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Effects of microplastics alone and with adsorbed benzo(*a*)pyrene on the gills proteome of *Scrobicularia plana*

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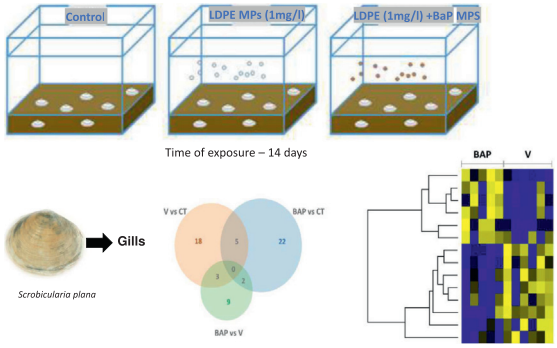
HIGHLIGHTS

- Polyethylene microplastics induce changes in *Scrobicularia plana* gills proteome.
- Different changes occur in gills proteome when microplastics contain benzo(*a*) pyrene.
- Protein pathways changes induced by both stressors affect gills health.

GRAPHICAL ABSTRACT



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ABSTRACT

Microplastics (MPs) are globally present in the marine environment, but the biological effects on marine organisms at the molecular and cellular levels remain scarce. Due to their lipophilic nature, MPs can adsorb other contaminants present in the marine environment, which may increase their detrimental effects once ingested by organisms. This study investigates the effects of low-density polyethylene (PE) MPs with and without adsorbed benzo[*a*]pyrene (BaP) in the gills proteome of the peppery furrow shell clam, *Scrobicularia plana*. Clams were exposed to PE MPs (11–13 μm; 1 mg L^{-1}) for 14 days. BaP was analyzed in whole clams' soft tissues, and a proteomic approach was applied in the gills using SWATH/DIA analysis. Proteomic responses suggest that virgin MPs cause disturbance by altering cytoskeleton and cell structure, energy metabolism, conformational changes, oxidative stress, fatty acids, DNA binding and, neurotransmission highlighting the potential risk of this type of MPs for the clam health. Conversely, when clam gills were exposed to MPs adsorbed with BaP a higher differentiation of protein expression was observed that besides changes in cytoskeleton and cell structure, oxidative stress, energy metabolism and DNA binding also induce changes in glucose metabolism, RNA binding and apoptosis. These results indicate that the presence of both stressors (MPs and BaP) have a higher toxicological risk to the health of *S. plana*.

1. Introduction

The increase of plastic in the marine environment as a result from its incorrect elimination or illegal dumping became a global threat to the ocean's health. It is estimated that around 10 million tons of plastics are disposed of every year, and an increase of an order of magnitude is predicted by 2025 (Barnes et al., 2009: Jambeck et al., 2015). Microplastics (MPs) whose size range is <5 mm, derive from the degradation and fragmentation of macroplastics and from the direct release of MPs used in personal care products present in sewage and industrial effluents. Therefore, the ubiquitous presence of MPs in the ocean is alarming (Ryan et al., 2009). They are

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detected in the water column and the sediments from polar areas (Obbard et al., 2014) to the deep-sea as well as in the biota (Avio et al., 2017). Once ingested, MPs can induce physical and chemical stress in aquatic organisms and several health effects (Ribeiro et al., 2017; O'Donovan et al., 2018).

Marine organisms are known to ingest MPs. The ingestion of these microparticles which are of a similar size range to the food particles they normally ingest are a cause of concern due to the possible biological effect that this ingestion may trigger.

Commercial marine species which are eaten whole, such as shrimps and bivalves, constitute potential transfer pathways of MPs to humans (Rochman, 2015; Santillo et al., 2017). Suspension and filter-feeders bivalves can ingest MPs and any organic and inorganic chemicals they contain or adsorb from the marine environment (O'Donovan et al., 2018; Islam et al., 2021). So, it is crucial to identify the effects of these microparticles on the health of the biota, their implications to the blue economy, and to human health.

The analysis of the effects of MPs in the biota at the molecular and cellular levels is scarce. Effects already detected include gut blockage, physical injury, oxidative stress, changes in feeding behavior, and energy metabolism (Galloway & Lewis, 2016; O'Donovan et al., 2018; Solomando et al., 2021). Toxic effects of virgin MPs in mussels *Mytilus* sp., clams *Scrobicularia plana* and oysters *Crassotrea gigas* include neurotoxicity, genotoxicity, oxidative stress and damage (Avio et al., 2015; Paul-Pont et al., 2016; Sussarellu et al., 2016; Ribeiro et al., 2017; O'Donovan et al., 2018), changes in lysosomal membrane stability, alterations in immune parameters (Pittura et al., 2018), formation of granulocytomas (Von Moos et al., 2012), and histopathological lesions (Paul-Pont et al., 2016; Bråte et al., 2018).

MPs can also be a vector for the accumulation of other contaminants such as metals and persistent organic compounds like polychlorinated biphenyls, polyaromatic hydrocarbons (PAHs), polybrominated diphenylethers and perfluoroctane sulfonic acid (PFOS) (O'Donovan et al., 2018, 2020; Islam et al., 2021), all of which possess endocrine-disrupting activity (O'Donovan et al., 2018). Their effects depend on MPs size, type as well as exposure time and conditions (Ribeiro et al., 2017; Town et al., 2018; Islam et al., 2021). Once ingested by the biota, retention time and fate of MPs influence the desorption of persistent organic compounds from MPs (Bakir et al., 2014; Batel et al., 2016, 2018). Both virgin polystyrene (PS) MPs in *S. plana* and virgin polyethylene (PE) MP in *M. galloprovincialis* induce a significant increase in DNA strand breaks in haemocyte cells (Avio et al., 2015; Ribeiro et al., 2017).

There is also evidence that exposure of bivalves *M. galloprovincialis* and *S. plana* to low-density polyethylene (LDPE) PM contaminated with benzo [*a*]pyrene (BaP) results in DNA damage (O'Donovan et al., 2018).

LDPE represents around 40 % of total plastic production and is one of the types of plastic most used as a component of single-use plastic packaging, and the polymer utmost detected in floating marine debris (Plastic Europe, 2019; Pittura et al., 2018). On the other hand, BaP, a five-ring aromatic hydrocarbon PAH, is the most toxic component of oil frequently detected in the ocean and in several biota species (Banni et al., 2017). Besides oil spills, other important anthropogenic sources of PAHs to the marine environment are through atmospheric deposition due to incomplete combustion of organic material and incineration. Due to its carcinogenic and mutagenic nature, BaP belongs to the list of priority substances of European legislation (Directive 2008/105/EC) and is used as a model compound in ecotoxicology to assess the biological effects and changes in metabolic pathways of PAHs in marine organisms (Liu et al., 2015). In mussels and clams, BaP cause peroxisome proliferation, oxidative stress, endocrine disruption and genotoxic effects (Venier et al., 2003; O'Donovan et al., 2020). In *M. galloprovincialis* exposed to BaP using a proteomic approach, BaP disrupts protein-protein signaling pathways, namely cytoskeleton and cell structure, transcription regulation and energy metabolism (Maria et al., 2013).

The molecular mechanisms by which bivalves modulate the effects of uncontaminated and contaminated MPs are scarce. Changes in different classes of proteins were detected in the gills of the freshwater zebra mussel *Dreissena polymorpha* exposed for six days to PS MP (10 μm). These changes were related to the cytoskeleton and structural activity, structure and function of ribosomes, energy metabolism, cellular trafficking, and RNA-binding, and are directly or indirectly involved with oxidative stress homeostasis confirming that oxidative stress is a major effect of MPs exposure (Magni et al., 2019). However, no modulation of protein changes were observed when zebra mussels were exposed to smaller MPs (1 μm) indicating that changes in the gills proteome of freshwater mussels may be related to size. In marine organisms, the only information available of MPs effects at the proteome level is a comparison between the effects of high-density polyethylene (HDPE) MPs and of biodegradable polymers, such as polylactic acid (PLA) MPs of a wide size range (0.48–316 and 0.6–363 μm, respectively). After a long-term exposure (52 days), MPs induce protein alterations in vital biological processes on the hemocytes of *M. edulis* namely immune regulation, detoxification, metabolism and structural development that were type of MPs dependent (Green et al., 2019). Moreover, MPs altered the haemolympph proteomic profile of the pacific white shrimp *Litopenaeus vannamei* after two weeks of exposure to five different color, types, shapes and size of MPs namely: PE (6–18 μm), PTFE (1–8 μm), PP (1.77–18 μm), (100–200 μm) and PVC (size: 1–13 μm). The alteration of the immune-related proteins is type dependent MPs (Duan et al., 2021). Therefore, it is crucial to assess the effects of MPs at the proteome level in other tissues and species.

