



Time-dependent metabolic disorders induced by short-term exposure to polystyrene microplastics in the Mediterranean mussel *Mytilus galloprovincialis*

Tiziana Cappello^{a,*}, Giuseppe De Marco^a, Gea Oliveri Conti^b, Alessia Giannetto^a, Margherita Ferrante^b, Angela Mauceri^a, Maria Maisano^a

^a Department of Chemical, Biological, Pharmaceutical and Environmental Sciences, University of Messina, Viale F. Stagno d'Alcontres 31, 98166 Messina, Italy

^b Environmental and Food Hygiene (LIAA) of Department of Medical, Surgical and Advanced Technologies "G.F. Ingrassia", University of Catania, Via Santa Sofia 87, 95123 Catania, Italy

ARTICLE INFO

Edited by: Professor Bing Yan

Keywords:

Microplastics
Early toxicity mechanisms
Mussels *Mytilus galloprovincialis*
NMR-based metabolomics
Digestive glands
Metabolite biomarkers

ABSTRACT

In the modern society, plastic has achieved a crucial status in a myriad of applications because of its favourable properties. Despite the societal benefits, plastic has become a growing global concern due to its persistence and bioavailability as microplastics (MPs) to aquatic biota. In order to provide mechanistic insights into the early toxicity effects of MPs on aquatic invertebrates, a short-term (up to 72 h) exposure to 3 µm red polystyrene MPs (50 particles/mL) was conducted on marine mussels *Mytilus galloprovincialis*, selected as model organism for their ability to ingest MPs and their commercial relevance. The use of protonic Nuclear Magnetic Resonance (¹H NMR)-based metabolomics, combined with chemometrics, enabled a comprehensive exploration at fixed exposure time-points (T24, T48, T72) of the impact of MPs accumulated in mussel digestive glands, chosen as the major site for pollutants storage and detoxification processes. In detail, ¹H NMR metabolic fingerprints of MP-treated mussels were clearly separated from control and grouped for experimental time-points by a Principal Component Analysis (PCA). Numerous metabolites, including amino acids, osmolytes, metabolites involved in energy metabolism, and antioxidants, participating in various metabolic pathways significantly changed over time in MP-exposed mussel digestive glands related to control, reflecting also the fluctuations in MPs accumulation and pointing out the occurrence of disorders in amino acid metabolism, osmotic equilibrium, antioxidant defense system and energy metabolism. Overall, the present work provides the first insights into the early mechanisms of toxicity of polystyrene MPs in marine invertebrates.

1. Introduction

In the modern society, plastic has achieved a crucial status with extensive use in a myriad of applications because of its favourable properties (Avio et al., 2017). Whilst the societal advantages of plastics are undeniable (Kramm and Volker, 2018), it is an emerging environmental issue as plastic debris, subjected to a gradual degradation till giving rise to microplastics (MPs, < 5 mm), is persistent and ubiquitously present in seas and oceans worldwide (Barnes et al., 2009). It is of growing scientific concern that MPs pose a serious threat to the aquatic environments and marine biota. Indeed, since MPs size overlaps with the size of planktonic organisms and sediments, these microplastic fragments are potentially bioavailable to a wide range of organisms,

particularly to indiscriminate feeders at the bottom of food webs (Vroom et al., 2017). Therefore, the size of MPs makes them bioavailable, facilitating their entry into the food chain at various trophic levels and their bioaccumulation (Oliveri Conti et al., 2020; Zuccarello et al., 2019a, 2019b). Many lower trophic organisms have a reduced ability to differentiate between plastic particulate matter and food and, therefore, may capture and ingest anything of proper size. In turn, higher trophic planktivores could passively ingest MPs through normal feeding behaviour or mistake plastic fragments for natural prey. As a matter of fact, MP ingestion has been reported in numerous taxa including plankton (Vroom et al., 2017), bivalves (Van Cauwenberghe et al., 2015), crustaceans (Devriese et al., 2015), echinoderms (Courtene-Jones et al., 2017), fishes (Bellas et al., 2016), elasmobranchs (Alomar

* Corresponding author.

E-mail address: tcappello@unime.it (T. Cappello).

<https://doi.org/10.1016/j.ecoenv.2020.111780>

Received 18 September 2020; Received in revised form 18 November 2020; Accepted 6 December 2020

Available online 19 December 2020

0147-6513/© 2020 The Author(s).

Published by Elsevier Inc.

This is an open access article under the CC BY-NC-ND license

(<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

and Deudero, 2017), and cetaceans (Zhu et al., 2019). Recently, some studies demonstrated also experimentally the trophic transfer of MPs between trophic levels following ingestion (Batel et al., 2016; González-Soto et al., 2019), which may result in serious implications for predators, including humans (Rochman et al., 2015).

Once accumulated into organisms, plastics may exert deleterious effects depending on their shape/size, in term of physical harm i.e. internal abrasions, blockage of feeding appendages or intestinal or digestive tract, or cause false satiation resulting in reduced food intake (Avio et al., 2017), that may lead to inhibited growth and development and increased mortality, with negative repercussions on the population fitness (Avio et al., 2017, 2015; de Sà et al., 2018, 2015). Among filter feeders, mussels have been extensively used as bioindicator species in programs of environmental monitoring and studies of ecotoxicology to evaluate the impact of contaminants (Caricato et al., 2019; Gornati et al., 2019; Maisano et al., 2017; Wu et al., 2016), including also MPs (Li et al., 2019). Overall, a range of ecotoxicological effects attributable to MP exposure has been documented so far in mussels, and includes altered immune response (Pittura et al., 2018), oxidative stress (Paul-Pont et al., 2016), neurotransmission dysfunction (Avio et al., 2015), and increase in granulocytoma formation (Von Moos et al., 2012). However, mostly of the published papers to date reported the adverse effects induced by MPs in mussels after one week or longer period of exposure, whereas the early (within three days) mechanistic pathways involved in MP toxicity have, to our knowledge, not yet been reported.

Proton nuclear magnetic resonance (^1H NMR) metabolomics has been implemented with success in the field of environmental research and aquatic ecotoxicology (Cappello, 2020) since it allows in-depth investigation into the interactions between organisms and the environment and simultaneous identification of changes in different metabolic pathways upon exposure to pollutants (Bembek-Bailey et al., 2019; Cappello et al., 2016, 2018a; Vignet et al., 2019). To the best of our knowledge, to date only very few studies used the NMR-based metabolomics to unravel the impact of MPs on aquatic biota, and solely on zebrafish (Lu et al., 2016; Qiao et al., 2019).

Therefore, taking into account the urgency to gain a comprehensive understanding of the metabolic impact of MPs on marine biota, the present work was designed to explore the early mechanisms (within three days) of MP toxicity in the Mediterranean mussels *Mytilus galloprovincialis*, selected as model organisms because of their ability to ingest MPs and also because of their relevance to the issue of seafood safety, being commonly included in human diet (Rochman et al., 2015). To this aim, a ^1H NMR-based metabolomic approach was herein applied for the first time in order to comprehensively examine the effects of short-term (up to 72 h) exposure to polystyrene MPs on a daily basis in marine mussels at metabolite level, with the further objective to reveal any potential time-dependent metabolic changes as different response mechanisms to MPs over time. It was chosen to investigate the digestive gland because it accumulates contaminants and actively takes part in the xenobiotic metabolism (Cappello et al., 2018b). Polystyrene is one of the most commonly used plastic polymers, and it has been often found in plastic debris in marine ecosystems (Barnes et al., 2009).

2. Material and methods

2.1. Mussel acclimation and experimental design

Adult mussels *Mytilus galloprovincialis* (mean shell length: 6.6 ± 0.4 cm) were obtained in June 2017 from a local aquaculture farm (S.A.Co. M.) located in Messina, southern Italy, and transferred to laboratory aquaria filled with filtered artificial seawater (ASW) at a salinity of 35 PSU (Practical Salinity Unit), under continuous aeration. For acclimation, mussels were maintained for two weeks at stable temperature of 18 ± 1 °C, pH 7.8 and with a monitored photoperiod of 12: 12 h light: dark cycle. Water was renewed daily and, after each water renewal, mussels were fed with a commercial solution of algal mixture (Liquizell, Hobby),

dosed according to the product specifications. The same controlled laboratory conditions were also applied to conduct the experimental exposures (Giannetto et al., 2015, 2017).

