Response to microplastic exposure: an exploration into the sea urchin immune cell proteome

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Abstract

It is now known that the Mediterranean Sea currently is one of the major hotspot for microplastics (MPs; < 5mm) pollution and that the risks will be even more pronounced in the coming years. Thus, the in-depth study of the mechanisms underlying the MPs toxicity in key Mediterranean organisms, subjected to high anthropic pressures, has become a categorical imperative to pursue. Here, we explore for the first time the sea urchins immune cells profile combined to their proteome upon *in vivo* exposure (72h) to different concentrations of polystyrene-microbeads (micro-PS) starting from relevant environmental concentrations (10, 50, 10^3, 10^4 MPs/L). Every 24h, immunological parameters were monitored. After 72h, the abundance of MPs was examined in various organs and coelomocytes were collected for proteomic analysis based on a shotgun label free proteomic approach. While sea urchins treated with the lowest concentration tested (10 and 50 micro-PS/L) did not show the presence of micro-PS in any tissue, in the specimens exposed to the highest concentration (10^3 and 10^4 micro-PS) there was an internalisation of 9.75 ± 2.75 and 113.75 ± 34.5 MPs/g, respectively. Proteomic analyses revealed that MPs exposure altered coelomocytes protein profile not only compared to the control group but also among the different micro-PS concentrations and these variations are micro-PS concentration dependent. The proteins exclusively expressed in the coelomocytes of specimens exposed to MPs are mainly metabolite interconversion enzymes, involved in cellular processes, indicating a severe alteration of the cellular metabolic pathways. Overall, these findings provide new insights on the mode of action of MPs in the sea urchin immune cells both at the molecular and cellular level.

Keywords: coelomocytes; microplastics; oxidative stress; nitrosative stress; proteomics; sea urchin

1. Introduction

It is commonly believed that the Mediterranean Sea became over the years the sixth gyre with accumulation of plastics debris due to the semi-enclosed geographical configuration of the Atlantic Ocean with limited outlet flow (Còzar et al., 2015; Suaria et al., 2016; Macias et al., 2019; Everaert et al., 2020). In fact, approximately 229 thousand tons of plastics items are expected to end up in the Mediterranean every year (Boucher & Bilard, 2020). More deeply troubling are the recent estimates which have identified the presence of approximately 3.2×10^{12}-28.2×10^{12} plastic particles floating on the surface of Mediterranean Sea, known as microplastics (MPs, < 5mm) (van Sebille et al., 2015; Suaria et al., 2016). During their permanence in sea water, MPs undergo different transformations involving their surface properties such as shape, roughness, and charge. In addition, MPs interact with the surrounding environment, bacteria and chemical contaminants that contribute in determining their ecological impacts driving the rapid changes in seawater (Galloway et al., 2017). Actually, most of these MPs seems to be accumulated especially on the coastlines and on the sea bottom, this latter probably representing the long term- sinks for MPs (Fries et al., 2013; Cózar et al., 2014; Nuelle et al., 2014; Courtene-Jones et al., 2017). Thus, in the benthic environment of coastal areas, the concentrations of MPs are much higher than in the rest of the basin due also to the close proximity to the potential sources (Soto-Navarro et al., 2021).

In light of the extraordinary richness of the Mediterranean Sea as a biodiversity hotspot, such levels of MPs highlight the potential risk for marine species up to marine coastal areas and benthic environments. Moreover, model-based studies evidenced that species with smaller home ranges are more likely to be exposed to plastic particles compared to species with larger home ranges (Compa et al., 2019). Until now, the presence of MPs has been recorded in different Mediterranean benthic species such as sea squirts, sea cucumbers, sea urchins, clams, oysters and mussels up to reaching concentrations of 23 MPs/individual, mostly fibres and fragments mainly of polyacrylamide and polyethylene (Vered et al., 2019; Bulleri et al., 2021; Expòsito et al., 2022; Murano et al., 2022).

Over the years, growing attention was paid on the potential effects of MPs on marine organisms. Different laboratory studies have been performed both *in vivo* and *in vitro* evaluating different endpoints for neurotoxicity, immunotoxicity, embryonic development, cytotoxicity, ingestion and egestion (Barboza et al., 2018; Gambardella et al., 2018; Tang et al., 2020; Capolupo et al., 2021). In most of these studies, polystyrene was chosen as proxy for MPs due to the fact that it's one of the most largely used non-biodegradable plastic worldwide and unlike other polymers, it shows a greater stability in sea water suspension with low styrene release (Cohen et al., 2002; Messinetti et al., 2018). Overall, the lethal and sub-lethal effects of MPs on various marine organisms, belonging to different

trophic levels, have highlighted the involvement of oxidative stress and pathways activation, including inflammatory responses (Hu & Palic, 2020).

However, in spite of the growing concern about MPs pollution, there are still several scientific issues to be addressed, especially related to the molecular mechanisms and the cellular processes that are activated in response to MPs exposure. The development of advanced "omics" technologies has allowed to explore the effects of numerous contaminants or natural toxins on the proteome of the different organisms examined through both in field and in laboratory experiments (Su et al., 2019; Balbi et al., 2021; Sànchez-Marin et al., 2021). As a matter of fact, proteomic approaches lead to a deeper comprehension of the response mechanisms against environmental stressors providing important information on the abundance of proteins, key players of the biological processes (Gouveia et al., 2019; Liang et al., 2020). To the best of our knowledge, very few studies have analysed the effects of MPs on benthic organisms through proteomics techniques. For instance, Teng and co-workers (2021) demonstrated that the oyster gland proteome was significantly affected by MPs exposure, PE and PET (10 and 1000 μg L^{-1}), especially in terms of cytoskeleton organisation, metabolic processes, signal transduction and protein synthesis. Green et al. (2019) discovered that both the conventional plastic HDPE, as well as the biodegradable alternative, PLA (both at 2.5μg L^{-1} and 25 μg L^{-1}), greatly affected the proteome of mussels haemolymph, the main effector of the innate immune response, after *in vivo* exposure.

