Chemical pollution and ecotoxicological effects of high-density polyethylene microplastics in *Mytilus galloprovincialis* from two Italian lagoon ecosystems

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A B S T R A C T

Transitional water ecosystems have low water exchanges and can trap chemicals and microplastics (MPs). In this study, MPs, trace elements, polycyclic aromatic hydrocarbon-PHAs levels and the oxidative stress response were assessed in *Mytilus galloprovincialis* from two Italian lagoon ecosystems (Orbetello and Varano). In addition, the ecotoxicological effects induced by the exposure of *M. galloprovincialis* to high-density polyethylene-HDPE MPs were also determined. Levels of trace elements were almost always comparable among the sites, whereas MPs were found only in mussels from Orbetello. PAHs were always under the limit of quantification. Glutathione peroxidase and malondialdehyde levels were significantly higher in mussels from Varano. As regard the exposure test, it was found a significant effect of treatment, site and their interaction on mortality and biochemical biomarkers in both fed and unfed mussels. However, principal component analysis suggests similar effects of both color and nourishment condition on biochemical biomarkers. These findings warrant further investigation.

1. Introduction

The contamination of marine ecosystems due to microplastic (MPs) particles (0.1 μm – 5 mm) is of significant and growing concern. Microplastics were found all over the world, from land (Wang et al., 2021) to ocean (Stenger et al., 2021), from atmosphere (Ding et al., 2021) to soil (Yang et al., 2021), and even in high-mountain remote ecosystems (Pastorino et al., 2022a, 2022b). Recent literature supports that MPs can cause toxic effects to aquatic organisms (Prokić et al., 2021; Aliko et al., 2022a; Multisanti et al., 2022; Savuca et al., 2022; Zhang et al., 2022). Microplastics could also act as vectors for pathogens and environmental contaminants, having additional adverse effects on the entire aquatic ecosystems (i.e., Amelia et al., 2021; Burgos-Aceves et al., 2021; Hanslik et al., 2022; Impellitteri et al., 2022). Due to their small size, MP particles entry into feeding strategy for many filter-feeding organisms, such as bivalves, which are certainly exposed to MP particles (Van Cauwenberghe and Janssen, 2014). Due to their lifestyle and physiology, mussels are globally recognized to be one of the best marine bioindicators for a long time (Cappello et al., 2018; Provenza et al., 2022). Their wide geographical distribution paired to their filter-feeding activity make them a good choice to evaluate and quantify extensive polluted area and different contamination levels (Curpan et al., 2022). Due to their low metabolism, bivalves as *Mytilus galloprovincialis* have been found to be the most suitable organisms for biomonitoring programs and for MPs pollution, providing a snapshot of the environmental conditions (Bolognesi et al., 2004; Ribeiro et al., 2017;

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Provenza et al., 2022). Indeed, Ward et al. (2019) affirmed that mussels filter selectively some particles, by using palps, based on size, shape and surface characteristics and the selection of MP particles is related to the mouth size.

A lot of studies have quantified the presence of MPs in freshwater and marine sediments (Klein et al., 2015; Fuller and Gautam, 2016; Peng et al., 2017; Scheurer and Bigalke, 2018; Townsend et al., 2019; Zhang et al., 2020; Ziajahromi et al., 2020), but few data are available on trophic web accumulation of MPs in species. Some papers reported that mussels can accumulate MP fibres at high levels even if biochemical effects of MPs exposure were not evaluated (Renzi et al., 2018a, 2018b).

Mussels are also used as resource species in aquaculture, easy to breed and with high economic value (Provenza et al., 2022). Services provided by mussels are also related to the provisioning of food biomass, supporting ecosystems, providing sheltering and new habitats (diversity enhancing), regulating atmospheric CO_2 and O_2 through carbon sequestration and nutrient recycling by feeding (Radulovich et al., 2015; Jiang et al., 2020; Van den Burg et al., 2022). Indeed, mussel farming in suspended culture represent a natural mechanism to mitigate eutrophication within the marine environment, filtering large fractions of suspended organic matter from the water column (Ward and Shumway, 2004; Box et al., 2007; Strand and Ferreira, 2019). Therefore, natural shellfish farms, such as lagoon systems, and shellfish species represent an adequate and crucial "early warning" system for ecotoxicological studies (Pagano et al., 2017; Freitas et al., 2020).

It has been demonstrated that the exposure of mussels to MP particles causes notable changes of the antioxidant enzyme system (Avio et al., 2015; Provenza et al., 2022). An increased reactive oxygen species (ROS) concentration in the tissues of the exposed organism occur as a first response, stimulating the production of antioxidant biomarkers involved in critical pathways of ROS detoxification, such as superoxide dismutase (SOD), glutathione S-transferase (GST), glutathione peroxidase (GPx), and lipid peroxidation (LPO) (Lam and Gray, 2003; Elia et al., 2020; Pastorino et al., 2020). Shellfish farming is the main production sector for Italian aquaculture and is mainly based on the mussel's production (Esposito et al., 2021). Lagoons naturally provide essential habitat for many fish and shellfish species and are considered biodiversity hotspots due to the massive presence of important species (Perennou et al., 2020). One of the main good provided by coastal lagoons is the shellfish farming, deserving notable attention due to the generated socioeconomic effects (FAO, 2015). However, increased human pressure paired to low water exchange make the lagoons highly efficient traps not only for chemicals but also for MPs. Therefore, fisheries and aquaculture activities could be significantly affected by the accumulation of microplastic particles in farmed organisms, which in turn could be a source for humans through food.

In this study, chemical and microplastic analyses were performed in *M. galloprovincialis* collected from two lagoon systems to define baseline levels of pollutants and biochemical biomarkers. Moreover, the biochemical effects induced by the of exposure of *M. galloprovincialis* (collected from two lagoon systems) to different types and color of high-density polyethylene-HDPE were determined. In particular, the following H_0 hypothesis were tested: i) the exposure of *M. galloprovincialis* to 0.05 g/L of HDPE MPs is not able to induce biochemical effects ii) *M. galloprovincialis* responses are not related to the site of origin, color and feeding condition. In this study, high-density polymer was selected as MPs model due to its large use (Renzi et al., 2018a, 2018b; Piccardo et al., 2021) and consequent abundance in aquatic environments (Renzi et al., 2019). Similarly, many different colors are available and disposed in the environment; among them, pink, blue and white colors are the largest used ones and recorded in the lagoon ecosystems (Fastelli et al., 2016; Guerranti et al., 2017). Most of plastic particles present in aquatic environments belongs to filament, while fragment results to be the second widespread shape present in aquatic environments (Renzi et al., 2018b). It is note that the deleterious impact on marine species from the accumulation of small fragments is not completely understood, and studies are needed to fill this knowledge gap (Reddy et al., 2006). Due to this, fragment particles were selected for this study. Mussels were collected from Orbetello (Tuscany, north Italy) and Varano (Apulia, South Italy) lagoons, which represent two areas where shellfish farming is a very popular activity, with *M. galloprovincialis* as the dominant species.

2. Material and methods

2.1. Sampling sites and sample collection

During December 2019, 500 specimens of *M. galloprovincialis* were collected (authorization request was not mandatory) from two different Italian lagoon ecosystems: Orbetello (north Tyrrhenian Sea) and Varano (south Adriatic Sea) (Fig. 1). These two lagoons have a long shellfish farming tradition, but the critical issues related to the mussel production are different and based on different geomorphological characteristics, hydrodynamics, and pollution history (Specchiulli et al., 2008, 2010, 2011; Renzi et al., 2013; Molinaroli et al., 2014; Renzi and Guerranti, 2018).

Farmed *M. galloprovincialis* were collected with the help of the local shellfish farmers, transported to the laboratory in tanks with brackish water within few hours, and carefully washed in filtered seawater to remove the associated debris. Then, mussels from each lagoon were measured with a caliber ($\pm$ 0.1 mm), and dimensional classes were calculated.