The aim of the present study was to use a proteomic approach, Sequential Window Acquisition of all Theoretical fragment ion spectra (SWATH) and Data Independent Acquisition (DIA) approach - or SWATH/DIA analysis to provide new insights on the effects at the proteome level of both virgin and BaP contaminated LDPE MPs in the gills of the peppery furrow shell clam, *S. plana*. *S. plana* was selected because is a gonochoric suspension-feeder, ubiquitous in the Atlantic, Mediterranean, and North African coast that feeds on particles of surface deposits, but also obtains part of the food by filtering particles up to 40 μm from the overlying water (Hughes, 1969). This species is a good bioindicator to evaluate the health of the marine environment because is sedentary with relatively long life and importance in the food chain (Mouneyrac et al., 2008). MPs of size 11–13 μm were adsorbed with environmentally relevant BaP concentrations (16.87 ± 0.22 μg g^{-1}) to evaluate the effect of MPs with and without BaP adsorbed at the proteome level. SWAT analysis combines shotgun and targeted data extraction approaches, allowing deep proteome analysis (Anjo et al., 2015). The gills were selected as they are the first tissue to be in contact with MPs and a tissue that could be a target for MPs ingestion assessment. To our knowledge, this is the first time that SWATH/DIA technology was applied to assess the effect of MPs on the gills tissue proteome of a marine bivalve.

2. Materials and methods

2.1. Preparation and characterization of microplastics

White LDPE MPs of spherical shape and nonfluorescent (0.96 g cm^{-3}) (MPP-635G) of size range 11–13 μm were purchased from Micro Powders Inc. (NY-USA). BaP (CAS 50-32-8, purity ≥96 %) (2500 μg L^{-1}) was obtained from Sigma Aldrich. To reach a final concentration of 16.87 ± 0.22 μg $g^{-1}$of BaP adsorbed to LDPE (11–13 μm), MPs (125 g L^{-1} of plastic) were spiked with BaP into separate 250 mL narrow-mouth Septa bottles (Thermo scientific) filled with double-deionized water. Bottles were placed on a 20 rpm rotary shaker for two days. Samples were filtered using a ceramic funnel and glass microfiber filters (1.0 μm, Whatman® glass microfiber filters, GE Healthcare Life Sciences), rinsed with double-deionized water, dried by vacuum evaporation, and extracted in hexane (≥98 %, SupraSolv). Extracts were sonicated and centrifuged at 2000 RCF and filtrated through fiberglass and transferred to toluene (purity 96 %, SOLVECO). A nitrogen stream was used to reduce the volume to 500 μL. The concentration of BaP adsorbed to MPs was quantified using a high-resolution GC–MS system (Micromass Autopspec Ultima), separation on a

30 m (0.25 mm i.d., 25 μm film thickness) DB-5MS column (J&W Scientific, Folsom, USA). Details about the instrumental method can be found in Larsson et al. (2013). Benzo[*a*]pyrene-d12 in toluene was added as an internal standard. Quantification was performed against perylene-d12 recovery standard, dissolved in toluene (O'Donovan et al., 2018).

2.2. Experimental design

Clams *S. plana* (4 ± 0.5 cm) were collected from the Ria Formosa lagoon, Southern coast of Portugal (37°7′59.75″ N 7 36′34.95″ W), transported alive to the laboratory, and acclimatized in glass aquaria for a week in natural seawater, at constant aeration with a photoperiod of 12:12 h light and dark. This site was selected because previous studies did not identify any MPs in clams from this site (Islam et al., 2021). The experimental design was described in detail in O'Donovan et al. (2018). The exposure experiment consisted of six aquaria that include control, virgin LDPE (11–13 μm, density 0.96), and BaP contaminated MPs in a duplicate design. All aquaria, excluding controls, were exposed to LDPE MPs (11–13 μm) virgin and with BaP adsorbed at a concentration of 1 mg L^{-1} for 14 days. The concentration of MPs selected was based on measured environmental concentrations of MPs in both water and sediments of the world's ocean (Li et al., 2019). The selection of the BaP concentration (16.87 ± 0.22 μg g^{-1}) load into MPs was environmentally relevant.

Clams were randomly sampled from each aquarium at the beginning of the experiment (day 0) and after 14 days of exposure and the gills were immediately dissected, flash-frozen in liquid nitrogen, and stored at −80 °C for later proteomic analysis. The condition index was determined in six individuals per treatment as the percentage (%) of the ratio between dry weight of the soft tissues and the dry weight of the shell (Walne, 1976) and the whole clam soft tissues used for chemical analysis were frozen at −20 °C until they were processed.

2.3. BaP analysis in clam tissues

To assess if MPs were a vector of organic contaminants, BaP levels were quantified (6 clams per treatment and per time (at days 0 and 14)) in the whole soft tissues of freeze-dried clams by Gas Chromatography mass spectrometry (GC-MS) (Agilent 7890A- 5975C MS) according to the method previously described by De Witte et al. (2014), with some modifications described in O'Donovan et al. (2018). The analysis is accredited by BELAC under the ISO/IEC 17025 standard, with a limit of quantification of 1.65 ng g^{-1} d.w.

2.4. Quantitative proteomics of clam gills

A proteomic approach was carried out in the gills of *S. plana* unexposed and previously exposed to LDPE MPs (11–13 μm) virgin and with BaP adsorbed for 14 days. The Short GeLC-SWATH approach (Anjo et al., 2015) was used to perform the proteomic screening (both data-dependent acquisition – DDA- and data-independent acquisition - DIA).

Clam gills uncontaminated and exposed to MPs without and with BaP adsorbed were homogenized using a Polytron PT1200E with a solution of Tris-HCl at pH 7.2 containing a protease inhibitor cocktail added in a proportion of 1:4 (w/v) (Table 1).

A sonication step was performed for 1 min (40 % Amplitude, cycle: 3 s ON and 2 s OFF) followed by sample dilution (100 μL) in 6× concentrated Laemmli sample buffer (20 μL) for a denaturation step at 95 °C for 5 min. A centrifugation at 14,000 RCF was performed for 15 min, and the supernatant collected for protein quantification using the Pierce 660 nm kit.

The Short GeLC-SWATH approach was used for protein identification and relative quantification (Anjo et al., 2015). For protein identification, samples were pooled by grouping control (CT), virgin (V) MPs, and BaP adsorbed MPs (BAP), and for the relative quantification, samples were analyzed separately. Protein content from each sample was separated in different lanes by SDS-Page using 70 μg of protein from each pool for protein identification and 50 μg of protein from each sample for relative protein quantification. Proteins were stained in the gel with Coomassie Brilliant Blue G-250 (Manadas et al., 2006), and each lane was excised into three gel pieces. After a de-staining step, gel bands were incubated overnight with trypsin for protein digestion, and peptides were extracted from the gel using three solutions composed of different percentages of acetonitrile (30, 50 and 98 %) with 1 % formic acid. A cleaning step using C18 Omix tips was performed, and peptides were re-suspended in 30 μL of a solution containing 2 % acetonitrile and 0.1 % formic acid (Anjo et al., 2015).

Table 1
Sample description with the respective weight, volume of homogenization solution and protein quantification using the Pierce 660 nm Kit.

Condition	Sample name	Weight (mg)	$V_{sol.\ Homog.}$ (μL)[a]	Protein concentration (μg/μL)
Control	G1_CT_T14	104.9	400	10.1
	G2_CT_T14	73.0	300	10.5
	G3_CT_T14	95.7	400	9.1
	G4_CT_T14	105.6	400	9.7
	G5_CT_T14	152.2	600	8.2
	G6_CT_T14	103.9	400	7.5
V	G2_V_T14	204.9	800	6.6
	G3_V_T14	187.3	800	6.6
	G4_V_T14	99.1	400	8.0
	G5_V_T14	67.5	270	9.8
	G6_V_T14	80.6	300	10.1
BAP	G1_BAP_T14	46.4	200	6.9
	G2_BAP_T14	102.6	400	6.0
	G3_BAP_T14	58.9	200	10.1
	G5_BAP_T14	103.2	400	7.4
	G6_BAP_T14	101.6	400	5.3
	G1_BAP_T14	46.4	200	6.9

[a] The homogenization solution was Tris-HCl at pH 7.2 containing serine and cysteine protease inhibitors (cOmplete, EDTA-free).

2.4.1. LC-MS methodology

Samples were analyzed on a NanoLC Ultra 2D separation system (Eksigent) coupled to a Triple TOF™ 6600 System mass spectrometer (Sciex). The chromatographic separation was performed on a ChromXP C18CL 0.3 × 150 mm, 3 μm, 120 Å (Eksigent) at 50 °C. The volume of injection was 10 μL for all samples. The flow rate was set to 5 μL/min, and mobile phases A and B were 5 % DMSO plus 0.1 % formic acid in water and 5 % DMSO plus 0.1 % formic acid in acetonitrile, respectively. The LC program was performed as followed: 5–30 % of B (0–46 min), 30–98 % of B (46–47 min), 98 % of B (47–55 min), 98–5 % of B (55–56 min) and 5 % of B (56–66 min). The ionization source (ESI DuoSpray™ Source) was operated in the positive mode set to an ion spray voltage of 5500 V, 25 psi for nebulizer gas 1 (GS1), 25 psi for the curtain gas (CUR). For information-dependent acquisition (IDA or DDA) experiments, the mass spectrometer was set to scanning full spectra (*m/z* 350–1250) for 250 ms, followed by up to 100 MS/MS scans (*m/z* 100–1500) with an accumulation time of 30 ms. Candidate ions with a charge state between +2 and +5 and counts above a minimum threshold of 10 counts per second were isolated for fragmentation and one MS/MS spectrum was previously obtained before adding those ions to the exclusion list for 15 s (mass spectrometer operated by Analyst® TF 1.7, Sciex®). A collision energy spread of 5 was used as rolling collision. For DIA, the mass spectrometer was in a looped product ion mode specifically tuned to a set of 167 overlapping windows, covering the precursor mass range of 350–1250 *m/z*. At the beginning of each cycle, a 50 ms survey scan (350–1250 *m/z*) was performed, and SWATH-MS/MS spectra were collected from 100 to 1500 *m/z* for 19 ms that resulted in a cycle time of 3.3 s.