Cell-mediated immune response can be considered as the first target of contaminant-exerted toxicity in aquatic organisms. Among the benthic invertebrates of the Mediterranean Sea, the sea urchin *Paracentrotus lividus* stands out for its intrinsic and unique immune system (Smith et al., 2018). This peculiar immune system exhibits very similar features of the non-adaptive system or the innate system of vertebrates and can be considered the baseline of the original deuterostome ancestor (Smith & Davidson, 1992; Pancer et al., 1999). The innate immunity system of sea urchin is a very complex network based on cellular and humoral factors which together are able to counteract pathogens, foreign substances and other kinds of environmental challenges (Smith, 2010). In the coelomic cavity, the immune cells, named as coelomocytes, circulating freely in the coelomic fluid also reaching tissues and organs, control the cellular response (Buckley & Rast, 2019). In recent years, these cells generated great interest as prominent biosensors for environmental monitoring not only for their features but also for the sea urchin's strategic phylogenetic position. Indeed, thanks to the availability of the full sea urchin genome sequence (Sea Urchin Genome Sequencing Consortium, 2006), an extraordinary and also unexpected relationship to humans was disclosed. In particular, these strong similarities between sea urchin and humans mainly involve the immune system in terms of alternative

adaptive and anticipatory immune functions (Hibino et al., 2006; Rast et al., 2006). Being recognised as sensitive tools for investigating sea urchin health status, coelomocytes have been used for studying the effects of environmental conditions, such as ocean acidification, marine pollution including emerging contaminants as MPs and nanoplastics (Falugi et al., 2012; Pinsino et al., 2015; Marques-Santos et al., 2018; Migliaccio et al., 2019; Alijagic et al., 2020; Milito et al., 2020; Murano et al., 2020; Murano et al., 2021a). As evidence of this, field observations have shown that *P. lividus* is currently a species subject to contamination by MPs in coastal marine areas and in particular by microfibers, fragments and films and also plasticizers (Murano et al., 2022; Raguso et al. 2022). In this context and considering the knowledge-gap on immunological responses upon MPs exposure, this study aims to explore for the first time the sea urchin's immune cells profile combined to their proteome upon *in vivo* exposure using different concentrations of polystyrene-microplastics starting from relevant environmental concentrations.

2. Methods

2.1 Sea urchin's collection and handling

Adult specimens of *P. lividus* (Lamark, 1816) (diameter 5.32 ± 0.51 cm) were collected from a coastal site (40°42.335' N; 13°57.351' E) in the Gulf of Naples by the scuba diving staff of the Stazione Zoologica Anton Dohrn of Naples. This site is 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). Once in laboratory, sea urchins were acclimated for one weeks in glass tanks filled with circulating natural seawater (NSW) (temperature 17.7 ± 1.4 °C, salinity 40 ± 1, dissolved O_2 7 mg/L, pH 8.2; all the parameters remained constant during the experiment) and fed *ad libitum* with *Ulva lactuca*.

2.2 Polystyrene microbeads

Fluorescent-labelled polystyrene microbeads (45 µm micro-PS) (441excitation/485 emission) were purchased from Polysciences (Warrington, PA, U.S.A.). According to the supplier, the particles were packaged as 2.5% aqueous suspension (5 x 10^5 micro-PS/mL) without biocides or stabilisers. The beads size is in agreement with the data sheet provided by the manufacturer and the polystyrene composition of the particles was previously confirmed by FTIR analysis (please refer to Murano et al., 2021b). Micro-PS working solutions (10^4 particles mL^{-1} and 10^2 particles mL^{-1}) were prepared in deionised water and then added to filtered natural sea water (NSW, 0.22 µm) to reach the final concentration of 10, 50, 10^3 and10^4 particles L^{-1}. Stock and working solutions were vortexed for 3 min prior to use.

2.3 Sea urchin's in vivo exposure

Specimens of adult sea urchin were placed in 5L glass tanks (ratio of 1 specimen per liter) supplied with filtered NSW (0.22 µm) from the coastal site in the Gulf of Naples in a closed flow-through system constantly aerated. Micro-PS exposed treatments (10, 50, 10^3 and10^4 particles L^{-1}) were set up by directly adding the micro-PS into the tanks from the previously prepared and vortexed stock solutions. The sea urchins were exposed to NSW only (control) and to micro-PS for 72h, during which the urchins were not fed.

2.4 Micro-PS internalisation

The micro-PS extraction from sea urchin's tissues/organs was set according to our previous studies Murano et al. (2020, 2021b). Briefly, 1M KOH was added to the tissues/organs (digestive system, water vascular system and gonads) under examination (1:20, w:v) in glass flasks and the samples were then slowly stirred for 48h at room temperature. After 48h, the digested solution was filtered through a vacuum system on cellulose acetate membrane filters (0.45 µm) and then analysed under optical microscopy to quantify micro-PS.

