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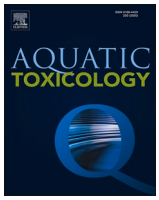

Short-term exposure to polystyrene microplastics hampers the cellular function of gills in the Mediterranean mussel *Mytilus galloprovincialis*

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A R T I C L E I N F O

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A B S T R A C T

Plastic is undoubtedly the most useful and versatile polymeric material that man has developed in the last two centuries Despite the societal benefits, plastic is now a serious global issue because it is persistent and may bioaccumulate into aquatic biota as microplastics (MPs). This study was designed to evaluate the daily uptake and cellular effects due to a short-term (up to 72 h) exposure to 3 μm red polystyrene MPs (50 beads/mL) in the gills of the Mediterranean mussel *Mytilus galloprovincialis*, chosen as model species for its ecological and commercial relevance. After measuring the daily uptake of MPs and detecting their presence within the branchial epithelium at all the exposure time-points (T24, T48, T72), some cleaning mechanisms were observed by neutral and acid mucous secretions at mussel gills. The protonic Nuclear Magnetic Resonance (^{1}H NMR)-based metabolomics, combined with chemometrics, allowed to comprehensively explore the time-dependent metabolic disorders triggered by MPs in mussel gills over the short-term trial. Specifically, the clear clustering between MP-treated mussel gills and those from control, together with the grouping for experimental time-points as depicted by the Principal Component Analysis (PCA), were due to changes in the amino acids and energy metabolism, disturbances in the osmoregulatory processes, as well as in the cholinergic neurotransmission. Moreover, as evidenced by enzymatic assays, even the oxidative defense systems and lipid metabolism were hampered by MP exposure. Overall, these findings provides the first insights into the early time-dependent mechanisms of toxicity of polystyrene MPs in marine mussels, and underline the potential environment and human health risk posed by MPs contamination.

1. Introduction

Plastic is undoubtedly the most useful and versatile polymeric material that man has developed in the last two centuries (Andrady and Neal, 2009). Its properties adaptable to various and different fields of use (packaging, textile, electrical, construction, cosmetics, chemical products) (Geyer et al., 2017; Schmid et al., 2021) make it common and widespread, with about 367 Mt of plastics produced in the world (PlasticsEurope, 2021). Unfortunately, today, also as a consequence of the spasmodic use of disposable materials due to the needs during the SARS-CoV-2 pandemic (Chen et al., 2021; Tagorti and Kaya, 2022), the excess and speed of production of plastics are not balanced by rapid disposal and adequate recycling activities. Therefore, this results in huge amounts of plastic waste accumulating in the environment since they are scarcely managed and little retained by the current wastewater treatment systems (Hahladakis, 2020; Uddin et al., 2020; Corsi et al., 2021; Xu et al., 2021; Kibria et al., 2023).

Plastic under the anthropogenic pressure is facilitated to being illegitimately introduced into the aquatic ecosystem, where it is subjected to a depolymerization process into smaller fragments, referred to as

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microplastics (MPs) as they reach the size < 5 mm. These small plastic polymers are distributed in a ubiquitous way, and can easily being in contact with the marine biota (H. Choi et al., 2022; Chen et al., 2021; Rice et al., 2021; Solomando et al., 2022; Krikech et al., 2023). Therefore, concerns are raised on the damage that organisms can undergo due to MPs uptake or ingestion (Abouda et al., 2022; De Marco et al., 2022a; Ferrari et al., 2022; Missawi et al., 2022; Romdhani et al., 2022; Elisoet al., 2020, M.C. 2023), which would imply repercussions along the food chain with negative consequences in terms of biodiversity, besides for the environment and human health (Della Torre et al., 2020; Zhang et al., 2021).

To date, cytotoxicological effects have been ascertained and associated with the size of plastic polymers since by this depends the ability of MPs to be internalized, pass across cell membranes and translocate to different body sites, triggering specific cellular mechanisms (Alimba et al., 2021). In our previous study, the 3 day-exposure of mussel *Mytilus galloprovincialis* to 3 μm polystyrene MPs revealed that MPs were quickly taken up and accumulated in mussel digestive tubules already after 24 h of exposure, to then exhibit a drastic reduction in the number of MPs after 48 h of exposure likely due to the activation of cytoprotective mechanisms for the detoxification and/or elimination of MPs at hepatic level, that were not counterbalanced by the high and constant amount of MPs reaching out the digestive gland over time, thus resulting in a further increase in the number of MPs bioaccumulated in mussels after 72 h of exposure (Cappello et al., 2021). As expected, this resulted in a time-dependent metabolic disorder at level of the mussel digestive gland over the short-term (3 days) exposure to polystyrene MPs, that may be summarized with alterations in amino acid and energy metabolism, osmoregulatory processes, and onset of oxidative stress (Cappello et al., 2021).

With the aim to gain a more comprehensive understanding of the impact of MPs on aquatic organisms, and to extend our previous findings on the digestive glands of mussels (Cappello et al., 2021), this study was designed to investigate the uptake of MPs and early responses occurring in the same individuals at level of the branchial tissue, being the first organ to suffer from pollutant toxic effects due to their large surface directly and constantly exposed to seawater and its substances, harmful or not (de Oliveira David et al., 2008). Indeed, the gills of marine bivalves play a key role in nutrient uptake, osmoregulation and gas exchange, and being mussels indiscriminate filter-feeders they may capture and ingest by gills anything of proper size (Van Cauwenberghe et al., 2015; Cappello et al., 2018). This implies that any response to environmental contaminants may be easily observed through investigation of neutral and acidic mucous secretions on the gill epithelium as a biomarker of effect for an overall assessment of the general health status of animals (Fasulo et al., 2012; Freitas et al., 2022). In order to carry out in-depth investigations to unravel any potential change in specific metabolic pathways upon exposure to pollutants, in the last few decades the proton Nuclear Magnetic Resonance (^{1}H NMR) metabolomics has been implemented with success in the field of environmental research and aquatic ecotoxicology (Cappello, 2020) since it allows to identify low molecular weight compounds (< 1.5 kDa) to be used as metabolic biomarkers, thus providing an overview of the metabolic status of a biological system (Cappello et al., 2020; Fujita et al., 2021; Hani et al., 2021; Zitouni et al., 2022; De Marco et al., 2022b, G. 2023). Moreover, as it is nowadays established that MPs are capable of inducing oxidative stress, lipid oxidative damage, and neurotoxicity in aquatic organisms (Barboza et al., 2020; Li et al., 2022), it is of primary importance the assessment of these cellular pathways and their time-dependent modulation following exposure to MPs. Overall, the relevance of this study is further increased by the choice of marine mussel *M. galloprovincialis* as model organisms, as this bivalve species occupies a privileged place into the food chain, especially within the Mediterranean diet (Walkinshaw et al., 2020), thus reflecting potential concerns for the environment and human health.

2. Material and methods

2.1. Mussel acclimation and experimental design

Adult mussels *Mytilus galloprovincialis*, with an average length of the shells of 6.6 ± 0.4 cm, were acquired from the aquaculture farm S.A.Co. M. in Messina, southern Italy. The Mediterranean mussels were then placed in laboratory glass aquaria containing filtered artificial seawater (ASW) at a salinity of 35 PSU (Practical Salinity Unit). From the beginning and during the two weeks of acclimation, the specimens were kept under continuous aeration, at a constant temperature of 18 ± 1 °C, and pH 7.8. The aquaria were monitored daily, assuring a controlled photoperiod cycle of 12 h light: 12 h dark, during which the renewal of the ASW took place daily, combined with the administration of a food solution of a mixture of algae, commercially obtained (Liquizell, Hobby) and provided according to the manufacturer's instructions (Cappello et al., 2021).

Following the acclimatization period, a total of 72 animals were randomly selected and placed, in groups of 12 specimens, in each aquarium containing 10 L of filtered ASW. As described in detail in our previous work (Cappello et al., 2021), the experimental plan, conducted in triplicates, consisted in the exposure of mussels to 50 beads/mL of red polystyrene MPs with a diameter of 3 μm (Sigma-Aldrich, Italy), and to ASW with no MPs for the control group (CTRL), for three days (72 h) and daily collection of samples (T24, T48, T72). The rationale for the dose of MPs herein tested was to approach realistic environmental concentrations of MPs in laboratory exposures (Vroom et al., 2017), in order to mimic an environmental scenario. No mortality was recorded during the experimental exposures.

The temperature and pH conditions were kept constant during the exposure, with daily ASW changes, administration of feed mixture and MPs re-dosed. No mortality was recorded during the entire duration of the experimental exposure. The collection of 9 mussels per each experimental condition took place at the beginning of the exposure (T0), after 24 h (T24) and 48 h (T48), and at the end of the exposure after 72 h (T72). The gills were then excised, quickly frozen in liquid nitrogen, and stored at −80 °C prior to processing for MP accumulation, metabolomics and biochemical analyses. For histological and histochemical analysis, a small piece of each gill tissue was immediately immersed in paraformaldehyde (PFA 4 %) fixing solution to preserve the tissue.

