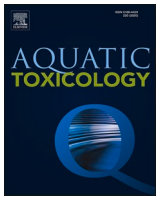
Aquatic Toxicology 262 (2023) 106669

Contents lists available at ScienceDirect

Aquatic Toxicology

journal homepage: www.elsevier.com/locate/aqtox



Study of the ageing and the sorption of polyaromatic hydrocarbons as influencing factors on the effects of microplastics on blue mussel

Romaric Moncrieffe [a], Maria Masry [b], Binbin Cai [a], Stéphanie Rossignol [b], Abderrahmane Kamari [a], Laurence Poirier [a], Samuel Bertrand [a,c], Pascal Wong-Wah-Chung [b], Aurore Zalouk-Vergnoux [a,*]

[a] Nantes Université, Institut des Substances et Organismes de la Mer, ISOMer, UR 2160, Nantes F44000, France
[b] Aix Marseille University, CNRS, LCE, Marseille, France
[c] ThalassOMICS Metabolomics Facility, Plateforme Corsaire, Biogenouest, Nantes, France

ARTICLE INFO

Keywords:
Polyethylene
PAH
Weathering
Biomarkers
Lipidomics
Blue mussel

ABSTRACT

The mussels are species with high socio-economic weights and are often used as bioindicators of biological and chemical contamination. In the field and aquaculture, they can intake microplastics during filter-feeding, and the microplastics can have a negative impact on their health, even at low concentrations. The effects of microplastics have yet to be fully examined on the blue mussel (*Mytilus edulis*), considering the factors of ageing and sorption of some polyaromatic hydrocarbons (PAHs), ubiquitous environmental contaminants. In this work, 5 different exposure conditions were studied: pristine microplastics, microplastics aged for 1000 days under UV radiation, microplastics sorbing PAHs, as well as microplastics both aged and sorbing PAHs, in parallel to controls. The microplastic changes after ageing were studied with spectroscopic and chromatographic methods. Then, 8-day laboratory exposures of mussels at 10 μg/L of microplastics were performed. The oxidative stress, as well as neurotoxic and immunological responses of *M. edulis*, were measured using a battery of biomarkers (catalase/CAT, superoxide dismutase/SOD, glutathione S-transferases/GST, acetylcholinesterase/AChE) in 3 different organs (digestive gland, gills and mantle), and acid phosphatase in hemolymph. Then, a study of lipid impairments on the digestive gland was performed through the use of lipidomic tools. No significant difference of oxidative stress activity was observed for all the tissues of mussels exposed to pristine microplastics at 10 μg/L, compared to controls. The ageing and the PAH soption onto microplastics were influencing factors of the oxydative stress in mussels with increased CAT activities in the digestive glands and decreased SOD activities in the mantles. The neurotoxicity was highlighted by higher AChE activities measured in the mantle of mussels exposed to all the microplastic treatments, compared to controls. Concerning lipidomics, no compound was determined as a biomarker of microplastic exposure. The study demonstrated a low toxicity of microplastics at environmental relevant concentration with a 8-day exposure and using the chosen biomarkers. However, some microplastic changes seemed to lead to specific effects on mussels.

1. Introduction

Historically, the word 'plastic' was only an adjective, meaning easily shaped or molded. Now, the word has evolved to describe the millions of materials which are made from polymers – long chain macromolecules which can be pressed, molded, heated, and hardened easily, quickly, and efficiently. With a size ranging from 1 to 5 mm, the plastic particles are called 'microplastics' (Thompson et al., 2004). Primary microplastics are micro-sized particles used in daily products such as fibers, pellets or microbeads (Andrady, 2011). Moreover, plastics being unreactive, not easily biodegradable or degraded by physical processes, such as erosion or weathering, they degrade into smaller and smaller pieces (Andrady, 2011; Barnes et al., 2009), which correspond to the secondary microplastics. Most of primary and secondary microplastics enter the environment and are carried easily through waterways and to the sea or other large bodies of water when they become small enough.

In aquatic media, microplastics can either sink into the sediment layer, or float on the surface, where their hydrophobic nature and high

* Corresponding author.
E-mail address: aurore.zalouk-vergnoux@univ-nantes.fr (A. Zalouk-Vergnoux).

https://doi.org/10.1016/j.aquatox.2023.106669
Received 5 May 2023; Received in revised form 21 August 2023; Accepted 24 August 2023
Available online 25 August 2023

surface area to volume ratio give them a tendency to attract and concentrate hydrophobic organic pollutants (Engler, 2012; Pittura et al., 2018), such as polyaromatic hydrocarbons (PAHs). PAHs are ubiquitous in the marine environment, also known to lead to adverse effects on humans and organisms (Grimmer, 2018), explaining why 16 of them are registered on European and American lists (U.S. Environmental Protection Agency, EPA) of priority pollutants and must be monitored in the environment. Their production is diffuse and unavoidable since it corresponds to the incomplete combustion of organic matter – meaning it can be both natural (forest fires, volcanism) and anthropogenic.

During their transport, microplastics are susceptible to UV radiation, which could degrade them, changing their surface area and their reactivity leading to releases of potentially toxic compounds into the water, such as additives like Bisphenol A (Pflugmacher et al., 2020), or sorbed environmental organic compounds which can induce different ecotoxicities (Hanun et al., 2021; Rodrigues et al., 2019). However, a gap of knowledge still remains about the effects of aged microplastics on aquatic organisms compared to pristine ones. Some studies showed that the ageing of microplastics can (i) promote the ingestion by marine zooplakton (Vroom et al., 2017), (ii) amplify the growth inibition of *Chlorella vulgaris* (Fu et al., 2019) and (iii) impact the gene expression levels of the marine mussel *Mytilus edulis* (Jaouani et al., 2023).

Plastic being ubiquitous in the water column, no aquatic species is preserved from its presence. This suggestion is further supported by hundreds of researches, which have studied filter feeder species, such as worms, crabs, bivalves, and lobsters (Lusher et al., 2013). Filter feeders are focal points for environmental toxicants which makes them an effective indicator species. Because they tend to accumulate environmental contaminants, studying them gives us a snapshot of the overall contamination of the ecosystem. The environmental contaminants can negatively impact organisms that consume them. Therefore, those organisms are sentinel species as well as biological models for laboratory exposures.

The blue mussel, *Mytilus edulis*, is one of the most important commercial species of mussel. Besides its commercial importance, the blue mussel also serves as a first warning for environmental monitoring. They can also be used for bay rehabilitation programs to help clean contamination from unhealthy bodies of water (Kotta et al., 2020). Furthermore, the potential of industry disruption keeps the blue mussel constantly at the forefront of scientific research, as understanding how the organism responds to emerging changes can help prepare hatcheries for upcoming tragedies and a changing climate. Unlike many other commercially important organisms in aquaculture, such as fish and crustaceans, mussels are nearly completely sedentary. They are easy to find, reproduce rapidly, and have a fully mapped life cycle. They are relatively simple organisms while also producing stress enzymes directly linked to environmental disturbances. All of these factors make them an experimentally cost-effective organism, perfect for dissection and experiments (Lusher et al., 2013).

The purpose of the experiment is to shed a bit of light on the effect of polyethylene (PE) microplastics on the blue mussel, taking into account potential influencing factors: ageing and environmental organic compounds sorption, *i.e.* PAHs. The processes of environmental ageing decrease the particle size, change the surface reactivity and increase the roughness and the specific surface area (Luo et al., 2020). These physico-chemical alterations have consequences on the behavior of the plastic items and also on their ecotoxicity. PAHs were here chosen as environmental contaminant models because they are ubiquitous, persistent, naturally produced, unintentionnaly produced by anthropogenic activities and toxic for living beings (Johnsen and Karlson, 2005; Shin et al., 2006). Moreover, a previous study showed PAHs as among the predominant families of persistent organic pollutants sorbed onto microplastics in the environment (Frias et al., 2010). The effects of PAH pollution have been previously studied (Speciale et al., 2018). However the assessment of their effects when in combination with microplastics and aged microplastics, is still incomplete (Martín et al., 2022). From the best of our knowledge, it is the first published work performing laboratory exposures combining both ageing and PAH sorption onto microplastics. To explore these scientific questions, *M. edulis* was used as a biological model. After an 8-day exposure with different treatments of microplastic contamination at 10 μg/L, a battery of biomarkers was measured in digestive gland, gills and mantle, such as enzymes involved in oxidative stress – catalase (CAT), glutathione S-transferase (GST), superoxide dismutase (SOD) – and another traducing neurotoxicity, *i.e.* acetylcholinesterase (AChE). As a marker of immunotoxicity, acid phosphatase (AcP) was studied in the hemolymph. For an in-depth assessment of the potential effects of these microplastics on the blue mussel, a lipidomic untargeted approach was performed on the digestive gland to highlight potential features of interest related to the different exposure conditions, corresponding to lipid metabolites, either down- or up-regulated.

2. Materials and methods

2.1. Pristine microplastics

The microplastics used in the experiment were commercial PE microplastic beads (PE Cospheric: Blue Polyethylene Microspheres 1.08 g/cc; size distribution: 27–45 μm). The size was chosen because it can be ingested by the blue mussel (Phuong et al., 2018). Moreover, PE corresponds to the second most produced plastic type, after polypropylene. Its density allowed quite a floating behavior, adequate to ensure the contact between microplastics and mussels during the exposure, thanks to the air diffusion in the tanks. PE is also a plastic polymer characterized by low crystallinity with a high surface area and free volume, which means it is a great sorbent (Rochman et al., 2016). The representativity of the microplastic environmental exposure is not fully ensured by the use of these spherical commercial microplastics, but it was required to study the effect of the ageing and to make the replicates reproducible.

2.2. Microplastic stock solution preparations

The stock solution of pristine microplastics was performed by the addition of 4 mg of microplastics and 20 μL of methanol in 200 mL of water. Some of the pristine microplastics were first irradiated for 1000 h in an accelerated weathering chamber SEPAP 12/24 (Atlas) to simulate an environmental ageing. The corresponding stock solution was performed the same way as for pristine microplastics (4 mg of aged microplastics + 200 mL of water + 20 μL of methanol). To simulate PAH sorption, 4 mg of each of the pristine and irradiated microplastics were suspended in 200 mL of water with the addition of 20 μL of methanol containing four PAHs, constituting two other stock solutions. The PAHs were chosen as representatives of those found in environmentally contaminated plastics (Hirai et al., 2011; Rios et al., 2007). It was a mix of two low molecular weight (LMW) PAHs – phenanthrene and fluoranthene (Phe-and Fluo) – and two high molecular weight (HMW) ones – benzo(a)anthracene and benzo(a)pyrene (BaAnt and BaPyr). The final concentrations of PAHs in the medium were 1 μg/L of Phe-and Fluo and 0.5 μg/L of BaAnt and BaPyr. The PAHs and aged microplastics were mixed after the ageing of the microplastics in the accelerated weathering chamber. For the control tank contamination, 20 μL of methanol were added to 200 mL of water. All these five stock solutions were used for spiking the tanks with mussel during the exposure. As 20 μL of methanol were added in each stock solution, including the control, the potential effects were not attributed to the presence of methanol, needed for the dissolution of the PAHs.