After acclimation, mussels were randomly placed into glass aquaria filled with 10 L filtered ASW (12 individuals per aquaria), and then two experimental conditions were set up in triplicates: exposure for 72 h to 3 μm red polystyrene MPs (Sigma-Aldrich, Italy) at the concentration of 50 particles/mL, and to ASW with no MPs for the control group. The experimental design was conducted in triplicates, therefore after acclimation mussels were distributed into a total of six aquaria, three for each experimental condition. The size of the unlabeled polystyrene microspheres was chosen taking into account that the size of MPs plays an important role in the transport and fate of MPs into mussels, as it was documented in *M. edulis* that over 60% of 3 μm MPs were found in hemolymph than larger MPs (9.6 μm) when exposed at their mixed concentrations (Browne et al., 2008). Selected concentrations of MPs were previously used to assess MP uptake and biological effects in mussels (Browne et al., 2008), and were chosen in order to approach realistic environmental concentrations in laboratory exposures (Vroom et al., 2017). During the short-term exposure, MPs were provided to mussels together with algae, and aeration was used to keep both plastics and algae in suspension. Water was changed daily in each aquaria and MP re-dosed. No mortality was observed during the experimental trial, and organisms did not spawn. Mussels ($n = 9$ per condition) were collected immediately before their random distribution into aquaria (T0), and then daily during the 72 h short-term exposure to MPs (T24, T48, T72). Mussel digestive glands were rapidly excised, flash-frozen in liquid nitrogen, and then stored at -80 °C prior to further analysis.

2.2. Characterization of polystyrene MPs and tissue accumulation

The red polystyrene MPs used in this study were purchased from Sigma-Aldrich (Italy). All MPs were spherical, with the particle size of 3 μm and density of 1.05 g/cm³, and no plasticizers have been added during their production according to the manufacturer indications. The MPs size of 3 μm was further confirmed by optical microscopy (Phase - Contrast Nikon Eclipse E400) and no aggregation of particles was observed, neither in the solution media nor into mussel digestive gland tissues, as showed in Fig. S1 (supported by Ribeiro et al., 2017).

The same optical microscope was also used to assess the number of MPs within mussel specimens. Quantification of MPs in digestive glands was achieved by direct MPs counting under the light microscope to pH2 of fresh samples at 20x. Data were then transformed as MPs/mg of mussel digestive glands for the presentation of results.

2.3. ^1H NMR-based metabolomics

2.3.1. Extraction of tissue metabolites

Polar metabolites were extracted from digestive glands of single mussels ($n = 9$ per each experimental group, after collection of 3 mussels per replicate aquarium at each selected time-point) by applying a “two-step” methanol/chloroform/water protocol, as reported previously (Cappello et al., 2018b). In brief, 100 mg sub-sample of single mussel tissue were homogenized in ice-cold solvent (methanol: water = 4: 0.85 mL/g) using a TissueLyser LT bead mill (Qiagen) with stainless steel beads (3.2 mm), operating at 50 vibrations/s for 10 min. Homogenates were put into glass vials with 4 mL/g chloroform and 2 mL/g water, and then vortexed, kept on ice for 10 min for partitioning between polar and non-polar layers, and then centrifuged at 2000g at 4 °C for 5 min. The supernatant phase with hydrophilic metabolites (600 μL) was dried in a centrifugal vacuum concentrator (Eppendorf 5301) and kept at -80 °C. Before the NMR analysis, the polar fractions were resuspended in a 0.1 M sodium phosphate buffer (600 μL ; pH 7.0, 10% D₂O; Armar AG, Döttingen, Switzerland) with 1 mM 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) (Sigma-Aldrich Co) as internal reference, and transferred into a 5 mm diameter NMR tube.

2.3.2. ^1H NMR metabolomics and spectral pre-processing

Polar extracts of mussel digestive glands were examined using a Varian-500 NMR spectrometer working at a spectral frequency of 499.74 MHz at 298 K. To obtain one-dimensional (1-D) ^1H NMR spectra, a PRESAT pulse sequence was applied to suppress the resonance of water, with a 6 kHz spectral width and a 2.0 s relaxation delay. 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 prior to Fourier transformation. Phasing, baseline-correction, and calibration (DSS at 0.0 ppm) of all ^1H NMR spectra were performed manually by Chenomx Processor (Chenomx NMR Suite version 5.1; Chenomx Inc., Edmonton, Canada) software. The metabolite resonances were identified according to known chemical shifts and peak multiplicities, using public databases and in conjunction with the Chenomx 500-MHz library database. The latter was also utilized to quantify the metabolites as it uses the concentration of a known DSS signal to define the levels of individual metabolites (Cappello et al., 2019, 2017c; Maisano et al., 2017).

After converting all 1-D ^1H NMR spectra to a data matrix by Chenomx Profiler, also included into the Chenomx NMR Suite software, each spectrum was subdivided into chemical shift buckets of 0.005 ppm width within 0.8 and 8.8 ppm, from which the regions between 4.5 and 5.1 ppm were removed to prevent any spurious interference of the residual water resonances in the subsequent multivariate statistical analysis. Moreover, in order to facilitate spectra comparison, the integrated spectral area of the remaining bins was normalized to the total integrated area of spectra. Data were mean-centered prior to carry out multivariable analyses.

2.4. Data statistical analysis

Data from accumulation of MPs in mussel digestive glands were subjected to one-way analysis of variance (ANOVA) followed by Tukey's multiple post-hoc comparisons to test for significance amongst different exposure groups or between single exposure and control conditions, by using the Graph Pad software (Instat, La Jolla, CA, US). The Principal Components Analysis (PCA), an unsupervised chemometric technique, was applied by MATLAB to reduce the dimensionality of metabolomic data and to distinguish among control and MP-exposed mussel groups on the exposure time-points. PCA allows multivariate NMR data to be visualized in a score plot, clustering together the samples with a similar metabolic profile. Metabolites responsible for group separation were identified using the corresponding loading plot. Metabolite changes were then calculated via the peak area ratio between the averages of the exposure and control groups. Univariate statistical analysis was also performed by using the Graph Pad software. All NMR data were statistically analysed by ANOVA, applying the Dunnett's post-test to determine the effects on time of single treatment groups compared to controls. The threshold for significance was $p < 0.05$, recognized as the criterion of statistical significance. No statistically significant differences were found among the control metabolite sets over time.

3. Results

3.1. MPs accumulation in mussel digestive glands

The amount of 3 μm polystyrene MPs accumulated into mussel digestive glands at the selected time-points is reported in Fig. 1. The highest number of MPs was observed in mussels after 24 h of exposure. A drastic reduction in the amount of MPs was found in mussels exposed for 48 h, whereas at T72 the number of MPs accumulated in mussel digestive glands increased in respect to that observed at T48. As expected, no MPs were detected in the digestive glands of mussels from control groups. Also, the median values of MPs accumulated in exposed mussels were all significantly ($p < 0.05$) higher than those of controls.

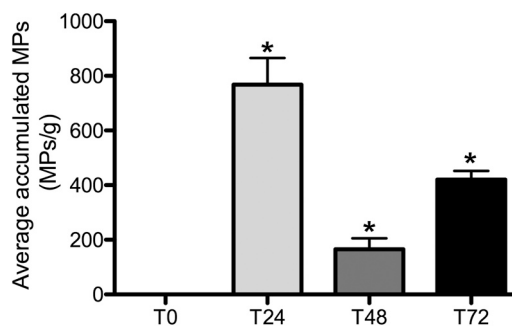


Fig. 1. Accumulation of MPs in mussel digestive glands (MPs/g, expressed as means \pm SD; $n = 6$) at the selected time-points. Asterisks indicate statistically significant differences relative to control and among groups (Tukey's test; $*p < 0.05$).

3.2. ^1H NMR metabolic profile of mussel digestive gland extracts

A representative 1-D ^1H NMR spectrum of digestive gland tissue extracts of mussels from the control group is depicted in Fig. S2. Numerous metabolites were identified, but all spectra were found to be dominated by taurine (3.25 and 3.41 ppm) and betaine (3.25 and 3.89 ppm), which were ca. 76 and 72 times higher in intensity than other metabolites, respectively. Other major classes of compounds included amino acids (e.g. glycine, alanine), osmolytes (e.g. hypotaurine), metabolites involved in the energy metabolism (e.g. glucose, glycogen), and tricarboxylic acid cycle intermediates (e.g. malonate).

