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How sea urchins face microplastics: Uptake, tissue distribution and immune system response☆

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ABSTRACT

Plastic pollution represents one of the major threats to the marine environment. A wide range of marine organisms has been shown to ingest microplastics due to their small dimensions (less than 1 mm). This negatively affects some biological processes, such as feeding, energy reserves and reproduction. Very few studies have been performed on the effect of microplastics on sea urchin development and virtually none on adults. The aim of this work was to evaluate the uptake and distribution of fluorescent labelled polystyrene microbeads (micro-PS) in the Mediterranean sea urchin *Paracentrotus lividus* and the potential impact on circulating immune cells. Differential uptake was observed in the digestive and water vascular systems as well as in the gonads based on microbeads size (10 and 45 μm in diameter). Treatment of sea urchins with particles of both sizes induced an increase of the total number of immune cells already after 24 h. No significant differences were observed among immune cell types. However, the ratio between red and white amoebocytes, indicative of sea urchin healthy status, increased with both particles. This effect was detectable already at 24 h upon exposure to smaller micro-PS (10 μm). An increase of intracellular levels of reactive oxygen and nitrogen species was observed at 24 h upon both micro-PS exposure, whereas at later time these levels became comparable to those of controls. A significant increase of total antioxidant capacity was observed after treatment with 10 μm micro-PS. Overall data provide the first evidence on polystyrene microbeads uptake and tissue distribution in sea urchins, indicating a stress-related impact on circulating immune cells.

1. Introduction

Over the last decade, the anthropogenic pressure on the marine environment has resulted in the widespread occurrence, distribution and accumulation of mismanaged plastic waste in both abiotic and biotic compartments leading to a growing concern on their ecological impact (Còzar et al., 2014; Eriksen et al., 2014; Geyer et al., 2017).

According to recent model-based projections, large rivers (>100 Km^2) represent the major sources of plastic (91%) into the sea, thus marine species living in coastal areas could be more at risk than those living in the open-sea (Lebreton & Andrady, 2019).

Semi-enclosed basins as the Mediterranean Sea are considered among the most polluted areas with estimates of a range between 873 and 2576 tons of plastic particles floating on sea surface, among which between 3.2×10^{12} and 28.2×10^{12} items of a small dimension, i.e. microplastics (<1 mm, Hartmann et al., 2019) (Còzar et al., 2015; Van Sebille et al., 2015; Suaria et al., 2016).

Recent plastic fate models show that the distribution of micro- and nanoplastics (<1 μm) on the sea surface, water column, and sediment is driven partly by polymers size and density, which affect their sinking or floating behaviour/capacity (Kooi et al., 2017). Once in natural waters, biofouling, chemicals adsorption (including contaminants) and incorporation into fecal pellets, and marine aggregates can significantly affect their buoyancy or precipitation

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to seafloor (Qian et al., 2007; Lobelle & Cunliffe, 2011; Rochman, 2015; Cole et al., 2016). Moreover, due to environmental conditions, polymer type and weathering, fragmentation of plastic can originate smaller particles, down to nanometer (nm) size (nanoplastics), as those recently detected in seawater below the North Atlantic sub-tropical gyre (Andrady, 2017; Ter Halle et al., 2017; Ekvall et al., 2019).

Microplastic exposure could pose a high risk to marine species, according to their ecological and behavioural traits (home range and feeding strategies), as well as their position in the food web (pelagic, demersal or benthic). Microplastic-associated effects on marine biota have been increasingly investigated, especially for what concerns the mechanism of interaction at the cellular level (Prinz & Korez, 2020). Size-dependent effects have been reported in different organisms such as *Danio rerio* (Lu et al., 2016), *Brachionus koreanus* (Jeong et al., 2016), *Mus musculus* (Deng et al., 2017), *Paracyclopina nana* (Jeong et al., 2017), *Caenorhabditis elegans* (Lei et al., 2018) in terms of uptake, oxidative damage and neurotoxicity.

Several impairments have been associated with microplastic ingested both in larvae (development and survival) (Sussarellu et al., 2016) and in adult stages (inflammation, growth and fecundity) (Lee et al., 2013; Besseling et al., 2014; Cole et al., 2015). Species living in highly urbanised sites in which domestic and industrial sewages exist together with other local sources (tourism, fishery, aquaculture) are likely more threatened by both micro- and nanoplastics. In particular, Mediterranean coastal species are considered at higher risk of microplastic ingestion than those living in the open sea according to the model of Compa and co-authors (2018), based on data on plastic ingestion, species distribution, and plastic dispersion. Small home range species are more exposed to plastic closer to their habitat, while long-range migrating species will catch those more highly distributed in the environment.

Mediterranean benthic marine species have been overlooked for plastic ingestion compared to filter-feeders, fish, and large mammals (Fossi et al., 2012; Romeo et al., 2015; Bellas et al., 2016; Güven et al., 2017; Compa et al., 2018; Digka et al., 2018; Duncan et al., 2018; Phuong et al., 2018; Giani et al., 2019; Lefebvre et al., 2019). The few available studies reported the presence of plastics in biota from a Norwegian fjord and in deep sea invertebrates, belonging to different phyla, including cnidarians, echinoderms, arthropods and molluscs (Taylor et al., 2016; Bour et al., 2018; Cau et al., 2019; Courtene-Jones et al., 2019).

Among the benthic species, sea urchins are key grazers structuring kelp forest ecosystems and represent an important element of trophic cascade (Steneck, 2013). In fact, when sea urchin predators are scarce, the number of sea urchins greatly increased forming the so called “urchin barrens”, thus limiting the extent of seagrass beds (Sala et al., 1998; Eklöf et al., 2008). Recently, sea urchins have been shown to act also as shredders, in capturing and converting the coarse kelp litter to fine fragments utilised by benthic detritivores (Yorke et al., 2019).

