; $p < 0.05$ *.

4. Discussion

Plastic pollution represents one of the main threats to aquatic ecosystems: the high production level and the slow degrading time of plastic have led to an increasing amount of plastic-derived debris in oceans and seas. After degradation, these remains are fragmented into micro sized particles that can be ingested by marine organisms and enter the food web (Andrady, 2015).

In this study, we evaluated the effects of polystyrene MPs on the development of two ecologically relevant marine invertebrates: the ascidian *C. robusta* and the sea urchin *P. lividus*. Our results add new important information about the effects of this emerging pollutant on early life stages of invertebrates with different feeding strategies.

As already reported for many other taxa, the feeding stages of both the analyzed species are able to efficiently ingest MPs. In particular, ascidian juveniles and plutei were fed with a suspension of MPs and algae and they appeared not to be able to discriminate between food and inorganic particles, as other invertebrates proved to do (Fernández, 1979; Paffenhofer and Vansant, 1985). Indeed, when ascidian juveniles are exposed to high particles concentrations, the MPs ingestion occurred as soon as beads were added to ASWH, suggesting that juveniles, filtering the water, did not make any kind of selection. However, some individuals were observed rejecting microbeads and algae from the oral siphon, with a series of whole body contractions. This is probably a reflex triggered by sensorial organs upon siphon tentacles (Mackie et al., 2006; Rigon et al., 2013) and due to the excessive intake of particles. In contrast, nauplii of the copepod *Calanus pacificus* strongly selected and consumed almost exclusively algal cells, when fed with mixtures of planktonic algae and plastic beads of different or similar sizes (Fernández, 1979). The ability of *C. pacificus* to select organic particles could be due to the restrictive diet of naupilii that feed only upon plant material (Fernández, 1979), while ascidian juveniles filter water uptaking any kind of particles. Thus, the different sensitiveness to MPs observed in these two animals could be related to their specific feeding behaviour. Furthermore, the disappearance of the rejection reflex in *Ciona* juveniles at high MPs concentration was probably caused by an adaptive behaviour of the sensorial organs. Indeed, different sensory structures have been described in ascidian siphons. In the atrial cavity, mechanoreceptors, the cupular organs, send their axons to the central nervous system. At the oral siphon base, the coronal organ, consisting of secondary neurons, borders the velum and tentacles. In addition, scattered sensory cells are located in siphons inner and outer wall (Mackie et al., 2006; Rigon et al., 2013).

Previous works reported that numerous marine organisms are able to ingest and expel plastic microspheres without any apparent negative effects (Thompson et al., 2004). On the contrary, our results suggested that MPs effects depend on animal feeding strategy and life stages.

After an hour of exposure, a significant percentage of ascidian juveniles and plutei showed plastic beads in their stomach at all tested concentrations. However, comparing MPs uptake in the two animal models, the higher ingestion ability of the filter-feeding juveniles appeared evident. Although at the two highest concentrations almost all individuals had ingested MPs, regardless of the species, at the lowest concentrations a significant higher percentage of ascidian juveniles ate microbeads compared to sea urchin plutei. This result suggested that the presence of MPs in the environment, even at low concentrations, can impact sessile filtering organisms more than pelagic suspension feeders. This consideration is strengthened by the fact that juvenile and adult ascidians employ the pharyngeal basket to filter a huge amount of water per day, estimated around 46.4 ml/min for adults with a total dry weight of 0.84 g (Randløv and Riisgård, 1979).

After 24 h, almost all ascidian juveniles, that ingested MPs at the concentrations of 0.125 µg/ml and 1.25 µg/ml, managed to expel them with faecal pellets. However, with the increase of particle concentration and, therefore of ingestion, it became more difficult for animals to expel them as demonstrated by the higher percentage of individuals that even after pellet expulsion retained particles in their stomach. Moreover, at the highest concentrations, some ascidian juveniles tried to expel their stomach content by rejecting it from the oral siphon, with a series of contractions of the whole body. Also this behaviour was not completely efficient to eliminate MPs from the digestive tract, suggesting that a higher particles intake can determine a higher risk of damages and digestive system block.