3.3. Pattern recognition analysis of ^1H NMR spectra

With the aim to assess group-wise differences among control and experimental MP-exposed mussel groups at each selected time-point, the multivariate statistics PCA was applied to all digestive gland samples, each represented by its whole NMR metabolite profile. The PCA scores plot of the ^1H NMR metabolic fingerprints of mussel digestive glands, depicted in Fig. 2, displays a clear clustering between mussels from the control groups and those exposed to polystyrene MPs along the PC2 axis (explaining 16% of variance), therefore indicating a distinct metabolome among the two experimental conditions. It is worthy to note that the metabolic profiles of mussels from control were comparable to each other over time since they overlapped at all the experimental time-points, thus suggesting that all control samples shared a very similar metabolome. Conversely, a distinct time-dependent metabolome was observed for MP-exposed mussel groups, which demonstrated a clear separation from each other, besides from controls.

The corresponding PC2 loading plot, shown in Fig. 3, was therefore analyzed in order to identify all the metabolites responsible for the observed grouping, which were then referred to as "metabolite biomarkers" because found to change following MP-exposure in respect to control group. Moreover, the direction of the changes of these metabolites was also detected. In detail, peaks with negative loadings represent metabolites with higher concentrations in MP-exposed mussels in respect to control, while positive loadings correspond to metabolites whose level is reduced in mussels treated with MPs than control. The analysis of the PC2 loading plot, combined with univariate statistics applied to one metabolite at a time, revealed that overall the metabolic profiles of the digestive glands of mussels exposed to MPs were mainly characterized by significantly elevated concentrations of branched-chain amino acids (BCAAs: isoleucine, leucine, valine), alanine, tyrosine, lactate, glycogen, glucose, betaine, taurine, homarine and glutathione, together with significantly decreased levels of dimethylglycine, glycine, acetoacetate, succinate, malonate, and hypotaurine.

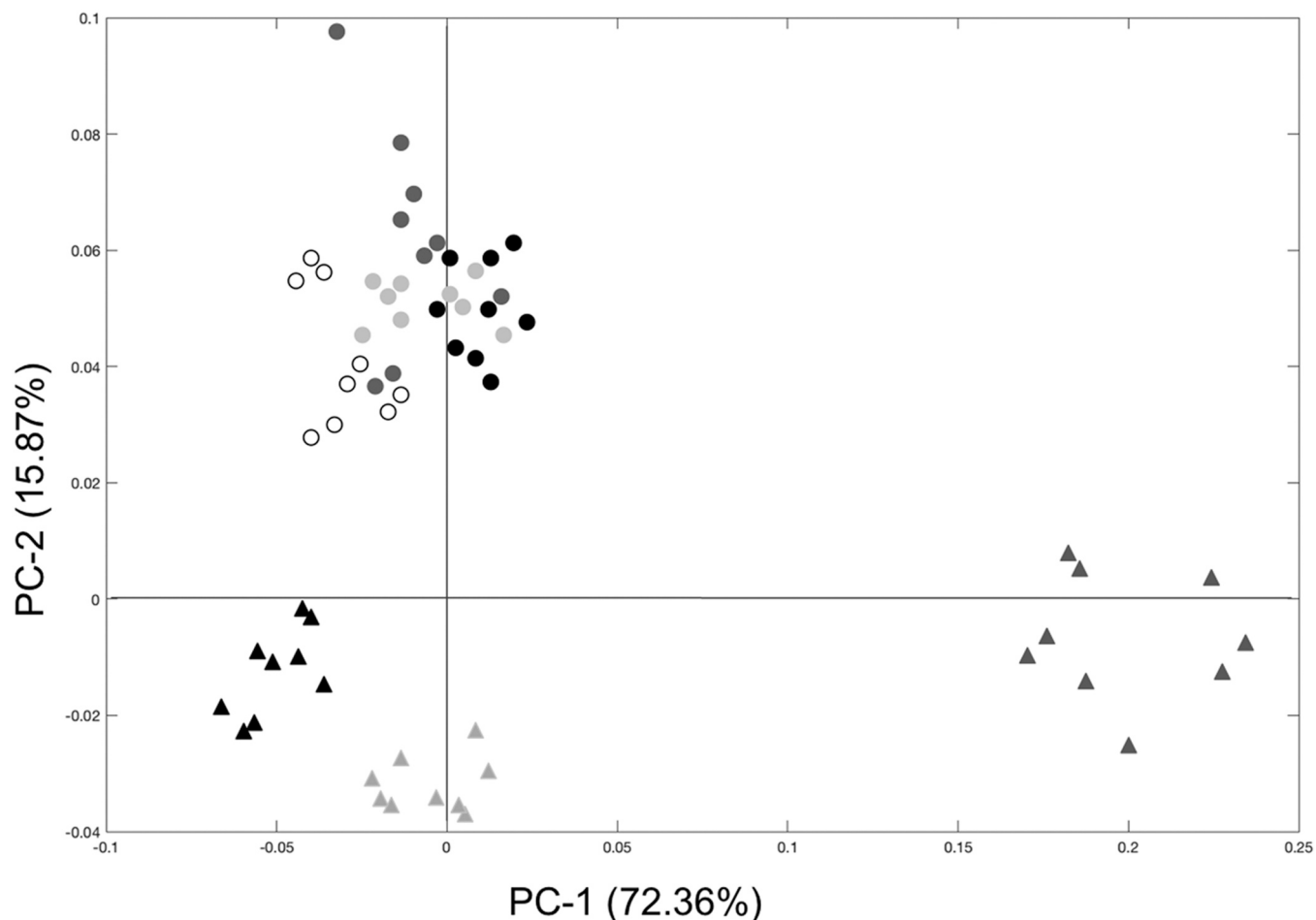


Fig. 2. Principal Components Analysis (PCA) of ^1H NMR spectra of digestive gland extracts ($n = 9$ per each group) showing separation (PC1 vs. PC2) between mussels from control group (circle) and exposed to MPs (triangle), at T0 (white), T24 (light grey), T48 (dark grey), and T72 (black) time-points.

3.4. Changing trend for key metabolites with time

From the PCA score plot shown in Fig. 2, it is evident that the MP-exposed mussels, besides being all clearly separated from controls along the PC2, were also clearly grouped according to their time of exposure, therefore demonstrating to have distinct time-dependent metabolic profiles. In order to unravel the differences in the metabolome among mussel digestive glands collected at T0, T24, T48 and T72, a quantitative analysis of the identified metabolite biomarkers was also performed. The recorded levels of all metabolites were therefore plotted against time and grouped into four major classes, namely amino acids (Fig. 4), metabolites involved in the energy metabolism (Fig. 5), osmolytes and antioxidants (Fig. 6). As the short-term exposure time increased, the majority of the key metabolites (Figs. 4, 5 and 6) increased steadily throughout T0 to T48, and then slightly decreased at T72, however showing values almost always higher than those recorded at T24. Only dimethylglycine (Fig. 4), after a sharp increase from T0 to T48 exhibited a drastic reduction in its concentration at T72, whereas glycine (Fig. 4) was the solely compound that decreased steadily over time. In contrast, some other metabolites including acetoacetate, succinate, malonate (Fig. 5), and hypotaurine (Fig. 6), showed an overall depletion in their level with a rapid rise detected only at T48. Overall, the percent change values of the key metabolites measured in the digestive gland of MP-exposed mussels at all time-points and their associated p -values are summarized in Table 1, together with their proton NMR chemical shift and peak shape.

4. Discussion

The aim of the present study was to characterize comprehensively the early time-dependent metabolic disturbances induced in marine mussels *M. galloprovincialis* after short-term (72 h) exposure to $3\ \mu\text{m}$ polystyrene MPs by using the ^1H NMR-based environmental metabolomics. This approach has been largely used in ecotoxicological studies for exploring changes in metabolite abundance and metabolic pathways in organisms upon exposure to environmental stressors (Cappello, 2020), as well as unravelling the time-dependent relationships between metabolite changes and physiological perturbations, including contaminants exposure (Lin et al., 2006).

