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Assessing the Impact of Chrysene-Sorbed Polystyrene Microplastics on Different Life Stages of the Mediterranean Mussel *Mytilus galloprovincialis*

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Abstract: The sorption of organic pollutants to marine plastic litter may pose risks to marine organisms, notably for what concerns their intake and transfer through microplastic (MP) ingestion. This study investigated the effects of polystyrene MP loaded with chrysene (CHR) on early-stage and physiological endpoints measured in the Mediterranean mussel *Mytilus galloprovincialis*. The same concentrations of virgin microplastics (MP) and MP loaded with 10.8 µg CHR/mg (CHR-MP) were administered to mussel gametes/embryos (25×10^3 items/mL) and adults ($5 \cdot \times 10^3$ items/L); further treatments included 0.1 mg/L of freely dissolved CHR and a second CHR concentration corresponding to that vehiculated by CHR-MP during exposure (3.78 µg/L and 0.73 ng/L for gamete/embryos and adults, respectively). None of the treatments affected gamete fertilization, while 0.1 mg/L CHR induced embryotoxicity. In adults, CHR-MP and MP similarly affected lysosomal membrane stability and neutral lipids and induced slight effects on oxidative stress endpoints. CHR affected tested endpoints only at 0.1 mg/L, with lysosomal, oxidative stress and neurotoxicity biomarkers generally showing greater alterations than those induced by CHR-MP and MP. This study shows that the CHR sorption on MP does not alter the impact of virgin MP on mussels and may pose limited risks compared to other routes of exposure.

Keywords: POPs; chemical sorption; bivalves; embryotoxicity; biomarkers

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1. Introduction

The demand for plastic products has globally risen over the past decades, resulting in the record of 368 million tonnes of plastics only produced in 2019 [1]. In addition to a prevalent use in modern society, the improper disposal of single-use items has progressively determined the widespread contamination of aquatic systems by plastic waste, which currently accounts for nearly the 80% of the oceanic litter [2,3]. According to recent appraisals, the rate of plastic input in the marine environment is around 9.5 metric tonnes per year, with a two- to ten-fold increase projected by the next ten years [4,5]. In this scenario, the potential environmental risks posed by microscopic plastic debris are capturing ever-growing attention [6–8]. Around five trillion < 5-mm plastic fragments, commonly referred to as microplastics (MP), are estimated to float into world's ocean [9]. MP mainly originates from the weathering-driven degradation of larger plastics, although a lower fraction is represented by micro-sized items designed for industrial/engineering processes (also known as primary microplastics) [10,11].

Several reports have documented the ingestion and tissue translocation of MP in marine taxa spanning from planktonic bacteria and algae to cetaceans and vertebrates at

the apex level of the trophic chain [10,12–15]. One of the effects most frequently associated to the MP ingestion is the physical blockage of the gastrointestinal tract, which produces outcomes of variable intensity: from impaired nutritional status to increased mortality rates [12,16,17]. Evidence of unspecific effects were also recorded in marine fish and invertebrates, including embryotoxicity, tissue inflammation, oxidative stress, transcriptional alterations, immunological and behavioural alterations [18–21].

A recurring paradigm in MP pollution is represented by their potential to act as vehicle of chemical pollutants, including either additives, degradation products or waterborne POPs [22–24]. Hydrophobic compounds, such as polychlorinated biphenyls (PCBs) and polycyclic aromatic hydrocarbons (PAHs) have been found to display an affinity for MP sorption up to two orders of magnitude higher than natural sediments [25,26]. According to Lee et al. [27], Log partition coefficients for PAHs on MP are comprised between 3 and 6 depending on chemical (molecular weight/Log K_{ow}) and physical (plastic polymer type/structure/charge) factors. Consistency has been observed between the concentrations of sorbed (legacy and emerging) pollutants in MP collected in natural systems and those experimentally loaded in batch experiments, with levels spanning from a few ng/g to tens of mg/g of particles [23,24,26].

A recent study by Nor and Koelmans [28] found MP to have a biphasic behaviour with respect to organic pollutants after ingestion by aquatic organisms: they tend to absorb chemicals in a relatively contaminated gut fluid and to release the chemicals under clean gut conditions. In this respect, experimental evidence suggests that the desorption of persistent organic pollutants (POPs) after ingestion may synergistically increase the toxicity of MP to aquatic species. For instance, hepatic stress associated to glycogen depletion and cell necrosis was reported in fish (*Oryzias latipes*) exposed to beach-collected MP contaminated by PAHs, PCBs and PBDEs [29]; reduced phagocytic activity was recorded in the lugworm *Arenicola marina* exposed to nonylphenol-loaded PVC MP [30], while mussels of the genus *Mytilus* exposed to MP loaded with benzo[a]pyrene, fluoranthene and pyrene showed increased body burden of tested chemicals, histopathological alterations, immune and physiological disorders [18,31,32].