Studies on MPs effects on animal development are particularly scarce, even if different stages of life cycle can have diverse sensitiveness, being the larvae usually the most vulnerable one. Thus, we explored the impact of MPs presence on the early life stages of ascidians and sea urchin. Although MPs exposure did not affect the survival rate of *C. robusta* juveniles and *P. lividus* embryos, plastic beads appeared to be detrimental during some developmental phases.

We analyzed three different standard parameters to characterized MPs effects on plutei body growth and we observed values that significantly differed from control group. A clear trend was not observable: the absence of a dose-dependent effect between bead concentrations and measured parameters is not unusual in aquatic organisms, being already reported in other species of sea urchin (Kaposi et al., 2013), copepods (Cole et al., 2013) and marine worms (Wright et al., 2013). In our case, the reduction of body and arm length and the increase of body width reported in plutei exposed to 25 μg/ml highlight that the co-ingestion of beads and algae compromised the correct development of the body shape. More controversial results are the increase of body length at 12.5 μg/ml, the increase of arm length at 0.125 and 12.5 μg/ml and the reduction of body width at 0.125 μg/ml. The high variability in particles uptake and release occurring at the lower tested concentrations may be responsible for the variability of the measured responses. In contrast, at the highest tested concentration, MPs distribution was probably more homogenous making the exposure more uniform and the effects more consistent. Moreover, the same kind of results have been found for the echinoderm species *Tripneustes gratilla* in which a reduction of body width was observed at the lowest and highest concentration tested but not at the intermediate ones (Kaposi et al., 2013).

In *Ciona,* the metamorphosis proved to be the most sensitive process to MPs presence. Exposed juveniles showed a significant slowdown of metamorphosis, displaying a high percentage of individual at an earlier developmental stage than controls. Since MPs did not affect the embryonic development of the non-feeding ascidian larvae, we supposed that the effects observed on the other stages were mainly due to the physical presence of the beads rather than to chemical released by plastic degradation.

It was proposed that the physical presence of microbeads can alter the development of a planktotrophic larva, probably reducing the food intake: in copepods, MPs presence appeared to decrease food ingestion (Cole et al., 2013), eventually leading to fertility reduction (Lee et al., 2013). Moreover, it was suggested that the selective feeding behaviour of *C. pacificus* nauplii could be an energy input optimization, so the animals could achieve a larger input of energy per unit time than would be if no selection occurred (Fernández, 1979). Thus, the non-selection behaviour displayed by ascidian juveniles and plutei suggested that MPs ingestion can cause a reduction of nutritional uptake, responsible for the developmental and growth alterations observed in ascidians and plutei.

These considerations suggested that sensitiveness to MPs differ among species, indicating the necessity to extend the studies to a wider range of species in order to elucidate MPs impact on marine ecosystem.

5. Conclusion

According to our results, the developing stages of both ascidian and sea urchin are able to ingest MPs. Particularly, comparing the intake efficiency of the two species, the sessile filtering organisms appeared more vulnerable to MPs effects than pelagic suspension feeders. Even if MPs exposure do not seem to influence specimens survival, their development was affected as plastic beads ingestion slowed down metamorphosis of *C. robusta* and altered post-embryonic development or/and growth of *P. lividus*. These results appear particularly worrying when we consider that the ability to clear the gut of plastic decreases with the increase of MPs concentration. In long-term exposure, this could cause MPs accumulation in the digestive tract, definitively compromise the nutritional uptake and ultimately lead to the animal death. Thus, our results are particularly relevant as they revealed that developmental stages of different species are highly sensitive to MPs presence, prompting the necessity to monitor coastal invertebrate populations since MPs can alter generation recruitment.

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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.2017.11.030.

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