2.2. Chemical analyses

Chemical and microplastic analyses were performed at the beginning of the study (T_0) in animals from both lagoons. Three pools of mussels were composed of different number of organisms and different mean lengths for the two lagoon areas: 6 organisms per pool for Orbetello with a mean length of 44.5 $\pm$ 2.6 mm, and 10 organisms per pool for Varano with a mean length of 33.9 $\pm$ 1.4 mm.

2.2.1. PAHs and trace elements

Chemical levels of organic (Polycyclic Aromatic Hydrocarbon, PAHs) and inorganic (trace elements) contaminants were determined on the whole mussels (3 pools: n = 6 organisms per pool for Orbetello and n = 10 per pool for Varano) at time T_0. These types of chemicals were selected due to the wide knowledge on the studied ecosystems (Specchiulli et al., 2008, 2010, 2011; Renzi et al., 2013; Molinaroli et al., 2014; Renzi and Guerranti, 2018). Total PAHs (μg/kg; dry weight) content was determined as sum of acenaphthene (Ace), acenaphthylene (Acy), anthracene (Ant), phenanthrene (Phe), fluoranthene (Flu), fluorene (Fl), naphthalene (N), benzo[*a*]anthracene (BaA), benzo[*a*]pyrene (BaP), benzo[*b*]lfluoranthene (BbF), benzo[*g,h,i*]perylene (BghiP), benzo[*j*]fluoranthene (BjF), benzo[*k*]fluoranthene (BkF), dibenzo[*a,h*] anthracene (DBA), indeno[*a*,2,3-*c,d*]pyrene (IP), pyrene (Py), chrysene (Chry). The determination of PAHs was performed by gas chromatography/mass spectrometry (Thermo Fisher Scientific), following standardised procedures reported in EPA 3546 + EPA 8270 D 2014. EPA 3546 was used as reference for the extraction. LOQ of these analyses was 0.05 μg/kg dry weight for singles PAH and 0.1 μg/kg dry weight for PAHs summatory.

Detection (mg/kg; dry weight) of arsenic (As), cadmium (Cd), total chrome (Cr), chrome VI (Cr^{6+}), mercury (Hg), nickel (Ni), lead (Pb), copper (Cu), and zinc (Zn) were also performed on mussels. Measurements were performed by optical emission spectrometry (Thermo Fisher Scientific), following standard procedures reported in EPA 3050 B 1996 + EPA 6010 D 2014. EPA 3050B was used as reference for the acid digestion extraction. Values of the limits of quantification (LOQ; Table 1) were defined referring to the instrumental baseline obtained on blanks while quality check and quality control procedures were based on repeated blanks during the analytical process and by recovery obtained

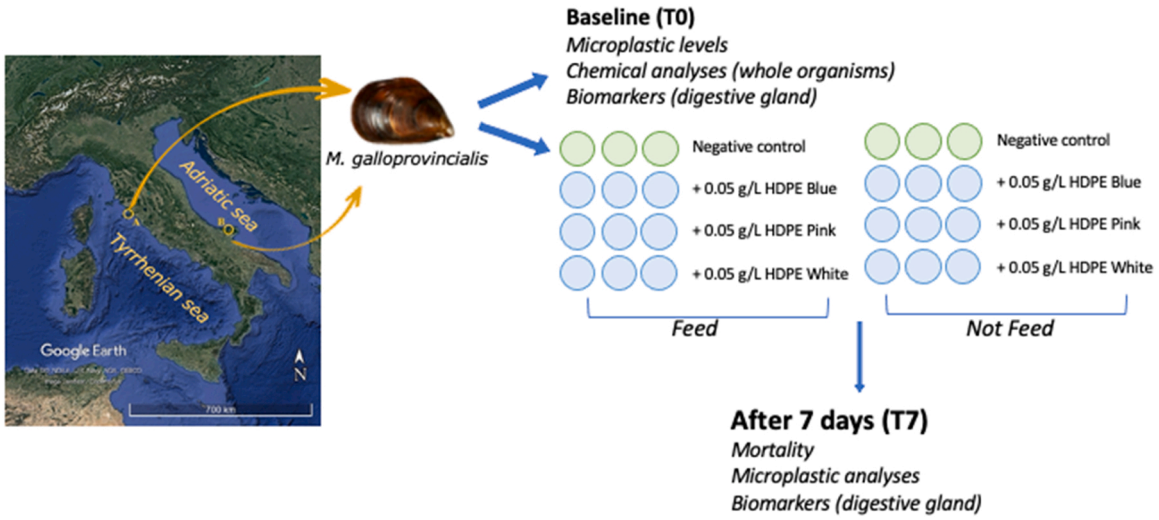

Fig. 1. Map (extracted from Google Earth Pro) of the Italian peninsula showing the position of the studied areas (yellow circles). Orbetello lagoon (A) along the Tyrrhenian coast and Varano lagoon (B) along the Adriatic coast. GPS coordinates of the mussel's collection were 42°28′15.48′' Lat. N - 11°11′52.24′'Long. E and 41°53′38.75″ Lat. N - 15°42′38.82″ Long. E for Orbetello lagoon and Varano Lagoon, respectively. Green circles represent vessels of negative control (marine water + 10 mussels per vessel); blue circles represent vessels of treatments (marine water + 10 mussels per vessel + HDPE microplastics).

from the analyses of standard reference materials (Mussel tissue SRM 2977; BRC-682) and recoveries were always higher than 85%. Results were not corrected for the recovery.

2.2.2. Microplastic determination

Mussels were carefully sectioned avoiding contamination and visually observed by microscopy (Nikon, mod. P-DSL32) connected with a camera (Nikon, mod. DSFi3). After that, tissues were completely digested with Creon (37 °C; TRIS-buffered pH) and filtered on paper fiber filter disk (pore diameters 0.6 μm) by vacuum pump according to literature (Renzi et al., 2020). Recorded particles were measured by stereomicroscopy integrating data with NS-Elements D.4.60 Software for micrometric measurements. Selected particles were measured and chemically analyzed by microscopy associated to Fourier-Transformed Infrared spectroscopy (μFT-IR), using a Thermo i-10 Nicolet MX infrared imaging microscope (Thermo Fisher Scientific), equipped with a detector optimized to work at room temperature in the spectral range 7600–450 cm^{-1} and with liquid nitrogen in the range 1800–650 cm^{-1}. Plastic particles were counted, chemically determined, and classified using a library included in the software, Thermo Scientific™ OMNIC™ Picta™.

2.2.3. Quality Assurance/Quality Control (QA/QC)

The QA/QC approach followed in this study is summarized using the main criteria reported in literature (Hermsen et al., 2018; Table 2). The BsRC research center is an ACCREDIA-certified laboratory for conducting microplastics analyses in various matrices, including biota, and ensures that the laboratory environment, equipment, and procedures used are appropriate to minimize sample contamination and improve the quality of the overall analytical process. Analyzes for the detection of microplastics were performed using a boxing glove (Iteco engineering, mod. SGS20–13599, serial number 103421) that ensures the absence of microplastic contamination from air and other sources. The materials, equipment, laboratory surfaces and gloves were carefully cleaned after the analysis of each sample. The water and reagents used to rinse and extract the samples were pre-filtered using a 0.45-micron cellulose acetate filter disk (Millipore®). The absence of airborne contaminants was monitored by exposing blank fiber filter disks (n = 5) to air in the glove box and blank samples used to evaluate the entire process (n = 5) as negative controls of the method. Samples were processed entirely in the glove box, from excision of the animal through digestion (the whole animal was digested), extraction, and identification under the microscope. Positive controls were performed with targeted microplastic particles (n = 3) subjected to the same treatment as the tested animals. The recovery rate of the particles was 100%. All pre-sorted particles were analyzed using the FT-IR technique as described in Section 2.2.2. The instrument was qualified annually by a Thermo Fischer® technician. In addition, instrument calibration (pass/fail) was checked every six months by a qualified BsRC researcher using a reference standard polystyrene material (Thermo®, Instrument Qualification Kit). Prior to the start of analyzes (daily), various certified plastic particles (PP, PE, PVC, PET) were tested and used as an internal standard to ensure instrument performance with various polymers before chemical identification of particles from tested samples began.