2.3. Microplastic characterization

To characterize the microplastics before and after ageing, infrared spectroscopy, thermo-desorption coupled to gas chromatography-mass spectroscopy (TD-GCMS–) and gas chromatography coupled to mass

spectroscopy (GC–MS) were used to determine the carbonyl index of aged microplastics (AMP), the volatile organic compound (VOC) contents and the organic compound contents respectively.

Regarding the infrared analysis, a FRONTIER spectrometer (PekinElmer) was used in attenuated total reflectance mode to acquire the infrared spectra of MP and AMP and to determine the carbonyl index of aged microplastics. The carbonyl index was calculated according to the following equation:

Carbonyl index = A_{1712} / A_{2916}

A_{1712} is the absorbance at 1712 cm^{-1} relative to the photodegradated products (carbonyl group C=O) and A_{2916} is the absorbance at 2916 cm^{-1} corresponding to the PE reference peak (methylene group $-CH_2-$).

The detailed protocols for analysing VOCs and hexane extracted organic compounds from the microplastics are given in Supplementary Material 1.

The quantification of PAHs sorbed onto the PE microspheres was not possible due to the small amounts of microplastics and PAHs used for the exposure, insufficient for the detection limits of analytical methods. Nevertheless, theoretical values based on PE-water partition coefficients allowed to assess the concentrations of PAHs in the water and sorbed onto PE microspheres in tanks used for the mussel exposure.

2.4. Experimental design

The experimental design was very similar to the experiment of a previous study performed by Khalid et al. (2021). All samples of live *M. edulis* from Ireland were selected in the fresh arrival of mussels in a supermarket (Super U, Nantes, France). Then, they were weight, sized and randomly sorted into 15 separate and cleaned tanks, with dead or cracked mussels discarded. The organism density was 1 mussel per liter of artificial seawater, prepared using sea salt (InstantOcean, Aquarium systems, France) solubilized in distilled water at 33 PSU (Practical Salinity Unit). The tanks were made of glass, with a 20 L total volume, and were equiped with an air diffusion system. To ensure the good health of the mussels before exposure and to acclimate them to the experimental conditions, the first step was an 8-day acclimation period. During the acclimation, they have been fed with a solution of pre-coral phyton (Tropic marin®, Germany) every 2 days, just after the change of water, which occurred every 2 days as well. The mussels were daily checked, particularly during the feeding, checking the filtration activity. Throughout the experiment the conditions were maintained constant in a controlled room with a 12:12 photoperiod and a temperature of 15°C.

After the step of acclimation, the 8-day exposure step followed the contamination of the tanks with the different types of microplastics at 10 μg/L. This concentration was chosen to be close to environmental ones, like in the Mediterranean Sea where it reaches 26 μg/L (Suaria et al., 2016). The contamination was performed by the addition of 10 mL of the suitable stock solution prepared as previously mentioned (2.1 section) to the corresponding tank. The five different treatments were performed in triplicates (R1, R2 and R3). These treatments corresponded to tanks contaminated with either pristine PE microplastics (MP), or PE microplastics irradiated (AMP), or pristine PE microplastics sorbing polyaromatic hydrocarbons (MP-PAH), or irradiated PE microplastics sorbing polyaromatic hydrocarbons (AMP-PAH). Controls were also performed in triplicates by the addition of 10 mL of the corresponding solution (200 mL water + 20 μL methanol). The feeding and the water changes were kept the same than for the acclimation step – every 2 days – with the air diffusion allowing the distribution and the suspension of the microplastics in the tanks. After the changes of water performed every 2 days, the tanks were re-contaminated with 10 mL of the stock solution corresponding to the treatment to mimic a constant exposure. The aim of the work was not to determine acute toxicity by exposing organisms to high concentrations of pollutants. As the concentrations of microplastics were chosen as relevant to the environment, an exposure duration of more than few hours was required. Furthermore, previous publications on the effects of microplastics on mussels have shown effects after one week of exposure (Alnajar et al., 2021); Avio et al., 2015; Khalid et al., 2021; Paul-Pont et al., 2016). These reasons explain why an 8-day experiment was carried out for this work.

After 8 days of exposure and without a depuration period, the mussels were removed and frozen in liquid nitrogen at −80°C to quickly kill them as ethically as possible and avoiding a potential stress caused by a slow death. Once frozen, they were kept in long-term storage at −20°C.

After being frozen, 5 mussels were randomly selected from each tank, then thawed, weighed and dissected, constituting a total of 75 mussels, *i.e.* 15 per treatment. Dissected organs included the digestive glands (DG), cut into two equal halves (for individual biomarker analysis and lipidomics), gills (G), mantle (M), and hemolymph (H), constituting 375 subsamples. Hemolymph was collected puncturing the posterior adductor muscle with a sterile syringe. Extracted organs were separated and labeled according to the exposure treatment and stored at −20°C until they were required for further analysis.

2.5. Biochemical markers

2.5.1. Biological sample preparation

The three dissected organs – DG, G, M – were prepared in the same way. About 1/3rd of each sample was removed from storage and weighed. Except for the GST analysis, the tissues were prepared by the addition of a solution of Trisma buffer (pH 7.4) and PMSF (1 mM; Sigma-Aldrich, USA), in a ratio of 4:1 (solution:sample weight (v/w)) into Eppendorf tubes. Trisma with PMSF is a buffer designed to protect the proteins and DNA found in the solution by keeping it at a stable pH. Then, the samples were ground to ensure an even mix with the Trisma and the protein extraction. After grinding, the tubes were placed in a centrifuge at 14,000 rpm for 30 min before individual biomarker measures. Rather than a Trisma buffer, a sodium phosphate buffer was employed for the extraction of GST from tissues with the same ratio 4:1. The sodium phosphate buffer was found to work more efficiently with GST and withstood the significant pH drop that adding reagents induced.

2.5.2. Total protein analysis

Total Proteins (TP) were determined using Bradford protocol (Bradford, 1976) and 96-well plates read at 595 nm with a UV–Vis spectrophotometer and using the bovine serum albumin (BSA) for the calibration curve. After the subtraction of the blank values, concentrations were calculated from the calibration curve. TP contents were analyzed in digestive gland, gills and mantle in triplicates. The following enzyme activities were expressed with reference to TP content in sample tissues, corresponding to specific activities.

2.5.3. Catalase analysis

Catalase (CAT) analysis was adapted from the Clairborne method (1985). CAT is an enzyme produced in nearly all living organisms, and when exposed to Reactive Oxygene Species (ROS) will rapidly decompose it. Therefore, CAT was measured in the samples during a kinetic with the addition of H_2O_2, measured every minute at 240 nm over 3 min.

2.5.4. SOD analysis

Superoxide dismutase (SOD) analysis was adapted from Beauchamp and Fridovich (1971) and Sim Choi et al. (2006). In cells, SOD is an enzyme which breaks down ROS and is produced in stressed organisms to aid in decrease oxidative stress. The amount of SOD produced is directly related to the stress of the organism, so it is a helpful biomarker. The reaction was followed at 560 nm with the addition of nitrotetrazolium bluechloride riboflavin, methionine and EDTA after being exposed to a light source.

2.5.5. GST analysis

The protocol for Glutathione S-transferases (GST) was adapted from Habig et al. (1974). GST is a detoxifying agent in the cell and helps protect cells from several potentially harmful compounds. In the presence of 1-chloro-2–4 dinitrobenzene (CDNB) and reduced glutathione (GSH), it conjugates the two at a linear rate.

2.5.6. AChE analysis

The protocol for the analysis of acetylcholinesterase (AChE) activity was adapted from Ellman et al. (1961). AChE, biomarker of neurotoxicity, is an enzyme responsible for the deactivation and subsequent breakdown of acetylcholine, a neurotransmitter responsible for signaling muscle contraction. The absorbance of the samples was measured at 405 nm over 15 min, with readings taken every minute after the addition of Ellmann's reagent.

2.5.7. Acid phosphatase

An Acid Phosphatase (AcP) Kinetic Method Kit (Biolabo Reagents) was used for its determination in mussel hemolymph, as a biomarker of immunotoxicity. Acid Phosphatase is an antigen, which helps activate portions of the immune system in response to stress triggers in stressed and sick organisms, increasing levels of AcP. The hemolymph was used for this biomarker analysis. Added to a commercial reagent (Biolabo Reagents), the absorbance at 405 nm was measured over 3 min, with readings taken every minute.

2.6. Lipidomics

Lipids play an important role in homeostasis of organisms. In *M. edulis*, lipids could show how the organism reacts to chronic and acute exposure to stressors. Lipidomics was employed as an untargeted approach and an extraction protocol allowing the extraction of the maximum of hydrophobic compounds from the samples. By this way, the method allowed the potential identification of new stress compounds, and a more in-depth analysis of stress responses. In this particular case, the aim was to determine if specific lipid compositions, as broaden as possible, were related to mussel responses to microplastics as performed by Khalid et al. (2021) and presented in Supplementary Material 1.

2.7. Statistical data treatment

To highlight the significant differences of weights and biomarkers (TP, SOD, GST, CAT, AChE and AcP) between the exposure treatments (MP, AMP, MP-PAH, AMP-PAH, control), statistical analyses were done using XLStat software. A one-way non-parametric Anova, Kruskal-Wallis test was conducted. Significance was determined if a p-value < 0.05 was found.