2.4.2. Data analysis

2.4.2.1. Library construction (IDA-MS). A specific library of the precursor masses and fragment ions was created by combining all files from the IDA experiments and used for subsequent SWATH processing. Protein

identification and library were obtained by ProteinPilot™ software (v5.0, Sciex) using the following parameters: search against the *Crassostrea gigas* database from NCBI, cysteine alkylation by acrylamide and digestion using trypsin. An independent False Discovery Rate (FDR) analysis was used to assess the quality of identifications using the target-decoy approach provided by Protein Pilot™. The mass spectrometry proteomics data have been deposited to the Proteome Xchange Consortium via PRIDE (Perez-Riverol et al., 2019) partner repository with the dataset identifier PXD030068.

2.4.2.2. Relative quantification of proteins (SWATH-MS). SWATH-MS data processing was performed using the SWATH™ processing plug-in for PeakView™ (v2.0.01, Sciex®). Briefly, peptides were selected automatically from the library, and up to 15 peptides with up to 5 fragment ions were chosen per protein. Quantitation was performed for all proteins from the library file that were identified below 5 % local FDR from ProteinPilot™ searches and by extracting the peak areas of the target fragment ions of those peptides which had an FDR% below 1.

2.5. Statistical analysis

The protein fold change (FC) was calculated using the median, and considering the comparisons CT vs V, CT vs BAP, and BAP vs V. The non-parametric test Mann-Whitney was applied for each comparison and proteins with $p < 0.05$ were selected for further analysis. Clustering and heat map representations were performed by PermutMatrix software (Caraux and Pinloche, 2005). Venn-diagram was calculated using an online bioinformatic tool (http://bioinformatics.psb.ugent.be/webtools/Venn/) and drawn using the Venn Diagram Maker Online (http://bioinformatics.psb.ugent.be/cgi-bin/liste/Venn/calculate_venn.htpl).

The gene ontology (GO) annotations for the statistically altered proteins were performed using the Blast2GO (version 5.2.5), where the respective protein sequences were loaded as a FASTA file. The BLAST was performed against the non-redundant (nr) protein database NCBI using the BLASTP method, considering a statistical significance threshold of 1×10^3. The graphical representation of the GO counts was generated for molecular function (MF), biological processes (BP) and cellular component (CC) for the proteins altered between conditions CT and V or the proteins altered between conditions CT and BAP. Pathway-maps were loaded from KEGG using the Blast2GO software for the two comparisons (V vs. CT and BAP vs. CT). Pathway maps were loaded from KEGG using the Blast2Go software for the comparisons (V vs. CT and BAP vs. CT).

3. Results

3.1. BaP in S. plana whole soft tissues

In control clams and in those exposed to LDPE mortality was 7 % while in those exposed to LDPE with BaP adsorbed was 3 %. The condition index was not significantly different between treatments (7.6 ± 0.4, 7.7. ± 0.3, 7.4 ± 0.5 % for controls, virgin MPs and MPs adsorbed with BaP, respectively). BaP levels in the whole soft tissues of *S. plana* in clams unexposed (controls) and exposed to virgin LDPE MPs were below detection limit (<1.6 ng g^{-1} d.w.). In those exposed to MPs with BaP adsorbed, levels reached 7.3 ± 2.0 ng g^{-1} d.w. after 14 days ($p < 0.05$) indicating that BaP levels were significantly higher in MPs contaminated with BaP ($p < 0.05$), and that *S. plana* accumulated BaP from contaminated MPs.

3.2. Effects of MPs in the clam gills proteome

The exposure to MPs virgin and adsorbed with BaP significantly altered the gills proteome when compared to controls and changes were significantly different between treatments. The short GeLC-SWATH approach identify 588 proteins (Supplementary Table 1) and quantify 435 proteins in *S. plana* gills (Supplementary Table 2). A subset of 59 proteins significantly different among treatments was selected ($p < 0.05$) (Supplementary Table 3). Moreover, significant differences exist for 20 proteins between unexposed, virgin and BaP contaminated MPs ($p < 0.05$). The Venn diagram depicting protein changes between the tested conditions (Fig. 1; Supplementary Table 3) shows that the number of differentially expressed proteins was higher in clam gills exposed to MPs with BaP adsorbed compared to controls. Five proteins were common in clam gills exposed to virgin and to BaP contaminated MPs in relation to controls. These proteins were related to DNA binding (histone H3), endoplasmic reticulum (surfeit locus protein 4) and ribosome structure (40 ribosomal proteins S3a and S26 and 60S ribosomal protein L8-like isoform X2). Three other proteins (coatomer subunit gamma-2, tryptophan 5-hydroxylase 1, and clathrin heavy chain 1 isoform X3) were common between virgin versus control and BaP contaminated MPs versus virgin. Moreover, two proteins (cytoplasmic dynein 1 heavy chain 1 isoform X11 and transmembrane emp24 domain-containing protein 9 isoform X2) were common between BaP MPs versus control and BaP MPs versus virgin MPs. From the common proteins differentially expressed in the gills exposed to both treatments, histone 3 was the most over-expressed in MPs contaminated with BaP while surfeit locus protein 4 was over-expressed in the gills exposed to virgin MPs. Regarding the ribosomal proteins, they were all downregulated in both treatments. In what concerns the three common proteins differentially expressed between virgin MPs versus controls and BaP MPs versus virgin MPs, coatomer subunit gamma-2 was more over-expressed in the gills exposed to virgin MPs, while the expression of cytoplasmic dynein 1 heavy chain 1 isoform X11 was similar between both cases and transmembrane emp24 domain-containing protein 9 isoform X2 was over expressed in the gills exposed to BaP MPs.

3.2.1. Proteins differentially expressed in S. plana gills exposed to virgin MPs

When clams were exposed to virgin MPs, twenty-six proteins (16 up-regulated and 10 down-regulated) were differentially expressed in relation to controls (Fig. 2). The exposure of the gills to virgin MPs altered the expression of proteins associated with endocytosis (clathrin heavy chain 1 isoform X3), cytoskeleton and cell structure (actin-related protein 2, coatomer subunit gamma-2, clathrin heavy chain), metabolism (propionyl-CoA carboxylase beta chain, glutamate dehydrogenase and tryptophan 5-hydroxylase 1), catabolic process (S-phase kinase-associated protein 1), proteasome (proteasome subunit alpha type-7), sulfur metabolism (bifunctional 3′-phosphoadenosine 5′-phosphosulfate synthase isoform X2, splicing factor, arginine/serine-rich 4), carbohydrate metabolism (leucine-rich repeat-containing protein 57 and glycogen phosphorylase, muscle form-like isoform X3), DNA binding (histone H3), ribosome structure (40 ribosomal proteins S3a and S26 and 60S ribosomal protein L8-like isoform

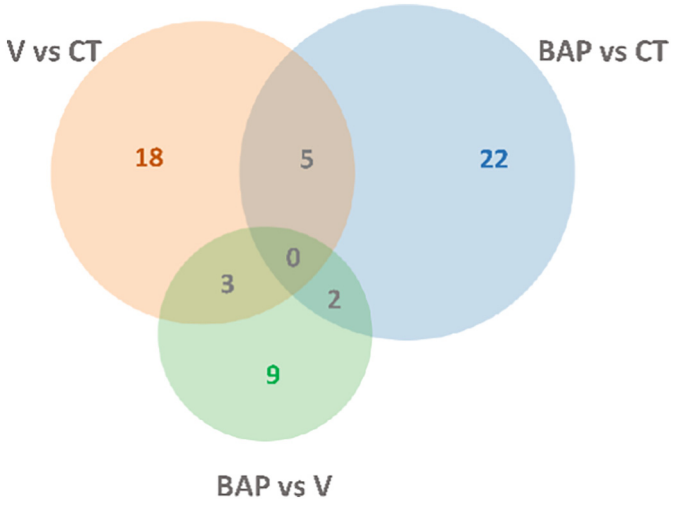

Fig. 1. Venn diagram showing the number of specific and common proteins between LDPE MPs (V) vs controls (CT), LDPE MPs contaminated with BaP (BAP) vs CT and BaP vs V in the gills *S. plana* after 14 days of exposure.