The abundance of *P. lividus* has been reported in Northwestern Mediterranean Sea, in the area around Corsica and Tuscany (Palacin et al., 1998; Sala et al., 1998; Jacinto et al., 2013; Duchaud et al., 2018). The growing demand and market values of gonads as food significantly affect both sea urchin abundance and population size in the Gulf of Naples, that receives terrestrial inputs from a highly urbanised area, with over 2 million inhabitants. Urban, industrial and agricultural discharges exert a strong pressure on the coastal area, including the contribution of the Sarno river, one of the most polluted rivers in Europe (Montuori et al., 2013). Therefore, *P. lividus* population living in the area is continuously threatened by a variety of pressures, acting alone or simultaneously, including microplastics.

Data on floating microplastic concentrations in the Gulf of Naples are scarce. In 2017, a cruise along the Italian coasts sampled at coastal (Portici) and offshore (Punta Campanella) stations, reporting 3.56 and 0.26 items per m^3 (CNR-ISMAR, 2017). Distribution appears to be strongly affected by the general water circulation and residency times, being strongly spatially and seasonally variable. These concentrations lie within those reported from other areas of the Mediterranean Sea, ranging from 0.15 to 7.68 items m^3 (Cincinelli et al., 2019), and are strongly influenced by hydrodynamic features such as currents, vertical movements, gyres and fronts. However, these data refer to microplastics greater than 200–780 μm depending on the mesh net used for sampling (Cincinelli et al., 2019).

A few papers have hitherto reported the effects of microplastics on sea urchin embryo development. However, no studies have been performed on adults. Contrasting results have been reported on sea urchin embryo development probably due to differences in the type, concentration and size of the polymers as well as in the developmental stage chosen for the treatment (Kaposi et al., 2014; Martínez-Gómez et al., 2017; Trifuoggi et al., 2019). The possible toxicity of microplastics has been related to different processes like larval ejection, the absorption of contaminants, or the release of plastic additives (Kaposi et al., 2014; Nobre et al., 2015; Martínez-Gómez et al., 2017; Beiras & Tato, 2019).

Adult sea urchins could accumulate microplastics via at least two different pathways, food ingestion and water vascular system, with putative differential impacts on the animal physiology and on the highly sensitive immune system, known as a sentinel of environmental stress (Pinsino & Matranga, 2015). In addition, sea urchin *P.lividus* show distinctive features for macro-plastic fragmentation producing smaller plastic pieces (Porter et al., 2019).

The aim of the present study was to investigate the uptake and tissue distribution of polystyrene microplastics of different size in *P. lividus* adults and their potential impact on immune cells.

2. Methods

2.1. Sea urchins collection and maintenance

Adult specimens of *P. lividus* (Lamark 1816) (diameter 4.68 ± 0.46 cm) were collected in the Gulf of Naples from sites (40°46′50″ N 14°12′03″ E) that are not privately-owned nor protected in any way, according to the authorisation of Marina Mercantile (DPR 1639/68, 09/19/1980, confirmed on 01/10/2000). Sea urchins were acclimated for two weeks in glass tanks filled with circulating natural seawater (temperature 18 ± 1 °C, salinity 38 ± 1, dissolved O_2 7 mg/L, pH 8.1; all the parameters remained constant during the experiment) and fed ad libitum with *Ulva lactuca*. Although no authorisation is required for sea urchins, all procedures were performed according to the European Directive 2010/63/EU on the protection of animals used for scientific purposes by reducing at minimum the number of specimens used and any pain or distress of animals during exposure.

2.2. Sea urchin in vivo exposure

2.2.1. Morphology of P. lividus madreporite

Before starting uptake experiments, the pore size of sea urchins madreporite was determined using scanning electron microscopy available at the Advanced Microscopy Center (AMC), at Stazione Zoologica Anton Dohrn of Naples. The madreporite was removed using scissors from three sea urchins with same dimensions of those used for the experiments. Madreporite specimens were washed two times in deionised water and then placed overnight in a NaClO solution (1:4 in deionised water) (Tamori et al., 1996). After further washing in deionised water, they were placed in a 70%

ethanol, air dried, sputter coated with gold, using a Leica ACE200 vacuum coater (Leica Microsystems, Inc. Buffalo Grove, IL). Observations were performed with a SEM JSM-6700F (JEOL USA, Inc. Peabody, MA).

2.2.2. Polystyrene microbeads

Fluorescent-labelled polystyrene microbeads (micro-PS) (441 excitation/485 emission) of 10 μm and 45 μm were purchased from Polysciences (Warrington, PA, U.S.A.). According to the supplier, the particles were packaged as 2.5% aqueous suspension without biocides or stabilisers. Besides, the fluorophore was embedded inside the particles, thus providing the fluorescent beads high stability and resistance. Micro-PS working solutions (10^4 particles mL^{-1}) were prepared in deionised water and then added to filtered natural sea water (0.22 μm) to reach the final concentration of 10 particles mL^{-1}. Stock and working solutions were vortexed for 3 min prior to use. Size and shape of micro-PS was further confirmed by light microscopy (Supplementary data, Fig. S1).

2.2.3. Experimental design

Control and micro-PS exposed treatments (10 particles mL^{-1}) were set up by placing sea urchins in 4L experimental glass tanks (1 specimen per liter) supplied with natural filtered seawater (0.22 μm) in a closed flow-through system, constantly aerated. Waters were renewed every 24h. Sea urchins were exposed for 72h and not fed. Experiments were run three times. In absence of reliable data on the environmental levels of MPs in the size range, 10 μm and 45 μm, we chose to expose the animals to 10 particles mL^{-1}, on the base of the micro-PS concentrations used in the last 5 years in similar ecotoxicological studies (see Supplementary data, Table S1). This concentration also ensures that detectable MP amounts could be resolved following uptake and organ distribution by sea urchins.