From lipidomic data, the matrices were filtered and the peak areas were taken into account only when the signal was 3-times higher than within the blanks and when the QC signals were proportionnal to their concentrations. Finally, an auto-scaling was done (Van den Berg et al., 2006) on the matrices containing 419 and 550 features for positive and negative ionization modes respectively, to perform an unsupervised analysis by Principal Component Analysis (PCA), and a supervised one by Partial Least Square regression with Discriminant Analysis (PLS-DA) using the online software MetaboAnalyst 5.0, online platform (Pang et al., 2021). The PLS-DA analysis gathering each treatment, on the one hand, and the control, on the other hand, gave a list of features "Very Important for Projection" (VIP) with a corresponding score which could correspond to lipids impacted by the microplastic contamination (Chong et al., 2019; Kind et al., 2013). Finally, the putative annotation of the features of interest was achieved based on accurate mass using the Lipid Blast Database (Kind et al., 2013) and Lipid Maps® Database, of which the searching engine can be accessed in the website (Liebisch et al., 2020, 2013).

3. Results

3.1. Microplastic characterization

3.1.1. Carbonyl index

Infrared spectra obtained from pristine and aged microplastics (Supplementary Material 2, Fig. S2) showed an additional band around 1700 cm^{-1} for the aged polymer compared to pristine one. This band reflects the presence of carbonylated compounds and thus traduces an observable photodegradation of the microplastics after irradiation in an accelerated weathering chamber during 1000 h. A carbonyl index of 0.036 was calculate for aged microplastics. However, this value is relatively low compared to those found in environmental samples, which typically range from 0.1 to 1.2 (Prata et al., 2020). According to the supplier Cospheric LLC, this was due to the stabilizing formula of the PE that can contain up to 30% of stabilizers.

3.1.2. Organic compound content

The lists of VOCs and organic compounds detected by TD-GC–MS and GC–MS, respectively are given in Supplementary Material 3. As presented in the Table S3–1, VOCs in pristine microplastics consisted mainly of hydrocarbons (linear, ramified, aromatic) containing up to 23 carbon atoms. These hydrocarbons were most likely non-intentionally added-substances leftover compounds that did not react to form longer polymer chains during the synthesis. Oxygenated compounds as well as a nitrogen-containing compound were also detected from pristine microplastics. Those could be added by the manufacturer during polymer synthesis to enhance the plastic properties. However, while a significant number of compounds were detected from pristine microplastics, only four VOCs remained in after UV exposure. This may be due to their photodegradation and/or to their volatilization caused by the aeration and temperature (60°C) of the SEPAP chamber.

The majority of the hexane extracted compounds (Supplementary Material 3, Table S3–2) was hydrocarbons with minor differences between pristine and aged microplastics. Nevertheless, some compounds were detected in pristine microplastics but not in aged ones, such as pentadecane, hexadecane and 2-methyl-2-phenyl-tridecane while the aged microplastics showed more oxygenated compounds than pristine ones, such as 7-hexadecenal, 2-[(hexadecyloxy)methyl]oxirane and 2-hexyl-1-decanol. It is reasonable to assume that the latter are photodegradation products while the former are photodegraded and/or are lost by volatilisation in the weathering chamber.

These results, together with those on the carbonyl index, suggest that differences in the effects induced by pristine and aged microplastics on blue mussels, if detected, could be driven by both the evolution of the surface state (reflected by the change in the carbonyl index, even if this value is much lower here than in environmental samples) and the change in the microplastic composition in terms of leachable compounds.

3.1.3. PAH sorption

In this study, PAHs containing stock solutions were prepared adding the PAHs solubilized in methanol (final concentrations in the tank : 1 µg/L of Phe-and Fluo, 0.5 µg/L of BaAnt and BaPyr) to 4 mg of PE microspheres in 200 mL of water. A first PAH partitioning between water and microplastics occurred in these stock solutions. Then, another equilibrium was achieved in the tanks after the use of 5 mL of stock solution to contaminate the tanks.

According to the diffusion coefficients (D) of PAHs in low-density PE (Rusina et al., 2010), the time required to reach the sorption equilibrium ranges from 8 min to 7 h. The fastest system corresponds to the lightest PAH, Phe ($\log D = -12.45$ $m^2.s^{-1}$), sorbing into the smallest microsphere (27 µm diameter) and the slowest system corresponds to the heaviest PAH, BaPyr ($\log D = -13.72$ $m^2.s^{-1}$) sorbing into the largest microsphere (45 µm diameter).

As the quantification of PAH sorbed onto the PE microspheres was

not possible due to the environmental doses used for both microplastics and PAHs, theoretical water and PE concentrations were calculated for both stock solutions and mussel tanks, based on microplastic-water partition coefficients ($K_{PE/W}$) determined by Choi et al. (2013). The data are summarized in Table 1. The levels of PAHs theoretically sorbed onto the PE microspheres increased with the increase of the molecular weight, ranging from 6.29 to 319.62 ng/g of PE for Phe-and BaPyr, respectively (Table 1). However, it is important to note that these theoretical values can only give an order of magnitude of real concentrations since polymer size, shape, density, color, chemical composition and weathering are properties that can influence sorption dynamics of organic compounds (Fisner et al., 2017; Munoz et al., 2021), as well as pH, salinity, organic matter and temperature of water (Martín et al., 2022; Ziccardi et al., 2016). It was thus impossible to differentiate the quantities sorbed onto pristine microplastics and aged ones. Commercial plastics are usually hydrophobic as most of the organic contaminants (Syberg et al., 2015) and changes induced by photoageing can lead to different sorption levels of these contaminants (Lin et al., 2020; Müller et al., 2018; Zhang et al., 2018). On the one hand, the ageing can be responsible for cracks and pores on the surface, increasing the specific surface area and enhancing the sorption of contaminants. On the other hand, UV irradiation increases the content of oxygen-containing functional groups, which also increases the polarity, hydrophilicity and charge of the microplastic surfaces (Fan et al., 2021). The ageing process can also weaken the π-π interaction, the main mechanism of interaction with aromatic compounds such as PAHs, due to chain scissions (Ding et al., 2020; Mailhot and Gardette, 1992). These two oposite effects of the photoageing on the sorbing capacities of PE microplastics preclude any definitive conclusion. Nevertheless, a recent study showed higher adsorption of Phe-on aged HDPE microplastics than on pristine ones (Bhagat et al., 2022).

3.2. Biometric characteristics of mussels

Table 2 shows the average weights and standard deviations of the whole mussels measured before dissection. The lowest average weight was 6.27 ± 1.85 g and the highest 7.89 ± 2.67 g for AMP and MP-PAH treatments, respectively. No significant difference was highlighted by a Kruskal-Wallis test which meant that the variances were homogenous among the different treatments.

3.3. Biochemical markers

3.3.1. Total protein contents

Fig. 1A, B and C show box and whisker plots for TP contents in digestive gland, gills, and mantles, respectively. No significant difference was highlighted for the gills and the mantles between the treatments. In DG, TP was significantly higher in AMP compared to MP-PAH treatment, as determined by the Kruskal-Wallis test ($p < 0.05$). Comparing the different organs, the values of TP were in the same range.

3.3.2. Oxidative stress

Catalase

Fig. 2A, B and C show box and whisker plots for CAT specific activities for digestive gland, gills and mantle, respectively (expressed related to the total protein contents). No significant differences were highlighted in gills and mantle between the different exposure treatments. In DG, the AMP-PAH group showed CAT activities significantly higher than the control group. Comparing the 3 studied organs, the values of catalase activity were of the same order of magnitude.

Superoxide dismutase

Fig. 2D, E and F depict the results of the SOD specific activities measured on digestive gland, gills and mantle of mussels, respectively. No significant difference was observed for the gills between the different exposure treatments. About the digestive gland, the SOD activities were significantly lower in the mussels from the tanks contaminated by aged microplastics (AMP) compared to those from tanks containing microplastics sorbing PAHs (MP-PAH). In the case of the mantle, the SOD activity was significantly lower for the AMP-PAH treatment compared to the control. Comparing the 3 studied organs, the values of SOD activity were of the same order of magnitude.

Glutathione S-transferase

Fig. 2G, H and I show the GST specific activities in digestive gland, gills and mantle of the mussels, respectively. No significant differences were found for the gills and the mantle between the different treatments. Concerning the digestive gland, the activity of the MP group was significantly higher than the activities of both AMP and MP-PAH groups. Comparing the 3 organs, a great difference of activities was observed with the highest values for the digestive gland.

3.3.3. Neurotoxicity - acetylcholinesterase activity

Fig. 3A, B and C is an overview of the AChE results. No significant differences were found for the digestive gland and the gills. Nevertheless, in the mantle, the AChE activities were significantly lower for the control group compared to all the other groups with microplastic contaminations.

3.3.4. Immunotoxicity - acid phosphatase activity

The acid phosphatase (AcP) activity was measured from the hemolymph of the mussels. Median AcP activities were 31.65 (14.65–64.47), 20.22 (10.57–67.78), 31.12 (8.65–67.22), 34.57 (7.42–56.36), and 28.89 (8.02–49.87) UI/L for the controls, MP, AMP, MP-PAH, and AMP-PAH treatments, respectively. No significant differences were observed between the treatments.

3.3.5. Lipidomics

After FIA-HRMS analysis of lipid extracts of the mussels' digestive glands, lipidomic data matrices were obtained for negative and positive ions from the generated fingerprints using automatic peak picking open source software (Pluskal et al., 2010). As a first step, an unsupervised data analysis was performed using PCA (MetaboAnalyst 5.0) to explore

Table 2
Average whole mussel weights and standard deviation (SD) by treatment, in grams.