2.2. Characterization of polystyrene MPs and gill tissue accumulation

The red coloured and spherical shaped polystyrene MPs with a diameter of 3 μm were acquired from Sigma-Aldrich (Italy). The size of the plastic beads was confirmed by optical microscopy (Phase - Contrast Nikon Eclipse E400) and no aggregation of MPs was observed in ASW, as we previously reported (Cappello et al., 2021). According to the product specifications, the MPs had a density of 1.05 g/cm^3 and no plasticizers were added during their production process.

Using the same optical microscope, it was ascertained that aggregation of the single MPs did not occur even among the gill lamellae of the specimens treated. A quantitative analysis to assess the uptake of MPs into gill tissues was performed under the light microscope to ph2 of fresh samples at 20x. Subsequently, the data were expressed as number of MPs/mg of mussel gills.

2.3. Histological analysis

To observe the presence of MPs into mussel gills, PFA 4 % in 0.1 M phosphate buffered solution (PBS; pH 7.4) was used for 4 h at 4 °C to fix mussel gill samples ($n = 9$ per experimental condition). After this step, a series of increasing ethanol concentrations were used for dehydration, followed by buthanol clarification, and embedding in Paraplast (Bio-Optica, Milano, Italy). Sections of 5 μm thickness were then obtained from paraffin-embedded gills using a rotary automatic microtome (Leica

Microsystems, Wetzlar, Germany). After rehydration in decreasing series of ethanol, five randomly selected fields in each slide were observed using a 63x oil immersion objective under an Axio Imager Z1 microscope (Carl Zeiss AG, Werk Göttingen, Germany) to examine the presence of MPs within the mussel branchial epithelium. The AxioCam digital camera (Zeiss, Jena, Germany) associated to the microscope allowed the acquisition of the images (Maisano et al., 2017).

2.4. Histochemical analysis

To assess the presence of neutral and acid mucous secretions on branchial epithelium of mussels, a combined method based on Periodic Acid Schiff (PAS) staining for polysaccharides and on Alcian Blue (AB; pH 2.5) staining for acid mucopolysaccharides was applied on histological sections of gills (Cappello et al., 2019). Five fields (size of 250 μm each one, selected as field of view using the objective 10x) of one section per sample were therefore examined under a 40x oil-immersion objective using a motorized Zeiss Axio Imager Z1 microscope (Carl Zeiss AG, Werk Göttingen, Germany) equipped with an AxioCam digital camera (Zeiss, Jena, Germany).

2.5. ^{1}H NMR-based metabolomics analysis

2.5.1. Gill tissue metabolite extraction

The extraction of cellular metabolites was performed according to the protocol reported in detail in Cappello et al. (2018), employing the "two-phase" methanol/chloroform/water method to obtain only the polar metabolites from each mussel gill sample. In brief, 150 mg of each branchial tissue were taken and homogenized in a ice-cold solution of methanol : distilled water in the ratio 4 : 0.85 mL/g), with a stainless steel bead (3.2 mm size) for each tube to be used in a TissueLyser LT bead mill (Qiagen) working at 50 vibrations/s for 10 min. Once entirely homogenized, gill tissues were placed into glass tubes with addition of 4 mL/g of chloroform and 2 mL/g of distilled water, to be vortexed and then maintained into ice for 10 min to obtain an initial partitioning of the polar and non-polar cellular components. A centrifugation phase was then followed, executed at 4 °C at 2000 g for 5 min, resulting in a three clear phase partition from which about 600 μL of the methanol supernatant containing the polar metabolites were collected to be dried by centrifuge in a vacuum concentrator (Eppendorf 5301), and then stored at −80 °C until the NMR analysis. The polar fraction was therefore resuspended in a 0.1 M sodium phosphate buffer (600 μL; pH 7.0, 10 % D_2O; Armar AG, Döttingen, Switzerland) containing 1 mM 2,2-dimethyl-2-silapentane-5-sulfonate (DSS; Sigma-Aldrich Co), as internal reference. NMR tubes of 5 mm in diameter were then used for the successive metabolomic analysis to the NMR spectrometer.

2.5.2. ^{1}H nmr metabolomics and spectral pre-processing

The metabolomic analysis was performed with a Varian-500 NMR spectrometer operating at the spectral frequency of 499.74 MHz at 298 K. To delete the resonance of water from each one-dimensional (1-D) ^{1}H NMR spectrum obtained, a PRESAT pulse sequence was applied, with a spectral width of 6 kHz and a relaxation delay of 2.0 s. Transients (n = 128) were collected into 16,384 data points and all data sets were zero filled to 32,768 data points, with application of 0.5 Hz exponential line-broadenings before application of the Fourier transformation. Phasing, baseline-correction and calibration of the DSS at 0.0 ppm, of all ^{1}H NMR spectra were executed manually by the use of the software Chenomx Processor (Chenomx NMR Suite version 5.1; Chenomx Inc., Edmonton, Canada). The resonances of polar metabolites were recognized in accordance to known chemical shifts and peak multiplicities, employing public databases as well as the Chenomx 500-MHz library database. Quantification of the level of each identified polar metabolite was also performed using the same software, with reference to the known concentration of the DSS signal introduced in each sample (Cappello et al., 2019, 2017a; Maisano et al., 2017).

Once the 1-D ^{1}H NMR spectra were converted in a data matrix by the use of Chenomx Profiler, another module included in the Chenomx NMR Suite software, all spectra were split into chemical shift bins of 0.005 ppm width between 0.9 and 9.1 ppm, without including the regions between 4.5 and 5.4 ppm to prevent any interference of the residual water resonance in the following multivariate statistical analysis. To compare all the spectra acquired, the integrated spectral area of the remaining bins was normalized to the total integrated area of spectra. Data were mean-centered before conducting multivariable analyses.

2.6. Biochemical analysis

For the biochemical assays, 60 mg of gill tissue samples were homogenised in 600 μL (10 vol w/v) of 100 mM Tris–HCl buffer, pH 7.5, using the Qiagen TissueLyser LT bead mill, with 3.2-mm stainless steel beads, at 50 vibrations/s for 7 min. After centrifugation at 9000 g for 15 min at 4 °C, the supernatant was collected from each homogenate to perform the biochemical assays using the Shimadzu UV-1240 spectrophotometer (De Marco et al., 2022c; S. Afsa et al., 2023), operating at 25 °C. Moreover, total protein content was measured by applying the Pierce BCA Protein Assay Kit (Thermo Scientific), using the bovine serum albumin (BSA) as a reference (Bradford, 1976).

2.6.1. Biotransformation and antioxidant activity

In order to assess the activity of glutathione *S*-transferase (GST; nmol/min/mg protein), the thioether generation with a high absorbance at 340 nm, resulting from the conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) with glutathione, was monitored for 5 min as previously described in Habig et al. (1974).

For the evaluation of the enzymatic activity of catalase (CAT; μmol/min/mg protein) the decomposition of H_2O_2 at 240 nm was evaluated for 90 s by application of a colorimetric approach (Sureda et al., 2011).

The assessment of the activity of superoxide dismutase (SOD; U/mg protein) was performed in accordance with the protocol introduced by Paoletti and Mocali (1990), based on the quantification of the oxidant action of superoxide radicals against NADH, as measured at 340 nm for 15 min.

2.6.2. Lipid peroxidation

To evaluate the lipid peroxidation (LPO), the levels of malondialdehyde (MDA; nmol/mg protein) were quantified by executing the thiobarbituric acid reactive (TBARS) method (Wills, 1966). Briefly, an aliquot of 500 μL of mussel gill supernatants was added to 1 mL of trichloroacetic acid (TCA)-thio-barbituric acid (TBA) (15 % w/v TCA; 0.375% w/v TBA), and heated for 15 min in a boiling water bath at 90 °C. After cooling, the solution was measured using the spectrophotometer at 532 nm. Moreover, a MDA calibration curve was prepared using the 1,1,3,3-tetraethoxypropane (TEP), following the instructions reported in Botsoglou et al. (1994).

2.6.3. Neurotoxicity

The method of Ellman et al. (1961) was employed to estimate the activity of acetylcholinesterase (AChE; μmol/min/mg protein). This protocol relies on the ability of AChE to hydrolyse thiocholine. Indeed, the reaction with 5,5-dithiobis-2-dinitrobenzoicacid (DTNB) produces the yellow anion 5-thio-2-nitrobenzoic acid that absorbs strongly at 412 nm. According to the protocol applied, this reaction was monitored for 3 min.

2.7. Data statistical analysis

Results from all the approaches herein applied were expressed as mean ± standard deviation (SD). Data on the bioaccumulation of MPs in mussel gills were processed by one-way analysis of variance (ANOVA) followed by Tukey's multiple post-hoc comparisons to assess the significance amongst the different experimental conditions or between

single exposure and control groups, by using the Graph Pad software (Prism 8.0, San Diego, CA, USA).

Histochemical data were processed by two-way ANOVA followed by Dunnett's multiple comparison test to differentiate between the number of neutral and acid mucous cells detected in treated mussel gills in respect to those of control, by using the GraphPad software.

To reduce the amount of data obtained from the metabolomic analysis and to clearly discern among control and MP-exposed groups at the three selected time-points, the Principal Components Analysis (PCA), an unsupervised chemometric technique, was applied using MATLAB software. PCA allows multivariate NMR data to be shown in a score plot, in which samples sharing similarities in their metabolic profiles tend to be grouped together. To recognize the cellular metabolites responsible for clustering, analysis of the corresponding loading plot was performed. Metabolite changes were then measured through the peak area ratio between the averages of the exposure and control groups. The Graph Pad software was therefore used to perform univariate statistical analysis, processing NMR data by ANOVA applying the Dunnett's post-test to describe the effects on time of single treatment groups in respect to controls. No statistically significant differences were revealed among the control metabolite sets over time.