Based on the above considerations, this study aimed at assessing the adverse effects of polystyrene MP loaded with the PAH chrysene (CHR) on different life stages of the Mediterranean mussel *Mytilus galloprovincialis*. Mussels of the genus *Mytilus* have been suggested as a global bioindicator of MP pollution for coastal areas based on their sessile ecology, widespread distribution, high filtration/accumulation rate and responsiveness to physical and chemical stressors [33]. CHR is one of the sixteen PAHs included in the US EPA list of high priority substances due to their prevalence/persistence in the environment and potential toxicity to humans and biota [34]. It shows a relatively high molecular weight (228.3) and a Log K_{ow} of 5.73 [35], indicating affinity for organic matter and possible migration into/across biological systems. To accurately assess whether (and to what extent) the CHR sorption affects the mussel biological parameters, treatments involved single exposure to virgin MP, CHR and CHR-loaded MP. The analysed endpoints encompassed early life stages endpoints (gamete fertilization, embryo-larval development), and a battery of lysosomal, oxidative stress and neurotoxicological biomarkers analysed in adult mussels following a 7-day exposure to selected treatments.

2. Materials and Methods

A stepwise approach represented by (i) preliminary sorption trials, (ii) selection of CHR-MP co-incubation timing and (iii) mussel early stages/adult exposure was implemented in this study to evaluate the potential effects of CHR-sorbed MP on the *M. galloprovincialis* ontogeny and physiology. This technical framework was undertaken to establish the timing of CHR absorption and its concentration per mass of MP, aiming at regularly exposing organisms to particles loaded with a known CHR concentration. To reliably estimate the effects caused by the uptake of CHR-sorbed MP on the mussel ontogeny

and physiology, data were compared with those obtained following single exposure to both virgin MP and freely-dissolved CHR.

2.1. Sorption of Chrysene on Polystyrene MP

An experimental method was implemented in this study to determine a CHR sorption on MP which might be reliably based on time-dependent sorption analyses, quantifiable by chemical measurements and suitable to be employed for mussel experimental exposure. Preliminary trials aimed at characterizing the CHR sorption on MP were performed according to Batel et al. [36] in order to identify the suitable incubation time to generate CHR-sorbed MP to be subsequently used for experimental exposures. A solution containing 0.5 mg of 3-µm polystyrene MP (described in detail in SM, Section S1) and 0.5 mg/L chrysene (CHR, Merck Life Science, Milan, Italy) was prepared in 20 mL Milli-Q® water (in Pyrex® glass beakers) and kept in agitation at room temperature for 0, 3, 6, 12 and 24 h. At each interval, the CHR-MP mixture was filtered on a 0.45-µm nylon filter (in a sterile 25-mL syringe) to isolate MP, and the extracted aqueous medium was stored at −20 °C for subsequent analysis. After washing the syringe filter with Milli-Q® water, MP were recovered in 40 mL of 0.22 µm-filtered seawater (FSW) and used as stock of CHR-loaded particles for experimental purposes. In line with recent investigations on POPs sorption on MP [32,36,37], the amount of sorbed CHR was preliminarily quantified by comparing the concentration of freely dissolved CHR in the extracted aqueous medium with that of a "control" solution of 0.5 mg/L CHR (with no MP added) kept in agitation parallelly to the CHR-MP mixture and subjected to the same filtering steps described above.