2.3. Extraction and quantification of microplastics from sea urchin organs

After 72h, the abundance of micro-PS was examined in various organs, including the esophagus, the digestive system, and the vascular system (ring canal, stone canal and ampullae), and the whole gonads. Sea urchins were sacrificed by cutting off the peristomial membrane. Esophagus, digestive system, water vascular system and gonads were removed, weighed and kept at 4 °C until further processing. Microplastics were extracted from organs according to the method of Kühn et al. (2017) by placing fresh tissue in KOH 1M (1:20, w:v) at room temperature for two days under continuous orbital shacking (IKA KS250). After 48h, the obtained solution was filtered on cellulose acetate membrane filters (0.45 μm) and then analysed under optical microscopy to quantify micro-PS (Supplementary data, Figs. S2 and S3). As quality control of measures, a recovery test of the extraction method was performed as follows: a known aliquot of the working micro-PS solution (equivalent to 200 particles) was added to KOH 1M, and treated as the tissue extracts, and at the end the micro-PS quantified on the filter.

2.4. Coelomic fluid collection and analysis

Every 24h, 1 mL of the coelomic fluid was withdrawn from each specimen (control and treated) through a puncture (needle 26 gauge) in the peristomial membrane using anticoagulant solution CCM 2X (NaCl 1M, $MgCl_2$ 10 mM, EGTA 2 mM, Hepes 40 mM, pH 7.2) at a ratio of 1:1 (anticoagulant: coelomic fluid) (Pinsino & Matranga, 2015; Migliaccio et al., 2019). Coelomocytes were immediately counted. Aliquots of the coelomic fluid was used to measure ROS and RNS, according to protocols described below. At the end of the exposure period (72h), coelomic fluid was again withdrawn from each specimen (control and treated) and observed under a fluorescence microscope to assess the presence of micro-PS (Zeiss Axioscope). An aliquot of coelomic fluid was washed twice in CCM 1X and the pellet was stored at −80 °C until total antioxidant capacity analysis.

2.4.1. Coelomocytes counts

Coelomocytes were then counted using Neubauer chamber (Bright-Line Hemacytometer) under optical microscope (Zeiss) and cells were morphologically identified according to Pinsino & Matranga (2015) as phagocytes, vibratile cells, white and red amoebocytes.

2.4.2. Reactive oxygen species (ROS) and nitrogen species (RNS)

Intracellular levels of ROS and RNS were determined using specific probes: DCFH-DA (2′,7′-dichlorohydro-fluoresceindiacetate) and DAF-DA (4-amino-5-methylamino-2′,7′-difluororescein diacetate), respectively. Sea urchin coelomocytes (about $1.5 \cdot 10^6$) were incubated for 1h in the dark at room temperature with 20 μM DCFH-DA or 20 μM DAF-DA in 1 mL of CCM anticoagulant. Control samples ($1.5 \cdot 10^6$ coelomocytes) were incubated with DMSO. After incubation, cells were collected by centrifugation at 8000 rcf for 10 min at +4 °C, briefly rinsed twice with CCM1x without DCFH-DA/DAF-DA and stored at −80 °C. An aliquot of coelomocytes suspended in CCM1x was used in order to verify the specificity of the intracellular signal through observation with a fluorescence microscope (Zeiss Axioscope). Frozen cells were resuspended in 0.5 mL Tris−HCl buffer 40 mM, pH7.0, vortexed for 1 min, and finally centrifuged for 10 min at 8000 rcf at +4 °C. The supernatant was harvested, and the fluorescence was measured using the spectrofluorometer (Tecan) at ex 488/em 525 nm for DCFH-DA and at ex 495/em 515 nm for DAF-DA. Fluorescence values were normalised by subtracting the autofluorescence of unlabelled extracts (DMSO). For the detection of ROS and RNS 16 organisms were used for each experimental group and triplicate measurements for each sample were performed. Results are expressed as fluorescence intensity referred to $1.5 \cdot 10^6$ cells.

2.4.3. Total antioxidant capacity (TAC)

TAC was determined according to our previous study (Milito et al., 2020) evaluating the decolorisation of 2,2′-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radical cation (ABTS•+), generated by oxidation of ABTS with hydrogen peroxide in the presence of horseradish peroxidase, by scavenging ability of antioxidants in the samples. The TAC is quantified by measuring the absorbance at 730 nm using as a reference standard curve of ascorbic acid (1−15 μM) and then the values were normalised versus total protein content. Total proteins were measured at 595 nm according to Bradford (1976) using a Tecan spectrometer and bovine serum albumin as standard. Also in this case, 16 organisms were used for each experimental group and triplicate measurements for each sample were performed.

2.5. Statistical analysis

The data on quantitative and qualitative analysis of coelomocytes were analysed by two-way analysis of variance ANOVA followed by Bonferroni's multiple comparisons test. Intracellular levels of ROS/RNS and TAC were analysed by two-way analysis of variance (ANOVA) ($P < 0.05$) followed by Tukey's multiple comparison test. Data are presented as mean ± SD and statistics was performed using GraphPad Prism version 7.00 for Windows.

3. Results

3.1. Madreporite pores size

SEM analysis performed on three madreporite samples revealed that the mean diameter of pores is between 65 μm and 70 μm (Fig. 1). Therefore, the micro-PS selected for the study were of 10 μm and 45 μm, thus able to pass through pores.