Biochemical data were processed by two-way ANOVA followed by Sidak's multiple comparison test for the two experimental conditions and the three different time-points of exposure, by using the GraphPad software and Excel.

For all data, the threshold for significance was $p < 0.05$, recognized as the criterion of statistical significance.

3. Results

3.1. MPs accumulation in mussel gills

The amount of 3 μm polystyrene MPs accumulated into mussel gills at the three selected time-points is reported in Fig. 1. The lower number of MPs was observed in mussel gills after 24 h of exposure. Interestingly, a notable increase in the amount of MPs was found in mussels exposed for 48 h, whereas a slight lowering in the number of plastic beads was revealed at T72 in respect to what observed at T48. As expected, no MPs were detected in the gills of mussels from the control group, and neither at the beginning of the experimental exposure (T0). Also, the median values of MPs accumulated in the exposed mussels were all significantly ($p < 0.05$) higher than those recorded in controls.

3.2. Presence of MPs at histological level

The histological observations of gills of mussel *M. galloprovincialis* from the group of control (Fig. 2A) revealed a regular morphology of the branchial tissue, and the absence of MPs, as expected. Conversely, the gills of mussels exposed to MPs exhibited the presence of 3 μm red polystyrene MPs within the branchial epithelium at all the three selected time-points, already after 24 h from the exposure (Fig. 2B-D), as detectable both within the lateral cells (LC), ciliated and not, and within the haemolymphatic sinus (S) in the intermediate zone of gill filaments.

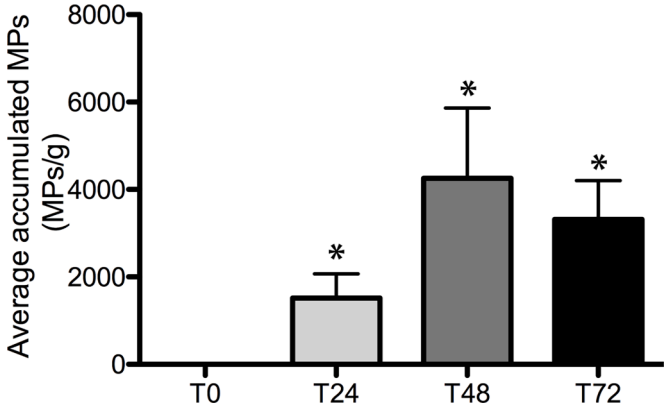

Fig. 1. Accumulation of MPs in mussel gills (MPs/g, expressed as means ± SD; $n = 9$) at the selected time-points. Asterisks indicate statistically significant differences relative to control and among groups (Tukey's test; $*p < 0.05$).

3.3. Mucous cells in mussel gills

The AB/PAS staining was performed on mussel gill tissue sections in order to visualize the presence of mucous cells. Neutral mucopolysaccharides were observed in the branchial epithelium of specimens from control (Fig. 2E) and exposure groups at all the selected time-points (Fig. 2F-H), whereas a higher number of acid mucous cells was detected in all the gills of mussels exposed to MPs, as clearly shown in Fig. 2F-H, both along the intermediate zone and in the frontal cells (FC) and postlatero-frontal cells (PLFC). The increase in mucous production, both neutral and acid mucous, was statistically significant after 48 h MP-

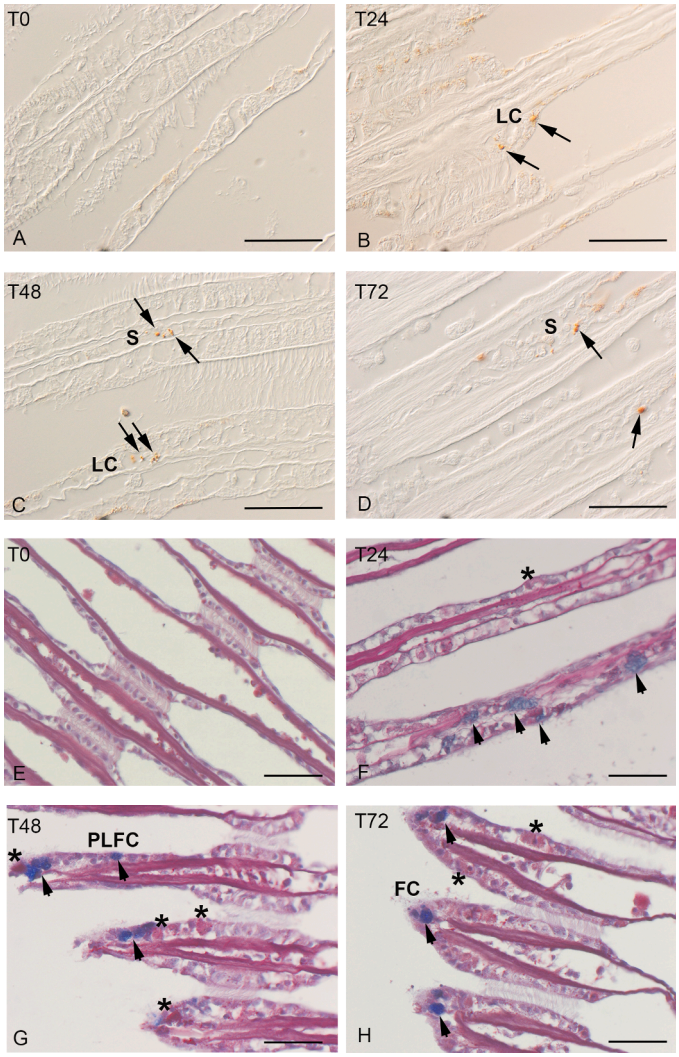

Fig. 2. Representative histological sections of gills of mussel *Mytilus galloprovincialis* from the group of control (A), and from those exposed to MPs for 24 h (B), 48 h (C) and 72 h (D), showing the presence of red MPs within the branchial epithelium, as indicated by arrows. (LC: lateral cells; S: haemolymphatic sinus). AB/PAS reaction showing the presence of neutral (asterisk) and acid mucous (arrow head) cells in gills of mussels from the group of control (E), and from those exposed to MPs for 24 h (F), 48 h (G) and 72 h (H). (PLFC: postlatero-frontal cells; FC: frontal cells). Scale bar = 20 μm.

exposure in respect to control (Fig. 3).

3.4. Metabolome of mussel gill extracts

A representative 1-D ^{1}H NMR spectrum of the hydrophilic extract from the mussel gill tissue from the control group is shown in Fig. 4. Overall, all the spectra of mussel gills resulted to be dominated by the intense resonances stemming from two organic osmolytes, namely taurine (triplets at 3.25 and 3.41 ppm) and betaine (singlets at 3.25 and 3.89 ppm). These molecules are typically accumulated in the cells of euryhaline species for counteracting changes in the salinity of water (Yancey, 2005), especially in bivalve gills primarily involved in osmoregulatory processes. Within the metabolome of mussel gills, other prominent classes of compounds were detected, including amino acids (*e.g.*, alanine, glutamate), osmolytes (*e.g.*, hypotaurine, homarine), energy metabolism-related metabolites (*e.g.*, glucose), and tricarboxylic acid cycle intermediates (*e.g.*, malonate), and neurotransmitters (*e.g.*, serotonin).

3.4.1. Pattern recognition analysis of ^{1}H NMR spectra

In the PCA score plot of the ^{1}H NMR metabolic profiles of mussel gills (Fig. 5), a clear sample groupings can be observed, where mussels challenged with polystyrene MPs form separate and distinct clusters in respect to control along the PC2 axis (27.30 % of variance), describing a different metabolome in gills of mussels from the two experimental conditions. It is also interesting to note that while mussel gills from the control groups cluster together sharing a similar metabolic profile, a clear groupings of mussels exposed to MPs at the three selected time-points was revealed, suggesting a distinct time-dependent gill metabolome induced by MP insult.

To recognize the cellular metabolite biomarkers responsible for the clustering of samples as revealed by PCA, as well as the direction of their changes among groups, the corresponding PC2 loading plot (Fig. 6) was analysed. Specifically, peaks with positive loadings correspond to metabolites with higher concentrations in MP-exposed mussels in respect to control, whereas those with negative loadings refer to metabolites whose level decreased following exposure to MPs than control. The analysis of the PC2 loading plot, associated with quantitative analysis and univariate statistical analysis applied to one metabolite at a time for each selected time-points (Table 1), revealed that overall the metabolic profiles of the gills of mussels exposed to MPs were mainly characterized by significantly elevated levels of energy-related metabolites (*e.g.*, acetoacetate, ATP/ADP and mytilitol), and osmolytes (*e.g.*, betaine, taurine, homarine), together with significantly decreased concentration of amino acids (*e.g.*, alanine, glycine), Kreb's cycle intermediates (*e.g.*, succinate), and neurotransmitters (*e.g.*, acetylcholine).