2.2.4. Biomarkers of oxidative stress

Biomarkers of oxidative stress (superoxide dismutase: SOD; glutathione peroxidase, GPx; glutathione S-transferase: GST; lipid peroxidation: LPO) were performed at T_0 on the digestive gland of each mussel, as it has a high potential for reactive oxygen species (ROS) production (Elia et al., 2020; Magara et al., 2021). Collected tissues were put into a test tube, the weight was recorded, and the tube was put in liquid nitrogen till analyses.

Extraction of protein fraction: the analyses were performed using the protein fraction of the collected tissues. The buffer used for the extraction was a phosphate buffer 50 mM and EDTA 2 mM (Vidal-Liñán and Bellas, 2013), added to the samples (digestive gland) in a 1:4 (w/v) ratio. The samples were homogenized using an Ultraturrax and then ultracentrifuged at 12,000 x g for 12 min at 4 °C. The supernatant was recovered and aliquoted in 2 mL test tubes and then put in liquid nitrogen. This fraction was used to determine protein content of the tissues and biochemical parameters.

Protein content (Lowry colorimetric method): protein content was quantified according to the colorimetric method based on Lowry analysis (Dubois, 1956). Briefly, the protein contents were quantified spectophometrically at 750 nm, after the reaction with NaOH 0.5 M, Folin-Ciocalteau reactive and mix of reactive ($CuSO_4$ * 5 H_2O, Rochelle salt and Na_2CO_3). Results are expressed as μg/mL.

Activity of superoxide dismutase (SOD): the activity of SOD was quantified following the method of Gao et al. (1998). This method is based on the ability of the enzyme to inhibit the autoxidation of pyrogallol. The autoxidation of pyrogallol in the presence of EDTA at the

Table 1
Baseline levels (mean values ± standard deviation) of chemical contaminants quantified in Mytilus galloprovincialis from both Varano and Orbetello lagoons. Abbreviation list and measurement units are also reported. LOQ denotes limit of quantification, d.w.: dry weight. Lowercase letters denote significant differences ($p < 0.05$) revealed by the Mann–Whitney U test.

Variable	Symbol	Unit	LOQ	Varano	Orbetello	p-value
Arsenic	As	mg/kg d.w.	0.1	11.6 ± 2.3[a]	16.7 ± 1.34[b]	0.032
Cadmium	Cd	mg/kg d.w.	0.1	1.7 ± 0.89[a]	0.4 ± 0.08[b]	0.012
Chrome	Cr	mg/kg d.w.	5.0	< 5.0	< 5.0	-
Chrome^{6+}	Cr(VI)	mg/kg d.w.	5.0	< 5.0	< 5.0	-
Mercury	Hg	mg/kg d.w.	0.1	< 0.1	< 0.1	-
Nichel	Ni	mg/kg d.w.	5.0	8.1 ± 1.23[a]	< 5.0[b]	0.021
Lead	Pb	mg/kg d.w.	2.5	< 2.5	< 2.5	-
Copper	Cu	mg/kg d.w.	0.2	8.8 ± 0.76[a]	9.9 ± 1.45[a]	0.071
Zinc	Zn	mg/kg d.w.	1	121 ± 4.45[a]	104 ± 2.23[b]	0.037
Acenaphtene	Ace	µg/kg d.w.	0.05	< 0.05	< 0.05	-
Acenaphtylene	Acy	µg/kg d.w	0.05	< 0.05	< 0.05	-
Anthracene	Ant	µg/kg d.w	0.05	< 0.05	< 0.05	-
Phenanthrene	Phe	µg/kg d.w	0.05	< 0.05	< 0.05	-
Fluoranthene	Flu	µg/kg d.w	0.05	< 0.05	< 0.05	-
Fluorene	Fl	µg/kg d.w	0.05	< 0.05	< 0.05	-
Naphthalene	N	µg/kg d.w	0.05	< 0.05	< 0.05	-
Benzo[*a*] anthracene	BaA	µg/kg d.w	0.05	< 0.05	< 0.05	-
Benzo[*a*]pyrene	BaP	µg/kg d.w	0.05	< 0.05	< 0.05	-
Benzo[*b*] lfluoranthene	BbF	µg/kg d.w	0.05	< 0.05	< 0.05	-
Benzo[*g,h,i*] perylene	BghiP	µg/kg d.w	0.05	< 0.05	< 0.05	-
Benzo[*j*] fluoranthene	BjF	µg/kg d.w	0.05	< 0.05	< 0.05	-
Benzo[*k*] fluoranthene	BkF	µg/kg d.w	0.05	< 0.05	< 0.05	-
Dibenzo[*a,h*] anthracene	DBA	µg/kg d.w	0.05	< 0.05	< 0.05	-
Indeno[*a*,2,3-*c*, *d*]pyrene	IP	µg/kg d.w	0.05	< 0.05	< 0.05	-
Pyrene	Py	µg/kg d.w	0.05	< 0.05	< 0.05	-
Chrysene	Chry	µg/kg d.w	0.05	< 0.05	< 0.05	-
Sum of PAH	PAH	µg/kg d.w	0.1	< 0.1	< 0.1	-

specific pH used (pH 8.2) is 50%. Absorbance was measured at 420 nm. SOD activities are expressed as Units/mL (U/mL). One unit is defined as amount of enzyme required to cause 50% inhibition of pyrogallol autoxidation.

Activity of glutathione S-transferase (GST): the activity of GST was quantified following the method described by Habig et al. (1974). GSTs catalyze the conjugation of the substrate 1-chloro-2,4-dinitrobenzene (CDNB, 60 mM) with glutathione (10 mM). The samples were put in a mix of reaction composed by phosphate buffer 0.1 M at pH 6.5, GST and CDNB. The absorbance was measured at 340 nm for 5 min. The activity of GSTs was determined using the extinction coefficient of 9.6 mM^{-1} cm^{-1} for CDNB. Results were related to protein content and time and were expressed in nmol/(µg*min).

Level of glutathione peroxidase (GPx): the level of GPx was quantified following the method of Badary et al. (2005). Reaction of the S9 fraction was performed with a mixture of GSH (reduced glutathione) 10 mM, GSSG reductase 2.4 U/mL, and NADPH 1.5 mM. After incubation at 37 °C for 5 min in the presence of hydrogen peroxide, GPx kinetics were quantified spectrophotometrically at 340 nm for 2 min. The measurements were obtained by calculating a delta value and expressed in nmol/(mg*min). Results are related to protein content and expressed in µmol/mg.

2.3. Laboratory exposure

Based on their length, mussels were divided into groups, and the classes with the higher number of organisms (41–55 mm and 26–45 mm, for Orbetello and Varano, respectively) were selected to set up the experiment.

Mussels (n = 240) were acclimatized for 15 days at standard environmental conditions (salinity 36‰; pH 8.12; temperature 20 ± 1 °C) (Provenza et al., 2022); then, 10 specimens (n = 5 from Orbetello; n = 5 from Varano) for each treatment (2 nourishment conditions: fed and unfed; 3 HDPE color; 2 controls) were exposed under controlled conditions for 7 days using a single glass jar per animal. Three replicates were processed for each treatment. A summary of the experimental design is reported in Fig. 1.

In each vessel, one liter of natural marine sea water filtered at 0.45 µm was added. The physicochemical water parameters were: salinity 36‰; pH 8.12; temperature 20 ± 1 °C. Mussels were exposed at a single concentration (0.05 g/L) of MPs fragments of HDPE. The concentration of 0.05 g/L is one hundred-fold of the "realistic" seawater concentration (Bour et al., 2018), and it was selected based on previous experimental exposure tests on marine mussels (i.e., von Moos et al., 2012; Pedersen et al., 2020). Three colors (pink, blue, white) were selected for the purpose of this study.