2.2. Chemical Quantification of CHR Sorbed on MP

The CHR concentration was measured in the aqueous extracts recovered after 0, 3, 6, 12 and 24 h of MP-CHR co-exposure and in the CHR control solution following the modified US EPA Method 8272 [38]. An aliquot (1.5 mL) of the aqueous extract solution was added to a vial with a small Teflon-coated stir bar and spiked with 0.1 mL of chrysene-d_{12} solution (5 mg/L in acetone) as an internal standard. Direct immersion solid phase microextraction (SPME) analyses were performed by directly exposing the commercially available polydimethylsiloxane (PDMS, polydimethylsiloxane-coated fused silica fiber, Supelco, Darmstadt, Germany) fiber into the sample solution under magnetic stirring. After 30 min of exposure, the fiber was inserted into the injector port maintained at 300 °C in the splitless mode of a Shimadzu GC–MS-QP2010 (Shimadzu, Kyoto, Japan). Separation was conducted with a Zebron ZB-35 column (95% dimethyl, 5% diphenylpolysiloxane 30 m, 0.25 mm, 0.25 µm film; Phenomenex Inc., Torrance, CA, USA), using helium as the carrier gas. The following thermal program of the capillary column was used: from 50 °C to 310 °C at 10 °C/min, then a hold for 2 min at 310 °C. Acquisition was performed under electron ionisation at 70 eV using the selected ion monitoring mode of the ion m/z 228 for the detection of chrysene and m/z 240 for the detection of chrysene-d_{12}. Prior to sample analysis, blanks were performed first with a thermal desorption of the fiber alone and then after a 30 min exposure to a solution of deionized water. Further details on CHR calibration and recovery are reported in SM (Section S2, Figure S1). After absolute quantification, the concentration of CHR sorbed on MP was assessed by normalizing data per gram of co-incubating MP. A decrease in CHR concentration was generally detected between the CHR control solution and the aqueous solution extracted after MP/CHR incubation at all the intervals comprised between 3 and 24 h (Table 1). The CHR sorption on MP over time fitted with a non-linear (three-parameter) regression model (R^2 = 0.971; SM, Figure S2). An exponential trend of sorption was detected between 6 and 12 h of MP/CHR co-incubation, while it tended to stabilize at 10.8 µg CHR per mg of MP at 24 h (SM, Figure S2). On these bases, 24 h of incubation was selected as the CHR/MP co-exposure timing to produce the CHR-loaded MP used in the following exposure treatments.

Table 1. Chrysene (CHR) concentrations measured using GC-MS in filtered water after incubation of 0.5 mg of 3-µm MP in 0.5 mg/L CHR solution and in a CHR 'control' solution having the same CHR nominal concentration and incubated for the same time intervals in the absence of MP. Data are expressed as mean ± SD (n = 3).

Sample	Incubation Time (Hours)	[CHR] mg/L ± SD
0.5 mg/L CHR	0	0.64 ± 0.01
0.5 mg/L CHR + MP		0.63 ± 0.03
0.5 mg/L CHR	3	0.63 ± 0.01
0.5 mg/L CHR + MP		0.53 ± 0.02
0.5 mg/L CHR	6	0.64 ± 0.03
0.5 mg/L CHR + MP		0.52 ± 0.08
0.5 mg/L CHR	12	0.74 ± 0.08
0.5 mg/L CHR + MP		0.35 ± 0.05
0.5 mg/L CHR	24	0.45 ± 0.02
0.5 mg/L CHR + MP		0.15 ± 0.03

2.3. Mussel Acclimation and Selected Experimental Treatments

Sexually mature mussels (*M. galloprovincialis*) were harvested from fishermen of the COPRALMO cooperative in Cesenatico (NW Adriatic Sea, Italy), and acclimatised in laboratory for ten days in artificial seawater (ASW, prepared according to ASTM, [39]) at controlled physico-chemical conditions (16 °C and at a density of 1 mussel L ASW^{-1}). Subsequently, exposures were performed involving early (gametes and embryos) and adult mussel stages to screen the effects of CHR-loaded particles on different phases of the *M. galloprovincialis* life cycle. Irrespective to the mussel stage, organisms were exposed to virgin MP (MP) and to the same concentration of CHR-loaded MP (hereafter defined as CHR-MP). Notably, these were 25 × 10^3 MP/mL FSW for early stages and 5 × 10^3 MP/L FSW for adult mussel exposure. These concentrations were selected based on the available knowledge on MP treatments able to induce a ready ingestion by larvae and/or sub-lethal effects in adult marine invertebrates, including bivalves [19,40]. The stock of CHR-sorbed MP to be used in bioassays or adult mussel exposures was freshly renewed before each administration by co-incubating MP and CHR in Pyrex beakers for 24 h as described previously. At each administration, MP concentrations were checked microscopically using a Bürker chamber (HBG, Giessen-Lützellinden, Germany); based on these measurements, the real tested concentrations were 23.67 ± 2.46 × 10^3 MP/mL (corresponding to a mass/volume concentration of 0.35 mg MP/L) for early stages tests and 4.57 ± 0.30 × 10^3 MP/L (i.e., 67.7 ng MP/L) for adult in vivo exposure, indicating consistency with selected nominal concentrations. Further treatments included 0.1 mg/L CHR, chosen as a positive control based on literature information on CHR toxicity to aquatic species (i.e., scallops [41,42] and shrimps [43]), and a second CHR treatment representing the actual concentration vehiculated by CHR-loaded MP in the CHR-MP condition. The latter was included to comparatively assess the effects of CHR-loaded MP with respect to an identical dosage of freely dissolved CHR and was given by the product of the amount of CHR sorbed per milligram of MP (10.8 µg) and the amount (in mg) of MP/L ASW^{-1} used during early life stages and adult mussel tests (0.35 and 0.0000677, respectively). Notably, these treatments were 3.78 µg/L CHR for early life stages tests and 0.73 ng/L for adult mussel in vivo exposure. In all performed tests, a control condition represented by ASW-only exposure was performed in parallel to treatments. All experiments were conducted at 16 ± 1 °C, as reported previously [44]. Replicate numbers (n) adopted in each test are reported in the sections below.