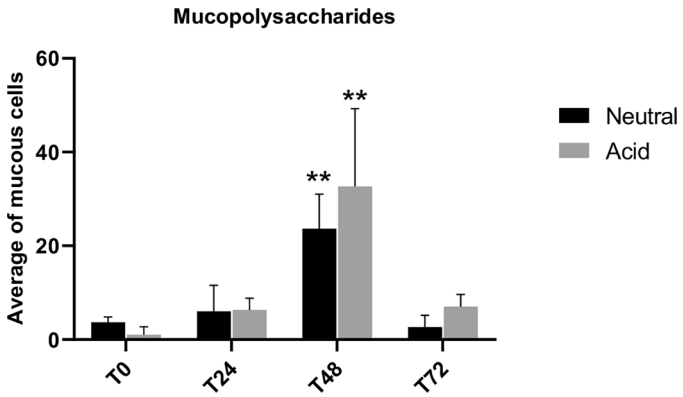

Fig. 3. Average of neutral and acid mucous cells detected in the gills of MP-exposed mussels at the selected time-points in respect to control (T0). (Dunnett's test; $^{**}p < 0.01$).

3.4.2. Changing trend for key gill metabolites with time

The MP-exposed mussels, besides being all clustered from controls along PC2, were also clearly grouped for experimental time-points, suggesting a distinct time-dependent gill metabolome that shared the similar sub-set of compounds identified as key metabolites towards MP challenge but with significant ($p < 0.05$) differences in their magnitudes. According to the quantitative analysis performed, as also reported in Table 1, the metabolite biomarkers were plotted against time and grouped into four major classes, namely amino acids, energy-related metabolites, osmolytes and neurotransmitters (Fig. 6). In regard to amino acids, a significant drastic reduction in the levels of alanine and glycine was recorded from T24 and throughout the entire period of the experimental exposure. Relevant alterations were detected in the energy-related metabolites. Specifically, as the short-term exposure time increased, the level of the majority of the key energy metabolites, namely acetoacetate, ATP/ADP and mylitilol, significantly raised steadily throughout T0 to T72 except for acetoacetate, whose concentration slightly decreased after T48. Interestingly, only succinate exhibited a significant sharp depletion from the beginning of the exposure, which was maintained over the 72 h of exposure to MPs. Furthermore, MPs induced significant changes in the osmoregulatory processes of mussel gills since the levels of taurine and homarine showed significant exponentially increases during the entire period of exposure. Conversely, only betaine, after a significant sharp reduction from T0 to T24, exhibited a rapid rise in its concentration reaching values comparable to those of control at T72. Worthy of note, the exposure to polystyrene MPs provoked in mussel branchial tissues also a marked and significant depletion in the level of acetylcholine, a validated biomarker of the cholinergic neurotransmission, especially after 24 h and 48 h from the beginning of the exposure. Overall, the percent change values of the key metabolites measured in the gills of MP-exposed mussels at all the selected time-points and their associated *p*-values are summarized in Table 1, together with their proton NMR chemical shift and peak shape, while the fluctuation in their levels over the time are depicted in Fig. 7.

3.5. Biochemical assays

3.5.1. Biotransformation and antioxidant activity

In mussels exposed to polystyrene MPs, no significant change in the GST activity was observed in the gills after 24 h of exposure, as shown in Fig. 8. Conversely, longer period of exposure induced a significant ($p < 0.05$) 1.61-fold and 1.79-fold increase at T48 and T72, respectively, in the activity of GST in mussel gills (Fig. 8).

In regard to the enzymatic activity of CAT, although no relevant alteration was noticed after 24 h of exposure to polystyrene MPs, it was significantly ($p < 0.05$) raised in the gills of MP-exposed mussels both at T48 and T72 with 1.66-fold and 1.40-fold increase, respectively, in respect to control (Fig. 8). Noteworthy, the increment in CAT activity at T48 was statistically ($p < 0.05$) higher than what recorded at T72.

Similarly, the antioxidant activity of SOD in mussel gills was not affected by the MPs exposure at T24. However, a significant ($p < 0.05$) 1.91-fold and 1.34-fold elevation in SOD activity was recorded respectively at T48 and T72 in respect to control mussels (Fig. 8).

3.5.2. Lipid peroxidation

In order to evaluate the lipid peroxidation caused by polystyrene MPs exposure in mussel gills, the MDA assay was performed. Interestingly, it was revealed a significant ($p < 0.05$) increment in MDA content only at T48, with levels 1.73-fold higher than those recorded in control mussel gills. On the contrary, no significant differences were detected in MDA levels between MPs-exposed and control mussels at the other selected time-points, namely T24 and T72 (Fig. 8).

3.5.3. Neurotoxicity

The polystyrene MPs generated in mussel gills no changes in the AChE activity after 24 h of exposure (Fig. 8), whereas a 1.16-fold

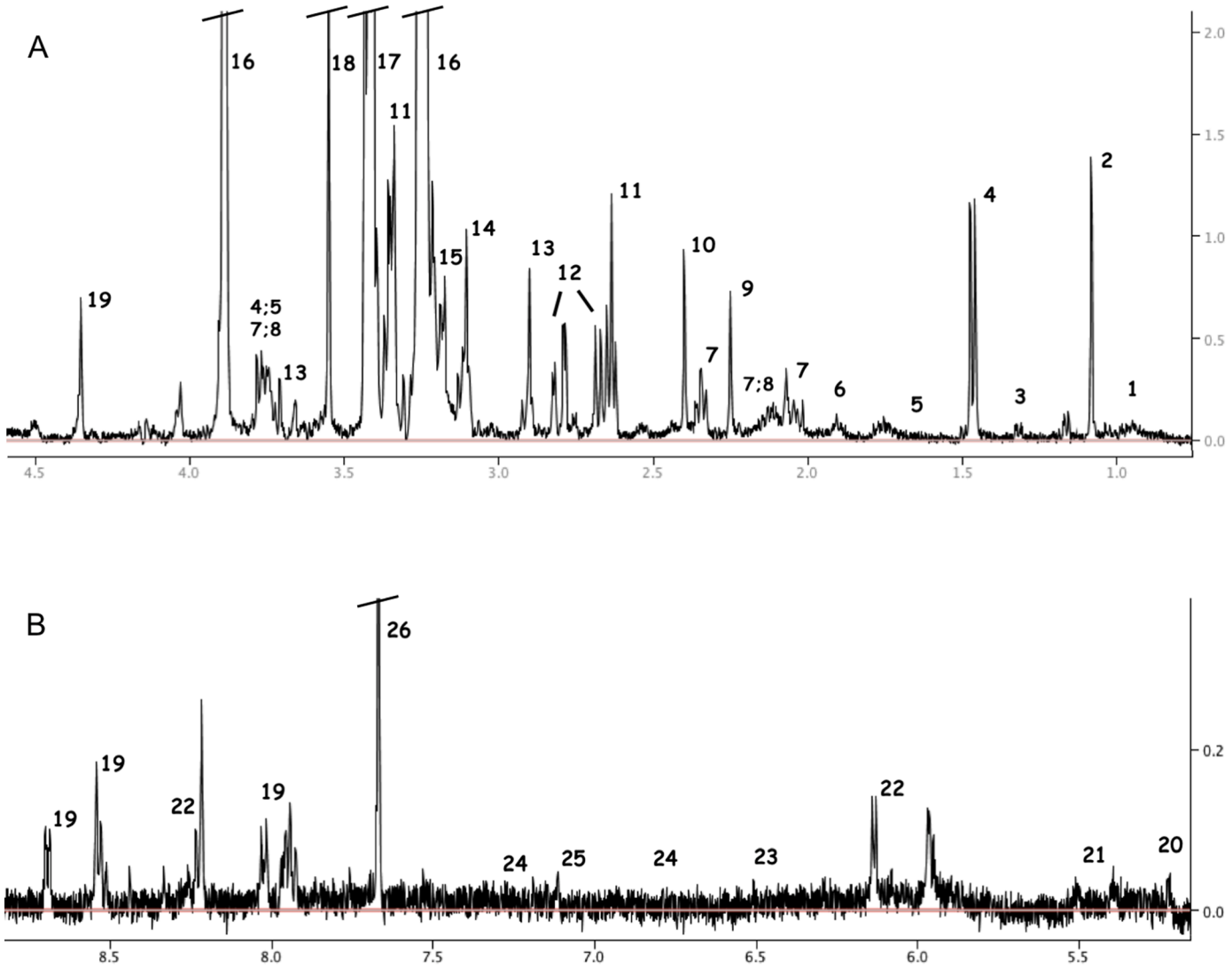

Fig. 4. A representative 1-D 500 MHz ^{1}H NMR spectrum of mussel gills, with (A) showing the aliphatic region, and (B) a vertical expansion of the aromatic region. Keys: (1) branched chain amino acids (BCAAs; isoleucine, leucine, valine), (2) mytilitol, (3) lactate, (4) alanine, (5) arginine, (6) acetate, (7) glutamate, (8) glutamine, (9) acetoacetate, (10) succinate, (11) hypotaurine, (17) aspartate, (13) dimethylglycine, (14) malonate, (15) acetylcholine, (16) betaine, (17) taurine, (18) glycine, (19) homarine, (20) glucose, (21) glycogen, (22) ATP/ADP, (23) fumarate, (24) tyrosine, (25) serotonin, and (26) unknown #1.

increase, even if not significant ($p = 0.84$) at T48 and a significant ($p < 0.05$) 1.30-fold enhancement at T72 were recorded in mussel gills in respect to control mussels (Fig. 8).