	Mussel Weights (g)				
	Control	MP	AMP	MP-PAH	AMP-PAH
Average	7.23	7.39	6.27	7.89	7.56
SD	1.79	1.74	1.85	2.67	1.65

Table 1
PAH distribution in the microplastic/water (PE-W) system. The values were calculated based on partition coefficient determined by Choi et al. (2013). The stock solution contained 4 mg of PE microspheres in 200 mL of deionized water and the mussel tank contained 200 µg of PE microspheres in 20 L of seawater.

	log $K_{PE/W}$ deionized water	Stock solution		log $K_{PE/W}$ seawater	Mussel tanks	
		$[PAH]_{Water}$	$[PAH]_{PE}$		$[PAH]_{Water}$	$[PAH]_{PE}$
	(L/kg)	(µg/L)	(µg/g)	(L/kg)	(ng/L)	(ng/g)
Phenanthrene	4.04	0.82	8.99	4.10	0.49	6.29
Fluoranthene	4.75	0.47	26.46	4.85	0.49	35.34
Benzo(a)anthracene	5.43	0.07	21.08	5.55	0.24	86.90
Benzo(a)pyrene	6.14	0.02	24.13	6.11	0.25	319.62

FIGURES

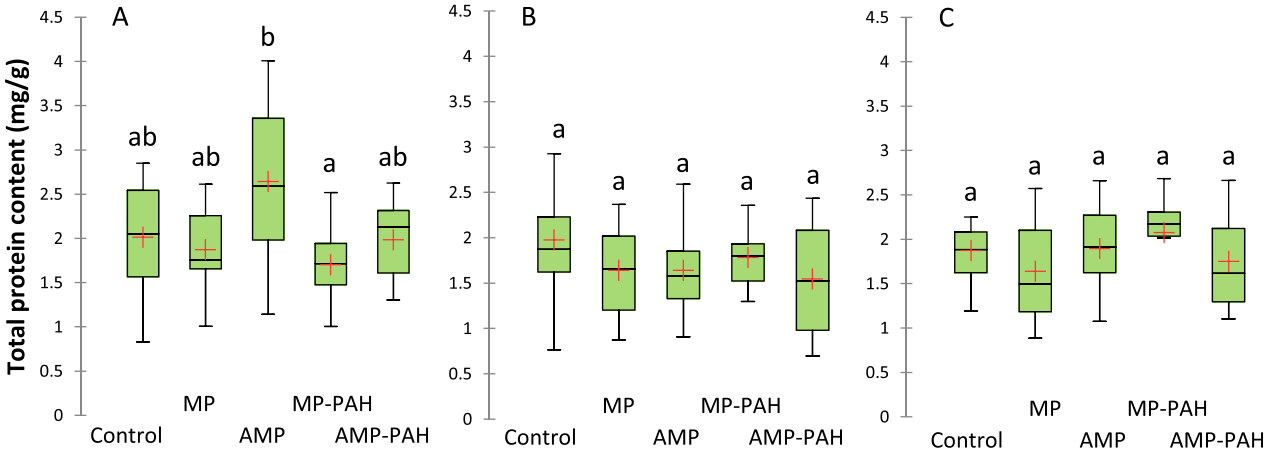


Fig. 1. Total protein contents measured in digestive glands (A), gills (B) and mantles (C) of *M. edulis* exposed to microplastics (MP), aged microplastics (AMP), microplastic sorbing PAHs (MP-PAH), aged microplastic sorbing PAHs (AMP-PAH) at 10 μg/L and controls. $N = 15$ for each treatment. Different letters correspond to significant differences between treatments (Kruskall Wallis test; $p < 0.05$). Red plus corresponds to the mean.

potential mussel treatment discriminations according to lipid impairments. The sample representations are shown in Supplementary Material 4, Fig. S4A and S4B, for positive and negative ions, respectively.

Using both the positive and negative ionization data, no clear clustering was observed in the PCA score plots for mussels exposed to the five treatments. This absence of clustering using an unsupervised method indicated that there was no major lipidic modification related to the exposure treatments.

For in-depth exploration of the data, and highlight minor lipidic alterations, a supervised data analysis (formerly PLS-DA) was performed using data matrixes from both ionization mode. For each mode, a total of 4 PLS-DA were achieved focusing on the discrimination of each microplastic treatment (MP, AMP, MP-PAH and AMP-PAH) compared to the controls. The 4 score plots and corresponding loading plots were analyzed to highlight potential ions responsible for such discrimination. The first 20 variables based on their VIP score (Variable Importance in the projection score) were selected (VIP > 2) as they corresponded to the most significant altered variable in each discrimination of treatments *vs* controls. No significant alteration was observed exploring the negative ionization mode data. Nevertheless, positive ionization data showed significant discrimination using PLS-DA (Fig. 4).A total of 16, 18, 18 and 19 features were found as features up- or down regulated in the organisms exposed to MP, AMP, MP-PAH and AMP-PAH, respectively *vs* controls.

In all PLS-DA, the component 1 was accounting for 3.8 to 4% of the total variance when clear and significant separations were observed. The most significantly altered features were both up- and down regulated during the exposure. The list of the ions with variable importance in the projections (VIP score > 2) was determined and used to build a Venn diagram (Heberle et al., 2015) displayed in Fig. 5A. The detailed *m/z* of the features shared between some treatments are also shown in the Fig. 5B. No feature with the same mass was shared by all the mussels exposed. To assess the potential effect of the influencing factors, *i.e.* ageing and sorption of PAHs by microplastics, Fig. 5B shows the features up- or down-regulated shared by the mussels from the different exposure treatments. Two features were shared by the organisms exposed to aged microplastics (AMP and AMP-PAH: *m/z* 466.437 and 643.667, up-regulated and down regulated, respectively) and none between conditions with microplastics sorbing PAHs (MP-PAH and AMP-PAH). Some other features were shared by different exposure treatments, but not depending on the studied influencing factors.

Only 5 features were further annotated (Table 3) based on high mass accuracy using the Lipid Blast Database (Kind et al., 2013). Four of them were putatively identified as diradylglycerolipids and the last one as triradylglycerolipids.

4. Discussion

M. edulis is an important suspension filter feeder that it has been used in several studies to assess the bioaccumulation of contaminants and to evaluate their effects on marine biota (Beyer, 2017). More recently, mussels were particularly used for the assessment of microplastic bioaccumulation in the field (Phuong et al., 2018) and of their effects after laboratory exposures (Alnajar et al., 2021; Choi et al., 2022; Green et al., 2019; Khalid et al., 2021; Li et al., 2022; Mkuye, 2022). As a good global bioindicator of pollution and particularly for microplastics (Li et al., 2019), mussel watch programs were organized in several countries and some biomarkers of microplastic exposure were identified in the Mediterranean mussel (*Mytilus galloprovincialis*) (Provenza et al., 2022). Marine mussel-based biomarkers were also recently used as a risk indicator of microplastic impacts (Chen, 2021).

Considering this state of the art, the blue mussel was chosen as the species used in the laboratory experiments of the present study. A classical battery of biochemical biomarkers was used to measure the effects of microplastics to have data easily comparable with those found in the literature, associated with lipidomics, as a promising tool for ecotoxicological concerns.

4.1. Biochemical markers

Regarding the biochemical markers results, few significant differences between the five treatments were observed for oxidative stress (CAT, SOD and GST), neurotoxicity (AChE) and immunotoxicity (AcP). Comparing the data of mussels from the control tanks and tanks contaminated with pristine microplastics (MP), no significant differences were observed on the oxydative stress, for all the studied tissues. Many studies were performed to determine the effects of microplastics on bivalves (Khalid et al., 2021; Mkuye, 2022; Tlili et al., 2020), and particularly the Mediterranean Mussel (*Mytilus galloprovincialis*) (Provenza et al., 2022). The comparison of the results of the present study with those from the literature is not easy because they depend on the experimental design, such as microplastic characteristics (form, size,

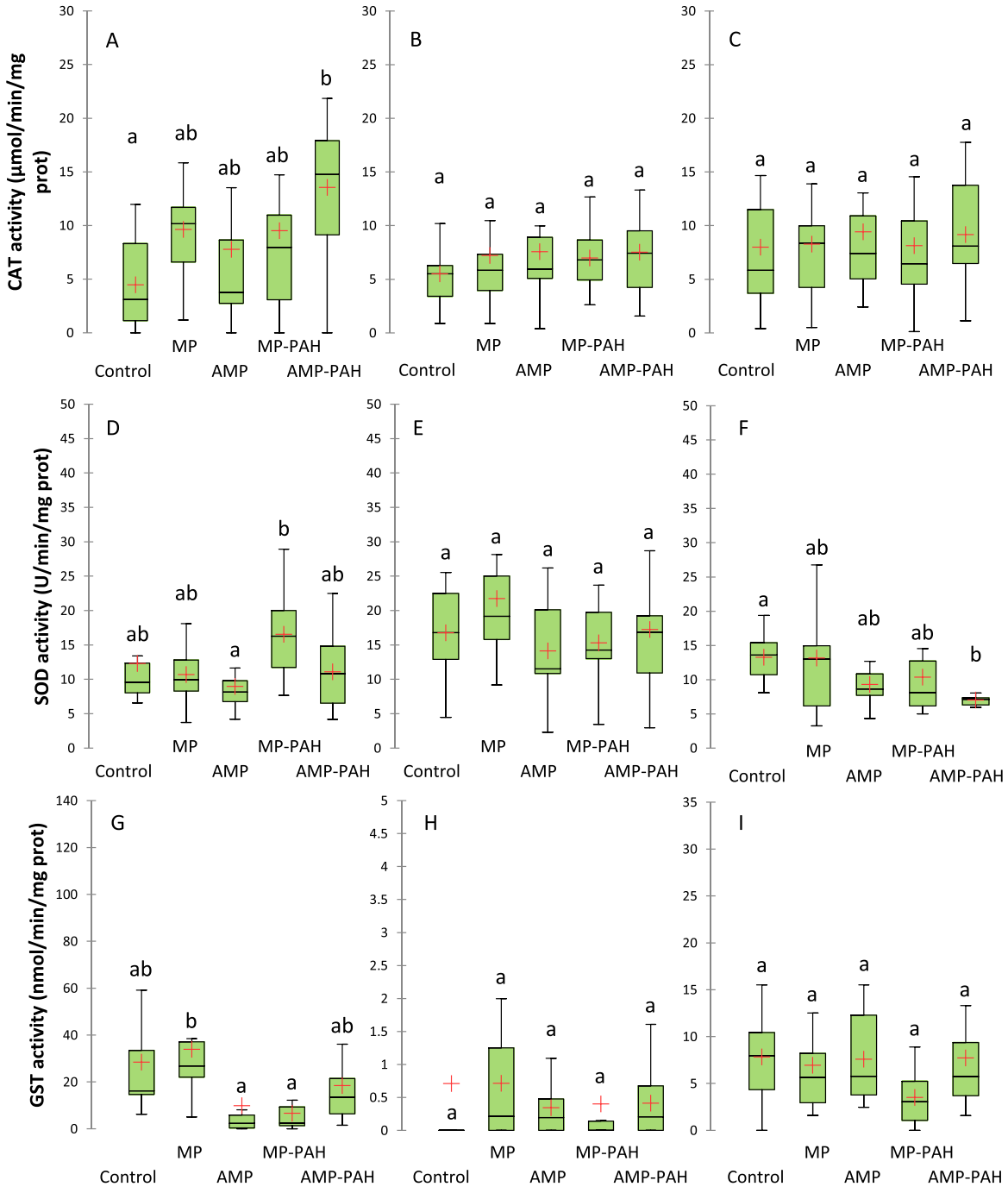


Fig. 2. Catalase activity measured in digestive glands (A), gills (B) and mantles (C), SOD activity measured in digestive glands (D), gills (E) and mantles (F) and GST activity measured in digestive glands (G), gills (H) and mantles (I) of *M. edulis* exposed to microplastics (MP), aged microplastics (AMP), microplastic sorbing PAHs (MP-PAH), aged microplastic sorbing PAHs (AMP-PAH) at 10 µg/L and controls. $N = 15$ for each treatment. Different letters correspond to significant differences between treatments (Kruskall Wallis test; $p < 0.05$). Red plus corresponds to the mean. Black dots are potential outliers.

composition, concentration), model species, biomarkers and duration of the experiments which are different from each other (Khalid et al., 2021). Laboratory exposures during 8 days, using PE pristine microspheres at 10 µg/L of concentration and with size ranging from 27 to 45 µm were not a threat for the mussel health. Nevertheless, it is important to keep in mind the limited exposure duration compared to the environment. Thus, effects could be possible in the field for mussels constantly exposed to microplastics during their whole life. Additionnally, the biomarker battery used for the present study is quite restricted and the use of others at many biological organization levels could conclude to different results. Large microplastics (27 to 45 µm) could have effects in tissues besides disruption the gut and assimilation of nutrients.