Exposure was performed under two different conditions (fasting and feeding) to determine differences introduced by nourishment. Mussels of the fed group were fed daily by the addition of 30 mg/L of commercial fish food mixture. Microplastics were added at the time T_0 and water were not changed for the entire exposure test, as detailed in Provenza et al. (2020). Temperature, light/dark cycle, oxygenation, and salinity were daily monitored using a multiparameter probe (HI98194, Hanna Instruments Inc., Woonsocket, Rhode Island, USA). For each treatment, a negative control (only filtered seawater) was performed. Mortality was checked every day and at the end of the exposure to calculate the total percentage of mortality for each experimental group. At the end of the experiment (T_7), the biochemical biomarkers were determined as reported in the Section 2.2.4.

Experiments were sized to reduce Type-I and Type-II errors, following a logical model (Benedetti-Cecchi, 2004) based on a nested hierarchical design developed according to a priori randomly defined factors of variability.

Tested factors were: i) origin of mussels (two fixed levels: Orbetello and Varano lagoons); ii) plastic colors of HDPE (three fixed levels: HDPE_P= pink high-density polyethylene; HDPE_B=blue high-density polyethylene; HDPE_W=white high-density polyethylene); iv) nourishment (two fixed levels: fed and unfed).

2.3.1. Microplastic features

Microplastic fragments of HDPE used for the experiment were made by mechanical fractioning certified materials and accurately washed before the experimental exposure to avoid any contamination. Particles of HDPE (n = 30) of different colors (blue, pink, white) were measured using a stereomicroscope (Nikon P-DSL32) connected to a camera (Nikon DSFi3) and software (NS-Elements D.4.60) calibrated with a micrometrical calibrated slide to correctly determine dimension.

The dimensional range were the follow (mean values; range): HDPE_B= 3031.8 μm (160.2–4896 μm); HDPE_W= 3214.5 μm (137.5–4889 μm); HDPE_P= 3189.2 μm (150–4757 μm) (Fig. 2). Analyses of chemical spectra were also performed before the laboratory exposure by Fourier transformed infrared detection (μFT-IR), Nicolet iN10, Thermo Scientific, Waltham, MA. Spectra are reported in Fig. S1.

2.4. Statistical analysis

Normality and homoscedasticity of data were assessed using the Shapiro-Wilk and the Levene test, respectively. The Mann-Whitney U test was used to compare baseline levels of chemical contaminants and biochemical biomarkers at T_0. As the data were normally distributed, the two-way ANOVA, with site (Orbetello and Varano), treatment group (control; HDPE_B; HDPE_P; HDPE_W), and site × treatment interaction as independent variables was used to test statistically significant differences in mortality percentage and biochemical biomarkers in mussels exposed to HDPE MPs. Dunnett's multiple comparison test was used to compare the mussels treated with HDPE MPs (control; HDPE_B; HDPE_P; HDPE_W) against the control group (natural seawater). Finally, principal component analysis (PCA) was used to check for trend in biochemical biomarkers in response to site of collection and HDPE color. Statistical significance was set at $p < 0.05$. Statistical analysis was performed using R software (RStudio, Inc., Boston, MA, USA, version 3.5.2).

3. Results

3.1. Baseline levels of chemicals and biochemical biomarkers at time T_0

3.1.1. Chemical pollution

Results of chemical analyses performed at time T_0 on mussels collected from the two lagoons are reported in Table 1. Some elements (total Cr, Cr^{6+}, Hg, Pb) were lower than the LOQ, except As, Cd, Ni, Cu and Zn. In particular, as showed significant higher concentration in mussels from Orbetello compared to Varano (Mann–Whitney U test; $p < 0.05$). On the contrary, Cd, Ni and Zn showed significant higher levels in Varano lagoon compared to Orbetello (Mann–Whitney U test; $p < 0.05$). Total PAHs and single PAH compound were lower than LOQ in both Orbetello and Varano lagoons.

3.1.2. Microplastics

Microplastics were also determined at T_0 in mussels from both lagoons. In Fig. 3, some examples of recovered items in both byssus and gills are reported. However, only items recovered in gills were determined and counted. In mussels (n = 6; 49.8 ± 4.7 mm) from Orbetello lagoon, an average of 1.00 ± 2.45 items/ind (range: 0–6 items/ind) were recorded. Items were mainly filaments of polyethylene terephthalate-PET (blue; 2640.9 ± 1266.8 μm), while only a fragment (blue; polyethylene-PE; 1566.0 μm) was recorded. In mussels (n = 6; 34.5 ± 2.3 mm) from Varano, no MPs item was recorded.

3.1.3. Biomarkers of oxidative stress

Levels of oxidative stress biomarkers in mussels collected at T_0 from the two lagoon systems showed similar baseline values for SOD: 2.4 ± 0.2 U/mg of protein and 2.2 ± 0.1 U/mg of protein for Orbetello and Varano, respectively (Mann–Whitney U test; U=2; p = 0.3). Significant higher levels of MDA were observed for Orbetello (10.8 ± 0.1 μmol MDA/min*mg of protein) compared to Varano (8.4 ± 0.1 μmol MDA/min * mg of protein) (Mann–Whitney U test; U=3; p = 0.034). GPx values were significant higher in Varano (13.7 ± 2.1 nmol/mg of protein) compared to Orbetello (5.5 ± 0.2 nmol/mg of protein) (Mann–Whitney U test; U=4; p = 0.026). A significant difference between the two lagoons was also observed for the GST levels (0.040 ± 0.02 nmol/μg*min and 0.049 ± 0.01 nmol/μg*min for Orbetello and Varano, respectively) (Mann–Whitney U test; U=6; p = 0.011).

3.2. Results after HPDE exposure (T7)

3.2.1. Mortality

Percentage of mortalities in both fed and unfed mussels exposed to HDPE are reported in Fig. 4. All the negative controls (sea water without HDPE particles) did not show dead organisms. Mortality of 63.3% was observed in both fed and unfed mussels from Varano exposed to pink HDPE MPs. HDPE-blue particles caused mortality of both fed (33.3%) and unfed (66.7%) mussels collected from Orbetello. Finally, HDPE-white particles caused mortality in both fed (33.3%) and unfed (36.7%) mussels from Varano and in unfed mussel (66.7%) from Orbetello. There was a significant effect ($p < 0.01$) of treatment (control; HDPE_B; HDPE_P; HDPE_W), site (Orbetello and Varano), and interaction treatment × site on percentage of mortality in both fed (Table S1) and unfed mussels (Table S2).

3.2.2. Microplastic recovery

In Fig. 5, some examples of blue fragments recovered in gills are reported. Average items recorded in tissues per mussel were 14 (Orbetello) and 10 (Varano). In mussels from Orbetello, recovered items showed an average size of 755.7 ± 650.1 μm (range: 138.9–2656.0 μm), whereas in mussels from Varano, recovered items showed an average size of 624.1 ± 419.2 μm (range: 143.0–1419.8 μm).

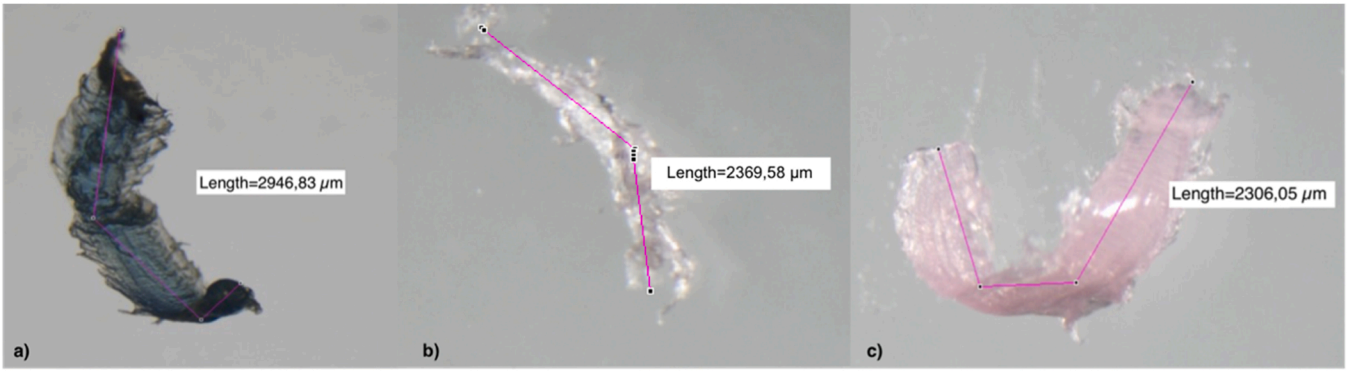

Fig. 2.. Examples of tested HDPE MPs particles (a: blue; b: white; c: pink).