4. Discussion

This study sheds lights on the early time-dependent metabolic disorders provoked in the gills of the Mediterranean mussel *M. galloprovincialis* after a short-term (72 h) exposure to 3 μm polystyrene MPs under an environmental scenario. In fact, the dose of MPs herein tested was chosen to approach realistic environmental concentrations of MPs in laboratory exposures (Vroom et al., 2017), in order to mimic an environmental condition that may represent a threat for aquatic biota. Indeed, according to literature, no studies have addressed the issue of short-term effects in mussels in the context of MP ecotoxicity up to now, as the biological impact due to MPs has been documented starting from at least one week of the experimental exposures (Avio et al., 2015; Paul-Pont et al., 2016; Pittura et al., 2018; González-Soto et al., 2019; J.S. Choi et al., 2022). In this study, with the aim to comprehensively assess the early biological impact due to MPs exposure, the branchial epithelium was selected as target tissue since gills are constantly in direct contact with the surrounding environment, and are therefore the first organ to be mechanically and physiologically affected by the presence of environmental pollutants. Besides evaluating the uptake and bioaccumulation of MPs in gill tissues daily over the three day-experimental exposure, an overall assessment of the general health status of mussels was obtained by investigation of the neutral and acidic mucous secretions by gills, while specific time-dependent early responses to MP insult were revealed by comprehensive insights to a range of cellular metabolic pathways, with a focus on the oxidative defense systems, lipid metabolism, and cholinergic neurotransmission at level of mussel gills.

4.1. MPs uptake in mussel gills

The uptake and bioaccumulation of MPs in mussel gill tissues was performed daily over the three day-experimental exposure, and the presence of 3 μm polystyrene MPs in the branchial tissues of the exposed mussels was confirmed at all the three selected time-points (T24, T48, T72) in respect to control mussels, where no MPs were found as expected. These findings were further supported by the histological observation of mussel gills, which exhibited the presence of the tested

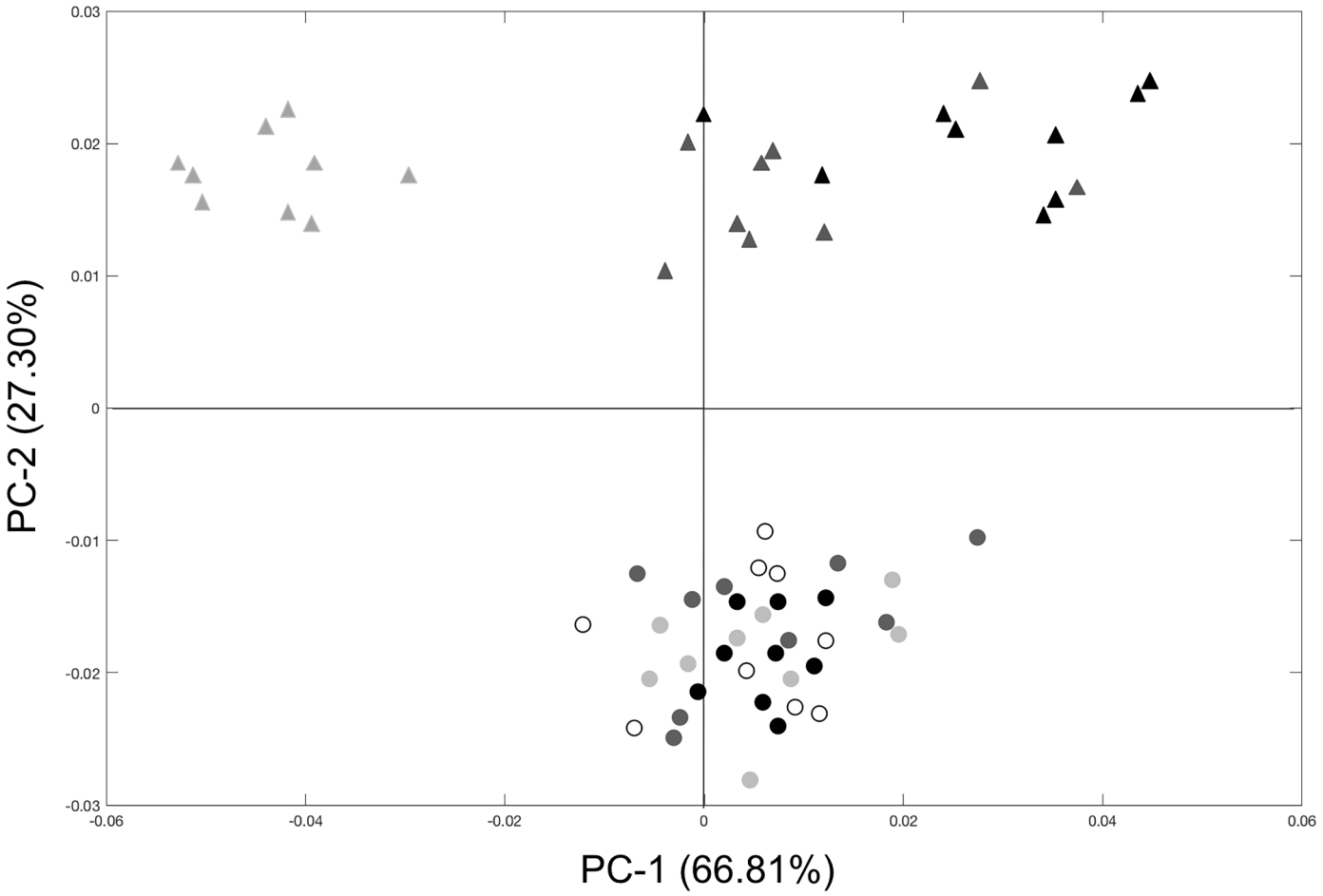

Fig. 5. Principal Components Analysis (PCA) of ^{1}H NMR spectra of mussel gills (n = 9 per each experimental condition) showing separation (PC1 *vs*. PC2) between mussels from control group (circle) and those challenged with MPs (triangle), at T0 (white), T24 (light gray), T48 (dark gray), and T72 (black) time-points.

red spherical MPs trapped among the branchial lamellae already after 24 h from the exposure until the end of the experiment.

Marine mussels feed themselves by performing an intense filtering activity operated by gills, acting as the mussel filter-pump since they, apart for respiration, are primarily food-collecting organs that ensure that suspended food items in the water are captured and transferred to the mussel mouth by the current generated by the frontal cilia (Riisgard et al., 2015). Nonetheless, the gills of mussels are able to indiscriminately collect small food items as well as anything of proper size present in the water, harmful or not (de Oliveira David et al., 2008; Van Cauwenberghe et al., 2015; Gornati et al., 2016; Freitas et al., 2022). This was also demonstrated in this study since the 3 μm polystyrene MPs were observed and quantified within the mussel gills already after 24 h from the exposure. At T24 the number of plastic beads counted into the branchial tissue was low likely due to the pathways for the uptake and tissue distribution of MPs occurring in filter-feeders bivalves, and previously described in mussels. Indeed, a first site of microplastics uptake was shown at the gill surface, mediated by microvilli that actively transport MPs into the gills by endocytosis. Additionally, the second pathway occurred via ciliae movement, which transferred MPs to the stomach, intestine and digestive tubules, followed by accumulation within the lysosomal compartment (Von Moos et al., 2012). Therefore, it is reasonable to hypothesize that polystyrene MPs, after taking up by mussels via gills, were quickly transported and distributed to other mussel tissues, as previously demonstrated for mussel digestive glands (Cappello et al., 2021), thus resulting in the low number of MPs detected in the branchial tissue at T24. Worthy of interest, a notable increase in the amount of MPs was found in mussels exposed for 48 h, whereas a slight lowering in the number of plastic beads was revealed at T72 in respect to what observed at T48. This may be explained by the fact that, although these plastic microspheres quickly translocate from the gills to the rest of the body, the branchial epithelium while filtrating seawater with a high and constant presence of MPs over time, tends to trap the MPs among the branchial lamellae, thus compromising the filter functioning of gills and bioaccumulating MPs throughout the exposure, as herein reported. Similar results were also previously reported in the gills of the Mediterranean mussel *M. galloprovincialis* when challenged with polymethylmethacrylate MPs of 10 μm, thus overlapping in size with that of items that are naturally ingested by filter-feeders, thus further supporting their rapid uptake, transport and retention in branchial tissues (Pavičić-Hamer et al., 2022).