While no effects of the treatments were observed for GST in gills and mantle, its levels in digestive gland were significantly higher in organisms exposed to MP compared to those exposed to AMP and MP-PAH. Nevertheless, the results are questionable since not significant differences were highlighted compared to the controls. The impossible PAH analysis in mussels cannot ensure that these organic compounds were transferred to the organisms and bioaccumulated, generating effects. Their hydrophobic properties could lead to their strong sorption onto the microplastics (Engler, 2012; Pittura et al., 2018). Or, the low

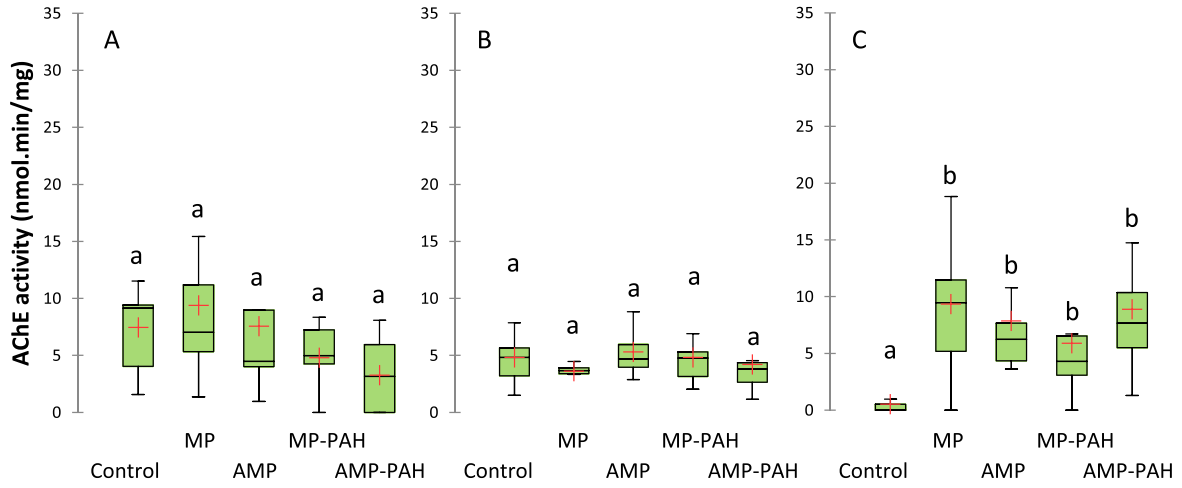


Fig. 3. Acetylcholinesterase activities measured in digestive glands (A), gills (B) and mantles (C) of *M. edulis* exposed to microplastics (MP), aged microplastics (AMP), microplastic sorbing PAHs (MP-PAH), aged microplastic sorbing PAHs (AMP-PAH) at 10 μg/L and controls. $N = 15$ for each treatment. Different letters correspond to significant differences between treatments (Kruskall Wallis test; $p < 0.05$). Red plus corresponds to the mean.

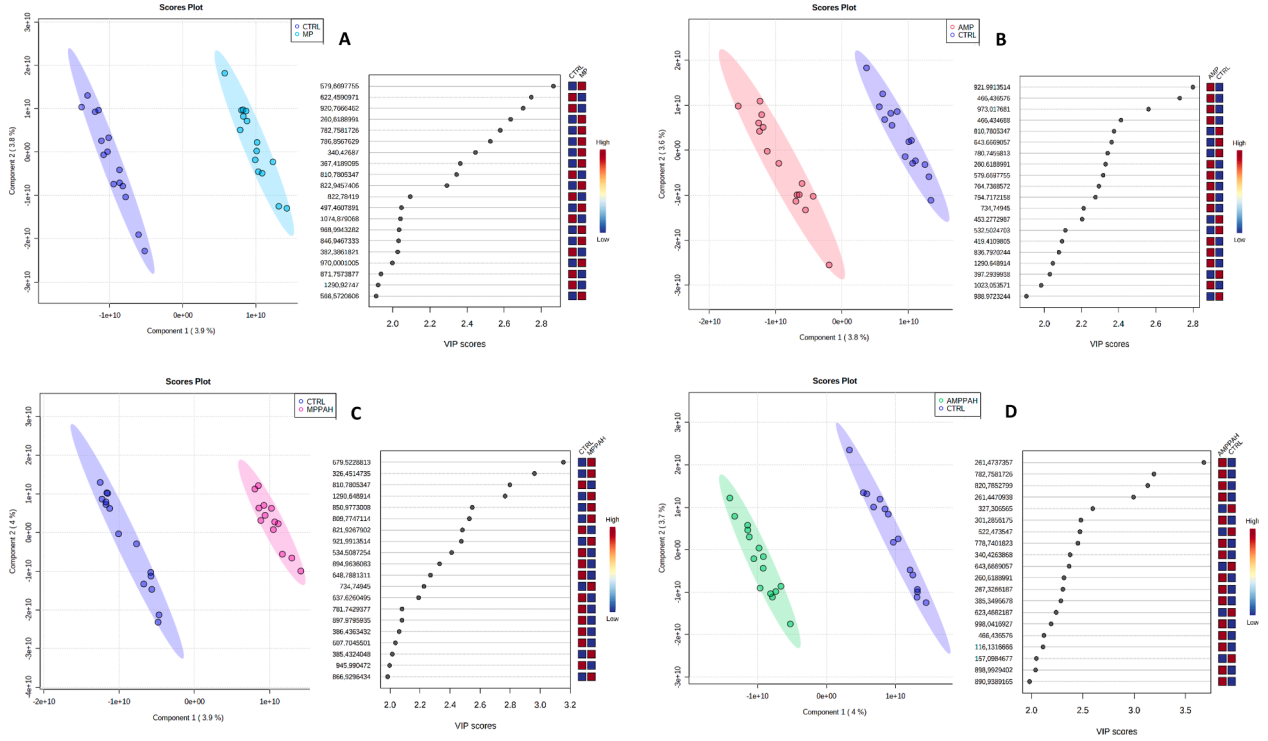
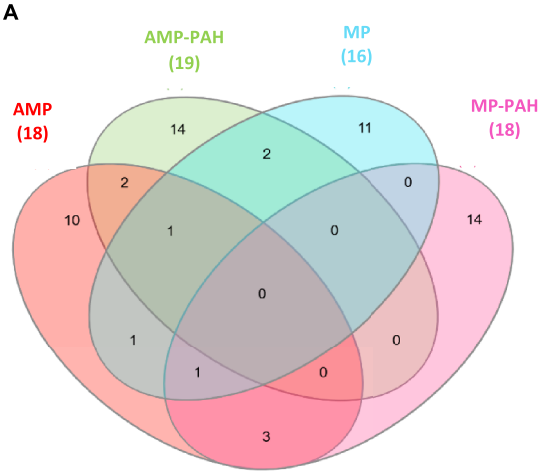


Fig. 4. Score plot of PLS-DA of FIA-HRMS positive ions from lipid extracts of the digestive gland from *M. edulis* in the controls compared with individuals exposed to: A: microplastics (MP), B: aged microplastics (AMP), C: microplastic sorbing PAHs (MP PAH), D: aged microplastic sorbing PAHs (AMP PAH) at 10 μg/L.

concentration of the PAHs did not reach toxic threshold in organisms. Other biomarkers could have demonstrated effect, such as DNA adducts which can be specific to PAHs (Venier and Canova, 1996). Concerning the state of the art, other results were published. On one hand, Pittura et al. (2018) concluded to none oxidative effect of co-exposure LDPE/-BaPyr on mussels exposed to microplastic concentrations with one order of magnitude higher (10 mg/L) than the present study. On the other hand, some articles described significant oxidative effects on mussels and other species (Avio et al., 2015; Détrée and Gallardo-Escárate, 2018; Paul-Pont et al., 2016; Ribeiro et al., 2017) with concentrations ranging from 0.5 to 1 mg/L. As for the effects of the pristine microplastics, it is difficult to directly compare the results between the studies because of the different experimental designs.

A significant oxidative stress was highlighted after the exposure of mussels to AMP-PAH treatment, measuring increased CAT activities in the digestive gland and decreased SOD activities in the mantle. González-Soto et al. (2019) also depicted a more toxic effect of BaPyr sorbed onto microplastics on CAT activity in hemocytes of mussels dietarily exposed.

The increased levels of AChE in the mantle were significant after the exposure of mussels to all the microplastic treatments (MP, AMP, MP-PAH and AMP-PAH) compared to the controls. This result was not observed for the other organs and it is of great interest since the mantle is not an organ usually studied (Provenza et al., 2022). Impairments on AChE were already measured after the exposure of aquatic species to microplastics (Avio et al., 2015; Oliveira et al., 2013; Ribeiro et al.,



B	Positive features in digestive gland lipids compared to the controls			
Features shared (*m/z*)	MP	AMP	MP-PAH	AMP-PAH
260.618	+	+		+
340.427	+			+
466.437		+		+
579.670	+	+		
643.667		-		-
734.750		+	+	
782.758	+			+
810.781	-	-	-	
921.991		+	+	
1290.649		+	+	

+: up-regulation of features in the treatments compared to controls;
- down-regulation of features in the treatments compared to the controls

Fig. 5. Positive features found as VIP>2 in different PLS-DA discriminating lipids of digestive gland in *M. edulis* from controls to exposure treatments: microplastics (MP), aged microplastics (AMP), microplastic sorbing PAHs (MP-PAH), aged microplastic sorbing PAHs (AMP-PAH). Concentration of microplastics at 10 μg/L. A: Venn diagram (Heberle et al., 2015); B: list of the features with their *m/z*.