3.2. Uptake and accumulation of microplastics in sea urchin organs

Micro-PS content analysis in sea urchin organs showed a size-dependent uptake and a different distribution among organs (Fig. 2 and Table 1). A greater abundance of micro-PS was found in sea urchins exposed to 45 μm PS-MPs (104.05 ± 43.3 particles g^{-1} whole fresh tissue) than in those exposed to 10 μm micro-PS (86.1 ± 23.4 particles g^{-1} whole fresh tissue) (Table 1). The bigger micro-PS (45 μm) resulted highly present in the esophagus and digestive system (67.5%), followed by the ring canal, the ampullae of the vascular system (20.3%) and finally in the gonads (12.2%) (Fig. 2). No 45 μm particles were found in the stone canal. By contrast, smaller micro-PS (10 μm) were mostly found in the ring and stone canal of the water vascular system (74.1%) than in the esophagus and digestive system (24.8%). Very low amount of 10 μm micro-PS was found in the gonads (1.0%). Apparently, no particles were found in the coelomic fluid after 72h of exposure to both micro-PS (10 and 45 μm). The recovery-effectiveness of particles using the KOH microplastics extraction method was about 75.25 ± 6%.

3.3. Sea urchin's immune cells response

Coelomocytes total count showed a significant increase already after 24h of exposure in both treatments compared to controls regardless the micro-PS size (45 μm and 10 μm) (Fig. 3). Phagocytes resulted the most abundant immune cells in both micro-PS and controls (Fig. 4). No significant differences were detected among immune cell types, except for an increase of red amoebocytes, more evident in sea urchins exposed to smaller micro-PS (10 μm). Indeed, the ratio between red and white amoebocytes (R/W) was significantly higher in specimens exposed to the 10 μm micro-PS at all experimental times compared to controls (Fig. 5). This increase was detectable after 48 and 72h of exposure also in sea urchins exposed to PS-MPs-45 μm (Fig. 5).

3.4. Intracellular levels of ROS and RNS

An increase of intracellular levels of ROS and RNS in coelomocytes was observed after 24h exposure to micro-PS regardless to their size. At 48h and 72h, the levels of both ROS and RNS in coelomocytes were comparable in control and micro-PS treated animals (Fig. 6, Supplementary data Figs. S4 and S5).

3.5. Total antioxidant capacity

Upon exposure to smaller micro-PS (10 μm), coelomocytes showed a significant increase of total antioxidant capacity compared to the controls. No effect was observed with micro-PS-45μm treatment (Fig. 7).

4. Discussion

The results reported in this study highlighted for the first time a size-dependent uptake and distribution of micro-PS in the adult sea urchin *P. lividus* and their impact on circulating immune cells through *in vivo* exposure.

The different localisation of micro-PS of different size is striking. While the 45 μm micro-PS were particularly abundant in the digestive system, more than 70% of the smaller micro-PS (10 μm) were found in the water vascular system. An important observation regards the higher amount of bigger micro-PS (45 μm) in the sea urchin' gonads compared to smaller ones (10 μm). These differences could be due to a potential sorting action of the madreporite, facilitating the penetration, as well as the consequent excretion, of the smaller micro-PS. However, also the dynamics of the micro-PS retention and egestion processes as well as the capability of organs to retain the particles of different size could play a significant role in the observed differential uptake. The presence of micro-PS in the sea urchin' aquifer system may affect important functions such as movement, feeding and breathing, although the continuous influx/efflux of seawater may reduce longer retention. Nevertheless, sea urchins in comparison with other echinoderms show relatively slow circulation in the aquifer system and therefore might be more affected by potential particles retention (Ferguson, 1996). As far as digestive system, a longer retention time could be predicted based on the estimates by Lawrence et al. (1989) which are between 8 and 40h.

No micro-PS was detected in the coelomic fluid after 72h of exposure suggesting that translocation across sea urchin tissues is

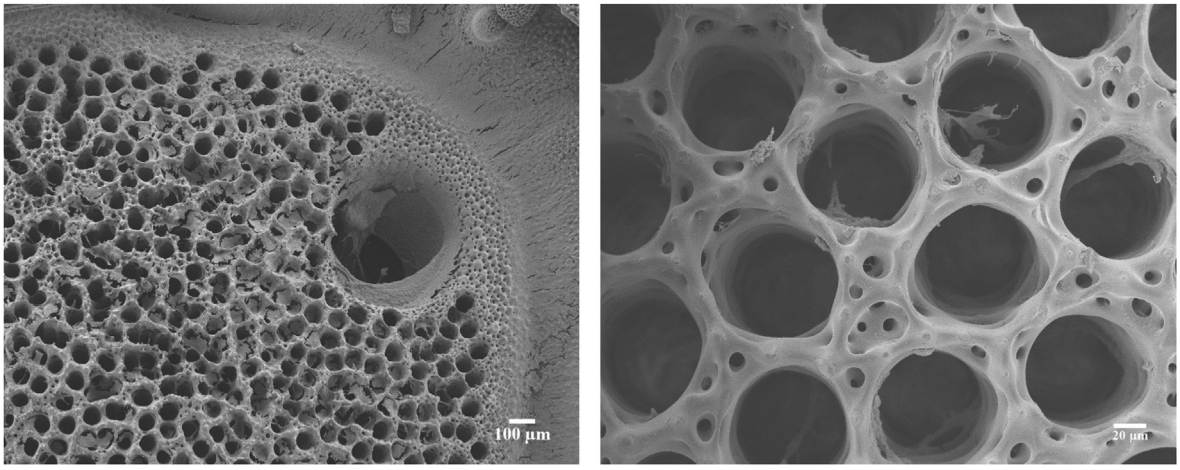

Fig. 1. Aboral views of *Paracentrotus lividus* madreporite examined with scanning electron microscope (SEM).