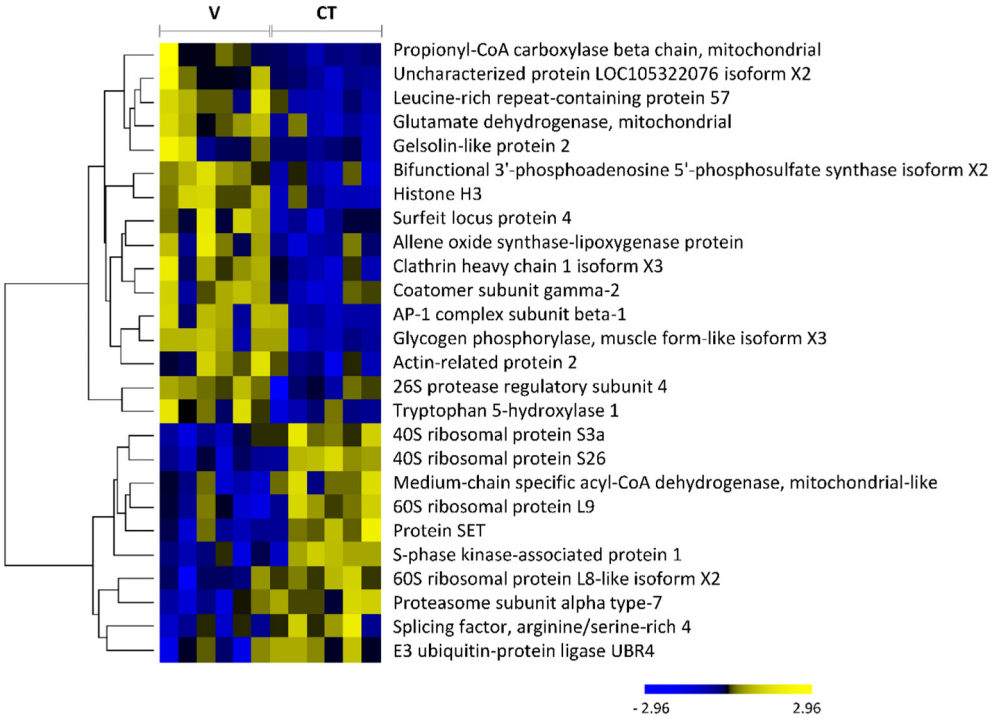

Fig. 2. Heat-map showing significant protein expression changes in the gills of *S. plana* after exposure to LDPE MPs (V) and controls (CT) for 14 days. All proteins identified as "Predicted" except 26S protease regulatory subunit 4 and Splicing factor, arginine/serine-rich 4.

X2), selenocompound metabolism (60S ribosomal protein L9), ATPase activity (26S protease regulatory subunit 4), endocrine and other factor-regulated calcium reabsorption (AP-1 complex subunit beta-1), fatty acid metabolism (medium-chain specific acyl-CoA dehydrogenase), calcium-binding (gelsolin-like protein 2), metal-binding (E3 ubiquitin-protein ligase UBR4 and allene oxide synthase-lipoxygenase protein), protein processing in endoplasmic reticulum (surfeit locus protein 4), apoptosis (protein SET) and uncharacterized protein LOC105322076 isoform X2. Within these, bifunctional 3′-phosphoadenosine 5′-phosphosulfate synthase isoform X2 was the most up-regulated while medium-chain specific acyl-CoA dehydrogenase was the most down-regulated (Fig. 2). GO counts for molecular function (MF), biological processes (BP) and cellular component (CC) for the proteins whose expression was altered between virgin MPs and controls are in Fig. 3. KEGG identified changes in the following pathways: selenocompound, purine, glyoxylate and dicarboxylate, glutathione, tryptophan, starch and sucrose, sulfur and thiamine metabolism, as well as monobactam, folate, and phenyl propanoid biosynthesis, whereby most of these processes are directly or indirectly related to oxidative stress.

3.2.2. Proteins differentially expressed in S. plana gills exposed to MPs contaminated with BaP

When clams were exposed to MPs with BaP adsorbed, 29 proteins were differentially expressed (12 up-regulated and 17 down-regulated) in the gills compared to controls (Fig. 4). MPs contaminated with BaP induced more and different protein changes when compared to those exposed to virgin. These changes were related to cytoskeleton and cell structure (actin, myosin heavy chain, and coiled-coil domain-containing protein 40), DNA binding (histone H3 and histone deacetylase 1-B), RNA binding (small nuclear ribonucleoprotein Sm D2), ribosome structure (40S ribosomal proteins SA, S3a and S26 and 60S ribosomal protein L8-like isoform X2), energy metabolism (glyceraldehyde-3-phosphate phosphorylation (G3PDH), isocitrate dehydrogenase (NADP), ATP synthase subunit beta and alpha-aminoadipic semialdehyde synthase), ATPase binding ubiquitination (UBX domain-containing protein1 and ubiquitin carboxyl-terminal hydrolase 7 isoform X4 protein) processing in endoplasmic reticulum (surfeit locus protein 4), sperm-associated antigen 6, apoptosis (programmed cell death 6 isoform X2 and guanine nucleotide-binding protein G(o) subunit alpha isoform X2), signal transduction (EF-hand domain-containing protein 1 and EF-hand domain-containing family member C2-like, dolichyl-diphosphooligosaccharide-protein glycosyltransferase subunit isoform X2), transmembrane proteins (transmembrane 9 superfamily member 1-like and transmembrane emp24 domain-containing protein 9 isoform X2), and three uncharacterized proteins (Supplementary Table 3).

GO counts for MF, BP and CC for the 29 proteins altered between MPs contaminated with BaP and controls is shown in Fig. 3. Changes in MF such as ATP binding, protein binding, structural constituent of ribosome, metal ion binding and protein heterodimerization activity were common to both treatments but more significant in clams exposed to MPs contaminated with BaP. In BP, only translation and oxidation-reduction process were similar to both treatments, but translation was more important in BaP contaminated MPs. Regarding CC, only changes in the integral component membrane, cytosolic small ribosomal unit, cytoplasm, nucleoplasm, and nucleus were common between treatments, but higher in the integral components, and cytosolic small ribosomal unit in BaP contaminated MPs while the nucleus was smaller.

KEGG analysis indicated that purine, glutathione, and thiamine metabolism pathways were common between treatments, but changes in the following pathways: glycolysis/glucogenesis, TCA cycle, purine, glutathione, carbon fixation and thiamine metabolism were specific to BaP contaminated MPs.

3.2.3. Differences in S. plana gills proteome between clams exposed to virgin and BaP contaminated MPs

Fourteen proteins were differently expressed between clam gills exposed to virgin MPs and MPs with BaP adsorbed (Fig. 5). Proteins related with cytoskeleton and cell structure (clathrin heavy chain 1 isoform X3, coatomer subunit gamma-2 and cytoplasmic dynein 1 heavy chain 1), metabolic pathway (TPH1), protein synthesis (elongation factor 2), cell growth (tyrosine-protein kinase), protein folding (heat shock 70 kDa protein 12B), and calcium-binding (plasma membrane calcium transport ATPase 2) were