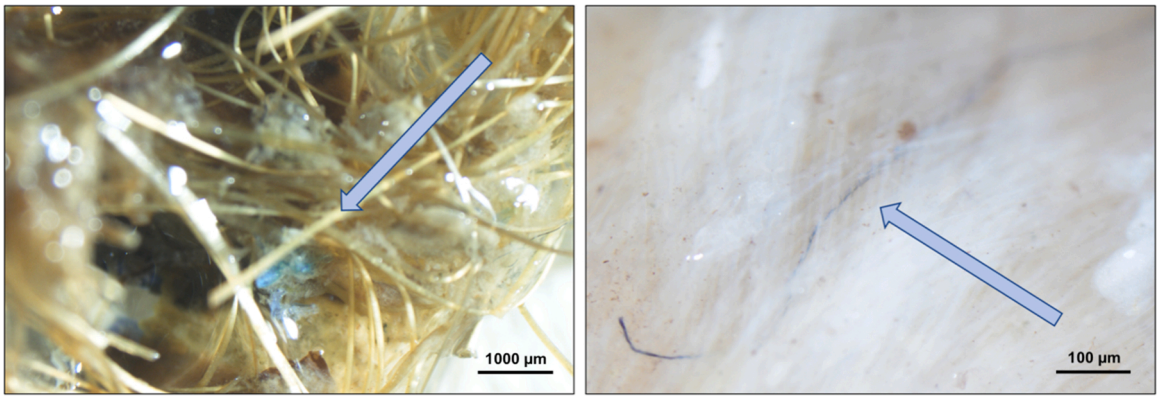

Fig. 3. Microplastic recovered in natural mussels from Orbetello at T_0. Microplastic fragment in byssus (left) and filament in gills (right).

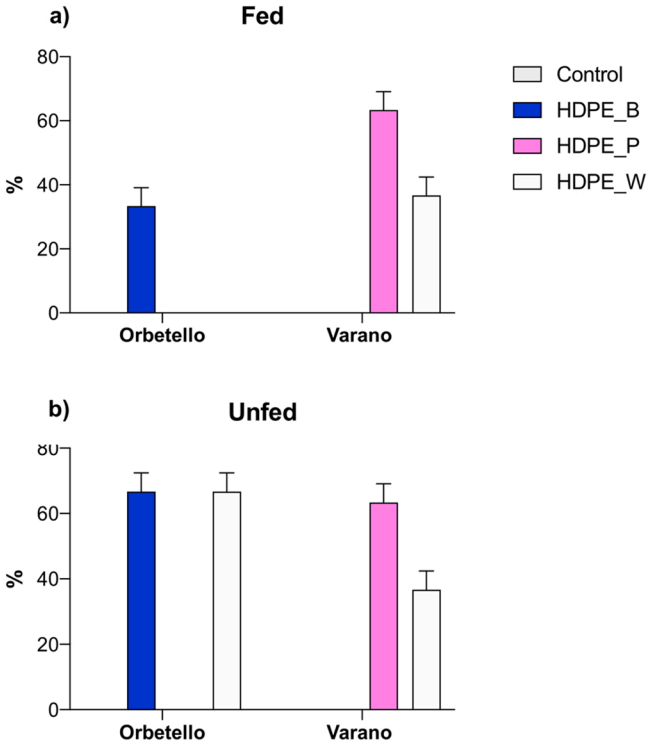

Fig. 4. Percentages of mortality (mean values ± standard deviation) in mussels exposed to HDPE (HDPE_P= pink high-density polyethylene; HDPE_B=blue high-density polyethylene; HDPE_W=white high-density polyethylene) at the end of the experiment (T7) for both fed (a) and unfed (b) mussels.

3.2.3. *Effects of site, treatment, and their interaction on biochemical biomarkers*

Table S1 and Table S2 present the results of two-way ANOVA for treatment (control; HDPE_B; HDPE_P; HDPE_W), site, and interaction treatment × site on biochemical biomarkers. Analysis showed a significant effect ($p < 0.05$) of treatment, site, and interaction treatment × site on GST, GPx and MDA levels, and a significant effect of site on SOD levels in fed mussels ($p < 0.05$) (Table S1). As regard the unfed mussels, there was a significant effect ($p < 0.05$) of both treatment and interaction treatment × time on MDA levels, and a significant effect of treatment, site, and interaction treatment × site on both GST and GPx levels ($p < 0.05$) (Table S2).

3.2.4. *Biochemical biomarkers in fed mussels*

In fed mussels, SOD levels in the treated mussels (HDPE_B; HDPE_P; HDPE_W) were comparable to those of the control group for both origin sites (Fig. 6). There was no significant difference in GST levels between the treated and the control group in mussels from Orbetello, whereas significant difference in GST levels was recorded between treated mussels and the control group ($p < 0.05$) (Fig. 6). Significant higher levels of GPx were observed for the control group compared to the treated mussels from Orbetello; on the contrary, for Varano site significant higher levels of GST were recorded for treated mussels compared to the control. MDA showed significant higher levels in mussels from Orbetello exposed to HDPE_B, whereas significant lower levels were recorded for the mussels exposed to HDPE_P and HDPE_W. Finally, significant lower MDA levels were recorded in mussels from Varano exposed to both HDPE_B and HDPE_P (Fig. 6).

3.2.5. *Biochemical biomarkers in unfed mussels*

In unfed mussels, SOD levels in the treated mussels (HDPE_B; HDPE_P; HDPE_W) were comparable to those of the control group for both sites of origin (Fig. 7). There were significant higher GPx levels in treated mussels from Orbetello compared to the control groups, whereas significant lower GPx levels were recorded in mussels exposed to HDPE_B and HDPE_P compared to the control group ($p < 0.01$) (Fig. 7). Significant higher levels of GST were observed in mussels from Orbetello exposed to HDPE_B and HDPE_P; on the contrary significant lower levels of GST were recorded for treated mussels from Varano compared to the control group. MDA showed significant higher concentrations in treated mussels from Orbetello compared to the control group; significant higher MDA levels were recorded for the mussels exposed to HDPE_B, whereas significant lower levels were recorded for mussels exposed to HDPE_W compared to the control (Fig. 7).

3.2.6. *Principal component analysis*

Principal component analysis (Fig. 8) was used to check for trend in biochemical biomarkers in response to HDPE type (color) (Fig. 8a) and feeding condition (Fig. 8b). The interpretation of principal component (PC) was assessed by considering their eigenvalue of the data matrix (only PCs with eigenvalue >1 were retained). PCA showed that the first (PC1) and the second (PC2) components accounted for meaningful amounts of the total variance (76.55%): PC1 explained 55.92% of the total variance and PC2 20.63%. The mussels exposed to HDPE MPs and the control groups are arranged by overall biochemical biomarkers. There was a clear separation between the two groups: the controls (red cluster) are located in the right part of the plot and the exposed groups in the left half. Moreover, the overlap of confidence ellipses (95%) of the three HDPE colours (pink, blue, white) (Fig. 8a) and the fed and unfed mussels (Fig. 8b) suggests similar effects of both color and nourishment on biochemical biomarkers.