2.5 Immune Cell Response

The impact of micro-PS exposure on the sea urchin immune system was evaluated by measuring different immunological parameters. In brief, after 24, 48, 72 h from incubation, the coelomic fluid was collected through the coelomic cavity using a sterile syringe (5 mL, needle 26 gauge) pre-loaded with the 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) as reported in Murano et al. (2021b). Heterogeneous coelomocytes were counted using a Neubauer counting chamber (Bright-Line Hemacytometer) under light microscope (ZEISS Apotome.2). At different times, aliquots of the coelomic fluid were washed twice in CCM 1X and the pellet was stored at -80 °C until levels of Reactive Oxygen Species (ROS), Reactive Nitrogen Species (RNS) and Total Antioxidant Capacity (TAC) were assessed.

Using two probes, DCFH-DA (2,7-dichloro dihydro-fluorescein-diacetate) and DAF-DA (4-amino-5-methylamino-2,7-dichlorofluorescein-diacetate), we specifically measured the intracellular levels of ROS and RNS respectively. From the aliquots, about $1.5 \cdot 10^6$ coelomocytes were isolated and subsequently exposed for 1h in the dark to a concentration of 20 µM DCFH-DA/ DAF-DA in anticoagulant cells/mL. After exposure and different washing, the cells were sonicated in Tris-HCl (40 mM- pH 7.4) and 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) and results are expressed as fluorescence intensity referred to $1.5 \cdot 10^6$ coelomocytes. The remaining aliquots were used to analyse the antioxidant capacity by exploiting the ability of hydrogen-donating antioxidants of the cells to induce a de-coloration of the pre-formed radical cation of 2,2-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS●+) during the reaction between ABTS and H_2O_2 in the presence of peroxidase. The TAC is quantified by measuring the absorbance at 730 nm using as a reference the standard curve of ascorbic acid (1-15 µM) and then the values were normalised versus total protein content. Total proteins were measured according to Bradford (1976) at 595 nm.

2.6 Proteomic analysis by a shotgun label free approach

The coelomocytes, obtained by centrifugation of the coelomic fluid in CCM 1X as reported above, were collected from animals exposed to different concentrations of micro-PS and from animals kept as control and analysed by a shotgun label free proteomic approach for the identification and quantification of expressed proteins. The samples were resuspended in urea 8 M /Hepes 20 mM pH 8.0 containing protease inhibitors cocktail (Roche), sonicated using an ultrasonic probe in bursts of 20–30 s and centrifuged at 16560 x g for 15 min at 16 °C to pellet the tissue debris as previously reported (Mortarino et al., 1998). The protein content was determined by the Bradford assay with bovine serum albumin as standard. Prior to proteolysis, proteins were reduced with 13 mM dithioerythriol (DTE; 15 min at 50 °C) and alkylated with 26 mM iodoacetamide (IAA; 30 min at room temperature, in the dark). Protein digestion was performed using sequence-grade trypsin (Promega) for 16 h at 37 °C using a protein: enzyme ratio of 20:1. The collected peptides were desalted using Zip-Tip C18 before mass spectrometric (MS) analysis as reported in Eberini et al. (2002). NanoHPLC coupled to MS/MS analysis was performed on Dionex UltiMate 3000 directly connected to an Orbitrap Fusion Tribrid mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) by a nanoelectrospray ion source. Peptide mixtures were enriched on 75µm ID × 150 mm EASY- Spray PepMap RSLC C18 column (Thermo Fisher Scientific) and separated using the LC gradient: 4% ACN in 0.1% formic acid for 3 min, 4–28% ACN in 0.1% formic acid for 100 min, 28–40% ACN in 0.1% formic acid for 10 min, 40–95% ACN in 0.1% formic acid for 1 min and 95–4% ACN in 0.1% formic acid for 3 min at a flow rate of 0.3 µL/min. Orbitrap-MS spectra of eluting peptides were collected over an m/z range of 375–1500 at resolution of 120000, operating in a data-dependent mode with a cycle time of 3 s between master scans. HCD MS/MS spectra were acquired in Orbitrap at resolution of 15000 using a normalised collision energy of 35%, and an isolation

window of 1.6 m/z. Dynamic exclusion was set to 60 s. Rejection of +1 and unassigned charge states were enabled.

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Vizcaino et al., 2016) partner repository, with the dataset identifier PXD033665.

A database search was conducted against the Uniprot *Strongylocentrotus purpuratus* database (34417 entries) (https://www.uniprot.org/proteomes, release 11/02/22) with MaxQuant (version 1.6.1.0) software. The initial maximum allowed mass deviation was set to 10 ppm for monoisotopic precursor ions and 0.5 Da for MS/MS peaks. Enzyme specificity was set to trypsin, defined as C-terminal to Arg and Lys excluding Pro, and a maximum of two missed cleavages were allowed. Carbamidomethylcysteine was set as a fixed modification, while Met oxidation and Asn/Gln deamidation were set as variable modifications. Quantification in MaxQuant was performed using the built-in label free quantification algorithms (LFQ) based on extracted ion intensity of precursor ions. False protein identifications (1%) were estimated by searching MS/MS spectra against the corresponding reversed-sequence (decoy) database. Statistical analysis was performed using the Perseus software (version 1.5.5.3) (Nicastro et al., 2015). Only the proteins present and quantified in at least 75% of the repeats were positively identified in a sample and used for statistical analysis. PCA was carried out by grouping quantitative data related to proteins in the coelomocytes from control group (CTR) and from animals exposed to different micro-PS exposed treatments: 10 micro-PS/L, 50 micro-PS/L, 10^3 micro-PS/L and 10^4 micro-PS/L, respectively. Focusing on specific comparisons, proteins were considered differentially expressed if they were present only in one condition or showed significant t-test difference (Welch's test $P \leq 0.05$). Bioinformatic analyses were carried out by Panther software (release 17.0) (Mi et al., 2021) to classify the proteins in the various data sets and to cluster enriched annotation groups of Biological Processes, Molecular Function, Pathways, and Networks within the set of identified proteins. Functional grouping was based on Fischer's exact test $P \leq 0.05$. Interaction networks were visualised using the "Search Tool for Recurring Instances of Neighbouring Genes" (STRING) (Szklarczyk et al., 2021) setting the minimum required interaction score at 0.7 and hiding disconnected nodes.