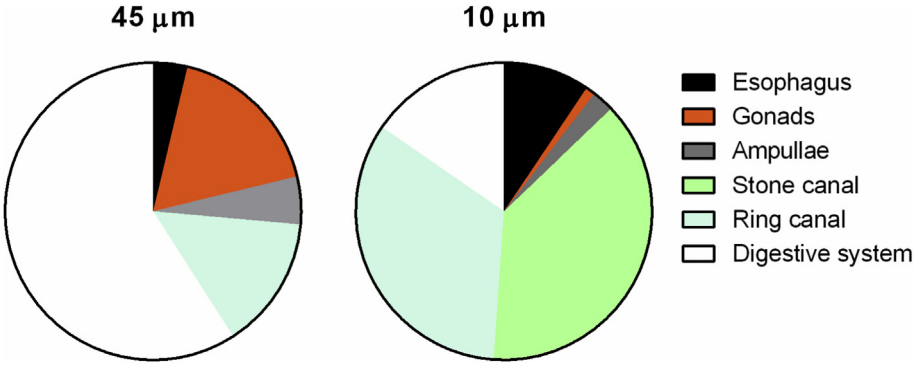

Fig. 2. Abundance of PS-MPs in different organs of sea urchins after 72h of exposure. The results are expressed as percentage of particles found in organs normalised by the weight of fresh tissue.

Table 1
Abundance of micro-PS (10 μm and 45 μm) in the organs of *P. lividus* upon waterborne exposure (72h). Data shown as mean ± SE.

	particles/g	
	10 μm	45 μm
Esophagus	8.25 ± 2.24	3.86 ± 1.76
Digestive system	13.5 ± 3.67	61.39 ± 27.90
Ring Canal	28 ± 7.34	15.09 ± 6.81
Stone Canal	33 ± 8.98	0 ± 0
Ampullae	2.14 ± 0.64	5.54 ± 2.57
Gonads	1.24 ± 0.57	18.17 ± 8.27

unlikely. The ability of 10 μm micro-MPs to penetrate biological barriers and to move in different tissues has been already described in both freshwater and marine mussels as *Dreissena polymorpha* (Magni et al., 2018) and *Mytilus edulis* (Browne et al., 2008). On the opposite, no translocation has been reported in the shore crab *Carcinus maenas* upon waterborne exposure to micro-PS of same size (Watts et al., 2014). Despite limited information, such processes appear to be species-specific and uptake and translocation dynamics might significantly affect micro-PS distribution.

Despite the sea urchin is a benthic grazer and not a filter-feeder, the micro-PS retention inside different organs clearly highlights potential ecological impacts (through trophic transfer) as well as human risks associated for instance to predation and the high consumption of sea urchins' gonads in the Mediterranean area (Carbery et al., 2018). The comparison of sea urchin micro-PS retention with what reported in other marine organisms (sea cucumbers, copepods, bivalves, crustaceans) is challenging due to differences in feeding strategies, exposure scenarios, particles concentration and size (Browne et al., 2008; Graham & Thompson., 2009; Cole et al., 2013, 2015; Setälä et al., 2015; Sussarellu et al., 2016; Welden & Cowie, 2016; Sun et al., 2017). On the other hand, our results showed that sea urchin has a good ability to accumulate micro-PS, considering that the total number of micro-PS for each gram of tissue ranged between 0.46 and 1.65% initial concentration. This is still relevant, even though it is less than what reported in other organisms such as shore crab gills (0.39–7.7% initial concentration) (Watts et al., 2014). The higher amount of the larger micro-PS (45 μm) in the gonads compared to the smaller micro-PS (10 μm) may significantly affect the reproductive success of sea urchin in analogy to what is reported in oysters, which showed negative effects on the reproductive health indices, quality and quantity of gametes, and fecundity (Sussarellu et al., 2016).

Our study showed for the first time the ability of adult sea urchins to accumulate micro-PS from sea water and distribute them to various organs according to their size. Although no data are currently available on the occurrence of microplastics in sea urchin specimens from the Gulf of Naples and generally from the Mediterranean Sea, such findings clearly suggest potential risk scenarios for sea urchin's local population. In fact, coastal areas of the Mediterranean Sea have been recently identified as hot spots of microplastic pollution (Compa et al., 2019). In the coastal environments *P. lividus* controls the dynamic, structure and composition of shallow macroalgal assemblages through its grazing activity and represents also a food source for fishes and other animals, including humans. Sea urchin predators which include gastropods, shore crabs, fish and star fish will then receive microplastics from their prey and then transfer them along the trophic food web

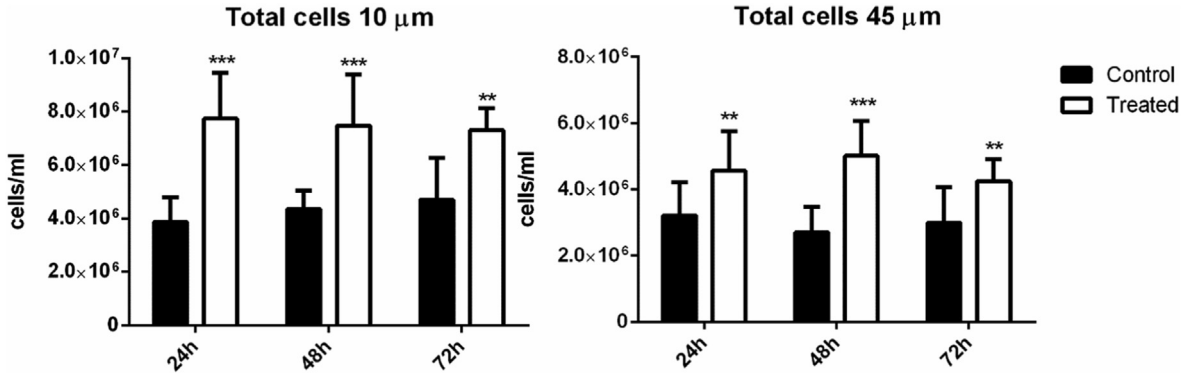

Fig. 3. Total immune cells count of sea urchin at different time of exposure to a PS-MPs (10 μm and 45 μm). All data were analysed by Two-way ANOVA followed by Bonferroni post-test compared with the respective control. Bars represent mean ± SD. Asterisks indicate values that are significantly different from the control, **P < 0.01, ***P < 0.001.