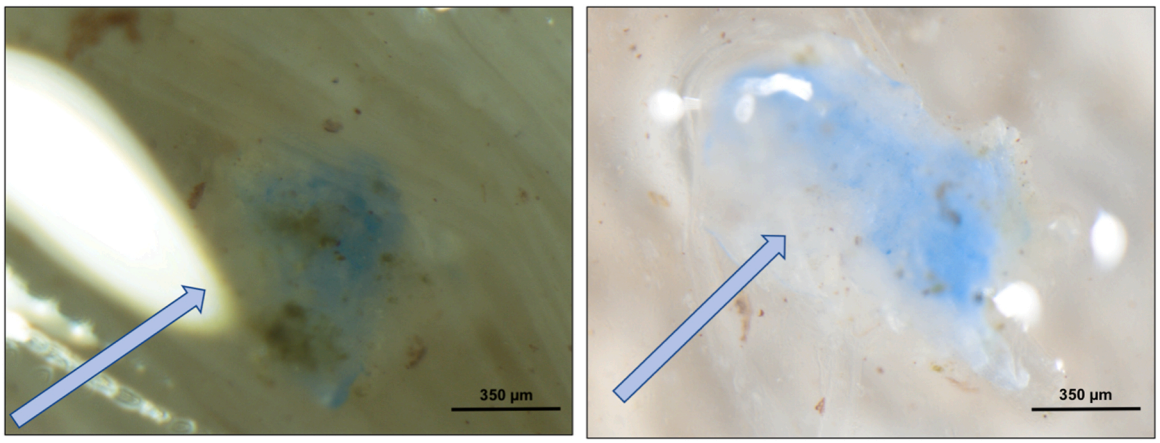

Fig. 5. Microplastic items recovered in exposed mussels at the end of the experiment (T_7): HDPE-blue fragments recovered in gills of exposed mussels from Orbetello lagoon.

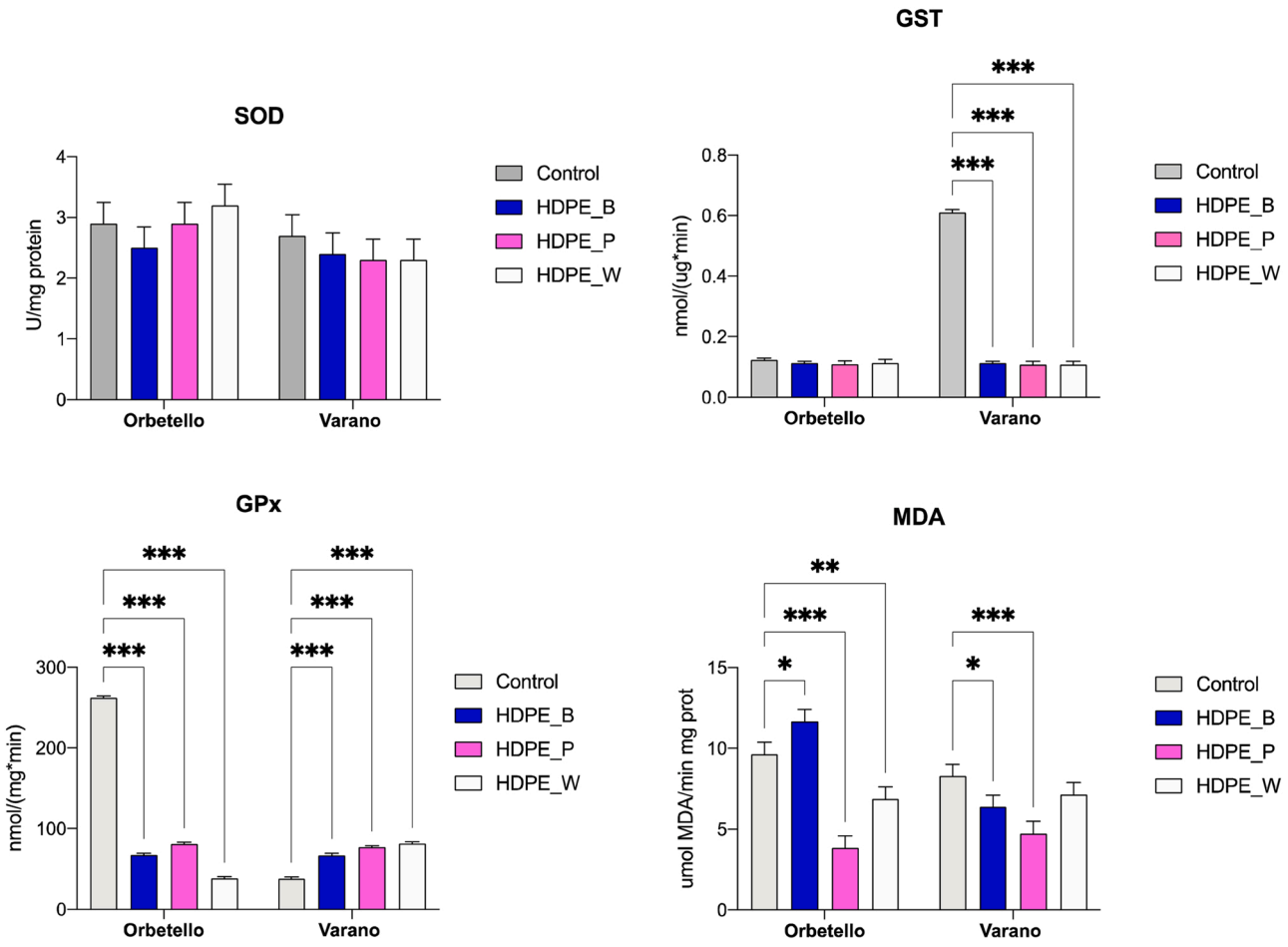

Fig. 6. Bars of biochemical biomarkers (mean values ± standard deviation) in fed mussels exposed to HDPE microplastics (HDPE_P= pink high-density polyethylene; HDPE_B=blue high-density polyethylene; HDPE_W=white high-density polyethylene). Asterisks indicate significant differences according to Dunnett's multiple comparison test (* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$). GST: glutathione S-transferase; SOD: superoxide dismutase; GPx: glutathione peroxidase; MDA: malondialdehyde.

4. Discussion

In this study, the baseline levels of chemical pollutants and biomarkers of oxidative stress levels in *Mytilus galloprovincialis* from Orbetello and Varano Lagoon were determined. Chemical analysis showed the absence of PAHs in mussels from the two lagoons and the presence of some trace elements at different concentrations, with differences among the two lagoons. A high concentration of arsenic was recorded in mussels from both ecosystems. On this path, mussels were collected in winter, and it is possible that arsenic values were higher than during other seasons, as also reported by Klarić et al. (2004). Zinc was higher in Varano mussels; nevertheless, levels were aligned with the