2.7 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 Micro-PS uptake

Micro-PS content analysis in the different organs of sea urchins showed a concentration dependent uptake after 72 h exposure. While sea urchins treated with the lowest concentration tested (10 and 50 micro-PS/L) did not report the presence of micro-PS in any tissue/organ, the specimens exposed to the highest concentration tested (10^3 and 10^4 micro-PS) revealed micro-PS internalisation of 9.75 ± 2.75 and 113.75 ± 34.5 particles normalised by the weight of fresh organs, respectively (Figure 1). As expected, the digestive system includes the organs most affected by internalisation which exceeds more than 70% of the total particles internalised in both cases, followed by gonads and esophagus. Interestingly, only at the highest concentration used (10^4 micro-PS), micro-PS were found at the level of the aquifer system (ring canal and ampullae) even if in a very small amount (total quantity below the 6% of the total particles).

3.2 Quantitative and qualitative analysis of coelomocytes

Starting from 24h exposure, a statistically significant increase of the total coelomocytes count was detected in specimens treated with 10^4 micro-PS/L compared to the control group ($4.6\cdot 10^6 \pm 3.3 \cdot 10^5$ *vs* $3.2\cdot 10^6 \pm 6.8 \cdot 10^5$ cells/mL, respectively) (Figure 2). This increase in specimens treated with 10^4 micro-PS/L occurred up to 72h. Conversely, after 24h of exposure, specimens treated with 10^3 micro-PS/L displayed a significant decrease in total number of coelomocytes compared to the control ($2.0\cdot 10^6 \pm 3.0\cdot 10^5$ *vs* $3.2\cdot 10^6 \pm 6.8 \cdot 10^5$ cells/mL, respectively). But after 24h, the cell concentration returned to be similar to the control. In the case of both low concentrations tested (10 and 50 micro-PS/L), no alterations in total cell number was observed compared to control values.

Figure 3 shows the morphological profile of the sea urchin's immune cells along different experimental groups. No variations between the different cell types was detected. However, the ratio between red and white amoebocytes resulted affected by exposure to micro-PS. In fact, a ratio between 0.5 and 1 values, indicative of an initial stressful condition, was found at different times of exposure to micro-PS (10, 50, 10^3,10^4). A more harmful situation with a value greater than 1 was detected after 48h of exposure to micro-PS 10^4 (Table 1).

3.3 Oxidative stress status in coelomocytes

By exploring the oxidative stress status of coelomocytes, increases in intracellular levels of both ROS and RNS as well as variations in antioxidant capacity were recorded upon exposure to micro-PS. In

details, at 24h coelomocytes of specimens treated with 10^4 micro-PS/L showed a significant increase of ROS levels compared to the control group ($2.5 \cdot 10^5 \pm 2.2 \cdot 10^3$ *vs* $1.5 \cdot 10^5 \pm 4.6 \cdot 10^3$ a.u.) which is still evident at 48h (Figure 4A). At this exposure time, a significant increase of ROS levels was also detected after treatment with 10 and 10^3 micro-PS/L ($3.1 \cdot 10^5 \pm 2.3$ and $2.1 \cdot 10^5 \pm 1.0$, respectively). Then, at 72h, the ROS levels were comparable to control. In the case of intracellular RNS levels, starting from 24h up to 72h, the coelomocytes of organisms treated with 10^4 micro-PS/L showed a statistically significant increase compared to the control (72h: $3.9 \cdot 10^5 \pm 4.2 \cdot 10^3$ *vs* $1.2 \cdot 10^5 \pm 5.7 \cdot 10^3$) (Figure 4B). On the other hand, for the other concentrations of microplastics, a significant increase in RNS at 72h was observed in the case of treatment with 10 and 10^3 micro-PS/L ($2.5 \cdot 10^5 \pm 3.2 \cdot 10^3$ and $2.2 \cdot 10^5 \pm 6.2 \cdot 10^3$ a.u., respectively). At the highest concentration tested (10^4 micro-PS/L) no alterations in the total antioxidant capacity of the coelomocytes was detected. At the lowest concentration (10 micro-PS/L) some imbalance was revealed. In fact, at 24h a statistically significant increase was first observed (1.20± 0.4 *vs* 0.70± 0.23 µmol eq. ascorbate/ µg protein) followed by a significant decrease at 48h compared to the control (0.3 ± 0.02 *vs* 0.96± 0.09 µmol eq. ascorbate/ µg protein, respectively) (Figure 4C). In addition, a decrease was detected at 48h and 72h after treatment with 10^3 micro-PS/L (0.44±0.22 and 0.27±0.09 µmol eq. ascorbate/ µg protein, respectively).