After conducting the 72 h experimental trial with mussels challenged with MPs, the presence of $3\ \mu\text{m}$ polystyrene MPs in the digestive gland tissues of exposed mussels was confirmed at all selected time-points (T24, T48, T72) in respect to control mussels where no MPs were found. The uptake and tissue distribution of MPs in filter-feeder bivalves may occur by two different pathways: at the gill surface, mediated by microvilli that actively transport MPs by endocytosis, or via ciliae movement, which transfer MPs to the stomach, intestine and digestive tubules (Von Moos et al., 2012). Therefore, it is reasonable to hypothesize that in this study polystyrene MPs were taken up by mussels via mouth, transported to the gastrointestinal tract and internalized by endocytosis into cells of the digestive system. Worthy of interest, similarly to the data on MPs accumulation herein reported, it was documented that MPs were quickly taken up and accumulated in mussel digestive tubules as early as from 3 h from the exposure to nearly 24 h, to then remain steadily high over 96 h exposure (Von Moos et al., 2012).

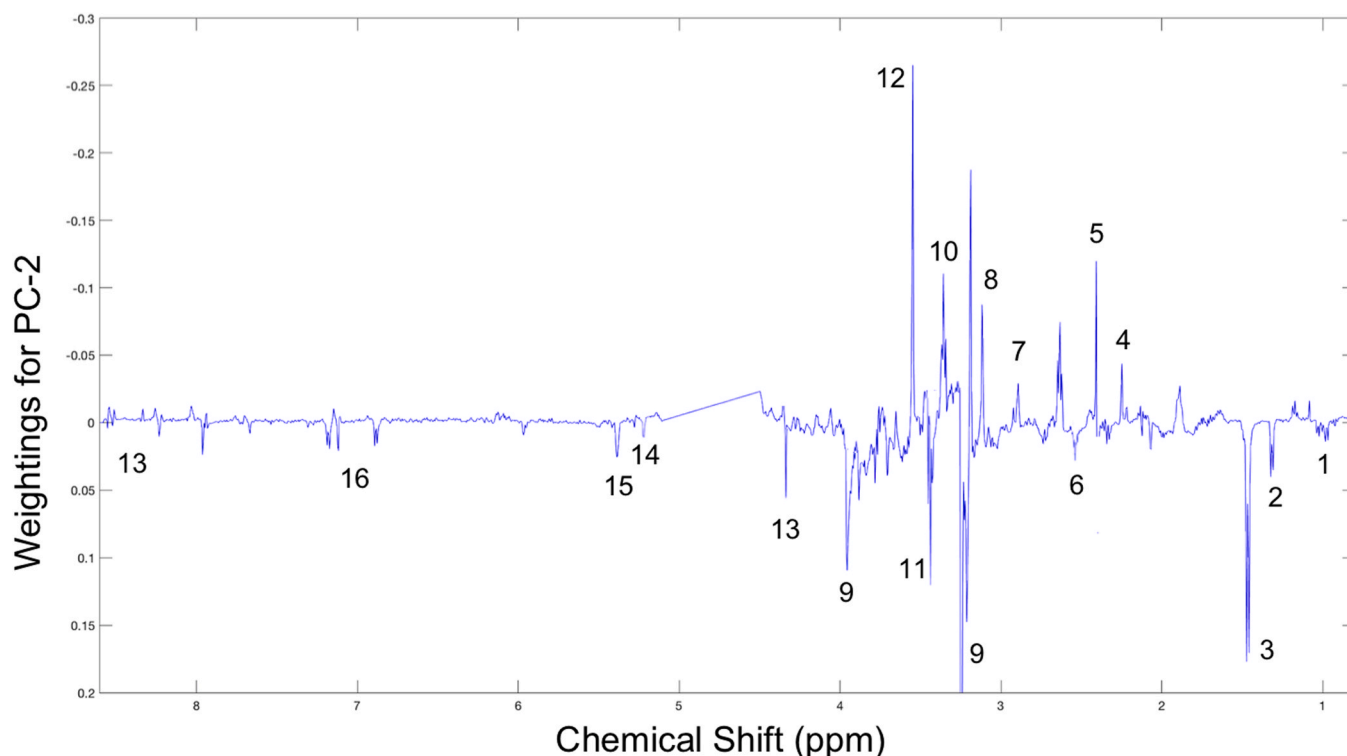


Fig. 3. Corresponding PC2 loading plot showing the metabolic differences between digestive glands of control and MP-exposed mussels. Keys: (1) branched-chain amino acids (BCAAs; isoleucine, leucine, valine), (2) lactate, (3) alanine, (4) acetoacetate, (5) succinate, (6) glutathione, (7) dimethylglycine, (8) malonate, (9) betaine, (10) hypotaurine, (11) taurine, (12) glycine, (13) homarine, (14) glucose, (15) glycogen, and (16) tyrosine.

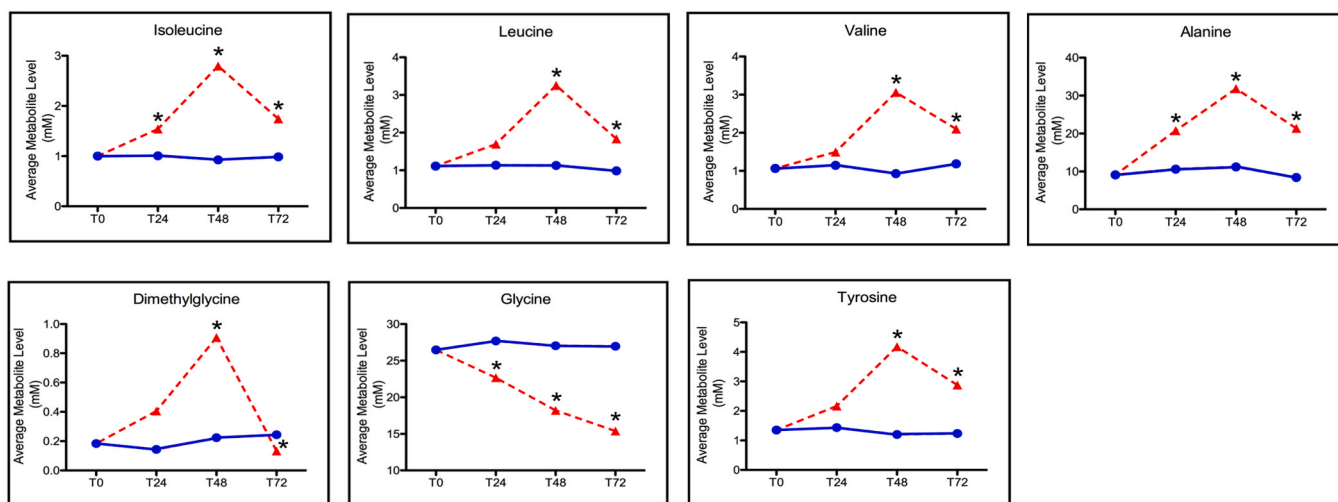


Fig. 4. Plots of the concentrations (mM, expressed as means; $n = 9$) of key amino acids in mussel digestive glands against time. The changes of the metabolites in the control and MP-exposed mussel groups are depicted with a blue solid line and a red dashed line, respectively. Asterisks indicate significant differences relative to control (Dunnett's test; $*p < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Moreover, in regard to ingestion and translocation of $3 \mu\text{m}$ polystyrene MPs in mussels, these microspheres may translocate from the gut cavity to the haemolymph in as little as 3 days (Browne et al., 2008), as supported by the presence of MPs herein observed in mussel haemolymph already at T24 (Cappello and Maisano, 2018).