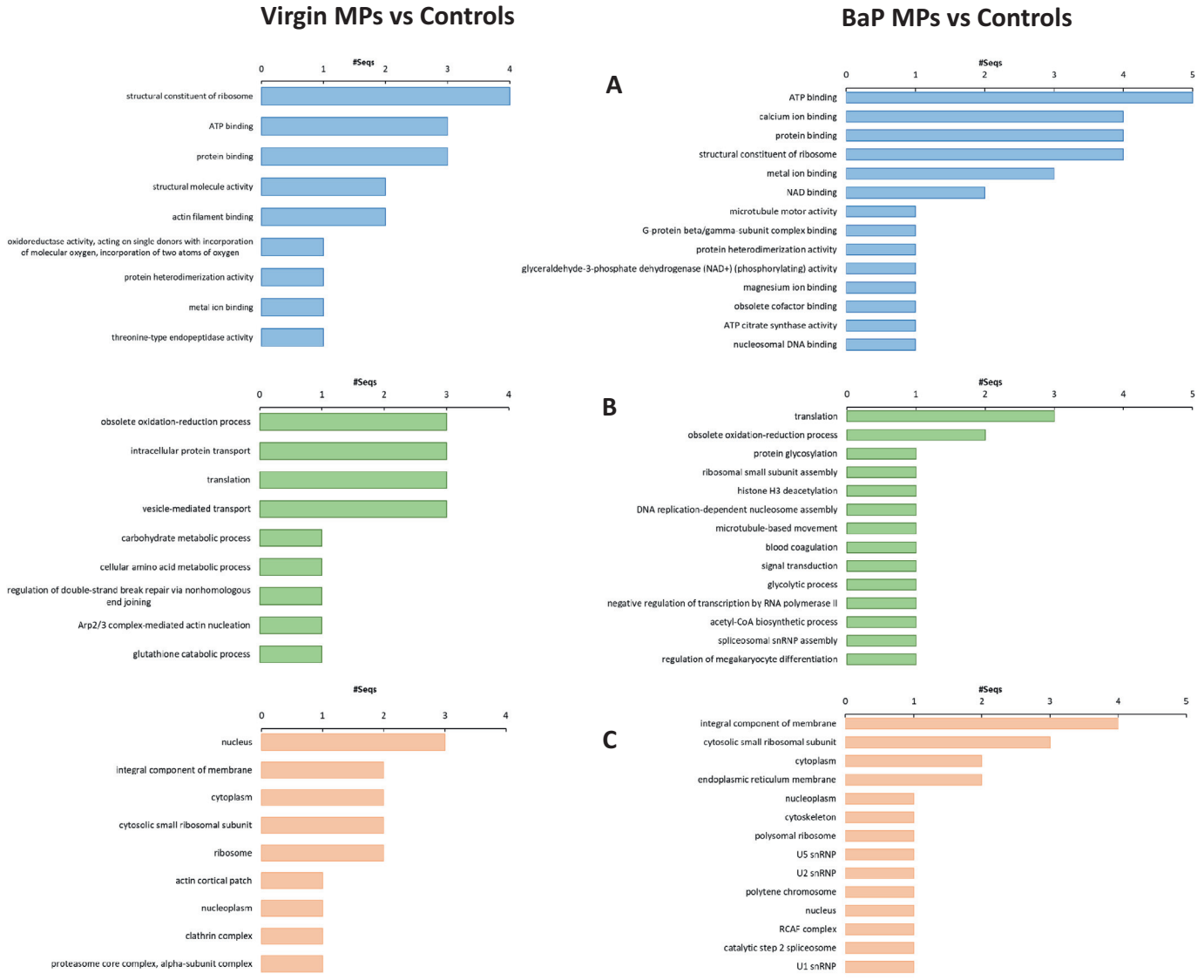

Fig. 3. Functional identification in *S. plana* gills identified by Blast2GO analysis of the Molecular Function (A), Biological processes (B) and Cellular Components of differential proteins expressed between MPs virgin and controls and between MPs with BaP adsorbed and controls.

over expressed in clam gills exposed to MPs while proteins related to intracellular metabolism (intraflagellar transport protein 74 homolog), energy metabolism (trifunctional enzyme subunit beta mitochondrial-like), ubiquitination (ubiquitin-like modifier-activating enzyme 1) were over expressed in clam contaminated with MPs and BaP.

4. Discussion

MPs in the marine environment became a global environmental issue due to the high amount of plastic that yearly reaches the ocean from different sources (Dalberg and Bigaud, 2019). Marine organisms, bivalves in particular, ingest MPs of several types, shapes and size (Avio et al., 2015; O'Donovan et al., 2018, 2020; González-Soto et al., 2019; Rivera-Hernández et al., 2019; Islam et al., 2021; Rodrigues et al., 2022). MPs size influence the ingestion and the sorption and desorption of chemicals to and from particles (González-Soto et al., 2019), and the smaller the particles, have a higher potential to be transported between tissues, (Ribeiro et al., 2017). *S. plana* is able to ingest the same type and similar shape of LPDE MPs within the size ranges 4–6 and 20–25 μm (Islam et al., 2021; Rodrigues et al., 2022) and although ingestion of MPs (11–13 μm) used in this experiment was not evaluated it is expected that ingestion also occurred. These microparticles act as a vector of other contaminants, such as PAHs present in the ocean at varying concentrations (Avio et al., 2015; O'Donovan et al., 2018, 2020). MPs contaminated with Hg and PAHs influence the ingestion and egestion pathways in *M. galloprovincialis* as well as the accumulation of BaP in *S. plana* (Oliveira et al., 2013; Avio et al., 2015; Barboza et al., 2018; O'Donovan et al., 2018). When *S. plana* was exposed to virgin MPs, BaP was not detected, but in those exposed to BaP adsorbed MPs, BaP was accumulated in whole soft tissues, indicating that MPs are acting as a vector of BaP accumulation. Although BaP was not measured in clam gills, Pittura et al. (2018) revealed that BaP was accumulated in *M. galloprovincialis* gills following an exposure to the same type and size of MPs adsorbed with the same BaP concentration, but BaP accumulation was lower when compared to the uptake from water or food (Paul-Pont et al., 2016; Pittura et al., 2018). Comparable results were obtained by Avio et al. (2015) with the same mussel species exposed to a size range of PS or PE MPs (1 to 100 μm) adsorbed with pyrene, another PAH compound. During the MPs ingestion process, contaminants can desorb from MPs (Bakir et al., 2014; Batel et al., 2018), and this desorption is size and residence time dependent (Engler, 2012; Lusher, 2015). These results show

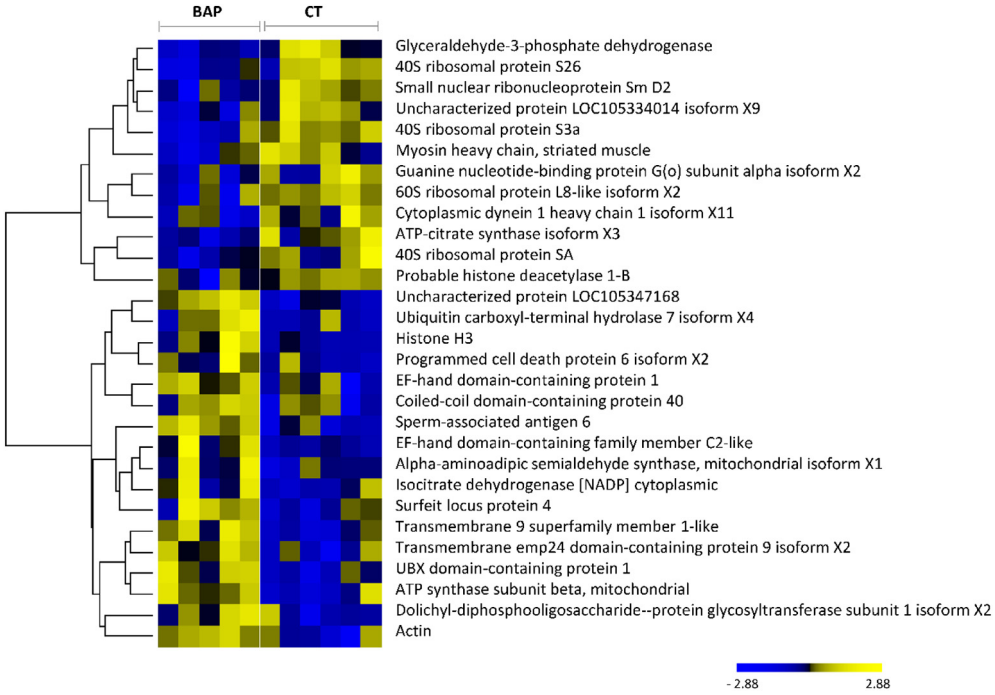

Fig. 4. Heat-map showing significant protein expression changes in the gills of *S. plana* after exposure to BaP contaminated LDPE MPs (BAP) and controls (CT) for 14 days. All proteins identified as "Predicted".

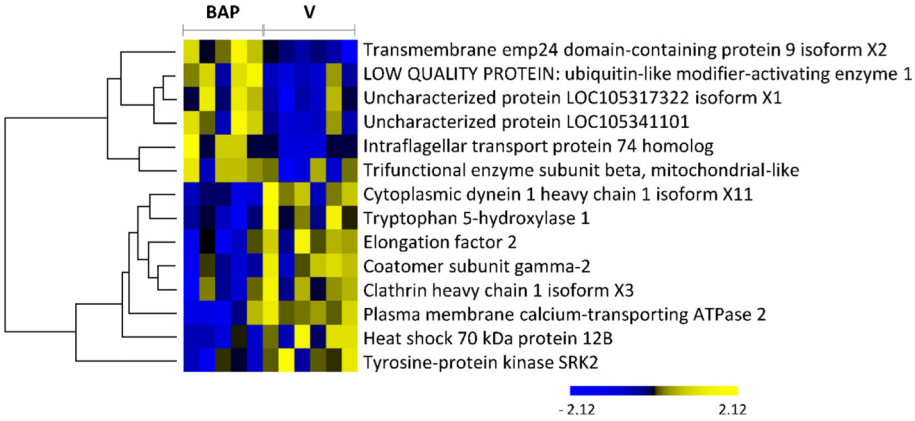

Fig. 5. Heat-map of protein changes between BaP contaminated (BAP) LDPE MPs and non-contaminated LDPE MPs after exposure of the gills of *S. plana* for 14 days. All proteins identified as "Predicted" except Heat shock 70 kDa protein 12B.

that clams were able to ingest MPs contaminated with BaP, and BaP desorption occurred, confirming that MPs act as a vector of BaP accumulation. However, the Trojan horse effect of MPs seems negligible (Koelmans et al., 2016) when compared to BaP accumulation from the natural environment. In fact, based on the data available about MPs concentrations in the ocean the bioaccumulation of BaP from microplastics seems lower in relation to the uptake from natural environmental contamination pathways (Koelmans et al., 2016). However, this might not occur in the future if MPs concentrations in the ocean continue to increase.

The exposure of *S. plana* to MPs either virgin or contaminated with BaP affect the proteome of the gills differently (Figs. 2, 4). SWATH-MS analysis allowed the detection of a large set of proteins (Supplementary Table 2), but its identification was biased for the nonexistence of this species genome. GO enrichment outlined different MF, BP and CC and KEGG pathways when comparing the effects of both stressors, some of which common to both types of MPs (structural constituent of the ribosome, ATP binding, protein binding and metal ion binding) (Fig. 3).