3.4 *Impact of micro-PS on the proteome of coelomocytes*

The coelomocytes were collected from specimens exposed to NSW (controls) and to those exposed to micro-PS/L and analysed by a label free shotgun proteomic approach for the identification and quantification of all the proteins expressed as previously described by Inguglia et al. (2020). In the present study this approach allowed to identify 1323, 1305, 1152, 1275 and 1152 proteins in CTR, 10 micro-PS/L, 50 micro-PS/L, 10^3 micro-PS/L and 10^4 micro-PS/L, respectively, as reported in the corresponding Supplementary Tables S1-S5. The proteomic analysis identified proteins common to all samples as well as proteins differentially expressed in the different conditions as reported in the Venn diagram of all the data sets (Figure 5A). The PCA analysis, reported in Figure 5B, clearly showed a marked effect on the proteome of coelomocytes from animals exposed to MPs in comparison to the control but also among different MPs concentrations. The result prompted us to compare by Perseus the proteins expressed in the control (1323 entries) and all the proteins expressed in the samples exposed to micro-PS/L (1341entries) (Figure 6A). The comparison allowed to identify 1218 common proteins, 105 proteins expressed only in the CTR and 123 proteins exclusively expressed in samples exposed to different concentrations of micro-PS/L (Table S6). These latter were further analysed by Panther for protein classification, biological function (GOBP) and molecular function (GOMF) as reported in Figure 6B. Based on the classification results, many proteins (34.2%)

are metabolite interconversion enzymes suggesting that micro-PS exposure heavily alter the metabolism response of sea urchin. More than 11% are proteins involved in transport and trafficking and almost 8% (7.6 %) are cytoskeletal proteins (Figure 6B). In keeping, the GOBP classification shows that 12.1% are proteins involved in localisation suggesting a cytoskeleton reorganisation and increased intracellular membrane trafficking (Figure 6B). In accordance, most of the coelomocytes are of the large phagocytes class (up to 80% in *P. lividus*), characterised and described by an important and complex cytoskeletal organisation.

The results are further confirmed by the Panther enrichment test analysis (Table S7, all micro-PS/L vs CTR) that underlines in the 123 proteins analysed a statistically significant enrichment of proteins involved in endosome transport via multivesicular body sorting pathway (35-fold enrichment) and in establishment of protein localisation (6.7-fold enrichment). Proteins involved in catabolic processes are also enriched in the analysis (5.3-fold enrichment).

To disclose the contribution of the micro-PS exposure concentration on the immune cells proteome, specific analyses were carried out by one to one comparison: 10 micro-PS/L vs CTR, 50 micro-PS/L *vs* CTR, 10^3 micro-PS/L *vs* CTR and 10^4 micro-PS/L *vs* CTR. According to the results, no proteins increased or decreased were identified with a statistical significance. On the contrary, the analysis allowed to identify either common proteins or proteins exclusively expressed in each condition as shown in the Venn diagrams reported in Figure 7A.

The bioinformatic analysis by Panther (Table S7) highlights that, starting from 50 micro-PS/L concentration on, there is an enrichment of terms related to protein transport, vacuole for ubiquitin-dependent catabolic processes as well as proteins involved in the cell aerobic respiration (Figure 7B). Upon increasing the micro-PS concentration at 10^3 and 10^4 micro-PS/L, a significant effect on the cytoskeleton became evident with enrichment of proteins involved in cytoskeletal regulation, cadherin signalling and integrin pathway. Some of these proteins are also involved in the inflammation pathway mediated by chemokine and cytokine and some are components of the endosomal sorting complexes required for transport complex assembly (ESCRT) and disassembly as classified by STRING (Figure 7C). The ESCRT complex enables a membrane remodelling, resulting in membranes bending/budding away from the cytoplasm. This machinery plays a vital role in a number of cellular processes including multivesicular body (MVB) biogenesis, cellular abscission, and is essential for cells to destroy misfolded and damaged proteins (Schmidt & Teis, 2012).

4. Discussion

Considering their dual chemical-physical nature facet, MPs represent a real challenge for marine organisms, especially for the immune system which is the first line of defence. Thus, a proper immune

response plays a vital role for preventing immunological disorders possibly caused by MPs. In light of this, the aim of this work was to study for the first time how exposure to different concentrations of micro-PS could alter the immunological response in adult sea urchins both from a functional point of view and more deeply at the level of the proteome.