Table 3
Features of interest, obtained in positive ionization mode and highlighted by the PLS-DA analysis, annotated using the Lipid Blast Database (Kind et al., 2013).

m/z	Exposure	Regulation compared to controls	Putative annotations
780.745	AMP	down	diradylglycerolipids (46:1)
782.758	MP ; AMP-PAH	up	diradylglycerolipids (46:0)
820.785	AMP-PAH	up	diradylglycerolipids (49:2)
822.784	MP	up	diradylglycerolipids (49:1)
920.767	MP	down	triradylglycerolipids (56:8)

AMP: aged microplastics; MP: microplastics; AMP-PAH: aged microplastic sorbing PAHs. Concentration of microplastics at 10 μg/L.

2017), traducing the influence of these contaminants on physiological and behavioral responses controlled by neurological mechanisms. In addition, Avio et al. (2015) mentioned that any anticholinesterase effect of microplastics in fish would be alarming considering the widespread presence of this stressor in the aquatic environment and the pivotal role of this enzyme in neurological function which is crucial to control several physiological (*e.g.* growth, reproduction) and behavioral (*e.g.* swimming) processes that directly or indirectly may influence individual and population fitness. For the previous studies, the impairment corresponded to decreases of the AChE activity. Nevertheless, some increases of AChE activities were also observed in the litterature, such as in heamolymph of *Patela vulgata* after the exposure to copper at 6.1 $\mu g.L^{-1}$ (Browne et al., 2004). Boukadida et al. (2022) also observed stimulated AChE activities in mussel larvae (*Mytilus galloprovincialis*) exposed to a combination of thermal and metallic stress for 48 h. They suggest that hypoactivity and hyperactivity of the larvae may be associated with AChE activity, since AChE activities were positively correlated with the maximum speed of the organisms. In the present study, the mussels could have their mantle muscle more contracted because of their contact with the plastic particles.

4.2. Lipidomics

Omic sciences including genomics, transcriptomics, proteomics and metabolomics were developed the last decades in many fields, such as in aquaculture (Laudicella et al., 2020) and more specifically to assess the toxicity of micro- and nanoplastics on fish and seafood species (Bhagat et al., 2022) for example. Among the metabolomic approaches, lipidomics is the method focusing on the lipidome, *i.e.* the lipids contained in a whole organism. Lipids constitute major components in biology since they are involved in membranes, energy reserves, reproduction, immunology and adaptation processes (Laudicella et al., 2020). The specific and targeted analysis of lipid classes was conventional and extended by innovative analytical developments using mass spectrometry, allowing the study of the entire spectrum of lipids extracted from organisms. This tool was notably employed on emblematic marine species, like the bottlenose dolphin (Ruiz-Hernández et al., 2022) or the common octopus (Gaspar et al., 2023), as well as on different species of oysters, mussels and clams in aquaculture. The objectives of the use of the lipidomics were varied: lipid composition (Gaspar et al., 2023), traceability (Boselli et al., 2012), life cycle (Facchini et al., 2018), early life stage (Young et al., 2016), effects of global warming (Mayor et al., 2015) or effects of contaminants (Khalid et al., 2021; Ruiz-Hernández et al., 2022).

From the lipidomic analysis, no feature with the same mass was shared by all the mussels exposed which could have been considered as a biomarker of microplastic exposure. The 2 features shared by the organisms exposed to aged microplastics could be marker of exposure but it was not possible to determine the lipid structure using the Lipid Blast Database. Since the other features were shared by different exposure treatments, not depending on the studied influencing factors, it is possible to conclude that the response of the mussels was quite exposure condition dependent. The annoted lipids impaired in organisms exposed to the different treatments were belonging to the diradylglycerolipid and triradylglycerolipid groups. These lipids were not previously mentioned in studies concerning shellfish. The same lipidomic analysis was previously performed on mussels exposed to bio-based and biodegradable microplastics in the study of Khalid et al. (2021). The only difference was the analyzed samples which were the whole mussels in that specific study, while only the digestive glands were used in the present study. Most of the 48 down-regulated lipids in organisms exposed to bio-based

and biodegradable microplastics were annoted as belonging to glycerophospholipids, major components of lipid membranes. The authors attributed this result to interactions between biological membranes and small plastic particles, ranging from 0.8 to 10 μm. Even if the species, the duration of exposure and the concentration of microplastics were the same (10 μg/L), the reasons of the different results between the studies were probably various. A first one could be the different plastic types, *i.e.* L-lactic acid polymer (PLLA) *vs* PE. The different shapes and sizes of the microplastics could also be another reason, *i.e.* fragments between 0.8 and 10 μm *vs* microspheres ranging from 27 to 45 μm. Finally, the investigation of the whole soft tissues of the organisms *vs* the digestive glands probably constituted the major reason of different results. The comparison of the data questioned about the interest of a sectioned tissue compared to the whole soft tissues, probably more relevant for a general biological response.

5. Conclusion

In the present study, the effects of microplastics on a representative marine filter feeder organism (*M. edulis*) were investigated considering the factors of ageing and sorption of some PAHs to simulate environmental exposure. Hence, blue mussels were exposed to five different laboratory conditions for 8 days: pristine microplastics, aged microplastics, microplastics sorbing PAHs, as well as microplastics both aged and sorbing PAHs, in parallel to controls. The results showed there was few induction of effects using lipidomics on digestive gland and a battery of biomarkers traducing oxidative stress, neurotoxicity and immunotoxicity on digestive gland, gills, mantle and hemolymph.

Regarding oxidative stress, no significant difference was observed in all the studied tissues of mussels exposed to pristine microplastics. The main results were a significant oxidative stress highlighted after the exposure to microplastics both aged and sorbing PAHs, As well as, a neurotoxicity in the mantle of mussels exposed to all the treatments compared to controls. This neurotoxicity was not observed for the other studied organs and it is of great interest since the mantle is not an organ usually studied in ecotoxicology. Concerning lipidomics, only 2 up- or down regulated ions were shared by the organisms exposed to aged microplastics and none between microplastics sorbing PAHs, traducing these factors were not of specific influence on lipid impairments of the digestive gland. Nevertheless, the difficulty of the annotation of the ions remains a limit of the lipidomic studies.

CRediT authorship contribution statement

Romaric Moncrieffe: Methodology, Formal analysis, Writing – original draft. **Maria Masry:** Resources, Writing – original draft. **Binbin Cai:** Methodology, Formal analysis. **Stéphanie Rossignol:** Resources, Formal analysis, Writing – original draft. **Abderrahmane Kamari:** Methodology. **Laurence Poirier:** Conceptualization, Writing – original draft. **Samuel Bertrand:** Methodology, Writing – review & editing. **Pascal Wong-Wah-Chung:** Conceptualization, Supervision, Resources, Project administration, Writing – review & editing. **Aurore Zalouk-Vergnoux:** Conceptualization, Methodology, Supervision, Resources, Project administration, Writing – original draft, Writing – review & editing.

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.

Aknowledgments

The authors thank Mickaël Croyal and the Corsaire CRNH mass spectrometry platform and the ThalassOMICS platform, both from BiogenOuest, for helping us with the FIA-HRMS analysis.

The authors also thank Joint Master Aquaculture Environment and Society (ACES+) for the funding of the dissertation projects of students involved in the project.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.aquatox.2023.106669.

References

Alnajar, N., Jha, A.N., Turner, A., 2021. Impacts of microplastic fibres on the marine mussel, *Mytilus galloprovinciallis*. Chemosphere 1, 28290.
Andrady, A.L., 2011. Microplastics in the marine environment. Mar. Pollut. Bull. 62, 1596–1605. https://doi.org/10.1016/j.marpolbul.2011.05.030.
Avio, C.G., Gorbi, 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. https://doi.org/10.1016/j.envpol.2014.12.021.
Barnes, D.K.A., Galgani, F., Thompson, R.C., Barlaz, M., 2009. Accumulation and fragmentation of plastic debris in global environments. Philos. Trans. R. Soc. B Biol. Sci. 364, 1985–1998. https://doi.org/10.1098/rstb.2008.0205.
Beauchamp, C., Fridovich, I., 1971. Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. Anal. Biochem. 44, 276–287. https://doi.org/10.1016/0003-2697(71)90370-8.
Beyer, J., 2017. Blue mussels (Mytilus edulis spp.) as sentinel organisms in coastal pollution monitoring: a review. Mar. Environ. Res. 28, 338–365. https://doi.org/10.1016/j.marenvres.2017.07.024.
Bhagat, K., Barrios, A.C., Rajwade, K., Kumar, A., Oswald, J., Apul, O., Perreault, F., 2022. Aging of microplastics increases their adsorption affinity towards organic contaminants. Chemosphere 298, 134238. https://doi.org/10.1016/j.chemosphere.2022.134238.
Boselli, E., Pacetti, D., Lucci, P., Frega, N.G., 2012. Characterization of phospholipid molecular species in the edible parts of bony fish and shellfish. J. Agric. Food Chem. 60, 3234–3245. https://doi.org/10.1021/jf205159a.
Boukadida, K., Banni, M., Romero-Ramirez, A., Clerandeau, C., Gourves, P.Y., Cachot, J., 2022. Metal contamination and heat stress impair swimming behavior and acetylcholinesterase activity in embryo-larval stages of the Mediterranean mussel, *Mytilus galloprovincialis*. Mar. Environ. Res. 179, 105677 https://doi.org/10.1016/j.marenvres.2022.105677.
Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254. https://doi.org/10.1016/0003-2697(76)90527-3.
Brown, R.J., Galloway, T.S., Lowe, D., Browne, M.A., Dissanayake, A., Jones, M.B., Depledge, M.H., 2004. Differential sensitivity of three marine invertebrates to copper assessed using multiple biomarkers. Aquat. Toxicol. 66, 267–278. https://doi.org/10.1016/j.aquatox.2003.10.001.
Chen, C.Y., 2021. Marine mussel-based biomarkers as risk indicators to assess oceanic region-specific microplastics impact potential. Ecol. Indic. 12, 106915. https://doi.org/10.1016/j.ecolind.2020.106915.
Choi, J.S., Kim, K., Park, K., Park, J.W., 2022. Long-term exposure of the Mediterranean mussels, Mytilus galloprovincialis to polyethylene terephthalate microfibers: implication for reproductive and neurotoxic effects. Chemosphere 299, 134317. https://doi.org/10.1016/j.chemosphere.2022.134317.
Choi, Y., Cho, Y.M., Luthy, R.G., 2013. Polyethylene-water partitioning coefficients for parent- and alkylated-polycyclic aromatic hydrocarbons and polychlorinated biphenyls. Environ. Sci. Technol. 47, 6943–6950. https://doi.org/10.1021/es304566v.
Chong, J., Wishart, D.S., Xia, J., 2019. Using MetaboAnalyst 4.0 for comprehensive and integrative metabolomics data analysis. Curr. Protoc. Bioinforma. 68, e86. https://doi.org/10.1002/cpbi.86.
Détrée, C., Gallardo-Escárate, C., 2018. Single and repetitive microplastics exposures induce immune system modulation and homeostasis alteration in the edible mussel Mytilus galloprovincialis. Fish Shellfish Immunol. 83, 52–60. https://doi.org/10.1016/j.fsi.2018.09.018.
Ding, L., Mao, R., Ma, S., Guo, X., Zhu, L., 2020. High temperature depended on the ageing mechanism of microplastics under different environmental conditions and its effect on the distribution of organic pollutants. Water Res. 174, 115634 https://doi.org/10.1016/j.watres.2020.115634.
Ellman, G.L., Courtney, K.D., Andres, V., Featherstone, R.M., 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem. Pharmacol. 7, 88–95. https://doi.org/10.1016/0006-2952(61)90145-9.
Engler, R.E., 2012. The complex interaction between marine debris and toxic chemicals in the ocean. Environ. Sci. Technol. 46, 12302–12315. https://doi.org/10.1021/es3027105.