4.1. Effects of virgin MPs on S. plana gills proteome

After two weeks of exposure, virgin MPs induce changes in *S. plana* gills proteome affecting several metabolic pathways (Fig. 2). Virgin MPs enter the cells via endocytosis, a major mode of action for intracellular trafficking of nanoparticles and MPs (Bouallegui et al., 2018). Endocytosis involves clathrin assembly in the formation of clathrin-coated pits and the destruction of lipids, carbohydrates, and nucleic acids (Moore, 2006; Bouallegui et al., 2018). When clam gills were treated with virgin MPs, clathrin heavy chain 1 isoform X3 was over-expressed (Fig. 2). In *M. galloprovincialis* gills exposed to nanoparticles, oxidative status was prevented when clathrin-mediated endocytosis was blocked, indicating that clathrin is needed for endocytosis and is also involved in oxidative stress (Bouallegui et al., 2018). Involved in membrane morphogenesis and in cytoskeletal organization, this protein has a protective role against MPs toxicity. AP-1 complex subunit beta-1 mediates clathrin recruitment to membranes and plays a role in the late-Golgi/trans-Golgi network and/

or endosomes was also over-expressed, confirming the need for clathrin heavy chain 1 isoform X3 for the endocytosis process. This protein was also over-expressed in *D. polymorpha* exposed to PS MPs (1 and 10 μm) (Magni et al., 2018), indicating an effect induced by MPs ingestion independent of its polymer chemical type.

The structure of *S. plana* gills is characterized by ciliated filaments and the internalization of virgin MPs physically damage its structure (Browne et al., 2008) by inducing changes in two cytoskeletal and cell structure proteins: actin-related protein 2 (ATP-binding component of the Arp2/3 complex) and gelsolin-like protein 2, a calcium-binding protein that binds actin and regulates the assembly and disassembly of actin filaments activated by calcium-binding (Rocher et al., 2015). These two proteins were over-expressed indicating that MPs exposure induce structural and possibly osmotic changes in clam gills. Similar changes were detected in the haemolymph of *M. edulis* exposed to two types of MPs (HDPE and PLA) and in *D. polymorpha* exposed to PS MPs (Green et al., 2019; Magni et al., 2019), indicating an MPs effect. In *M. galloprovincialis* exposed to two sizes (0.5 and 4.5 μm) of PS microspheres, MPs were found between gill filaments but not within the epithelial cells of the gills (González-Soto et al., 2019).

Another protein with a structural activity that mediates protein transport was the coatomer subunit gamma-2, a protein essential for the retrograde Golgi-to-ER transport of dilysine-tagged proteins (Lee and Goldberg, 2010). Changes in this protein indicate oxidative stress and an inflammatory response that leads to an accumulation of unfolded/misfolded proteins in the ER. In mammals, the coatomer can only be recruited by membranes associated with ADP-ribosylation factors (Hsia and Hoelz, 2010) but this needs to be confirmed in bivalves.

Cytoskeleton proteins are the first target of oxidative stress (Gomes et al., 2013; Artigaud et al., 2014). The imbalance of the oxidative defense was detected in *S. plana* gills exposed to the same type and concentration of virgin MPs by inducing glutathione peroxidation activity and oxidative damage (O'Donovan et al., 2018), confirming that this type of MPs induces oxidative stress in the gills of *S. plana*. Oxidative stress is followed by DNA damage and changes in carbohydrate, lipids and protein structures (Alimba and Faggio, 2019). The protein most up-regulated in the gills proteome was histone H3. Involved in DNA interactions, this protein undergoes methylation, acetylation and phosphorylation (Letendre et al., 2011), indicating that exposure to these two types of MPs induces changes in DNA binding. This protein was also over-expressed in the gills of *D. polymorpha* exposed to a mixture of PS MPs indicating another MPs effect (Magni et al., 2019). The over-expression of two other histone proteins (histone H1 and histone H2A) was also detected in the haemolymph of *M. edulis* exposed to HDPE and PLA MPs (Green et al., 2019), and a high DNA fragmentation was observed in *M. galloprovincialis* exposed to PE MPs (Avio et al., 2015) indicating that changes in DNA binding might be a disturbing effect of MPs.

On the other hand, RNA-binding proteins, the main components of stress granules formed in the cytoplasm, are crucial for splicing regulation, mRNA transport, translation, and stress response (Alves and Goldenberg, 2016). Serine/arginine-rich splicing factor 4 an essential pre-RNA splicing factor related to sulfur metabolism (Sanford et al., 2005) was down-regulated, indicating that MPs could impair the immune function of the gills by altering RNA processing or by the formation of stress granules. Ribosomal proteins (40S ribosomal protein S3a, 40S ribosomal protein S26, 60S ribosomal protein L8-like isoform X2) that play a role in immune responses associated with stress, in pre-RNA synthesis, and spliceosome (Venier et al., 2003; Wu et al., 2020) were down-regulated after exposure to both stressors (virgin and BaP contaminated MPs) along with 60S ribosomal protein L9 (whose expression only changed due to virgin MPs), indicating that MPs clearly affect the immune system. Nevertheless, in the gills of *D. polymorpha* exposed to a mixture of PS MPs (4×10^6 MP/L) of different sizes (1 and 10 μm) for a shorter period of time (6 days), these proteins were up-regulated and related to granules formation (Magni et al., 2019). However, this was not the case indicating that this behavior might be related to the chemical composition of MPs or to a different mode of action of MPs.

The energy balance was also affected by MPs exposure. In fact, glycogen phosphorylase muscle form-like isoform X3, implicated in glycogen on carbohydrate metabolism, was up-regulated indicating an increase of energy requirement Similarly in mussels increase of metabolic enzymes involved in glycolysis were also upregulated in the haemolymph of *Mytilus* spp. exposed to HDPE, PLA and PS MPs, indicating an increase in energy to cope with MPs ingestion (Van Cauwenberghe et al., 2015; Paul-Pont et al., 2016; Green et al., 2019). Leucine-rich repeat-containing protein 57 involved in carbohydrate metabolism was highly induced suggesting an association with antioxidant and detoxification responses.

Proteasome subunit alpha type-7, a member of the proteasome β-type family and of the 20S proteasome core complex that depends on ATP-cycling, oxidative stress, immune response and apoptosis (Ji et al., 2013; Rocher et al., 2015; Liu et al., 2015) was down-regulated indicating a low catalysis efficiency and a decrease in fatty acids (Wu et al., 2013). Moreover, medium-chain specific acyl-CoA dehydrogenase, a mitochondria protein that catalyses fatty acid oxidation to the main respiratory chain via electron transfer flavoprotein-ubiquinone oxidoreductase (Chora et al., 2008), was also down-regulated indicating that MPs ingestion cause pseudo-satiety in marine organisms thus lowering fatty acids (Kühn et al., 2015). Alteration in fatty acids was also noted in *C. gigas* and *D. polymorpha* exposed to PS MPs (Sussarellu et al., 2016; Magni et al., 2019).

Three mitochondrial proteins (propionyl-CoA carboxylase beta chain, glutamate dehydrogenase and surfeit locus protein 4) related to fatty acid and metabolic pathways were over-expressed which is consistent with the high energy demand of gill tissues to engage in water movement and food transport along with MPs (Rocher et al., 2015). The over expression of surfeit locus protein 4 after exposure to both types of MPs was higher in the gills exposed to virgin MPs indicating that the ingestion of MPs induces inflammatory process that seems to be slightly counter acted when BaP is present.

Although binding directly to cytoskeleton proteins, MPs can indirectly affect Ca^{2+} homeostasis by binding proteins via –SH group such as Ca^{2+} ATPases (Gomes et al., 2013). In fact, the highest up-regulated protein was bifunctional 3′-phosphoadenosine 5′-phosphosulfate synthase isoform X2, a bifunctional enzyme with both ATP sulfurylase and APS kinase activity involved in sulfur metabolism, indicating that the exposure of MPs induces changes in the thiol components. MPs exposure also influences the over-expression of allene oxide synthase-lipoxygenase, a metal-binding protein related to oxidation-reduction and the hydro peroxidation of fatty acids. In plants and in soft corals the overexpression of this protein indicates a defense against tissue injury (Lõhelaid et al., 2008) which in this case might be linked to the physical injury inflicted in clam gills as a result of MPs ingestion.

S-phase kinase-associated protein 1, involved in catabolic processes that mediate ubiquitination, was down-regulated. Ubiquitination requires the sequential action of three enzymes: ubiquitin first activated by E1, then transferred to E2 ubiquitin-conjugating enzyme and finally, an E3 ubiquitin-ligase (UBR4) attaches the ubiquitin moiety to the substrate (Rocher et al., 2015). UBR4, a zinc-binding protein that forms meshwork structures involved in membrane morphogenesis and cytoskeletal organization, together with clathrin, mediates ubiquitination of ATP-citrate lyase was down-regulated, leading to its subsequent degradation. Green et al. (2019) also detected an over expression of a metal-binding protein in mussels haemolymph exposed to fragments of HDPE 102.6 μm (range 0.48–316 μm) and PLA MPs 65.6 μm (range 0.6–363 μm). Conversely, 26S protease regulatory subunit 4 involved in the ATP-dependent degradation by ubiquitin-proteasome pathway (Ferrell et al., 2000; Glickman and Ciechanover, 2002) was over-expressed, indicating that virgin MPs besides inducing DNA damage also induce apoptosis in clam gill cells. In fact, protein SET involved in apoptosis, transcription, nucleosome assembly and histone chaperoning, was down-regulated (Rocher et al., 2015), confirming the presence of components of the apoptotic pathways in clam gills due to MPs exposure.