4.1 Microplastics exposure induces functional alterations in immune system

Our data indicate that the *in vivo* waterborne exposure of sea urchins to different concentrations of micro-PS reshapes the immunological profiles of the immune cells, coelomocytes. As expected, these changes concern especially the highest concentrations tested of 10^4 micro-PS/L although at the lowest concentration of 10 micro-PS/L the cellular homeostasis is altered. In detail, the total number of coelomocytes was nearly similar across all treatments except at the highest concentration in which a significant increase starting from 24h of exposure was observed, confirming our previous findings (Murano et al. 2020). The increase of coelomocytes, and thus the proliferative response, is a phenomenon that occurs in sea urchins usually in response to immunological challenges (Brockton et al., 2008). This process could derive from an increased production of coelomocytes in coelomic fluid or from an increase of their migration from surrounding tissues into the coelomic fluid (Golconda et al., 2019). However, the absence of micro-PS in the coelomic cavity suggests that the proliferation occurs in other organs such as the digestive system, whose internal walls are filled with coelomocytes (Holland, 2020). In fact, the highest amount of micro-PS was recorded in the digestive system at the two highest exposure concentrations both at 10^4 and 10^3 micro-PS/L. Interestingly, at this last concentration, the number of coelomocytes in the coelomic cavity at 24h is lower than that of the corresponding controls and then reaches the control levels in the following hours. In this case it is possible to hypothesise that the momentary decrease is due to a migration from the coelomic cavity. The cellular morphological profile confirms that in terms of composition, phagocytes represent the majority of the immune cells, exceeding about 80% in all treatments and at all times. Alterations mainly concern minor morphological cellular types, the red cells whose number increases as reflected by the increased ratio between red and white amebocytes, already detected at the lowest concentration. This ratio reflects the fitness of the sea urchin's populations. Indeed, an increase in red cells compared to white cells indicates that sea urchins are in a condition of injury (Matranga et al., 2005; Pinsino & Matranga, 2015). For example, it was demonstrated that in pollution or hypoxia conditions, after injuries or after MPs exposure, the homeostasis between red/white amoebocytes was visibly affected (Matranga et al., 2002; Matranga et al., 2005; Pinsino et al., 2007; Suh et al., 2014; Murano et al., 2020; Murano et al., 2021b). The common defence mechanisms used by most invertebrates to face injuries include phagocytosis, encapsulation and production of ROS and nitrogen

radicals, RNS (Canesi & Procházková, 2014). Our results indicate that the highest concentration tested caused a significant increase in both reactive species at all times compared to controls. Nevertheless, the cells try to counteract low concentration MPs exposure already at 24h, as revealed by the increase in the antioxidant system at 24h followed by a decrease at 48h and a concomitant increase in ROS and RNS levels at 48h and 72h, respectively. Our finding of the modulation of redox homeostasis starting from low concentrations certainly strengthens what it is commonly believed that oxidative stress represents the universal common factor of toxicity caused by exposure to MPs (Hu & Palic, 2020).

4.2 Microplastics exposure affects the immune cell proteome

An important outcome of this study concerns the use of the proteomic approach that allowed us to demonstrate, for the first time, that the MPs *in vivo* exposure affects the immune cell proteome in sea urchin. As also suggested by the PCA analysis, these variations are not only evident compared to the control group but also among the different micro-PS exposure concentrations. While the highest concentrations (10^3 and 10^4 micro-PS/L) cause similar changes, the lowest (10 and 50 micro-PS/L) differ from each other and from the highest, indicating a concentration dependent proteomic profile.
The set of proteins exclusively expressed in the coelomocytes of specimens exposed to MPs are mostly classified as metabolite interconversion enzymes, mainly involved in cellular processes, indicating a severe alteration of the cellular metabolic pathways.
This most likely depends on the fact that the immune defence action against external factors such as MPs includes the involvement of cellular detoxification processes which have a significant metabolic cost (Guderley & Pörtner, 2010; Gardon et al., 2020).
In fact, it is well known that MPs have the ability to cause metabolic disorders in various organisms belonging to different trophic levels (Paul-Pont et al., 2016; Kim et al., 2019; Magni et al., 2019; Green et al., 2019; Qiao et al., 2019; Duan et al., 2021). For instance, Duan et al. (2021) demonstrated that the haemolymph metabolic functions of the shrimp *Litopenaeus vannamei* were altered upon long-term MPs exposure. Similarly, Green et al. (2019) showed that the MPs exposure (HDPE and PLA) altered the abundance of metabolic as well as detoxification proteins in haemolymph of *M. galloprovincialis*. In accordance, mitochondrial proteins involved in the energetic metabolism are found among the differentially expressed proteins in coelomocytes of specimens exposed to micro-PS (Table S6). Under normal environmental conditions, energy allocations in organisms remain optimal (Sokolova, 2013), but in moderate environmental stress, such as upon MPs exposure, marine organisms potentially compensate the increased energy requirements by increasing energy generation and integration, as well as through metabolic regulation to meet the elevated ATP demands (Lannig

et al., 2010). This compensatory process is evident from our finding showing that proteins associated with energetic metabolic activities are among the protein expressed upon micro-PS exposure and not in CTR, like the mitochondrial ATP synthase d (Table S3), NADH dehydrogenase iron-sulfur protein 6 (Table S3, S4, S5, S6), cytochrome b-c1 complex subunit 8 (Table S3), cytochrome c domain-containing protein (Table S4), cytochrome c oxidase and cytochrome b5 heme-binding domain-containing protein (Table S5) and other proteins involved in redox activities, such as glutamate dehydrogenase, oxoglutarate dehydrogenase, 3-hydroxyisobutyrate dehydrogenase and D-3-phosphoglycerate dehydrogenase (Table S3, S4, S5, S6).

The increase in mitochondrial activity is related to the production of ROS that, under normal conditions, is associated with homeostatic regulation (Manduzio et al., 2005) but, when environmental conditions become unfavorable, tethers precariously towards an imbalance between the generation and removal of ROS by antioxidants, leading to oxidative stress. This can either be due to excessive ROS production or the impairment of antioxidant machinery, or both (Sheehan & McDonagh, 2008). Concerning this aspect, proteomic data identified superoxide dismutase and catalase among the proteins expressed upon exposure to micro-PS suggesting that the increase of intracellular ROS in specimens treated for 48h (Figure 4A) may be counteracted by antioxidant enzymes resulting in a decrease of ROS production upon 72h exposure (Figure 4A, Table S3, S4, S5, S6).