One of the early responses to the bioaccumulation of MPs observed at the level of mussel gills is represented by the secretions of acidic mucopolysaccharides along the branchial epithelium. Indeed, the presence of acid mucous cells was greater than that of neutral mucous cells in the gills of MP-exposed mussels at all the selected time-points, and specifically within the lateral cells (LC), ciliated and not, and within the haemolymphatic sinus (S) in the intermediate zone of gill filaments. In general, the production of acid mucosubstances generates a protective coat around the branchial epithelium that plays a lubricant role, besides having anti-bacterial properties (Sanchez et al., 1997; Natalotto et al., 2015; Freitas et al., 2022). Therefore, the secretions of acidic mucopolysaccharides may enhance the protective function of mucous against pathogens, natural toxins, and even environmental pollutants (Cappello et al., 2019), besides having a potential role in particle transport corresponding to a cleaning function (Beninger et al., 1993; Liberatori

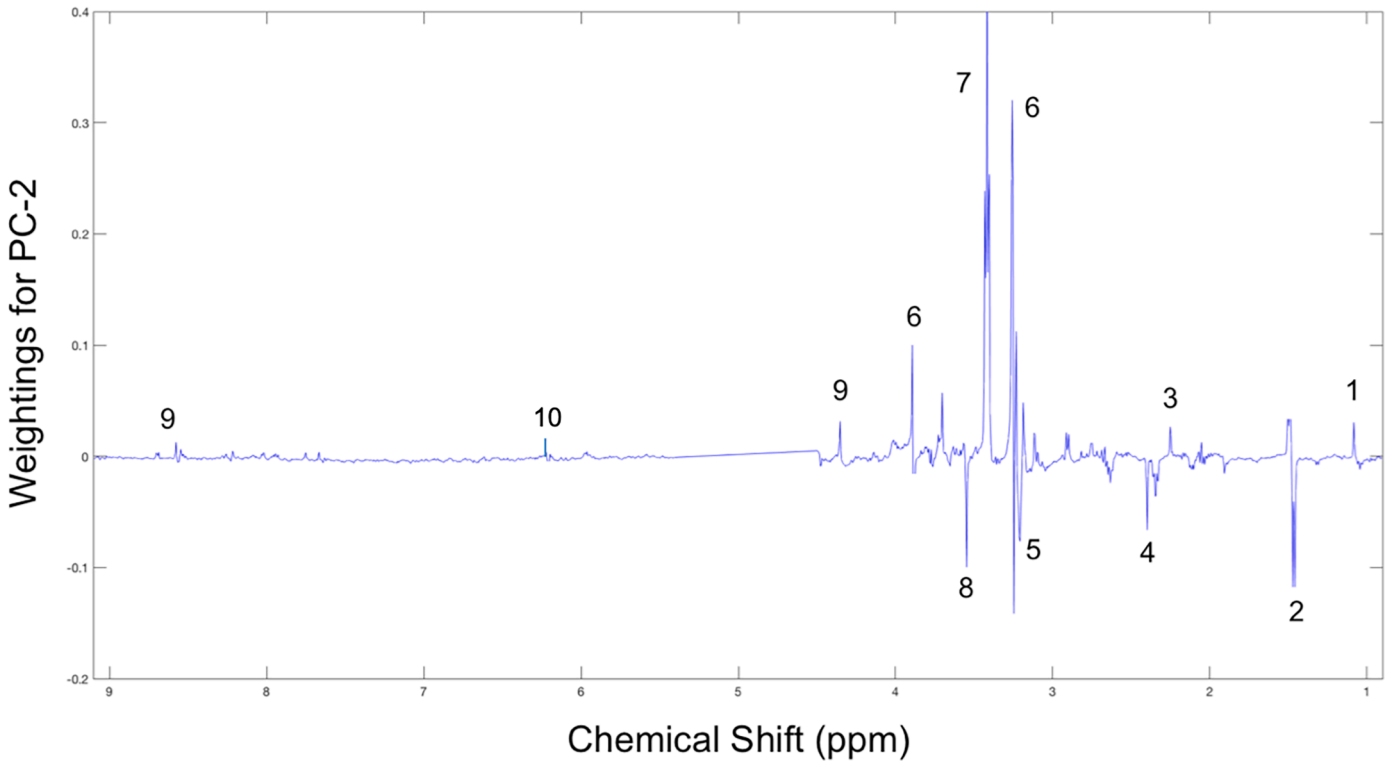

Fig. 6. Corresponding PC2 loading plot showing the metabolic differences between gills of control and MP-exposed mussels. Keys: (1) mytilitol, (2) alanine, (3) acetoacetate, (4) succinate, (5) acetylcholine, (6) betaine, (7) taurine, (8) glycine, (9) homarine, and (10) ATP/ADP.

Table 1
Percent changes in concentrations of metabolites between polystyrene MPs-exposed and control mussels ($n = 9$) at the selected time-points (Dunnett's test, $^*p < 0.05$; s: singlet; d: doublet; t: triplet; dd: doublet of doublets; m: multiplet).

	Chemical shift and peak shape, ppm	T24	T48	T72
Amino acids				
Alanine	1.46 (d), 3.76 (m)	↓ 56%*	↓ 44%*	↓ 42%*
Glycine	3.54 (s)	↓ 85%*	↓ 86%*	↓ 89%*
Energy metabolites				
Acetoacetate	2.22 (s), 3.41 (m)	↑ 35%	↑ 74%*	↑ 62%*
Succinate	2.41 (s)	↓ 93%*	↓ 88%*	↓ 90%*
ATP/ADP	4.21 (m), 4.28 (m), 4.39 (m), 4.51 (m), 4.62 (t), 6.13 (d), 8.24 (s), 8.53 (s)	↑ 15%	↑ 34%*	↑ 36%*
Mytilitol	1.10 (s), 3.36 (m)	↑ 42%*	↑ 136%*	↑ 247%*
Osmolytes				
Betaine	3.25 (s), 3.89 (s)	↓ 55%*	↓ 16%	↑ 15%
Taurine	3.25 (s), 3.41 (t)	↑ 44%*	↑ 118%*	↑ 112%*
Homarine	4.35 (s), 7.95 (dd), 8.02 (d), 8.53 (dd), 8.71 (d)	↑ 29%	↑ 36%*	↑ 42%*
Neurotransmitter				
Acetylcholine	2.15 (s), 3.20 (s)	↓ 76%*	↓ 75%*	↓ 53%*

et al., 2020), therefore suggesting the occurrence of adaptive and protective strategies by mussel gills again MP insult.

4.2. Metabolic disorders induced by MPs in mussel gills

The powerful and sensitive NMR-based metabolomics allowed us to successfully obtain an integrated description of the metabolic alterations provoked in mussel gills after exposure to 3 μm polystyrene MPs for three days. It was showed by PCA a clear clustering between MP-exposed and control mussels, therefore indicating that individuals may be distinguished according to the cell metabolome of their gills. The major cellular pathways hampered by the polystyrene MPs exposure in mussel gills were those related to protein and energy metabolism, osmoregulatory processes, and neurotransmission. It is also worthy to note that, among individuals exposed to MPs, it was also possible to cluster mussels among the different experimental time-points (T24, T48, T72), and this suggests a distinct time-dependent gill metabolome as a clear modulation over the exposure time, likely not in the metabolite composition since mussel gills shared the similar sub-set of compounds identified as key metabolites towards MP challenge but in their magnitudes as function of the exposure time.

From an in-depth investigation of the metabolomics profiles of the gills of mussels treated with MPs in respect to the control group, alteration in the protein metabolism were revealed, as suggested by the significant drastic reduction in the levels of alanine and glycine recorded from T24 and throughout the entire period of the experimental exposure. Marine bivalves possess a large portion of their metabolome constituted by free amino acids (Henry et al., 1980; Kube et al., 2007; Hani et al., 2021), considered as critical compounds for the synthesis of body proteins and for the regulation of protein turnover processes. Therefore, the overall depletion detected in the level of these two amino acids throughout the duration of the experiment may be related to the activation of defensive and cytoprotective mechanisms by mussels to counteract the toxic impact induced by MPs, likely promoting the induction of protein synthesis in order to repair or replace MP-damaged