Facchini, L., Losito, I., Cataldi, T.R.I., Palmisano, F., 2018. Seasonal variations in the profile of main phospholipids in Mytilus galloprovincialis mussels: a study by hydrophilic interaction liquid chromatography–electrospray ionization Fourier transform mass spectrometry. J. Mass Spectrom. 53, 1–20. https://doi.org/10.1002/jms.4029.

Fan, X., Zou, Y., Geng, N., Liu, J., Hou, J., Li, D., Yang, C., Li, Y., 2021. Investigation on the adsorption and desorption behaviors of antibiotics by degradable MPs with or without UV ageing process. J. Hazard. Mater. 401, 123363 https://doi.org/10.1016/j.jhazmat.2020.123363.

Fisner, M., Majer, A.P., Balthazar-Silva, D., Gorman, D., Turra, A., 2017. Quantifying microplastic pollution on sandy beaches: the conundrum of large sample variability and spatial heterogeneity. Environ. Sci. Pollut. Res. 24, 13732–13740. https://doi.org/10.1007/s11356-017-8883-y.

Frias, J.P.G.L., Sobral, P., Ferreira, A.M., 2010. Organic pollutants in microplastics from two beaches of the Portuguese coast. Mar. Pollut. Bull. 60, 1988–1992. https://doi.org/10.1016/j.marpolbul.2010.07.030.

Fu, D., Zhang, Q., Fan, Z., Qi, H., Wang, Z., Peng, L., 2019. Aged microplastics polyvinyl chloride interact with copper and cause oxidative stress towards microalgae *Chlorella vulgaris*. Aquat. Toxicol. 216, 105319 https://doi.org/10.1016/j.aquatox.2019.105319.

Gaspar, L., Ricardo, F., Melo, T., Domingues, P., Domingues, M.R., Calado, R., Rey, F., 2023. Lipidomics of common octopus' (Octopus vulgaris) arm muscle using untargeted high-resolution liquid chromatography-mass spectrometry. J. Food Compos. Anal. 115, 104871 https://doi.org/10.1016/j.jfca.2022.104871.

González-Soto, N., Hatfield, J., Katsumiti, 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. https://doi.org/10.1016/j.scitotenv.2019.05.161.

Green, D.S., Colgan, T.J., Thompson, R.C., Carolan, J.C., 2019. Exposure to microplastics reduces attachment strength and alters the haemolymph proteome of blue mussels (Mytilus edulis). Environ. Pollut. 246, 423–434. https://doi.org/10.1016/j.envpol.2018.12.017.

Grimmer, G., 2018. Environmental carcinogens: polycyclic aromatic hydrocarbons.

Habig, W.H., Pabst, M.J., Jakoby, W.B., 1974. Glutathione S-transferases: the first enzymatic step in mercapturic acid formation. J. Biol. Chem. 249, 7130–7139. https://doi.org/10.1016/S0021-9258(19)42083-8.

Hanun, J.N., Hassan, F., Jiang, J.J., 2021. Occurrence, fate, and sorption behavior of contaminants of emerging concern to microplastics: influence of the weathering/aging process. J. Environ. Chem. Eng. 9, 106290 https://doi.org/10.1016/j.jece.2021.106290.

Heberle, H., Meirelles, G.V., da Silva, F.R., Telles, G.P., Minghim, R., 2015. InteractiVenn: a web-based tool for the analysis of sets through Venn diagrams. BMC Bioinform. 16, 169. https://doi.org/10.1186/s12859-015-0611-3.

Hirai, H., Takada, H., Ogata, Y., Yamashita, R., Mizukawa, K., Saha, M., Kwan, C., Moore, C., Gray, H., Laursen, D., Zettler, E.R., Farrington, J.W., Reddy, C.M., Peacock, E.E., Ward, M.W., 2011. Organic micropollutants in marine plastics debris from the open ocean and remote and urban beaches. Mar. Pollut. Bull. 62, 1683–1692. https://doi.org/10.1016/j.marpolbul.2011.06.004.

Jaouani, R., Roman, C., Decaix, J., Lagarde, F., Châtel, A., 2023. Effect of aging of microplastics on gene expression levels of the marine mussel Mytilus edulis: comparison *in vitro/in vivo* exposures. Mar. Pollut. Bull. 189, 114767 https://doi.org/10.1016/j.marpolbul.2023.114767.

Johnsen, A.R., Karlson, U., 2005. PAH degradation capacity of soil microbial communities—does it depend on PAH exposure? Microb. Ecol. 50, 488–495. https://doi.org/10.1007/s00248-005-0022-5.

Khalid, A., Zalouk-Vergnoux, A., Benali, S., Mincheva, R., Raquez, J.M., Bertrand, S., Poirier, L., 2021. Are bio-based and biodegradable microplastics impacting for blue mussel (*Mytilus edulis*)? Mar. Pollut. Bull. 167, 112295 https://doi.org/10.1016/j.marpolbul.2021.112295.

Kind, T., Liu, K.H., Lee, D.Y., DeFelice, B., Meissen, J.K., Fiehn, O., 2013. LipidBlast *in silico* tandem mass spectrometry database for lipid identification. Nat. Methods 10, 755–758. https://doi.org/10.1038/nmeth.2551.

Kotta, J., Futter, M., Kaasik, A., Liversage, K., Rätsep, M., Barboza, F.R., Bergström, L., Bergström, P., Bobsien, I., Díaz, E., Herkül, K., Jonsson, P.R., Korpinen, S., Kraufvelin, P., Krost, P., Lindahl, O., Lindegarth, M., Lyngsgaard, M.M., Mühl, M., Sandman, A.N., Orav-Kotta, H., Orlova, M., Skov, H., Rissanen, J., Šiaulys, A., Vidakovic, A., Virtanen, E., 2020. Cleaning up seas using blue growth initiatives: mussel farming for eutrophication control in the Baltic Sea. Sci. Total Environ. 709, 136144 https://doi.org/10.1016/j.scitotenv.2019.136144.

Laudicella, V.A., Whitfield, P.D., Carboni, S., Doherty, M.K., Hughes, A.D., 2020. Application of lipidomics in bivalve aquaculture, a review. Rev. Aquac. 12, 678–702. https://doi.org/10.1111/raq.12346.

Li, J., Lusher, A.L., Rotchell, J.M., Deudero, S., Turra, A., Bråte, I.L.N., Sun, C., Shahadat Hossain, M., Li, Q., Kolandhasamy, P., Shi, H., 2019. Using mussel as a global bioindicator of coastal microplastic pollution. Environ. Pollut. 244, 522–533. https://doi.org/10.1016/j.envpol.2018.10.032.

Li, Z., Chang, X., Hu, M., Fang, J.K.H., Sokolova, I.M., Huang, W., Xu, E.G., Wang, Y., 2022. Is microplastic an oxidative stressor? Evidence from a meta-analysis on bivalves. J. Hazard. Mater. 423, 127211 https://doi.org/10.1016/j.jhazmat.2021.127211.

Liebisch, G., Fahy, E., Aoki, J., Dennis, E.A., Durand, T., Ejsing, C.S., Fedorova, M., Feussner, I., Griffiths, W.J., Köfeler, H., Merrill, A.H., Murphy, R.C., O'Donnell, V.B., Oskolkova, O., Subramaniam, S., Wakelam, M.J.O., Spener, F., 2020. Update on LIPID MAPS classification, nomenclature, and shorthand notation for MS-derived lipid structures. J. Lipid Res. 61, 1539–1555. https://doi.org/10.1194/jlr.S120001025.

Liebisch, G., Vizcaíno, J.A., Köfeler, H., Trötzmüller, M., Griffiths, W.J., Schmitz, G., Spener, F., Wakelam, M.J.O., 2013. Shorthand notation for lipid structures derived from mass spectrometry. J. Lipid Res. 54, 1523–1530. https://doi.org/10.1194/jlr.M033506.

Lin, L., Tang, S., Wang, X., Sun, X., Yu, A., 2020. Adsorption of malachite green from aqueous solution by nylon microplastics: reaction mechanism and the optimum conditions by response surface methodology. Process Saf. Environ. Prot. 140, 339–347. https://doi.org/10.1016/j.psep.2020.05.019.

Luo, H., Zhao, Y., Li, Y., Xiang, Y., He, D., Pan, X., 2020. Aging of microplastics affects their surface properties, thermal decomposition, additives leaching and interactions in simulated fluids. Sci. Total Environ. 714, 136862 https://doi.org/10.1016/j.scitotenv.2020.136862.