Notoriously, the effects of oxidative stress and excess ROS are associated with cytoskeletal damage with repercussions for processes such as protein transport and intracellular signaling (Raftos et al., 2016). Interestingly, the enrichment analysis revealed a significant variation of the "Rho GTPase pathway" only at the highest concentrations (10^3 -10^4 micro-PS/L). As well known, Rho GTPases are involved in different cellular functions mainly related to cytoskeletal alterations, intracellular trafficking, cell migration, gene expression and cell proliferation (Li et al., 2015). Precisely, they control intermediate filaments, the cortical actin cytoskeleton, myosin filaments and microtubules to regulate immune cell migration, activation, proliferation and phagocytosis (Ridley, 2001). These processes are supported by the action of the Wiskott-Aldrich syndrome protein family members, which controls cell polarity, required to generate and maintain migration, synapse formation and polarised cytokine secretion (El Masri & Delon, 2021). Notably, in this study these proteins were found exclusively expressed in the high concentrations tested. The most representative cell population of coelomocytes are phagocytes (>80 %) which occur in two morphotypes: petaloid and filopodial (Pinsino & Matranga, 2015). The phagocytes appear to be the core of immunity also thanks to their cytoskeleton that rearranges itself rapidly in association with immune response (Smith et al., 2006). Under stressful conditions or in response to different stimuli, a petaloid-filopodial transition occurs

which involves a significant change in the cytoskeletal morphology switching from bladder-like shape to filopodial (Smith et al., 2019). This phenomenon also occurs in other echinoderms such as sea stars in which proteomic studies have shown that the transition is regulated mainly by two major pathways: integrin signalling and the Rho GTPase (Franco et al., 2011; Andrade et al., 2021). These observations clearly suggest that exposure to these high concentrations influences the cytoskeletal rearrangement of immune cells inducing morphological changes predicting a condition of heavy stress. In fact, the morphological transition was observed in sea urchins upon UV-B radiation and post exposure to zinc and silver nanoparticles (Matranga et al., 2006; Pagliara & Stabili, 2012; Magesky et al., 2016) or in sea stars as post-traumatic response (Pinsino et al., 2007). Moreover, in the 10^4 micro-PS/L treatment there is the activation of the cadherin and integrin pathways that are closely linked to the Rho-GTPase in the cell-substratum and cell-cell adhesion processes, respectively, suggesting that in this scenario the alteration is in a more advanced step with more severe features (Fukata et al., 1999). At highest concentrations there is also the enrichment of "Huntington disease (6 proteins cytoskeleton)", a key regulator of several important phenomena, including immune synapse organisation, viral infection, cell migration, and the transformation and degradation of misfolded proteins (Valenzuela-Fernandez et al., 2008).

Interestingly, the enrichment of cadherin and integrin pathways was also described in the early life stages of *Oryzias melastigma* exposed to the endocrine disruptors 17α-ethinylestradiol and bisphenol A (Bhandhari et al., 2020), suggesting a similar mechanism of action between endocrine disruptors and MPs but more specifically with styrene oligomers (Choi et al., 2005).

The bioinformatic analysis by Panther of proteins differentially expressed in samples exposed to different concentrations of micro-PS, highlights that more than 11% are involved in transport and trafficking and 12.1% are involved in localisation. In addition, the analysis shows a significant enrichment of pathways, such as "endosome transport via multivesicular body sorting" and "late endosome to vacuole transport", related to the formation of multivesicular endosomes, known as compartments for receptor downregulation and intermediates in the formation of secretory lysosomes (Raiborg et al., 2003). To date, this type of mechanism was reported in coelomocytes of *S. purpuratus*, in response to LPS in which the upregulation in protein sorting and trafficking of transport vesicles was highlighted through microarray analysis of coelomocyte gene expression (Nair et al., 2005). More recently, the same mechanism was evidenced by a high throughput iTRAQ-based proteomics methodology in haemocytes of the South African abalone *Haliotis midae*, as molecular response to the exposure at acidic waters for prolonged periods, in which "Intracellular trafficking, secretion and vesicular transport" was among the most enriched functional classes (Carroll & Coyne, 2021).

5. Conclusions

In a scenario of continuously pronounced global changes, the priority of understanding how MPs compromise marine organisms becomes more evident, particularly by elucidating the toxicity mechanisms of action in species highly exposed to several anthropogenic pressures in coastal areas such as *P. lividus*.

For the first time with this study, it was shown that exposure to micro-PS causes an immunological challenge such as to remodel the structural and functional components of the coelomocytes concurrently to the activation of particular cellular compartments involved in trafficking and protein sorting. Changes in the morphological profile of immune cells have been recorded during exposure to MPs as well as a promotion of the ROS and RNS formation starting already from the lowest concentrations.

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Figure Legends

Figure 1. Abundance of micro-PS in different organs of sea urchins after 72h of exposure, expressed as percentage of micro-PS found normalised by the weight of fresh organs.

Table 1. Ratio between red (%) and white amoebocytes (%) of sea urchin at 24,48,72h after exposure to different concentrations of micro-PS (10, 50, 10^3, 10^4). Green background indicated a normal status (<0.5); orange background indicated a beginning stress status (between 0.5-1); red background indicated a harmful condition (>1).

Ratio R/W	**24h**	**48h**	**72h**
Control	0.22	0.30	0.49
10 micro-PS	0.43	0.50	0.63
50 micro-PS	0.54	0.62	0.74
10^3 micro-PS	0.62	0.35	0.40
10^4 micro-PS	0.44	1.28	0.87

Figure 2. Total immune cells count of sea urchin at 24, 48, 72h after exposure to different concentrations of micro-PS (10, 50, 10^3, 10^4). All data were analysed by Two-way ANOVA followed by Bonferroni post-test compared with the control. Bars represent mean ± SD. Asterisks indicate values that are significantly different from the control, *P < 0.05; **P < 0.01.