The exposure of *S. plana* gills to MPs also induces neurotransmission effects. Tryptophan 5-hydroxylase 1 (TPH1) a rate-limiting enzyme that requires Fe^{2+} in the synthesis of the neurotransmitter serotonin and a precursor of melatonin (Aiello et al., 2017) was up-regulated in gills exposed to both stressors indicating that these two stressors induce neurotransmitter effects. An increase of the neurotransmitter dopamine was also observed in the haemolymph of *D. polymorpha* exposed to PS MPs (1 and 10 μm), indicating that neurotransmitter effects intervene in the elimination of MPs by increasing the cilia movement in the gills (Magni et al., 2018). An increase in TPH1 was also identified in the brain of Wistar rats during the neonatal period as a result of malnutrition (Manjarrez-Gutiérrez et al., 2012). Neurotoxic effects were also detected in *S. plana* gills exposed to the same MPs (O'Donovan et al., 2018). One of the effects of MPs ingestion is food scarcity (Browne et al., 2008), so the induction of neurotoxic effects might be related to the elimination of MPs but further research is needed to clarify if this neurotransmission effect is related to egestion.

In conclusion, virgin MPs exposure induce changes in cytoskeleton and cell structure, oxidative stress, energy and fatty acid metabolisms, immune response, DNA binding, neurotoxicity, and possibly apoptosis highlighting the potential risk for clam health and survival when exposed to this size and shape of PE MPs.

4.2. Effects BaP contaminated MPs on S. plana gills proteome

SWATH analysis outlined that the proteome of *S. plana* gills exposed to MPs with BaP adsorbed shows more and diverse proteins differentially expressed when compared to controls or virgin MPs (Fig. 5). The two stressors combined induce changes in several pathways, namely: glycolysis/glucogenesis, TCA cycle, purine, glutathione and thiamine metabolism, and carbon fixation (Fig. 3). Changes in purine, glutathione and thiamine metabolism pathways were common between both treatments (virgin and BaP MPs).

Three cytoskeleton and cell structure proteins changed as a result of MPs contaminated with BaP but were different from the ones that changed when clams were exposed to virgin MPs. Actin, a redox-sensitive ubiquitous cytoskeleton protein (involved in microfilaments formation), with a role in intracellular trafficking, endocytosis, and a major target for glutathionylation and carbonylation under oxidative stress (McDonagh and Sheehan, 2007; Bouallegui et al., 2018) was over-expressed. In *M. galloprovincialis* and *P. martensii* gills exposed only to BaP, actin was downregulated, indicating that BaP exposure compromise cell structure, induce cellular injury, damage the cytoskeleton, and apoptosis (Maria et al., 2013; Chen et al., 2018). However, actin was over-expressed in *P. viridis* gills exposed to a mixture of BaP and 2,4 -DDT indicating that DDT played a central role in the cytoskeleton function (Song et al., 2016). In the present case, actin seems to play a central role in maintaining cell shape and in counteracting the cytoskeleton disturbances originated by BaP alone (Maria et al., 2013; Chen et al., 2016), indicating an antagonistic effect between MPs virgin or adsorbed with BaP. Oxidative modification of actin alters microfilaments structures and interacts with myosin on muscular contraction releasing Ca^{2+} ions bound to myosin (light or heavy chains) producing sliding effects (Dominguez and Holmes, 2011). Myosin heavy chain was downregulated indicating the need for a higher muscular contraction due to the exposure of BaP adsorbed to MPs. Coiled-coil domain-containing protein 40, a cytoplasmic cilia protein required for assembly of dynein regulatory complex and responsible for ciliary beat regulation (Becker-Heck et al., 2011), was up-regulated indicating that the exposure to these combined stressors induces stress in cilia motility. Another down-regulated cytoskeleton protein was cytoplasmic dynein 1 heavy chain 1 isoform X11 of the dynein heavy chain family that acts as a motor for the intracellular retrograde motility of vesicles and organelles along microtubules (Nie et al., 2020). Changes in these proteins seem to be related to maintenance of the cytoskeleton function.

MPs adsorbed with BaP affect energy metabolism through glycolysis and oxidative phosphorylation. Two proteins involved in the glycolysis/glucogenesis pathway were over expressed. Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) that modulates the organization and assembly of the cytoskeleton is involved in glucose degradation and energy yield, participates in RNA transport, DNA replication, and apoptosis (Romero-Ruiz et al., 2006) was downregulated, while isocitrate dehydrogenase NADP, a cytoplasmic protein that acts in the Krebs cycle and catalyses reversible oxidative/reduction reactions of NADP+ to NADPH was up-regulated. G3DPH was overexpressed in *D. polymorpha* exposed to PS MPs (10 μm) (Magni et al., 2019), in the haemolymph of *M. edulis* after a long-time exposure to PS and PLA MPs (Green et al., 2019) and in *S. plana* inhabiting high metal-contaminated sites (Romero-Ruiz et al., 2006). The down-regulation of G3DPH indicate that glucose metabolism changes the pentose phosphate pathway, enabling the cell to generate NADPH as a response to oxidative stress (Bernard et al., 2011; Duroudier et al., 2019). Similarly, in *Mytilus* spp. exposed to PS MPs contaminated with fluoranthene, P-glycoprotein was also down-regulated (Paul-Pont et al., 2016), indicating that the under-expression of G3PDH is related to oxidative stress and the overexpression of isocitrate dehydrogenase [NADP] seems to compensate the energy reduction that results from G3DPH inhibition. Conversely, this protein was inhibited in the gills of zebra mussels exposed to PS MPs (Magni et al., 2019), indicating that this might be a result of the different chemical composition of the MPs or a cumulative effect of MPs and BaP exposure that need further research.

Mitochondria proteins were also significantly altered in clam gills. ATP synthase isoform X3, responsible for the supply of energy to the cells through the production of ATP and a key regulator in the Krebs cycle (Nelson and Cox, 2004; Maria et al., 2013; Qiu et al., 2016) was over-expressed in *S. plana* gills. This overproduction suggests that the flux through the Krebs cycle is reduced, indicating a mitochondrial dysfunction in the gills. A similar effect was detected in *M. galloprovincialis* exposed to BaP only (Maria et al., 2013), suggesting that there is a need for additional energy to cope with MPs and BaP ingestion. Another over-expressed mitochondrial protein also was alpha-aminoadipic semialdehyde synthase, a bifunctional enzyme that catalyses lysine degradation (Yang et al., 2015), but the function of this protein in bivalves needs to be confirmed.

As mentioned, histone 3 was over-expressed after exposure to both types of MPs, but the induction was lower in BaP contaminated MPs. Histone variants are highly similar and in mussels exposed to BaP only undergo methylation, acetylation and phosphorylation that change their interaction with DNA and proteins (Letendre et al., 2011). In the present case, histone deacetylase 1-B responsible for the deacetylation of lysine residues on the N-terminal of histone 3 was down-regulated. The disruption of DNA methylation in cancerous cells related to BaP exposure (Sadikovic and Rodenhiser, 2006), indicate that could be linked to carcinogenesis which is a known effect of BaP. Therefore, the expression of the two histones due to MPs contaminated with BaP needs further clarification.

The exposure of clam gills to both stressors also induces changes in proteins involved in RNA translation and protein synthesis. Small-nuclear ribonucleoprotein Sm D2, with an important role in splicing cellular pre-mRNAs and in the formation of the core snRNP was down-regulated, indicating that protein was impaired by exposure to MPs absorbed with BaP. In *D. polymorpha*, this protein was also down-regulated in BaP contaminated PS MPs (Magni et al., 2019), suggesting a specific BaP effect. Like for clams exposed to virgin MPs, BaP contaminated MPs lower the expression of the same ribosomal proteins but also of 40S ribosomal protein, indicating that these proteins are related to the formation of stress granules. 40S ribosomal protein SA was also down-regulated in the gills of *M. edulis* and the digestive gland of *P martensii* exposed to BaP only (Letendre et al., 2011; Chen et al., 2016) suggesting that MPs alone or contaminated with BaP have a strong effect on the ribosomes and in the formation of stress granules.