An integrated description of the perturbations induced by $3 \mu\text{m}$ polystyrene MPs in the metabolome of mussel digestive glands over a 72 h exposure time was herein successfully delivered by NMR-based metabolomics. In detail, PCA indicated that mussels challenged with MPs clustered separately from control, indicating a clear distinction between their metabolic profiles. Metabolomics highlighted that MPs

caused metabolite fluctuations that were associated with disorders in amino acids metabolism, disturbances in the osmoregulatory processes, onset of oxidative stress, and alterations in energy metabolism. Noteworthy, in regard to MP-exposed mussels it was found a clear grouping among the different experimental time-points (T24, T48, T72), indicating differences in their metabolome over the exposure time, not in metabolite composition but in their concentration, with the same metabolite changes observed as functions of time. Overall, dynamic variation in the metabolic responses to MPs were highlighted throughout the exposure period, during which T48 was distinguished as a turning point since all the metabolites from different classes exhibited

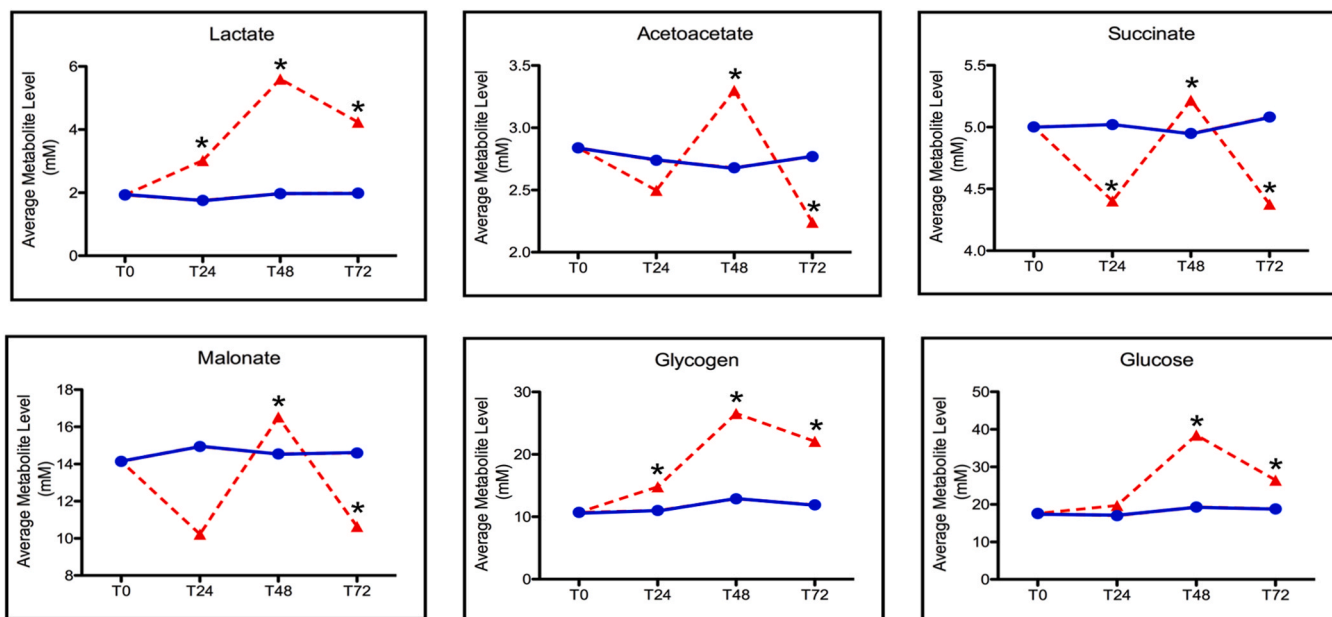


Fig. 5. Plots of the concentrations (mM, expressed as means; n = 9) of key metabolites involved in the energy metabolism in mussel digestive glands against time. The changes of the metabolites in the control and MP-exposed mussel groups are depicted with a blue solid line and a red dashed line, respectively. Asterisks indicate significant differences relative to control (Dunnnett’s test; *p < 0.05). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

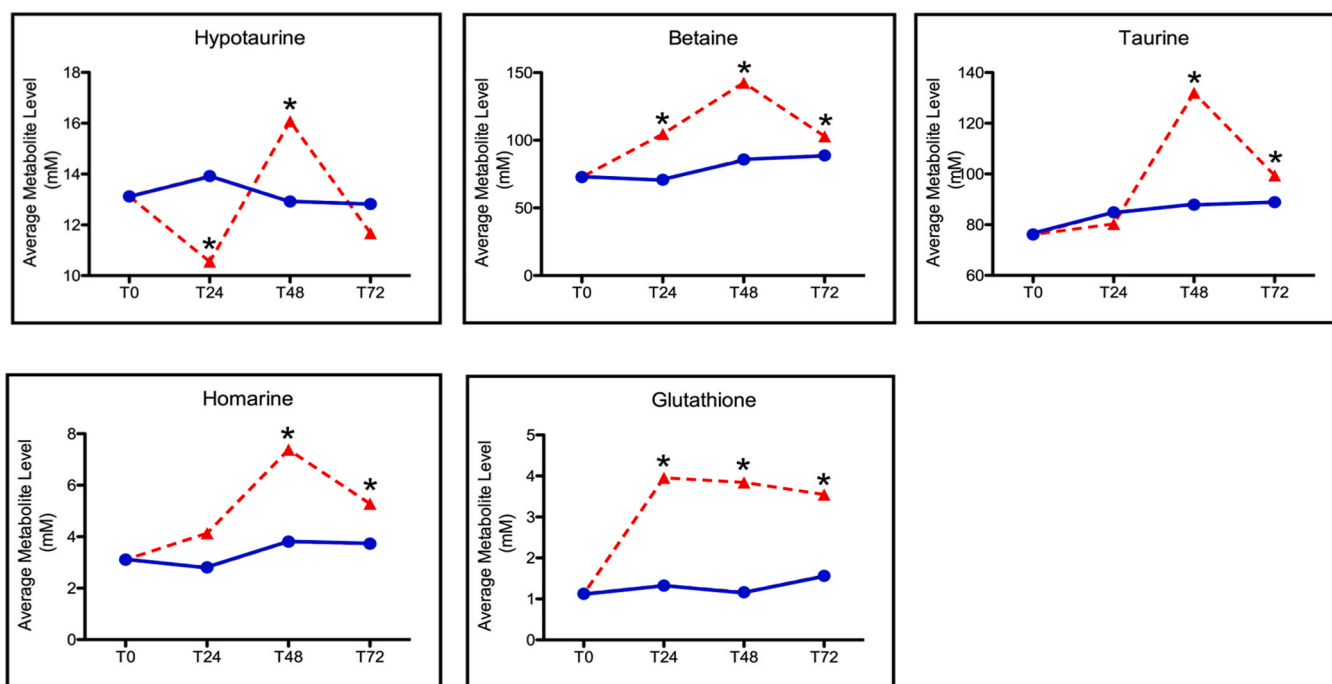


Fig. 6. Plots of the concentrations (mM, expressed as means; n = 9) of key osmolytes and antioxidant in mussel digestive glands against time. The changes of the metabolites in the control and MP-exposed mussel groups are depicted with a blue solid line and a red dashed line, respectively. Asterisks indicate significant differences relative to control (Dunnnett’s test; *p < 0.05). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

opposite changes at T72 relative to the initial 48 h of MP-exposure.

The most relevant metabolite alterations observed between MP-exposed and control groups were those related to amino acids, which increased steadily from T0 to T48, reaching their highest concentrations, and then slightly decreased at T72, except for dimethylglycine and glycine that showed a different trend over time. In marine bivalves, free amino acids constitute a large portion of the metabolome (Henry et al.,

1980), are essential substrates of body proteins and play a crucial role in regulating protein turnover process. The initial elevated levels of amino acids, including BCAAs that are essential proteinogenic amino acids, might result in an enhancement of protein catabolism as an early toxic response to the MP insult, which may induce the formation of reactive oxygen species (ROS) provoking protein damage (Prinz and Korez, 2020), or suggest a remarkable increase in the demand for BCAAs to be

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; q: quartet; m: multiplet).