Figure 3. Immune cells morphological profile of sea urchin at 24, 48, 72h after exposure to different concentrations of micro-PS (10, 50, 10^3, 10^4). Bars represent mean ± SD.

Figure 4. Oxidative stress status of sea urchin immune cells at 24, 48, 72h after exposure to different concentrations of micro-PS (10, 50, 10^3, 10^4). A) intracellular levels of ROS; B) intracellular levels of RNS; C) total antioxidant capacity. All data were analysed by Two-way ANOVA followed by

Bonferroni post-test compared with the control. Bars represent mean ± SD. Asterisks indicate values that are significantly different from the control, *P < 0.05; **P < 0.01; ***P<0.001.

Figure 5. Proteomic analysis of immune cells of sea urchins exposed to different concentrations of micro-PS/L. The coelomocytes were collected from control animals and from sea urchins exposed to different concentrations of micro-PS/L and analysed by a label-free shotgun proteomic approach. (A) Venn diagram of all the data sets. The proteomic analysis allowed to identify proteins common to all samples as well as proteins differentially expressed in the presence of 10 micro-PS/L, 50 micro-PS/L, 10^3 micro-PS/L and 10^4 micro-PS/L. (B) PCA analysis.

Figure 6. Venn diagram and bioinformatic analysis by Panther of the proteins exclusively expressed in the immune cells of sea urchins exposed to different concentration of microplastics in comparison to control samples (CTR). A) Venn diagram of all micro-PS/L *vs* CTR. (B) A total of 123 proteins exclusively expressed in the coelomocytes of sea urchins exposed to different concentration of microplastics in comparison to control samples were classified by Panther in terms of Functional Protein Classification, GO-Biological Process classification (GOBP) and GO-Molecular Function classification (GOMF).

Figure 7. Effect of the microplastics concentration on the immune cells proteome. A) Venn diagrams of the comparisons:10 micro-PS/L *vs* CTR, 50 micro-PS/L *vs* CTR, 10^3 micro-PS/L *vs* CTR and 10^4 micro-PS/L *vs* CTR. B) Network analysis by String of the proteins exclusively expressed in the comparison 50 micro-PS/L *vs* CTR and involved in aerobic respiration, ATP synthesis and TCA Cycle. In the figure the hortologs human gene are reported A0A7M7NI38: UQCRQ, A0A7M7N4E1: NDUFS6, A0A7M7RBT7, SUCLG. C) Network analysis by String of the proteins exclusively expressed in the comparison of all micro-PS/L *vs* CTR and involved in ESCRT complex assembly (Blue) and disassembly (Red). In the figure the hortologs human gene are reported (A0A7M7PCE8:SNF8, A0A7M7N202: VPS25, A0A7M7SU07:CHMP1A).

Supplementary Materials

Table S1. List of the proteins identified in the CTR samples. A database search was conducted against the Uniprot *Strongylocentrotus purpuratus* database with MaxQuant software. Statistical analysis was performed using the Perseus software. Proteins were considered unequivocally identified if present in 75% of the replicates.

Table S2. List of the proteins identified in the 10 micro-PS/L samples. A database search was conducted against the Uniprot *Strongylocentrotus purpuratus* database with MaxQuant software. Statistical analysis was performed using the Perseus software. Proteins were considered unequivocally identified if present in 75% of the replicates.

Table S3. List of the proteins identified in the 50 micro-PS/L samples. A database search was conducted against the Uniprot *Strongylocentrotus purpuratus* database with MaxQuant software. Statistical analysis was performed using the Perseus software. Proteins were considered unequivocally identified if present in 75% of the replicates.

Table S4. List of the proteins identified in the 10^3 micro-PS/L samples. A database search was conducted against the Uniprot *Strongylocentrotus purpuratus* database with MaxQuant software. Statistical analysis was performed using the Perseus software. Proteins were considered unequivocally identified if present in 75% of the replicates.

Table S5. List of the proteins identified in the 10^4 micro-PS/L samples. A database search was conducted against the Uniprot *Strongylocentrotus purpuratus* database with MaxQuant software. Statistical analysis was performed using the Perseus software. Proteins were considered unequivocally identified if present in 75% of the replicates.

Table S6. List of the proteins exclusively expressed in all the samples exposed to microplastics in comparison to control. A database search was conducted against the Uniprot *Strongylocentrotus purpuratus* database with MaxQuant software. Statistical analysis was performed using the Perseus software. Proteins were considered differentially expressed if they were present only in one condition or showed statistically significant difference (FDR≤0.05, Welch's t-test). Proteins were analysed using Perseus.

Table S7. Enrichment analysis by Panther of the proteins exclusively expressed in the samples exposed to microplastics in comparison to control. The statistical overrepresentation analysis for GO-Slim Biological Process, GO-Slim Molecular Function and Pathways was carried out by Panther on the proteins exclusively expressed in the samples exposed to micro-PS/L in the following comparison: all micro.PS/l *vs* CTR, 10 micro-PS/L *vs* CTR, 50 micro-PS/L *vs* CTR, 10^3 micro-PS/L *vs* CTR and 10^4 micro-PS/L *vs* CTR. Functional grouping was based on Fisher's exact test $P \leq 0.05$.

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