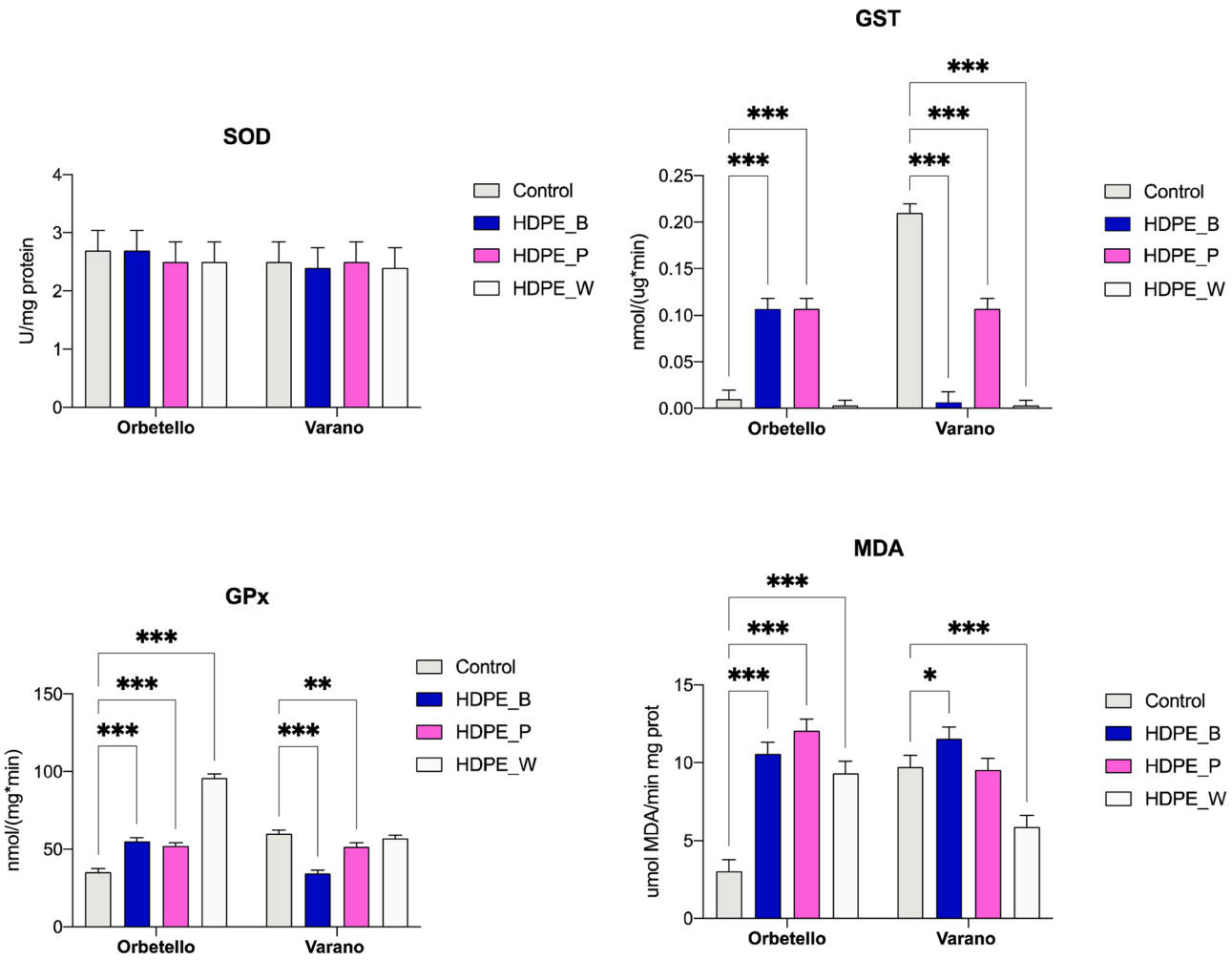

Fig. 7. Bars of biochemical biomarkers (mean values ± standard deviation) in unfed mussels exposed to HDPE microplastics (HDPE_P= pink high-density polyethylene; HDPE_B=blue high-density polyethylene; HDPE_W=white high-density polyethylene). Asterisks indicate significant differences according to Dunnett's multiple comparison test (* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$). GST: glutathione S-transferase; SOD: superoxide dismutase; GPx: glutathione peroxidase; MDA: malondialdehyde.

concentration usually present in Italian lagoon ecosystems, as reported by Adami et al. (2002) and Esposito et al. (2021).

Mussels were also characterized for the baseline levels of biochemical biomarkers. The exposure of bivalves to environmental pollutants and other abiotic factors causes the activation of their defensive system exposing antioxidant enzymes (Elia et al., 2020; Magara et al., 2021). In particular, levels of SOD and GST did not show relevant difference between ecosystems. On the contrary, mussels from Varano showed higher values of GPx and LPO. Thus, mussels from Varano were smaller in size, but more stressed than mussels from Orbetello. Environmental temperature in this case was not relevant, because similar temperatures were recorded during the sampling campaign (9.5 ± 2.3 °C and 10.4 ± 2.5 °C in Varano and Orbetello, respectively). It is known by literature that salinity fluctuations can cause a significant stress in mussels by direct damages, with the activation of the antioxidant enzymes (Elia et al., 2020). On this path, growth and filtration rate could be affected by suboptimal salinity condition (Pourmozaffar et al., 2020). Other studies (Blackmore and Wang, 2003; Heugens et al., 2001) also show how salinity is a driving factor in metal toxicity, in fact there is an inverse proportion between salinity value and free metal ion concentration. This can be related to the bioavailability and the toxicity in exposed organisms. Alteration of environmental parameters like salinity, oxygen concentration and temperature, could amplify physiological changes in aquatic organisms (Sacchi et. al, 2013) and in their ROS production (Abele et al., 1998). It is important to point out that in Varano lagoon during summer mussels are translocated by fisherman in sea and, at the end of the summer, again in lagoon. Thus, the sudden changes of both salinity and temperature during the seasons could be responsible of the high oxidative stress response recorded at T_0. Moreover, there were different sizes among mussels collected from the two lagoons. However, the size-dependent responses of mussels are less known than of other aquatic organisms like fish (Lau and Wong, 2003) and size-related differences in biomarker responses were observed in the freshwater mussels *Dreissena bugensis* affected by environmental pollution (Ács et al., 2016). Oxidative stress response could also derive from differences in bioaccumulation, uptake, elimination and leaching of chemical stressors that resulted associated to the body size of animals (Matthews et al., 2015). Another factor that can change response in biomarkers is the season. Ács at al. (2016) highlighted that during colder months there was a significant rise in lipid peroxidation (1.5–3.0-fold increase) and major DNA damage in mussels. In particular, some studies reported peeking of antioxidant enzymes activities in Dreissenidae species in late winter, when reproductive apparatus is in early spawning stage (Faria et al., 2010, 2014; Palais et al., 2012; Parolini et al., 2013). Variation in oxidative stress response of mussels among seasons is also related to changes in food availability (Leinio¨ and Lehtonen, 2005; Bocchetti and Regoli, 2006; Rank et al., 2007; Ochoa et al., 2012; Nahrgang et al., 2013).

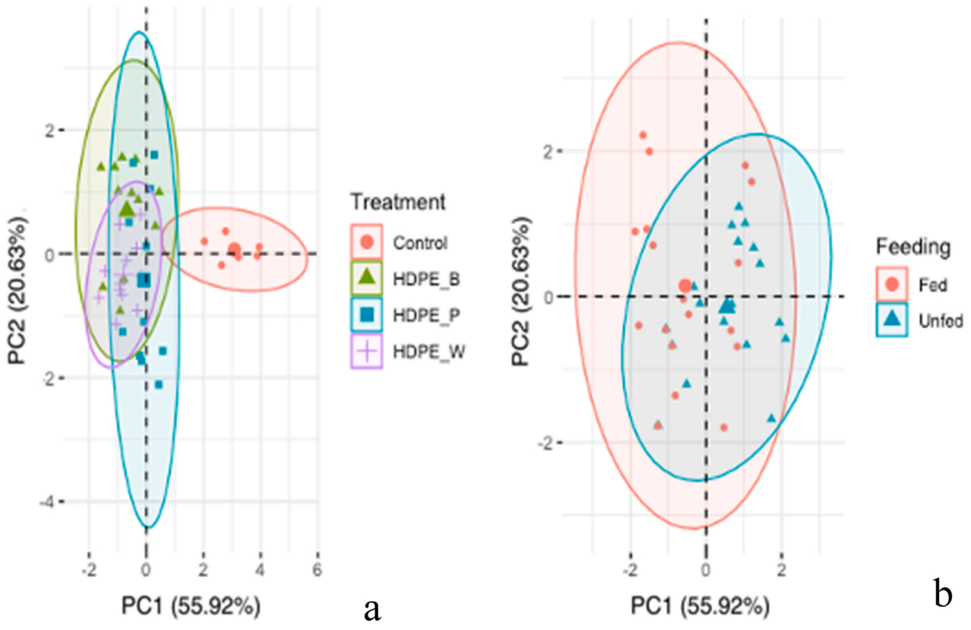

Fig. 8. Principal component analysis of overall biochemical biomarkers (SOD, GST, GPx, MDA) on mussels from Orbetello and Varano lagoon exposed to HDPE microplastics considering a) types of color (pink, blue, white) and b) feeding condition (fed and unfed). Confidence ellipses (95%) plot values for each group.