	Chemical shift and peak shape, ppm	T24	T48	T72
Amino acids				
Isoleucine	0.92 (t), 1.00 (d), 1.26 (m), 1.44 (m), 1.96 (m), 3.66 (d)	↑ 54%*	↑ 179%*	↑ 74%*
Leucine	0.94 (d), 0.96 (d), 1.66 (m), 3.71 (t)	↑ 52%	↑ 192%*	↑ 65%*
Valine	0.98 (d), 1.03 (d), 2.25 (m), 3.59 (d)	↑ 40%	↑ 187%*	↑ 96%*
Alanine	1.46 (d), 3.76 (m)	↑ 128%*	↑ 249%*	↑ 135%*
Dimethylglycine	2.92 (s), 3.70 (s)	↑ 121%	↑ 394%*	↓ 28%*
Glycine	3.54 (s)	↓ 14%*	↓ 31%*	↓ 42%*
Tyrosine	6.89 (d), 7.19 (d)	↑ 60%	↑ 208%*	↑ 112%*
Energy metabolites				
Lactate	1.33 (d), 4.12 (q)	↑ 56%*	↑ 189%*	↑ 119%*
Acetoacetate	2.22 (s), 3.41 (m)	↓ 12%	↑ 16%*	↑ 21%*
Succinate	2.41 (s)	↓ 13%*	↑ 4%*	↓ 12%*
Malonate	3.13 (s)	↓ 27%	↑ 17%*	↓ 25%*
Glycogen	3.40 (m), 3.60 (m), 3.80 (m), 3.96 (s), 5.40 (s)	↑ 38%*	↑ 147%*	↑ 106%*
Glucose	3.23 (m), 3.40 (m), 3.45 (m), 3.52 (dd), 3.73 (m), 3.82 (m), 3.88 (dd), 4.63 (d), 5.22 (d)	↑ 12%	↑ 118%*	↑ 50%*
Osmolytes				
Hypotaurine	2.64 (t), 3.36 (t)	↓ 19%*	↑ 23%*	↓ 11%
Betaine	3.25 (s), 3.89 (s)	↑ 44%*	↑ 95%*	↑ 41%*
Taurine	3.25 (s), 3.41 (t)	↑ 5%	↑ 73%*	↑ 30%*
Homarine	4.35 (s), 7.95 (dd), 8.02 (d), 8.53 (dd), 8.71 (d)	↑ 33%	↑ 137%*	↑ 70%*
Antioxidant				
Glutathione	2.13 (m), 2.54 (m), 2.97 (dd), 3.75 (m), 4.53 (m)	↑ 251%*	↑ 241%*	↑ 215%*

used as protein substrates. Subsequently, the overall reduction in amino acid levels observed at T72 may conversely indicate the onset of opposite mechanisms, such as the induction of protein synthesis in order to repair or replace MP-damaged proteins, as well as to activate cytoprotective and defensive responses to counteract MP toxicity. Taking into consideration the tendency of MPs to accumulate quickly in the digestive tissues at the initial exposure phases to then remain steadily high over time (Von Moos et al., 2012), the differential toxic mechanisms of MPs herein hypothesized to occur in mussels as functions of time gain plausibility. Unfortunately, no studies have addressed the issue of short-term (72 h) time-dependent effects in mussels in the context of MP ecotoxicity up to now, as the biological impact due to MPs has been documented starting from one week of the experimental exposures (Avio et al., 2015; González-Soto et al., 2019; Paul-Pont et al., 2016; Pittura et al., 2018). Nevertheless, evidences of early signs (after 6 h from exposure) of altered health status in mussels due to MPs were reported in digestive tissues with the formation of granulocytomas as an inflammatory cellular response (Von Moos et al., 2012). A significant increase in intracellular ROS level was also described in marine copepods treated with nano- and micro-sized polystyrene microbeads after 24 h exposure (Jeong et al., 2017).

In marine molluscs, free amino acids and their catabolites may also serve as osmolytes to regulate and maintain the intracellular osmotic balance (Yancey et al., 1982). Surprisingly, the concentration patterns of amino acids over the exposure time overlapped with those of osmolytes, except for hypotaurine. Therefore, the noticeably increased levels of amino acids and osmolytes up to T48, together with their successive slight depletion recorded at T72, are consistent with perturbations in the osmoregulatory processes triggered in mussel digestive glands as adaptive mechanisms to MPs. Alteration in osmotic regulation induced by other pollutants such as metals, petrochemical compounds or pharmaceuticals has been previously reported in mussels (Cappello et al., 2017a, 2017b; Wu et al., 2016). However, to the best of our knowledge, no studies have so far explored the impact of MPs on osmoregulatory mechanisms in aquatic biota.

Glycine was the only compound that decreased steadily over time in MP-exposed mussels relative to control. Glycine is a constituent of glutathione (GSH), and the plots depicting their concentrations against time showed an opposite trend of change. The level of GSH, which plays a prominent role in protective processes against oxidative insult (Lushchak, 2011), rose of 250% after 24 h exposure in respect to control, and

then remained constantly high throughout the 72 h experimental trial. The toxicity of MPs mainly arises from oxidative stress via the generation of ROS (Prinz and Korez, 2020), whose accumulation subsequently elicits different biological responses, including the activation of anti-oxidant defense system. Overall, the time of MP exposure, as well as the size, amount and type of MPs under examination, play a significant role in the toxicity mechanisms of MPs. In the present study, the sharp increase in GSH content in response to MPs over the short-term (72 h) exposure implies that its induction is likely to be an important defense mechanism against MP-induced oxidative stress in marine mussels.

In digestive glands, which serve also as a site for carbohydrate storage (Cappello et al., 2018b), alterations over time in the energy metabolism were also herein highlighted by NMR-based metabolomics in mussels challenged with MPs relative to control. The augmented levels of glucose and glycogen, both reaching their highest concentration at T48, may indicate a gluconeogenesis process occurring within the first 48 h of MP exposure as a sign that mussels may need more energy to cope with MP toxicity. Indeed, as the MP-related stress increased, an opposite change in their levels was recorded at T72 in response to the implementation of anti-oxidant and detoxification strategies. Evidences of consumption of energy resources for the activation of defensive mechanisms towards MP toxicity have been previously documented in mussels (Avio et al., 2015; Paul-Pont et al., 2016; Van Cauwenberghe et al., 2015). Additionally, alteration in the levels of lactate, succinate and malonate, the latter two known to be intermediates of the Krebs cycle, were also herein detected over the 72 h exposure period, thus further supporting the dynamic disturbance of energy metabolism in the digestive glands of mussels challenged with MPs.

5. Conclusions

Overall, the present work provides the first insight into the early mechanisms of toxicity of 3 µm polystyrene MPs in marine mussels *M. galloprovincialis* explored at metabolite level. The results presented herein confirmed that polystyrene particles of 3 µm size can accumulate in mussel digestive glands after 24 h exposure. Additionally, the NMR-based environmental metabolomics enabled to characterize comprehensively the time-dependent metabolic effects induced by MPs accumulation in mussel digestive glands over a 72 h exposure period. In detail, dynamic variations in the metabolic responses to MPs were highlighted throughout the exposure period since a different toxic

response to the MP-induced stress was revealed at T72 relative to the initial 48 h of MP-exposure. The metabolic pathways altered by MPs in mussel digestive glands may be summarized with disorders in amino acids metabolism, disturbances in the osmoregulatory processes, onset of oxidative stress, and alterations in energy metabolism. The variations observed at metabolite levels over the exposure time are adaptive and defense responses elicited by mussels challenged by the MPs insult that reflect the fluctuations recorded in the accumulation of MPs within mussel digestive glands over the short-time exposure. It may be supposed that by haemolymph, MPs can easily reach out the digestive tissues, where MPs accumulate as recorded at T24. The high presence of MPs induces in mussels processes for detoxification and/or elimination of MPs. Due to the very small size of the selected MPs (3 μm), the digestive gland is able to counteract very efficiently the presence of MPs by different adaptive and defense responses, as discussed in detail above, and in fact a reduced number of MPs was recorded at T48. Conversely, at T72 the number of MPs slightly increased again because, even if the digestive gland proceeds to excreting MPs, the amount of MPs reaching out the digestive tissue is high and constant over time, and therefore MPs tend to accumulate again in the tissue, inducing different metabolic responses in mussels, as herein observed. It is therefore evident a serious perturbation of the physiological functions of mussel digestive glands upon a short-term exposure to 3 μm polystyrene MPs. The findings of this study therefore provide novel insights into the early mechanisms of MPs ecotoxicity in aquatic invertebrates, highlighting the potential health risk of MPs exposure to aquatic biota.

CRedit authorship contribution statement

Tiziana Cappello: Conceptualization, Methodology, Investigation, Formal analysis, Data curation, Visualization, Supervision, Validation, Writing - original draft, Writing - review & editing. **Giuseppe De Marco:** Formal analysis, Investigation. **Gea Oliveri Conti:** Formal analysis, Investigation, Visualization. **Alessia Giannetto:** Formal analysis. **Margherita Ferrante:** Formal analysis, Investigation, Visualization. **Angela Mauceri:** Supervision, Resources. **Maria Maisano:** Conceptualization, Supervision, Resources, Visualization, Validation, Writing - Review & editing.