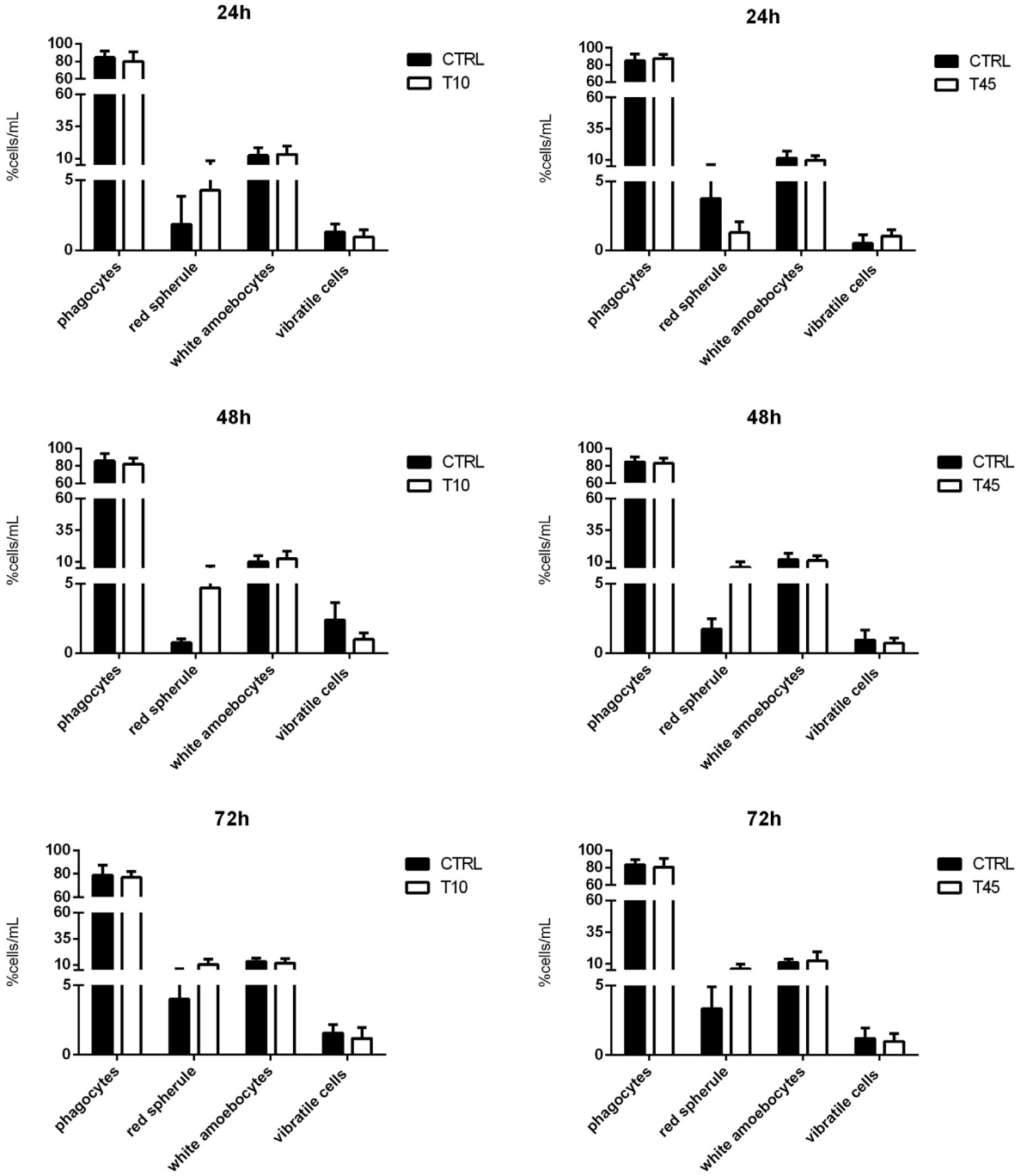

Fig. 4. Immune cells morphology of sea urchin at different time of exposure to a PS-MPs (10 and 45 μm). Bars represent mean ± SD.

allowing bioaccumulation and biomagnification (Boudouresque & Verlaque, 2001).