In our study, organisms were exposed to high-density polyethylene fragments of three different colours. This polymer was selected based on the literature, in fact a major part of the plastic polymer found in environment are polyethylene and polypropylene (Vianello et al., 2013; Aliko et al., 2022b). Vianello et al. (2013) reported a direct relationship between polyethylene (PE) and polypropylene (PP) MPs recovered in sediments and the percentage of mud in Venice Lagoon (Italy). Orbetello and Varano lagoons could be considered assimilable for sediment types to the Venice lagoon. HDPE was the most produced plastic in whole word, as reported in Andrady (2011). Furthermore, PE represented the main MP type recorded in mussels from the Orbetello lagoon sampled at T_0. Such assumptions are at the base of the decision to use this polymer type in our experimental research.

Exposure tests performed in this study showed that mortality percentage was absent in negative controls, suggesting that the exposure of mussels for seven days under controlled conditions did not induce a captivity stress. On the other hand, exposure of *M. galloprovincialis* to HDPE MPs was able to induce mortality after seven days of exposure. However, mortality percentage differed in animals from Orbetello and Varano in relation to nourishment. Generally, mortality percentage was higher in unfed mussels exposed to HDPE, with higher percentage in Orbetello compared to Varano. This finding suggests that *M. galloprovincialis* without nourishment could be more stressed than fed mussels. Furthermore, the difference in mussels' mortality percentage among the two lagoons could be also due to the difference in body size. Indeed, mussels form Varano were smaller in size than those from Orbetello. On this path, mussels from Orbetello could be able to better ingest larger MPs particles than those from Varano. This was confirmed by the ingestion of larger particles size (mean size: 755.7 μm) in mussels form Orbetello. *Mytilus galloprovincialis* is a suspension feeder and capture food particles from the water column (Rahman et al., 2020). In particular, it selects food based on particle size and shape (Ward and Shumway, 2004), but their capacity to identify non-food particles is limited; for this reason, mussels probably ingest MP particles (Van Cauwenberghe et al., 2015; Ward et al., 2019). The nutrition process is articulated in two phases: first, organisms inhale particles into the mantle cavity; then, they move particles through the mouth-opening into the digestive tract (Ward and Shumway, 2004).

The experiment was set up for a dose of 0.05 g/L. In literature, experimental exposure concentrations are between two to seven orders-of-magnitude higher than levels recorded in environment (Edo et al., 2020). Laboratory experiment set at environmentally relevant concentrations of MPs are scarce on mussels (Revel et al., 2019, 2020; Tlili et al., 2020). Goldstein et al. (2012) showed that concentrations between 10 μg/L and 0.1 mg/L of MPs can be considered representative of some marine polluted sites. Rosse and Loizeau (2003) reported an environmentally relevant dose of MPs ranged from 10 μg/L to 0.5 mg/L, but production trends are increasing, as well as attended levels in marine ecosystems (Gola et al., 2021). In our experiment, we used a high concentration of MPs to have a major probability to ingest MPs by mussels, as previously reported for *M. galloprovincialis* (Provenza et al., 2022). It is known that marine filter feeders are particularly susceptible to ingest suspended MPs, but it is not advisable to set up long-term studies on the potential effects of this uptake (Hamm and Lenz, 2021). In literature, the time of exposure of bivalves to MPs is quite variable (Trestrail et al., 2020; Abidli et al., 2021; Capolupo et al., 2021a, 2021b; Choi et al., 2022). It is also quite variable the frequency of water exchange and the type of exposition. For example, Capolupo et al. (2021a) exposed organisms to leachates and not directly to particles. In literature, it is well known that seven days are enough to allow mussels to ingest MP particles, but enough short to maintain organisms alive without changing water (Provenza et al., 2020, 2022).

The treatment with HDPE MPs causes biochemical stress in digestive glands of mussels. Magara et al. (2018) observed significant changes in antioxidant enzymes activity when *M. edulis* are exposed to polyethylene MP beads, in line with this study. Indeed, principal component analysis revealed how treated mussels with HDPE are clearly separated from the control group, so it means that MPs cause an effect on mussels. Our findings suggest that SOD activity was not affected by exposure to HDPE MPs. Thus, the production of superoxide anion (O_2^-) may not act a primary factor of oxidative impairment, in line with findings reported by Magara et al. (2018). On the other hand, GPx, which is involved in removal of H_2O_2, was generally the most active. Indeed, we observed a significant change of GPx levels (increasing or decreasing trend based on nourishment, treatment type and site of collection), as clearly highlighted by the two-way ANOVA. Such variability in biochemical response was also shared previously (Li et al., 2022). Part of the great variability find in literature could derive from different experimental

design and, especially, from the different time of exposure and MPs concentration (Li et al., 2022). However, GPx is very sensitive to MPs exposure, and it was proposed as a key biomarker to assess MPs toxicity (Li et al., 2022).

GST levels showed a similar trend, with increasing or decreasing activity based on site and type of treatment. This enzyme is essential for the phase II of detoxification, which involves conjugating GSH with phase I enzymes (Elia et al., 2020). As reported for GPx levels, a great variability of response is also reported in literature for GST (Li et al., 2022). For example, GST levels were found to have an enhanced activity in *M. galloprovincialis* exposed to plastic and tire rubber leachates (Capolupo et al., 2021a). Contrarily, neither the digestive gland, nor the gills of *M. galloprovincialis* exposed to chrysene-sorbed polystyrene MPs showed GST alterations (Capolupo et al., 2021c).

Malondialdehyde (MDA) levels, a by-product of lipid peroxidation, is also frequently used to evaluate oxidative stress since its determination in the mussel digestive gland has extensively been validated to ascertain the evolution of ROS-mediated effects of micro/nanoparticles (Capolupo et al., 2021c). In this study, MDA levels showed a significant change in all treated groups as reported in other studies. For examples, MDA was up-regulated in the digestive gland of *M. galloprovincialis* exposed to polystyrene MPs (3 μm) and nanoplastics (50 nm).

Finally, PCA did not show any differences when additional factors as feeding condition or color are take into consideration. Indeed, *Mytilus galloprovincialis* does not have an extreme selectivity for filtered particles, so the colours did not influence the selection of feeding resource. Indeed, particle colour is likely of low importance for bivalves, and their uptake may rather reflect patterns that occur within the surrounding aquatic environment (Joyce and Falkenberg, 2022).

5. Conclusion

In the first part of this study, mussels from two lagoon ecosystems were analyzed to assess levels of chemical pollution and oxidative stress. Findings suggest difference in contaminants uptake and oxidative stress between the mussels collected from the two-lagoon ecosystem. Assessing the baseline levels of contaminants and biomarkers is quite important since the site of origin of the organisms used to perform experiments (under controlled conditions) could influence the results. This was confirmed by the two-way ANOVA test, that showed a significant influence of the site of mussel collection on both mortality and oxidative stress biomarkers. The H_0 hypothesis that exposure of *M. galloprovincialis* to 0.05 g/L of HDPE MPs is not able to induce biochemical effects was rejected since it was observed a significant variation in biomarkers levels and mortality percentage at the end of the experiment. On the other hand, it was observed that color and feeding condition did not influence the biomarkers of oxidative stress. Further studies are needed to better understand the oxidative stress responses of mussels exposed to microplastics, also considering other types of polymers and leachates.

CRediT authorship contribution statement

Francesca Provenza: Conceptualization, Investigation, Methodology, Data curation, Writing – original draft. **Paolo Pastorino:** Conceptualization, Investigation, Data curation, Methodology, Writing – original draft. **Serena Anselmi:** Investigation, Methodology, Writing – review & editing. **Marco Leporatti Persiano:** Investigation, Methodology, Writing – review & editing. **Tommaso Scirocco:** Investigation, Methodology, Writing – review & editing. **Gianluca De Rinaldis:** Investigation, Methodology, Writing – review & editing. **Maria Cristina Fossi:** Conceptualization, Investigation, Writing – review & editing. **Cristina Panti:** Conceptualization, Investigation, Writing – review & editing. **Monia Renzi:** Conceptualization, Investigation, Methodology, Writing – review & editing, Supervision, Funding acquisition. **Antonietta Specchiulli:** Conceptualization, Investigation, Methodology, Writing – original draft, Supervision, 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.

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