Acknowledgments

Authors are thankful to the Gruppo Italiano Discussione Risonanze Magnetiche (GIDRM; Italy) and the Annalaura Segre's family for the scholarship "GIDRM/Borse Annalaura Segre 2017" provided to Dr. Tiziana Cappello.

Conflicts of interest

The authors declare no conflicts of interest in this work.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.ecoenv.2020.111780](https://doi.org/10.1016/j.ecoenv.2020.111780).

References

- Alomar, C., Deudero, S., 2017. Evidence of microplastic ingestion in the shark *Galeus melastomus Rafinesque, 1810* in the continental shelf off the western Mediterranean Sea. *Environ. Pollut.* 223, 223–229.
- Avio, C.G., Gorb, S., Milan, M., Benedetti, M., Fattorini, D., d'Errico, G., Pauletto, M., Bargelloni, L., Regoli, F., 2015. Pollutants bioavailability and toxicological risk from microplastics to marine mussels. *Environ. Pollut.* 198, 211–222.
- Avio, C.G., Gorb, S., Regoli, F., 2017. Plastics and microplastics in the oceans: from emerging pollutants to emerged threat. *Mar. Environ. Res.* 128, 2–11.
- Barnes, D.K., Galgani, F., Thompson, R.C., Barlaz, M., 2009. Accumulation and fragmentation of plastic debris in global environments. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 364, 1985–1998.
- Batel, A., Linti, F., Scherer, M., Erdinger, L., Braunbeck, T., 2016. Transfer of benzo[a]pyrene from microplastics to *Artemia nauplii* and further to zebrafish via a trophic food web experiment: CYP1A induction and visual tracking of persistent organic pollutants. *Environ. Toxicol. Chem.* 35, 1656–1666.
- Bellas, J., Martínez-Armenttal, J., Martínez-Cámara, A., Besada, V., Martínez-Gómez, C., 2016. Ingestion of microplastics by demersal fish from the Spanish Atlantic and Mediterranean coasts. *Mar. Pollut. Bull.* 109, 55–60.
- Bembek-Bailey, S.A., Niemuth, J.N., McClellan-Green, P.D., Godfrey, M.H., Harms, C.A., Gracz, H., Stoskopf, M.K., 2019. NMR metabolomic analysis of skeletal muscle, heart, and liver of hatchling loggerhead sea turtles (*Caretta caretta*) experimentally exposed to crude oil and/or Corexit. *Metabolites* 9, 21.
- Browne, M.A., Dissanayake, A., Galloway, T.S., Lowe, D.M., Thompson, R.C., 2008. Ingested microscopic plastic translocates to the circulatory system of the mussel, *Mytilus edulis* (L.). *Environ. Sci. Technol.* 42, 5026–5031.
- Cappello, T., 2020. NMR-based metabolomics of aquatic organisms. *eMagRes* 9, 81–100.
- Cappello, T., Maisano, M., 2018. Effects of polystyrene microplastics in marine mussel *Mytilus galloprovincialis* by NMR-based metabolomics. 47th National Congress on Magnetic Resonance (GIDRM), Torino, Italy.
- Cappello, T., Pereira, P., Maisano, M., Mauceri, A., Pacheco, M., Fasulo, S., 2016. Advances in understanding the mechanisms of mercury toxicity in wild golden grey mullet (*Liza aurata*) by ^1H NMR-based metabolomics. *Environ. Pollut.* 219, 139–148.
- Cappello, T., Maisano, M., Mauceri, A., Fasulo, S., 2017a. ^1H NMR-based metabolomics investigation on the effects of petrochemical contamination in posterior adductor muscles of caged mussel *Mytilus galloprovincialis*. *Ecotoxicol. Environ. Saf.* 142, 417–422.
- Cappello, T., Fernandes, D., Maisano, M., Casano, A., Bonastre, M., Bebianno, M.J., Mauceri, A., Fasulo, S., Porte, C., 2017b. Sex steroids and metabolic responses in mussels *Mytilus galloprovincialis* exposed to drosiprene. *Ecotoxicol. Environ. Saf.* 143, 166–172.
- Cappello, T., Vitale, V., Oliva, S., Villari, V., Mauceri, A., Fasulo, S., Maisano, M., 2017c. Alteration of neurotransmission and skeletogenesis in sea urchin *Arbacia lixula* embryos exposed to copper oxide nanoparticles. *Comp. Biochem. Physiol. C* 199, 20–27.
- Cappello, T., Giannetto, A., Parrino, V., De Marco, G., Mauceri, A., Maisano, M., 2018a. Food safety using NMR-based metabolomics: assessment of the Atlantic bluefin tuna, *Thunnus thynnus*, from the Mediterranean Sea. *Food Chem. Toxicol.* 115, 391–397.
- Cappello, T., Giannetto, A., Parrino, V., Maisano, M., Oliva, S., De Marco, G., Guerriero, G., Mauceri, A., Fasulo, S., 2018b. Baseline levels of metabolites in different tissues of mussel *Mytilus galloprovincialis* (Bivalvia: Mytilidae). *Comp. Biochem. Physiol. D* 26, 32–39.
- Cappello, T., Maisano, M., Giannetto, A., Natalotto, A., Parrino, V., Mauceri, A., Spanò, N., 2019. Pen shell *Pinna nobilis* L. (Mollusca, Bivalvia) from different peculiar environments: adaptive mechanisms of osmoregulation and neurotransmission. *Eur. Zool. J.* 86, 333–342.
- Caricato, R., Giordano, M.E., Schettino, T., Maisano, M., Mauceri, A., Giannetto, A., Cappello, T., Parrino, V., Ancora, S., Caliani, I., Bianchi, N., Leonzio, C., Mancini, G., Cappello, S., Fasulo, S., Lionetto, M.G., 2019. Carbonic anhydrase integrated into a multimarker approach for the detection of the stress status induced by pollution exposure in *Mytilus galloprovincialis*: a field case study. *Sci. Total Environ.* 690, 140–150.
- Courteney-Jones, W., Quinn, B., Gary, S.F., Mogg, A.O.M., Narayanaswamy, B.E., 2017. Microplastic pollution identified in deep-sea water and ingested by benthic invertebrates in the Rockall Trough, North Atlantic Ocean. *Environ. Pollut.* 231, 271–280.
- Devriese, L.I., van der Meulen, M.D., Maes, T., Bekaert, K., Paul-Pont, I., Frère, L., Robbens, J., Vethaak, A.D., 2015. Microplastic contamination in brown shrimp (*Crangon crangon*, Linnaeus 1758) from coastal waters of the southern North Sea and Channel area. *Mar. Pollut. Bull.* 98, 179–187.
- Giannetto, A., Maisano, M., Cappello, T., Oliva, S., Parrino, V., Natalotto, A., De Marco, G., Barberi, C., Romeo, O., Mauceri, A., Fasulo, S., 2015. Hypoxia-inducible factor α and Hif-prolyl hydroxylase characterization and gene expression in short-time air exposed *Mytilus galloprovincialis*. *Mar. Biotechnol.* 17, 768–781.
- Giannetto, A., Maisano, M., Cappello, T., Oliva, S., Parrino, V., Natalotto, A., De Marco, G., Fasulo, S., 2017. Effects of oxygen availability on oxidative stress biomarkers in the Mediterranean mussel *Mytilus galloprovincialis*. *Mar. Biotechnol.* 19, 614–626.
- González-Soto, N., Hatfield, J., Katsumi, A., Duroudier, N., Lacave, J.M., Bilbao, E., Orbea, A., Navarro, E., Cajaraville, M.P., 2019. Impacts of dietary exposure to different sized polystyrene microplastics alone and with sorbed benzo[a]pyrene on biomarkers and whole organism responses in mussels *Mytilus galloprovincialis*. *Sci. Total Environ.* 684, 548–566.
- Gornati, R., Maisano, M., Pirrone, C., Cappello, T., Rossi, F., Borgese, M., Giannetto, A., Cappello, S., Mancini, G., Bernardini, G., Fasulo, S., 2019. Mesocosm system to evaluate BF-MBR efficacy in mitigating oily wastewater discharges: an integrated study on *Mytilus galloprovincialis*. *Mar. Biotechnol.* 21, 773–790.
- Henry, R.P., Mangum, C.P., Webb, K.L., 1980. Salt and water balance in the oligohaline clam, *Rangia cuneata* II. accumulation of intracellular free amino acids during high salinity adaptation. *J. Exp. Zool.* 211, 11–24.
- Jeong, C.B., Kang, H.M., Lee, M.C., Kim, D.H., Han, J., Hwang, D.S., Souissi, S., Lee, S.J., Shin, K.H., Park, H.G., Lee, J.S., 2017. Adverse effects of microplastics and oxidative stress-induced MAPK/Nrf2 pathway-mediated defense mechanisms in the marine copepod *Paracyclopsina nana*. *Sci. Rep.* 7, 41323.
- Kramm, J., Volker, C., 2018. Understanding the risks of microplastics: a social-ecological risk perspective. In: Wagner, M., Lambert, S. (Eds.), *Freshwater Microplastics: Emerging Environmental Contaminants?*, 58 Springer, Heidelberg. *Env. Chem.*, pp. 223–238.