Two transmembrane proteins (transmembrane 9 superfamily member C2 like and transmembrane emp24 domain-containing protein 9 isoform X2) involved in the secretory pathway, and in intracellular membranes such as the ER-Golgi intermediate compartment and the Golgi apparatus were over-expressed. The over-expretion of two other proteins from the ubiquitin family (UBX domain-containing protein 1 and ubiquitin

carboxyl-terminal hydrolase 7 isoform X4) that modulates the immune response by inhibiting the TNFα-triggered NF-kB activity (Rezvani, 2016), suggests that the exposure to these two stressors inhibit NF-kB pathway that protects against the accumulation of damaged proteins. On the other hand, sperm-associated antigen 6, which mediates protein-protein interactions, was up-regulated suggesting a role in cell proliferation and division. In an embryonic mouse, this protein functions in neuronal differentiation and proliferation (Hu et al., 2016), but the function in *S. plana* needs to be identified.

The capacity of MPs adsorbed with BaP to induce oxidative stress and genotoxicity, disrupt the cytoskeleton, combined with Ca^{2+} ions fluctuations suggest that these two stressors induce apoptosis (Tian et al., 2021). EF-hand domain-containing protein 1 and EF-domain-containing family member C2-like and dolichyl-diphosphooligosaccharide-protein glycotransferase subunit 1 isoform X2 involved in signal transduction and in the apoptosis pathway were over-expressed. EF-hand domain-containing protein D1 acts as a calcium sensor for mitochondrial flash activation, characterized by superoxide production (Chin and Means, 2000), and plays a role in neuronal differentiation, indicating that MPs with adsorbed BaP damage Ca^{2+} homeostasis and stimulate programmed cell death. Superoxide dismutase activity increased in *S. plana* gills exposed to MPs with BaP adsorbed dismutating the superoxide anions produced due to this exposure (O'Donovan et al., 2018). In *P. martensii* gills exposed to BaP only, calcium-binding proteins like calmodulin and calumenin were down-regulated (Chen et al., 2018). Dolichyl-diphosphooligosaccharide-protein glycotransferase subunit 1 isoform X2 (DAD1), is a defender against cell death (Qiu et al., 2016), indicating that the ingestion of MPs contaminated with BaP elicits in clam gills a late response in the apoptotic pathway. In fact, programmed cell death protein 6 isoform X2, a marker of apoptosis (Cecconi et al., 1998), and guanine nucleotide-binding protein G(o) subunit alpha isoform X2 involved in cell signaling and apoptosis (Grassi et al., 2019) were over-expressed. Programmed cell death protein 6 isoform X2 was also over-expressed in female *P. viridis* exposed to only BaP (Qiu et al., 2016), indicating that this might be a specific BaP effect rather than a result of MPs ingestion. A mechanism underlying apoptosis was already postulated for BaP toxicity in the gills of *M. galloprovincialis* (Maria et al., 2013) that might also be a marker for MPs contaminated with BaP. Guanine nucleotide-binding protein G(o) subunit alpha isoform X plays a vital role in ATP-binding, contributing to the intracellular concentration of sugar and cAMP and to the release of cytoplasmic Ca^{2+} ions from ER (Herroeder et al., 2009) was down-regulated in *S. plana* gills exposed to BaP contaminated MPs but up-regulated in gills and down-regulated in the digestive gland of *P martensii* exposed to only BaP (Chen et al., 2016, 2018). This suggests a possible alteration of cellular signal transduction once MPs are internalized and an impact in the clam immune system implying an antagonistic effect between MPs and BaP.

In conclusion, the exposure of *S. plana* exposed to MPs contaminated with BaP besides changes in cytoskeleton and cell structure, oxidative stress, energy metabolism and DNA binding similar to MPs exposure also induce changes in glucose metabolism, RNA binding and apoptosis.

4.3. Comparison of the clam gills proteome exposed to virgin and to BaP contaminated MPs

The comparison of protein changes between MPs virgin and contaminated with BaP indicated that fourteen proteins were differently expressed (eight over-expressed and six inhibited in clam gills exposed to virgin MPs compared with those contaminated with BaP) between treatments (Fig. 5). Changes in cytoskeleton and cell structure proteins namely clathrin heavy chain 1 isoform X3, coatomer subunit gamma-2 related to membrane morphogenesis and cytoskeleton organization and cytoplasmic dynein 1 heavy chain 1, indicate inflammatory effects and oxidative stress induced by MPs ingestion that was counteracted in the presence of BaP. In fact, the accumulation of BaP is known to compromise the structure of the gill tissue (Maria et al., 2013). On the other hand Intraflagellar transport protein 74, a cytoskeleton protein from the intraflagellar 74 family that mediates transport of tubulin within the cilium required for ciliogenesis (Bhogaraju et al., 2015), was inhibited, indicating that the exposure to virgin MPs affects more the cytoskeleton than when exposed with BaP adsorbed.

TPH1 was also over-expressed in clams exposed to virgin MPs, indicating neurotransmission effects related to food satiety, while in clams exposed to both stressors neurotoxicity occurs. Maria and Bebianno (2011) also found that when mussels gills were exposed to BaP alone, BaP induced strong neurotoxic effects which is this case might be directly related to BaP contamination.

Elongation factor 2, another protein over-expressed in clam gills after exposure to virgin MPs, that catalyses the coordinated movement of mRNA, the conformational change in the ribosome is involved in the ubiquitination of proteins (Rocher et al., 2015) and is very sensitive to oxidative stress. This protein seems more affected by the physical stress induced by the ingestion of MPs, while when BaP is present an antagonistic effect seems to occur. Conversely, ubiquitin-like modifier-activating enzyme 1 that catalyses the first step in ubiquitin conjugation to mark cellular proteins for degradation through the ubiquitin-proteasome system was over expressed in BaP contaminated MPs. Tyrosine-protein kinase involved in cell growth was also over expressed in clam gills exposed to MPs only and in *C. gigas* gills as a response to ocean acidification (Timmins-Schiffman et al., 2014). This might indicate a shift away from repairing DNA damage by increasing oxidative stress. Conversely, the oxidative stress induced after exposure to MPs contaminated with BaP decreases the metabolic capacity of the gills either by disturbing the normal folding or by causing indirect damage suggesting an increase of apoptosis or independent caspase cell death as previously described.

The over-expression of HSP70, a stress-protein essential for protein folding/unfolding or trafficking, regulating proteolysis in cell cycle and signaling pathways (Fabbri et al., 2008; Bouallegui et al., 2018) confers protection against stress and apoptosis by inhibiting caspase activation (Parcellier et al., 2003). A down-regulation of HSP expression was detected in the pearl oyster *P. martensii* exposed to BaP alone (10 $\mu g\ L^{-1}$) (Chen et al., 2016), suggesting that the joint exposure of MPs contaminated with BaP represent a higher challenge for clam gills that initially was directed to activate the mechanisms of defense towards the physical stress provoked by MPs, while later, chemical stress assumed the major role in the disturbance of this protein.

Membrane proteins play a critical role in several cellular functions, including transport, cell-cell communication, and signaling. Plasma membranes are the first barrier against the outside environment and a response to external aggression and act as transport channels for ions and food (Matta et al., 2015). Plasma membrane calcium-transporting ATPase, a magnesium-dependent enzyme that catalyses the hydrolysis of ATP coupled with the transport of calcium out of the cell (Kirsikka Sillanpää et al., 2018) was down regulated in clams exposed to MPs contaminated with BaP. As calcium ion control is critical, this inhibition seems to impair critical cellular processes controlled by calcium. Transmembrane emp24 domain containing protein 9 isoform X2 was highly expressed in BaP contaminated with MPs indicating an increase in fatty acid oxidation needed for energy production on the ingestion of MPs contaminated with BaP. Trifunctional enzyme subunit beta, a mitochondrial enzyme that catalyses the β-oxidation of long-chain fatty acids (Xia et al., 2019) was also down regulated in clams exposed to MPs contaminated with BaP.

Therefore, combined effects of stressors and MPs is an environmental problem that needs further research to assess the antagonistic and synergistic effects of their implications towards the health of the marine organisms.

5. Conclusions

In this study a proteomic approach was applied to investigate the toxicological effects of spherical PE MPs of size range 11–13 μm virgin and adsorbed with BaP. Proteomic responses suggest that virgin MPs ingestion alter cytoskeleton and cell structure, energy metabolism, conformational changes, oxidative stress, fatty acids, DNA binding, neurotransmission that highlight the potential risk of this type of MPs for clam health and

survival. Conversely, when clam gills were exposed to MPs adsorbed with BaP there was a differentiation in protein expression that besides inducing changes in cytoskeleton and cell structure, oxidative stress, energy metabolism and DNA binding also induce changes in glucose metabolism, RNA binding, neurotoxicity, and apoptosis. Therefore, despite the changes that occur when MPs are ingested, the cumulative presence of MPs and BaP suggest that the toxicological risk of the combined effects of MPs and BaP might be higher to the health of the clams. As BaP concentrations increase at higher levels of the food chain, other experiments should be conducted at higher biological levels to assess the adsorption/desorption effects of MPs and BaP at the proteome level.

CRediT authorship contribution statement

M.J. Bebianno – conceptualization, data interpretation and paper writing.

Vera M. Mendes and Bruno Manadas – SWATH analysis and protein identification.

Sarit O'Donnavan – responsible for the experiment and biomarker analysis.

Camila, C. Carteny – BaP analysis in biological tissues.

Stephen Keiter − preparation of microplastics with BaP adsorbed.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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