Lusher, A.L., McHugh, M., Thompson, R.C., 2013. Occurrence of microplastics in the gastrointestinal tract of pelagic and demersal fish from the English channel. Mar. Pollut. Bull. 67, 94–99. https://doi.org/10.1016/j.marpolbul.2012.11.028.

Mailhot, B., Gardette, J.L., 1992. Polystyrene photooxidation. 2. A pseudo wavelength effect. Macromolecules 25, 4127–4133. https://doi.org/10.1021/ma00042a013.

Martín, J., Santos, J.L., Aparicio, I., Alonso, E., 2022. Microplastics and associated emerging contaminants in the environment: analysis, sorption mechanisms and effects of co-exposure. Trends Environ. Anal. Chem. 35, e00170. https://doi.org/10.1016/j.teac.2022.e00170.

Mayor, D.J., Sommer, U., Cook, K.B., Viant, M.R., 2015. The metabolic response of marine copepods to environmental warming and ocean acidification in the absence of food. Sci. Rep. 5, 13690. https://doi.org/10.1038/srep13690.

Mkuye, R., 2022. Effects of microplastics on physiological performance of marine bivalves, potential impacts, and enlightening the future based on a comparative study. Sci. Total Environ. 12, 155933. https://doi.org/10.1016/j.scitotenv.2022.155933.

Müller, A., Becker, R., Dorgerloh, U., Simon, F.G., Braun, U., 2018. The effect of polymer aging on the uptake of fuel aromatics and ethers by microplastics. Environ. Pollut. 240, 639–646. https://doi.org/10.1016/j.envpol.2018.04.127.

Munoz, M., Ortiz, D., Nieto-Sandoval, J., de Pedro, Z.M., Casas, J.A., 2021. Adsorption of micropollutants onto realistic microplastics: role of microplastic nature, size, age, and NOM fouling. Chemosphere 283, 131085. https://doi.org/10.1016/j.chemosphere.2021.131085.

Oliveira, M., Ribeiro, A., Hylland, K., Guilhermino, L., 2013. Single and combined effects of microplastics and pyrene on juveniles (0+ group) of the common goby Pomatoschistus microps (Teleostei, Gobiidae). Ecol. Indic. 34, 641–647. https://doi.org/10.1016/j.ecolind.2013.06.019.

Pang, Z., Chong, J., Zhou, G., de Lima Morais, D.A., Chang, L., Barrette, M., Gauthier, C., Jacques, P.É., Li, S., Xia, J., 2021. MetaboAnalyst 5.0: narrowing the gap between raw spectra and functional insights. Nucl. Acids Res 49, W388–W396. https://doi.org/10.1093/nar/gkab382.

Paul-Pont, I., Lacroix, C., González Fernández, C., Hégaret, H., Lambert, C., Le Goïc, N., Frère, L., Cassone, A.L., Sussarellu, R., Fabioux, C., Guyomarch, J., Albentosa, 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. https://doi.org/10.1016/j.envpol.2016.06.039.

Pflugmacher, S., Sulek, A., Mader, H., Heo, J., Noh, J.H., Penttinen, O.P., Kim, Y., Kim, S., Esterhuizen, M., 2020. The influence of new and artificial aged microplastic and leachates on the germination of Lepidium sativum L. Plants 9, 339. https://doi.org/10.3390/plants9030339.

Phuong, N.N., Zalouk-Vergnoux, A., Kamari, A., Mouneyrac, C., Amiard, F., Poirier, L., Lagarde, F., 2018. Quantification and characterization of microplastics in blue mussels: protocol setup and preliminary data on the contamination of the French Atlantic coast. Environ. Sci. Pollut. Res. 25, 6135–6144. https://doi.org/10.1007/s11356-017-8862-3.

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. https://doi.org/10.3389/fmars.2018.00103.

Pluskal, T., Castillo, S., Villar-Briones, A., Orešič, M., 2010. MZmine 2: modular framework for processing, visualizing, and analyzing mass spectrometry-based molecular profile data. BMC Bioinform. 11, 395. https://doi.org/10.1186/1471-2105-11-395.

Prata, J.C., Reis, V., Paço, A., Martins, P., Cruz, A., da Costa, J.P., Duarte, A.C., Rocha-Santos, T., 2020. Effects of spatial and seasonal factors on the characteristics and carbonyl index of (micro)plastics in a sandy beach in Aveiro, Portugal. Sci. Total Environ. 709, 135892 https://doi.org/10.1016/j.scitotenv.2019.135892.

Provenza, F., Rampih, D., Pignattelli, S., Pastorino, P., Barceló, D., Prearo, M., Specchiulli, A., Renzi, M., 2022. Mussel watch program for microplastics in the Mediterranean sea: identification of biomarkers of exposure using Mytilus galloprovincialis. Ecol. Indic. 142, 109212 https://doi.org/10.1016/j.ecolind.2022.109212.

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. https://doi.org/10.1016/j.marpolbul.2017.06.078.

Rios, L.M., Moore, C., Jones, P.R., 2007. Persistent organic pollutants carried by synthetic polymers in the ocean environment. Mar. Pollut. Bull. 54, 1230–1237. https://doi.org/10.1016/j.marpolbul.2007.03.022.

Rochman, C.M., Browne, M.A., Underwood, A.J., van Franeker, J.A., Thompson, R.C., Amaral-Zettler, L.A., 2016. The ecological impacts of marine debris: unraveling the

demonstrated evidence from what is perceived. Ecology 97, 302–312. https://doi.org/10.1890/14-2070.1.

Rodrigues, J.P., Duarte, A.C., Santos-Echeandía, J., Rocha-Santos, T., 2019. Significance of interactions between microplastics and POPs in the marine environment: a critical overview. TrAC Trends Anal. Chem. 111, 252–260. https://doi.org/10.1016/j.trac.2018.11.038.

Ruiz-Hernández, I.M., Nouri, M.Z., Kozuch, M., Denslow, N.D., Díaz-Gamboa, R.E., Rodríguez-Canul, R., Collí-Dulá, R.C., 2022. Trace element and lipidomic analysis of bottlenose dolphin blubber from the Yucatan coast: lipid composition relationships. Chemosphere 299, 134353. https://doi.org/10.1016/j.chemosphere.2022.134353.

Rusina, T.P., Smedes, F., Klanova, J., 2010. Diffusion coefficients of polychlorinated biphenyls and polycyclic aromatic hydrocarbons in polydimethylsiloxane and low-density polyethylene polymers. J. Appl. Polym. Sci. 116, 1803–1810. https://doi.org/10.1002/app.31704.

Shin, K.H., Kim, K.W., Ahn, Y., 2006. Use of biosurfactant to remediate phenanthrene-contaminated soil by the combined solubilization–biodegradation process. J. Hazard. Mater. 137, 1831–1837. https://doi.org/10.1016/j.jhazmat.2006.05.025.

Sim Choi, H., Woo Kim, J., Cha, Y., Kim, C., 2006. A quantitative nitroblue tetrazolium assay for determining intracellular superoxide anion production in phagocytic cells. J. Immunoassay Immunochem. 27, 31–44. https://doi.org/10.1080/15321810500403722.

Speciale, A., Zena, R., Calabrò, C., Bertuccio, C., Aragona, M., Saija, A., Trombetta, D., Cimino, F., Lo Cascio, P., 2018. Experimental exposure of blue mussels (Mytilus galloprovincialis) to high levels of benzo[*a*]pyrene and possible implications for human health. Ecotoxicol. Environ. Saf. 150, 96–103. https://doi.org/10.1016/j.ecoenv.2017.12.038.

Suaria, G., Avio, C.G., Mineo, A., Lattin, G.L., Magaldi, M.G., Belmonte, G., Moore, C.J., Regoli, F., Aliani, S., 2016. The mediterranean plastic soup: synthetic polymers in mediterranean surface waters. Sci. Rep. 6, 37551. https://doi.org/10.1038/srep37551.

Syberg, K., Khan, F.R., Selck, H., Palmqvist, A., Banta, G.T., Daley, J., Sano, L., Duhaime, M.B., 2015. Microplastics: addressing ecological risk through lessons learned. Environ. Toxicol. Chem. 34, 945–953. https://doi.org/10.1002/etc.2914.

Thompson, R.C., Olsen, Y., Mitchell, R.P., Davis, A., Rowland, S.J., John, A.W.G., McGonigle, D., Russell, A.E., 2004. Lost at sea: where is all the plastic? Science 304, 838. https://doi.org/10.1126/science.1094559, 838.

Tlili, S., Jemai, D., Brinis, S., Regaya, I., 2020. Microplastics mixture exposure at environmentally relevant conditions induce oxidative stress and neurotoxicity in the wedge clam Donax trunculus. Chemosphere 258, 127344. https://doi.org/10.1016/j.chemosphere.2020.127344.

Van den Berg, Hoefsloot, H.C., Westerhuis, J.A., Smilde, A.K., Van der Werf, M.J., 2006. Centering, scaling, and transformations: improving the biological information content of metabolomics data. BMC genomics 7, 1–15. https://doi.org/10.1186/1471-2164-7-142.

Venier, P., Canova, S., 1996. Formation of DNA adducts in the gill tissue of Mytilus galloprovincialis treated with benzo[*a*]pyrene. Aquatic Toxicol 34, 119–133.

Vroom, R.J., Koelmans, A.A., Besseling, E., Halsband, C., 2017. Aging of microplastics promotes their ingestion by marine zooplankton. Environ. Pollut. 231, 987–996. https://doi.org/10.1016/j.envpol.2017.08.088.

Young, T., Alfaro, A.C., Villas-Bôas, S.G., 2016. Metabolic profiling of mussel larvae: effect of handling and culture conditions. Aquac. Int. 24, 843–856. https://doi.org/10.1007/s10499-015-9945-0.

Zhang, H., Wang, J., Zhou, B., Zhou, Y., Dai, Z., Zhou, Q., Chriestie, P., Luo, Y., 2018. Enhanced adsorption of oxytetracycline to weathered microplastic polystyrene: kinetics, isotherms and influencing factors. Environ. Pollut. 243, 1550–1557. https://doi.org/10.1016/j.envpol.2018.09.122.

Ziccardi, L.M., Edgington, A., Hentz, K., Kulacki, K.J., Kane Driscoll, S., 2016. Microplastics as vectors for bioaccumulation of hydrophobic organic chemicals in the marine environment: a state-of-the-science review. Environ. Toxicol. Chem. 35, 1667–1676. https://doi.org/10.1002/etc.3461.