- Li, J., Lusher, A.L., Rotchell, J.M., Deudero, S., Turra, A., Brate, I.L.N., Sun, C., Hossain, M.S., Li, Q., Kolandhasamy, P., Shi, H., 2019. Using mussel as a global bioindicator of coastal microplastic pollution. *Environ. Pollut.* 244, 522–533.
- Lin, C.Y., Viant, M.R., Tjeerdema, R.S., 2006. Metabolomics: methodologies and applications in the environmental sciences. *J. Pestic. Sci.* 31, 245–251.
- Lu, Y., Zhang, Y., Deng, Y., Jiang, W., Zhao, Y., Geng, J., Ding, L., Ren, H.-Q., 2016. Uptake and accumulation of polystyrene microplastics in zebrafish (*Danio rerio*) and toxic effects in liver. *Environ. Sci. Technol.* 50, 4054–4060.
- Lushchak, V.I., 2011. Environmentally induced oxidative stress in aquatic animals. *Aquat. Toxicol.* 101, 13–30.
- Maisano, M., Cappello, T., Natalotto, A., Vitale, V., Parrino, V., Giannetto, A., Oliva, S., Mancini, G., Cappello, S., Mauceri, A., Fasulo, S., 2017. Effects of petrochemical contamination on caged marine mussels using a multi-biomarker approach: histological changes, neurotoxicity and hypoxic stress. *Mar. Environ. Res.* 128, 114–123.
- Oliveri Conti, G., Ferrante, M., Banni, M., Favara, C., Nicolosi, I., Cristaldi, A., Fiore, M., Zuccarello, P., 2020. Micro- and nano-plastics in edible fruit and vegetables: the first diet risks assessment for the general population. *Environ. Res.* 187, 109677.
- Paul-Pont, I., Lacroix, C., Fernández, C.G., Hégaret, H., Lambert, C., Le Goïc, N., Frere, L., Cassone, A.L., Sussarellu, R., Fabioux, C., Guyomarch, J., Albertosa, M., Huvet, A., Soudant, P., 2016. Exposure of marine mussels *Mytilus* spp. to polystyrene microplastics: toxicity and influence on fluoranthene bioaccumulation. *Environ. Pollut.* 216, 724–737.
- Pittura, L., Avio, C.G., Giuliani, M.E., d'Errico, G., Keiter, S.H., Cormier, B., Gorbi, S., Regoli, F., 2018. Microplastics as vehicles of environmental PAHs to marine organisms: combined chemical and physical hazards to the Mediterranean mussels, *Mytilus galloprovincialis*. *Front. Mar. Sci.* 5, 103.
- Prinz, N., Korez, Š., 2020. Understanding how microplastics affect marine biota on the cellular level is important for assessing e tion: a Review. In: Jungblut, S., Liebich, V., Bode-Dalby, M. (Eds.), *YOUMARES 9 - The Oceans: Our Research, Our Future*. Springer, Cham.
- Qiao, R., Sheng, C., Lu, Y., Zhang, Y., Ren, H., Lemos, B., 2019. Microplastics induce intestinal inflammation, oxidative stress, and disorders of metabolome and microbiome in zebrafish. *Sci. Total Environ.* 662, 246–253.
- Ribeiro, F., Garcia, A.R., Pereira, B.P., Fonseca, M., Mestre, N.C., Fonseca, T.G., Ilharco, L.M., Bebianno, M.J., 2017. Microplastics effects in *Scrobicularia plana*. *Mar. Pollut. Bull.* 122, 379–391.
- Rochman, C.M., Tahir, A., Williams, S.L., Baxa, D.V., Lam, R., Miller, J.T., Teh, F.C., Werorilangi, S., Teh, S.J., 2015. Anthropogenic debris in seafood: plastic debris and fibers from textiles in fish and bivalves sold for human consumption. *Sci. Rep.* 5, 14340.
- de Sà, L.C., Luis, L.G., Guilhermino, L., 2015. Effects of microplastics on juveniles of the common goby (*Pomatoschistus microps*): confusion with prey, reduction of the predatory performance and efficiency, and possible influence of developmental conditions. *Environ. Pollut.* 196, 359–362.
- de Sà, L.C., Oliveira, M., Ribeiro, F., Rocha, T.L., Futter, M.N., 2018. Studies of the effects of microplastics on aquatic organisms: what do we know and where should we focus our efforts in the future? *Sci. Total Environ.* 645, 1029–1039.
- Van Cauwenberghhe, L., Claessens, M., Vandegheuchte, M.B., Janssen, C.R., 2015. Microplastics are taken up by mussels (*Mytilus edulis*) and lugworms (*Arenicola marina*) living in natural habitats. *Environ. Pollut.* 199, 10–17.
- Vignet, C., Cappello, T., Fu, Q., Lajoie, K., De Marco, G., Clerandau, C., Mottaz, H., Maisano, M., Hollender, J., Schirmer, K., Cachot, J., 2019. Imidacloprid induces adverse effects on fish early life stages that are more severe in Japanese medaka (*Oryzias latipes*) than in zebrafish (*Danio rerio*). *Chemosphere* 225, 470–478.
- Von Moos, N., Burkhardt-Holm, P., Kohler, A., 2012. Uptake and effects of microplastics on cells and tissue of the blue mussel *Mytilus edulis* L. after an experimental exposure. *Environ. Sci. Technol.* 46, 11327–11335.
- Vroom, R.J.E., Koelmans, A.A., Besseling, E., Halsband, C., 2017. Aging of microplastics promotes their ingestion by marine zooplankton. *Environ. Pollut.* 231, 987–996.
- Wu, H., Xu, L., Yu, D., Ji, C., 2016. Differential metabolic responses in three life stages of mussels *Mytilus galloprovincialis* exposed to cadmium. *Ecotoxicology* 26, 74–80.
- Yancey, P.H., Clark, M.E., Hand, S.C., Bowlus, R.D., Somero, G.N., 1982. Living with water stress: evolution of osmolyte systems. *Science* 217, 1214–1222.
- Zhu, J., Yu, X., Zhang, Q., Li, Y., Tan, S., Li, D., Yang, Z., Wang, J., 2019. Cetaceans and microplastics: first report of microplastic ingestion by a coastal delphinid, *Sousa chinensis*. *Sci. Total Environ.* 659, 649–654.
- Zuccarello, P., Ferrante, M., Cristaldi, A., Copat, C., Grasso, A., Sangregorio, D., Fiore, M., Oliveri Conti, G., 2019a. Exposure to microplastics (< 10 µm) associated to plastic bottles mineral water consumption: The first quantitative study. *Water Res.* 157, 365–371.
- Zuccarello, P., Ferrante, M., Cristaldi, A., Copat, C., Grasso, A., Sangregorio, D., Fiore, M., Oliveri Conti, G., 2019b. Reply for comment on “Exposure to microplastics (<10 µm) associated to plastic bottles mineral water consumption: the first quantitative study by Zuccarello et al. [Water Research 157 (2019) 365–371]”. *Water Res.* 166, 115077.