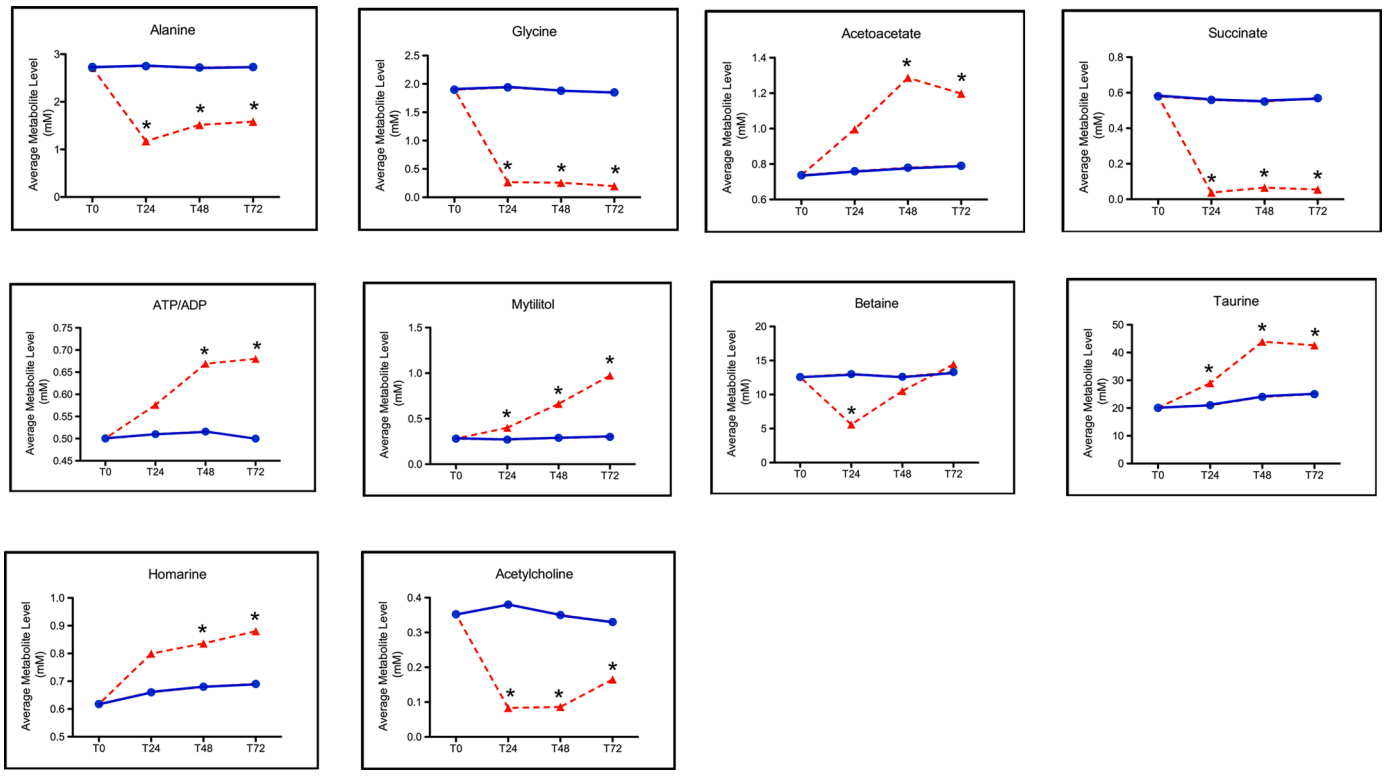

Fig. 7. Plots of the concentrations (mM, expressed as means; $n = 9$) of key metabolites in mussel gills against time. The changes of the metabolites in the control and MP-exposed mussel groups are depicted with a blue solid line and a red dashed line, respectively. Asterisks indicate significant differences relative to control (Dunnett's test; $*p < 0.05$).

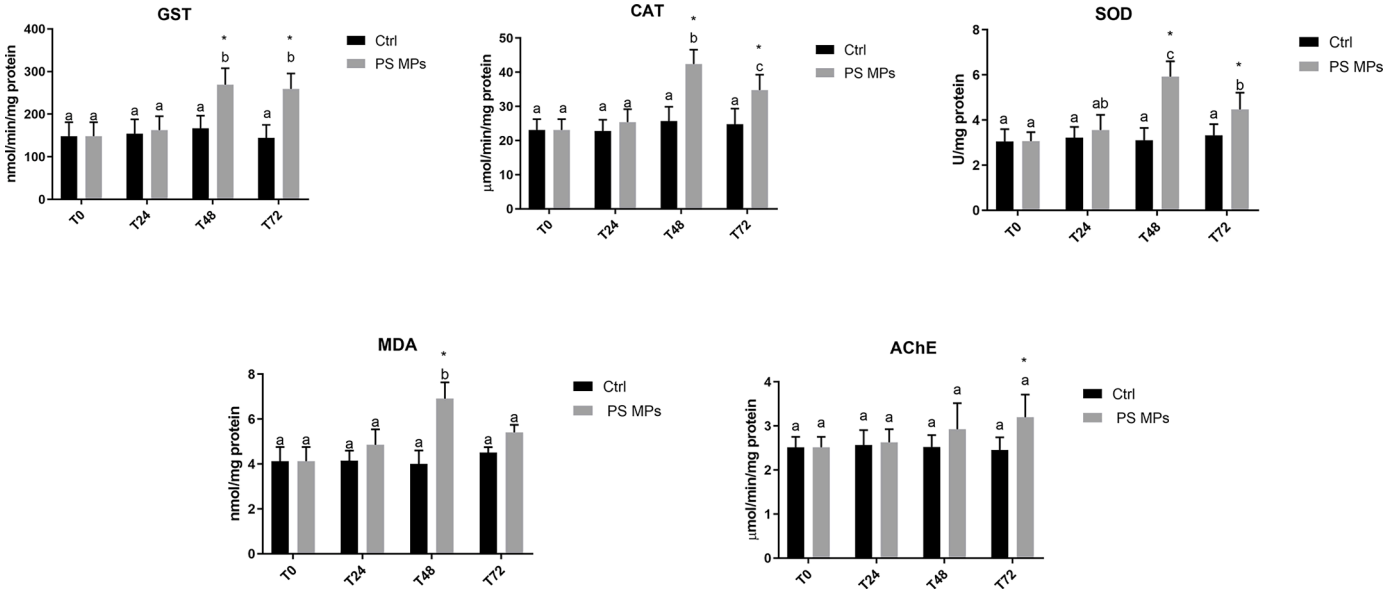

Fig. 8. Activity of the enzymes involved in the biotransformation and antioxidant defense system (GST: glutathione S-transferase, nmol/min/mg protein; CAT: catalase, µmol/min/mg protein; SOD: superoxide dismutase, U/mg protein), in lipid peroxidation (MDA: malondialdehyde, nmol/mg protein), and cholinergic neurotransmission (AChE: acetylcholinesterase, µmol/min/mg protein) in gills of mussel *M. galloprovincialis* challenged with 3 µm polystyrene (PS) MPs for 0 (T0), 24 (T24), 48 (T48) and 72 (T12) hours of exposure. Results are shown as means ± SD. Different letters denote significant differences among different exposure times within the same experimental group ($p < 0.05$). Asterisks indicate significant differences relative to control.

proteins (Cappello et al., 2017b; Maisano et al., 2017; Caricato et al., 2019). Interestingly, it must be pointed out the significant reduction in the concentration of alanine, which plays a key role in the transport of nitrogen that may be released as a result of protein catabolism, and that may be toxic for organisms. Generally, the presence of alanine at level of the branchial epithelium may be associated with an intense activity of nitrogenous waste excretion occurring in gills (Evans, 1987; Huang et al., 2020), finalized at preventing intoxication by ammonia. However, the depletion in alanine, as herein recorded during the overall duration of the experimental trial, is more plausible to be associated to the role of

osmolyte that alanine also plays in invertebrate species in order to regulate and maintain the intracellular osmotic balance (Yancey, 2005; Podbielski et al., 2022), as further reinforced by the fact that these data were recorded at the level of mussel gills, playing a major role in osmoregulatory processes. Noteworthy, it has been recently demonstrated in euryhaline teleosts that the transepithelial transport of nitrogenous waste in branchial tissues represents an essential component for the maintenance of ionic homeostasis under a hyperosmotic challenge (Huang et al., 2020).

In regard to the reduction in the level of glycine over time in MP-exposed mussels relative to control, it must be noticed that glycine is one of the compounds that forms glutathione (GSH), which serves mainly in the protective processes against the onset of oxidative stress at cellular level (Lushchak, 2011; Maisano et al., 2013). As reported by previous studies, the primary mechanism of toxicity triggered by MPs is the occurrence of oxidative stress following the generation of reactive oxygen species (ROS; Prinz and Korez, 2020), which may accumulate in cells and then elicit a variety of biological responses, including the activation of antioxidant defense system. Surprisingly, the presence of GSH was not detected within the NMR profile of mussel gills. A plausible explanation for this may be that the NMR spectroscopy allows us to identify only the reduced form of GSH, which may not be present in the early time of exposure to MPs if the available GSH is already complexed with MPs to minimize their biological impact. However, the absence of GSH from the NMR metabolic profiles of MP-exposed mussel gills may be related to the choice of gills as target organ since it is known that gills play a scarce antioxidant defense activities, which mainly occurs in mussel digestive glands. As a matter of fact, the induction of an important defense mechanism against MP-induced oxidative stress at level of the digestive glands of the same mussels was documented in our previous study, revealing a sharp increase in the GSH content in response to MPs over the short-term (72 h) exposure (Cappello et al., 2021).

Following the three-day exposure to MPs, relevant metabolic alterations were found in mussel gills in the energy-related metabolites. In detail, a time-dependent augmentation in the level of the majority of the key energy metabolites, namely acetoacetate, ATP/ADP and mytilitol, was recorded in mussel gills throughout the experiment, except for succinate that exhibited an opposite trend with a significant sharp depletion from the beginning until the end of the exposure to MPs. Acetoacetate is a ketone body synthesized from acetyl-coenzime A (acetyl-CoA) and it is the end product of the metabolism of fatty acid oxidation (Cappello et al., 2018). Therefore, acetoacetate is an important vector of energy transport during fasting or when the feeding activity of organisms is hampered (Wu et al., 2016), as it occurred herein because of MP insult that damaged mussel gills in their structure and function. Overall, the exponential increase in the level of acetoacetate detected in mussel gills from the beginning of the exposure, together with the concomitant marked depletion in the concentration of succinate, a Krebs cycle's intermediate, may be considered as indicators of alteration in the lipid metabolism triggered by the MP insult. Indeed, acetoacetate acts as a fuel to supply energy for cellular activity of various tissues. Accordingly, this hypothesis is further supported by the elevation in the level of ATP/ADP and mytilitol, a seven carbon (7-C) sugar C-methyl-scyllo-inositol (Aru et al., 2020), detected in the NMR metabolic profiles of the gills of MP-treated mussels, as a sign that organisms may need more energy to cope with MP toxicity. Taken together, all these findings suggest the occurrence of impairments in the energy metabolism of mussel gills due to MP exposure, with an altered lipid metabolism to meet the metabolic needs of organisms under unfavorable trophic condition (Cappello et al., 2013), also related to the compensatory mechanisms elicited by mussels in response to a compromised gill ciliary activity caused by trapping of MPs among branchial lamellae.