The presence of MPs in the seafood might represent a further threat to human health due to their consumption (Van Cauwenberghe & Janssen, 2014). Although sea urchin eggs consumption is heterogeneously distributed along the Mediterranean countries, it has been reported for instance an annual pro-capita consumption of about 1.1 kg in the Island of Sardinia, NW Mediterranean (Carboni et al., 2012). Based on our findings (see paragraph 3.2) on the micro-PS occurrence in sea urchin' gonads (about 12% of the 45 μm internalised particles/g fresh tissue), one year based consumption of sea urchin eggs could be around 5.5–19 particles g^{-1} fresh gonads. These findings are comparable to similar estimates for shrimp annual consumption of about 175 microplastic particles per person per year (considering scenario that 90% of microplastics will be removed) (Devriese et al., 2015). Van Cauwenberghe & Janssen (2014) estimated that in Europe molluscs consumers ingest up to 11,000 microplastics per year, a larger quantity compared to results obtained in this study considering only the edible part of the sea urchin without considering ejection mechanisms. Nevertheless, an annual dietary exposure based on species of high commercial value in the Mediterranean Sea, such as mussels and gonads of sea urchins, raises the issue of the impact of microplastics on seafood quality, even though it is still early for a

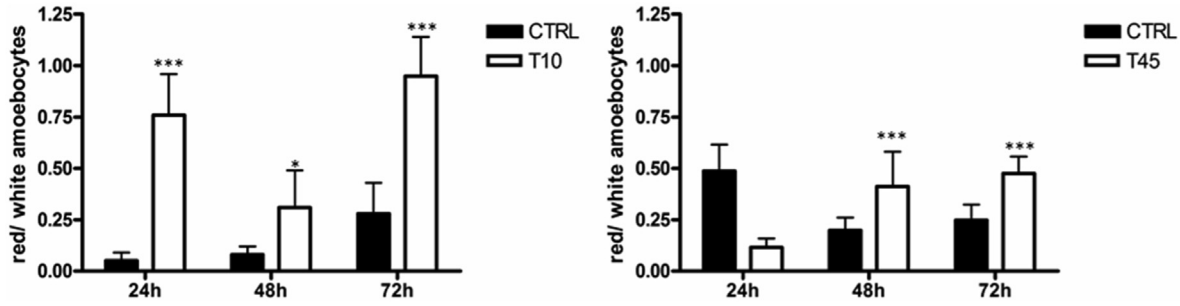

Fig. 5. Ratio between red (%) and white amoebocytes (%) of sea urchin at different time of exposure to a PS-MPs (10 and 45 μm). All data were analysed by Two-way ANOVA followed by Bonferroni post-test compared with the respective control. Bars represent mean ± SD. Asterisks indicate values that are significantly different from the control, *P < 0.05, ***P < 0.001.

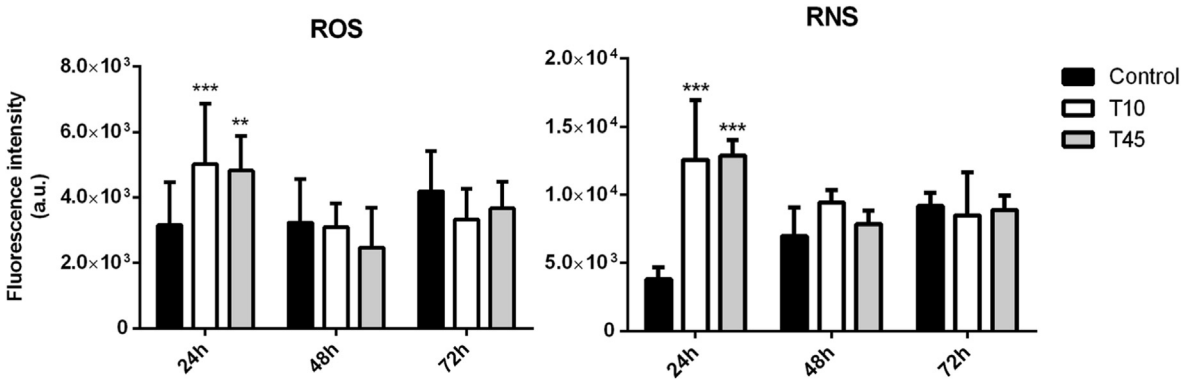

Fig. 6. Intracellular ROS and RNS levels at different time of exposure to a PS-MPs (10 μm and 45 μm). All data were analysed by Two-way ANOVA followed by Tukey's post-test compared with the respective control, **P < 0.01, ***P < 0.001. Bars represent mean ± SD.

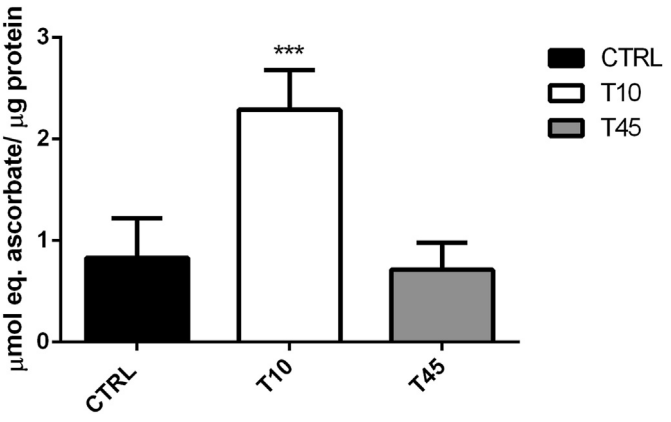

Fig. 7. Total antioxidant activity of coelomocytes after 72h of exposure to PS-MPs (10 μm and 45 μm). All data were analysed by Two-way ANOVA followed by Tukey's post-test compared with the respective control, ***P < 0.001. Bars represent mean ± SD.

proper risk assessment on human health. Field measurements of the presence of MPs inside sea urchin wild specimens collected from Mediterranean coastal areas, more densely populated as the Southern Thyrrenian coasts (i.e. the Gulf of Naples), will elucidate the real exposure scenarios and will allow to assess any potential risk for sea urchin populations and human health. Considering model-based projections, the coastal areas could be more impacted by MPs, reaching up higher levels than predicted due to specific regional and local input (Jambeck et al., 2015). For this reason, although egestion has not been investigated in the present study, we cannot disregard that the observed accumulation and toxicological effects might describe a real scenario under chronic exposure conditions.