Furthermore, MPs induced significant changes in the osmoregulatory processes of mussel gills since the levels of taurine and homarine showed significant exponentially increases during the entire period of exposure, whereas only betaine, after a significant sharp reduction at T24, raised during the exposure reaching values comparable to those of control at T72. These data are not surprising, as one of the major physiological functions of the gills of marine bivalves consist in their active involvement in the osmotic regulatory mechanisms because constantly dealing with the external saline environment (Jørgensen, 1974; Cappello et al., 2018; Huang et al., 2020), which may be perturbated by the presence of environmental pollutants, such as MPs as in this study. Indeed, the presence of organic molecules, such as betaine, taurine and homarine, serving as osmolytes within the mussel gill metabolome, is typical of aquatic mollusks, which are able to quickly adapt to any external fluctuations of salinity by promoting an active accumulation or release of these compounds at intracellular level in order to guarantee the maintenance of the cell volume (Somero and Bowlus, 1983; Podbielski et al., 2022), as also occurred herein in mussels challenged with short-term exposure to MPs.

Worthy of note, the exposure to polystyrene MPs provoked in mussel branchial tissues also a marked and significant neurotoxicity, as demonstrated by the depletion in the level of acetylcholine, a validated biomarker of the cholinergic neurotransmission, especially at T24 and T48. Firstly, the presence of acetylcholine within the metabolome of mussel gills confirm, among the others, the physiological role that gills of bivalves play in neuronal signaling (Jørgensen, 1974; Cappello et al., 2015, 2018). In fact, acetylcholine is a cholinergic neurotransmitter strictly related to the enzymatic activity of acetylcholinesterase (AChE), an enzyme that splits acetylcholine into choline and acetate. The AChE has been widely used in a number of ecotoxicological studies as a biomarker of neurotoxicity in aquatic bivalves (Tsangaris et al., 2010; Lionetto et al., 2013; Ciacci et al., 2012; Mancini et al., 2023), since its inhibition due to the effects of pollutants is followed by accumulation of acetylcholine and may thus compromise the normal function of nerve signal transmission in gills. However, in this study an opposite trend was recorded since the presence of MPs provoked a decreased in acetylcholine level within the mussel branchial tissues. Similar data were previously reported in the gills of mussel *M. galloprovincialis* following exposure to petrochemical contamination within a natural coastal area (Cappello et al., 2013). Although previously documented, the physiological mechanism that explains this trend is still poorly understood, but it is undoubtedly that the MP-induced cholinergic neurotoxicity contributes to perturbation of the normal ciliary motility of the branchial epithelium, with adverse consequences on filtering and nutritional activity of mussel gills.

4.3. Oxidative stress pathways and cholinergic neurotransmission

It is widely acknowledged how the exposure to several classes of pollutants, like pharmaceuticals (Freitas et al., 2019; Afsa et al., 2022, S. 2023; De Marco et al., 2022c), metal nanoparticles (Giannetto et al., 2018; Dellali et al., 2021; Noureen et al., 2022, A. 2023), pesticides (Yang et al., 2020; Sule et al., 2022), and also MPs (Cole et al., 2020; Fu et al., 2022), may entail to changes in the antioxidant system of aquatic organisms (Pereira et al., 2019; Benedetti et al., 2022).

In this work, 3 μm polystyrene MPs enhanced the activity of the enzymes CAT, SOD, and GST, especially at T48 and somewhat lower (for SOD and CAT) at T72, while the level of LPO increased only at T48. The enzymes SOD and CAT constitute two of the first enzymes in the cellular antioxidant system to be involved in the regulation of the levels of ROS, and have been validated as biomarkers to estimate the early pro-oxidant effects of contaminants (Regoli and Giuliani, 2014; Tang et al., 2018). An increase in their activity, mainly in the initial phases of exposure, represents an attempt by the organisms to counterbalance the rise in ROS triggered by the exposure to stressors (Li et al., 2020a). Interestingly, the slight reduction in the activity levels of SOD and CAT as observed at T72 compared to T48, would suggest an apparent condition of recovery promoted by the antioxidant system of mussel gills towards

the onset of oxidative stress, as further corroborated by the reduced MDA levels, a validated LPO biomarker, at T72 (Li et al., 2020b).

It is widely known that GST plays a predominantly biotransformation and detoxification role. Nevertheless some isoforms can reduce lipid hydroperoxides to alcohols, leading to the concurrent oxidation of reduced (GSH) to oxidized glutathione (GSSG; Regoli and Giuliani, 2014), thereby exerting a certain antioxidant activity particularly when the principal antioxidant enzymes become less active (Manduzio et al., 2004). Therefore, the increase in GST activity even at T72 might partially explain the reduction in LPO levels. The biological responses elicited by 3 μm MPs concerning the oxidative stress as herein observed closely resemble those of other aquatic organisms challenged by MP exposure (Lu et al., 2016; Missawi et al., 2021; Richardson et al., 2021; Zitouni et al., 2021; De Marco et al., 2022c; Fu et al., 2022), therefore supporting the assumption that mussels are capable to counteract, at least in the initial stages, the pro-oxidant effects induced by polystyrene MPs via enhancing the activity of the anti-oxidant and biotransformation enzymes (Li et al., 2022).

The evaluation of the AChE activity constitutes one of the most common biomarkers for testing the neurotoxic effect of a variety of classes of pollutants (Saidani et al., 2019; Parrino et al., 2021; De Marco et al., 2022a). Several authors (Avio et al., 2015; Ding et al., 2018; Usman et al., 2021) have highlighted how MPs exposure can lead to a neurotoxic effect on different aquatic species, usually manifested as an enzymatic inhibition of AChE. Nevertheless, in this case an augmentation of AChE activity was recorded in MP-exposed mussel gills at T48 and T72, which was consistent with the metabolomic data reporting a depletion in the level of acetylcholine. The rise in AChE, roughly concurrent to the enhanced LPO levels, may be linked to a LPO-induced disruption of acetylcholine-containing vesicles, as previously suggested by Barboza et al. (2020). Therefore, these data support the correlation between alterations in ROS metabolism and impairments in neurotransmission system, as reported in other aquatic organisms exposed to MPs and other environmental pollutants (Giménez and Nunes, 2019; Barboza et al., 2020; Usman et al., 2021; De Marco et al., 2022a). Anyway, the mechanism of toxicity behind the cholinergic neurotoxic effect elicited by MPs remain uncertain (Capolupo et al., 2021), and needs further investigations.

5. Conclusions

Overall, this work provides insights into the early time-dependent mechanisms of toxicity triggered by short-tern (72 h) exposure to 3 μm polystyrene MPs in the gills of the Mediterranean mussel *M. galloprovincialis*. It was demonstrated and quantified the daily uptake and bioaccumulation of 3 μm polystyrene MPs that enter and are retained within the branchial epithelium already after the first day of exposure, and the activation of defensive and cleaning mechanisms to excrete these plastic by the mixed neutral and acid mucous secretions produced by mussel gills. Additionally, the NMR-based metabolomics allowed us a comprehensive assessment of the time-dependent metabolic disorders triggered by MPs in mussel gills over the short-term trial, highlighting changes in amino acids and energy metabolism, disturbances in the osmoregulatory processes, as well as in the cholinergic neurotransmission. Among the variety of cellular metabolic pathways altered by the early exposure to MPs, also the oxidative defense systems and lipid metabolism were noticed at level of the mussel gills. Therefore, these findings shed lights on the hampered functioning of the mussel gills upon a short-term exposure to 3 μm polystyrene MPs, which may underline the environment and human health risk posed by MPs contamination. However, it must be pointed out that laboratory circumstances are not representative of current situations, where MPs may be found in the environment in a variety of polymers, shapes and sizes that may cause harm to organisms to varying degrees. As a result, future studies should therefore focus on understanding the mechanism of toxicity triggered by environmental MPs, combined or not with other pollutants, on aquatic biota.

Credit author statement

Giuseppe De Marco: Investigation, Methodology, Formal analysis, Experimental analysis, Data processing, Software, Writing – Original Draft. **Maria Concetta Eliso:** Investigation, Methodology, Formal analysis, Data processing, Software, Visualization. **Gea Oliveri Conti:** Resources, Methodology, Formal analysis. **Mariachiara Galati:** Formal analysis. **Barbara Billè:** Formal analysis. **Maria Maisano:** Conceptualization, Resources, Methodology, Visualization, Validation, Writing – review & editing, Supervision. **Margherita Ferrante:** Resources, Methodology, Formal analysis. **Tiziana Cappello:** Conceptualization, Resources, Methodology, Formal analysis, Experimental analysis, Data processing, Software, Visualization, Validation, Writing – Original Draft, Writing – review & editing, Supervision.

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.

Data availability

Data will be made available on request.

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