An important outcome of this study is the analysis of the impact of MPs on immune cells. In sea urchins, cell-mediated immune response is played by heterogeneous free circulating cells, coelomocytes (Smith, 2010). The three cell types of coelomocytes, phagocytes, vibratile cells and red and white amoebocytes, have been recognised to perform functions similar to those of the vertebrate blood cells (Ito et al., 1992; Smith et al., 1995; Pinsino & Matranga, 2015). Sea urchin immune cells have been proposed as biosensors of several environmental stressors such as temperature shock, sea water acidification, UV-B radiation, and more recently nanoplastics (Matranga et al., 2000; Matranga et al., 2006; Marques-Santos et al., 2018; Migliaccio et al., 2019). The major findings on the impact of micro-PS on sea urchin's immune cells underlined a stress syndrome as outlined below.

Although micro-PS were not found in the coelomic fluid, the number of immune cells increased after 24h compared to the control for both micro-PS suggesting the induction of proliferation. The increase in the number of coelomocytes has been also reported for the sea urchin *S. purpuratus*, as due to cell release from hematopoietic tissues in response to specific immune challenges and to a lesser extent, about 10%, to cell proliferation (Golconda et al., 2019). The higher increase of coelomocytes in *P. lividus* adults exposed to the smaller micro-PS (10 μm) than to the larger ones (45 μm) could be due to their presence in the stone canal which could stimulate coelomocytes production by an unknown mechanism. Indeed, the stone canal is located at the lateral edge of the axial organ which is recognised as one of the potential coelomocyte production site (Ramírez-Gómez and García-Arrarás, 2010;

Golconda et al., 2019).

A significant increase in the ratio of red to white amoebocytes compared to the controls was also observed after 24h of exposure to micro-PS 10 μm and after 48h and 72h to 45 μm micro-PS. These results are in line with previous studies showing an increase in the percentage of red amoebocytes under stress conditions even though the total number of coelomocytes remain constant (Matranga et al., 2005; Pinsino et al., 2008).

The significant increase of ROS and RNS levels in coelomocytes after 24h and their subsequent recovery to control values upon exposure to both micro-PS 10 and 45 μm suggest that coelomocytes can cope with micro-PS mitigating the concentration of these reactive species. Levels of ROS and RNS play an important role as regulatory mediators in signalling processes (González et al., 2015; Di Meo et al., 2016). Although it is widely known that MPs can induce ROS generation (Jeong et al., 2016, 2017; Prinz & Korez, 2020), this is the first report of an increase in RNS levels following micro-PS exposure. Increased levels of ROS have been also reported in haemocytes of *M. edulis* treated with micro-PS (2–6 μm) (Paul-Pont et al., 2016). Similar results were reported for haemocytes of the blue mussel *Mytilus* spp. after exposure to a mixture of micro polyethylene and polypropylene (20 μm) at different concentrations (Revel et al., 2019).

Generally, oxidative damage is the result of the imbalance between the formation of free radicals, including ROS and RNS, and the production of antioxidants (Ghiselli et al., 2000; de Almeida et al., 2007). When ROS and RNS are overproduced, they induce lipid oxidation/nitration and damage to proteins and DNA (Lesser, 2006; González et al., 2015). The observed differential increase in total antioxidant capacity levels in coelomocytes of sea urchins exposed to 10 μm micro-PS than in those exposed to 45 μm micro-PS suggests a size-dependent reaction, with smaller particles inducing a greater production of antioxidants than bigger ones. This could be due to the higher capacity of smaller particles to interact or aggregate with other cellular or extra-cellular components. The antioxidant capacity has a dynamic profile with increases or decreases depending on the time exposure and concentration of the stressors (Lushchak, 2011). Indeed, increased antioxidant enzymatic activities have been reported after exposure to micro-PS in gills and digestive gland of *Scrobicularia plana* (Ribeiro et al., 2017), in the whole body of *Brachionus koreanus* (Jeong et al., 2016), and *Paracyclopina nana* (Jeong et al., 2017). By contrast, a decrease in the antioxidant enzyme activities has been reported in the digestive system of mussels after exposure to micro-PS (Avio et al., 2015; Paul-Pont et al., 2016).

Overall our results on ROS/RNS and total antioxidant capacity suggest that the sea urchin is able to restore basal redox homeostasis at the level of coelomocytes after 72 h of PS-MPs exposure.

5. Conclusions

Although microplastic pollution has received considerable attention in recent years, no study has been carried out on their potential effects on adult sea urchins. In this context, this study acts as a pivotal investigation to understand the key factors that determine microplastics accumulation in sea urchin, thus providing also the necessary background for future biomonitoring campaigns. Precisely, the present study shows for the first time how sea urchin may represent suitable model organisms to highlight different uptake routes and toxic effects caused by the exposure to micro-PS of different sizes. The outcomes of this work provide first insights into the effects of MPs on marine benthic grazers with potential implications at ecological and human health levels. Also, these results will provide the basis for future studies to investigate how other particle sizes, polymers, and the biofilm may influence the uptake and biodistribution as well as immune-reactivity in *P. lividus*.

Declaration of competing interest

The authors declare no conflict of interest.

CRediT authorship contribution statement

Carola Murano: Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Visualization, Writing - original draft. **Claudio Agnisola:** Methodology, Visualization, Writing - review & editing. **Davide Caramiello:** Methodology, Validation, Visualization. **Immacolata Castellano:** Validation, Visualization, Writing - review & editing. **Raffaella Casotti:** Conceptualization, Funding acquisition, Project administration, Resources, Writing - review & editing. **Ilaria Corsi:** Data curation, Validation, Writing - review & editing. **Anna Palumbo:** Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing - review & editing.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2020.114685.

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