2.4. Gamete Fertilization and Embryotoxicity Tests

The gamete fertilization test was performed according to Capolupo et al. [45]. Aliquots of sperm collected from spontaneously spawning mussels were added to 12-well plates containing MP, CHR-MP or CHR at the above-mentioned concentrations. After 60 min, eggs were added at a 1:10 ratio with spermatozoa. The test was blocked after 30 min by adding 4% formol-calcium to each well. The egg fertilization success was identified using an inverted microscope (Carl Zeiss, PRIMOVERT series; Oberkochen, Germany; 40× magnification) by the presence of the polar lobe and/or cell division [45]. Data were expressed as the mean ± standard error of the mean (SEM) (n = 5) of the percentage of fertilization success attained at each tested condition. A mean fertilization rate >60% and ≤98% in controls was set as criterion to establish the test validity in line with Environment Canada [46].

The embryotoxicity test was performed according to Fabbri et al. [47], with slight modifications. Mussel eggs and spermatozoa were mixed (1:5 ratio) in 250 mL filtered seawater to allow for fertilization. Fertilized eggs were checked microscopically (>90% egg fertilization) and added to 96-well plates spiked with selected concentrations of MP, CHR-sorbed MP and CHR. After a 48 h incubation, samples were fixed with 4% formol-calcium and examined using an inverted microscope. Larvae were identified as abnormal in the presence of developmental defects (concave, malformed or damaged shell, protruding velum) or delayed stage (trocophore or earlier stages) and normally developed when they showed the typical morphology of the D-shaped veliger stage (straight hinge). Data were expressed as the mean ± SEM (n = 5) of the percentage of normally developed larvae. The test was considered acceptable if more than 70% embryos from control conditions showed a normal development [39].

2.5. Adult Mussel Exposure and Biomarker Analysis

2.5.1. Experimental Design

After acclimation, groups of ten adult mussels were placed into vessels containing 10 L ASW (1 mussel/L) and exposed to selected MP, CHR-MP and CHR concentrations. All conditions (including control) were carried out in triplicate (n = 3) and maintained at 16 °C, 12 h:12 h (light:dark) photoperiod, and constant aeration (>90% oxygen saturation). To ensure the constant exposure to selected conditions, all treatments were renewed daily after water change and feeding (Coral Diet Filtrator, Xaqua, Italy). At each renewal, mussels were carefully washed using ASW, dried rapidly and re-spiked with the same treatments, including MP, CHR and CHR-sorbed MP freshly produced in Pyrex glass beakers following 24 h of MP/CHR co-incubation (as described previously). The experiment was blocked after 7 days of exposure, which is recognized as the necessary time for *Mytilus* spp. to modulate cellular, biochemical or physiological parameters in response to chemical and physical stressors [31,48,49]. After exposure, mussels were dissected and tissues immediately used for toxicological assessments (haemolymph) or stored at −80 °C to be analysed subsequently (gills, digestive gland). To debate the possibility of biases related to the mussel inter-individual variability, data from each replicate (vessel) were expressed as the mean (± SEM) level of endpoints from four randomly selected mussels, as better described in the below sections.

2.5.2. Lysosomal Membrane Stability (LMS)

The LMS assessment was performed in mussel haemocytes using the Neutral Red Retention Assay (NRRA) described by Martínez-Gómez et al. [50]. Pools of haemolymph from four mussels per replicate (vessel) were placed onto slides for microscopy and spiked with a 0.01% solution of Neutral Red (NR) acidophile dye (Merck Life Science, Milan, Italy). Attached haemocytes were regularly checked using an optical microscope to evaluate the NR retention time (NRRT), which is the time (min) when more than 50% of the haemocytes' lysosomes released the NR into the cytosol.

2.5.3. Cytochemical Parameters

Lipofuscin (LF) and neutral lipid (NL) content was histologically assessed on cryosections of mussel digestive gland, which is a known target tissue of many classes of environmental pollutants [51,52]. Glands of four mussels per vessel (replicate) were dissected, grouped on aluminium tissue holders for microtomy (chucks), and immediately immersed in pre-cooled N-hexane. Thus, ten-µm gland sections were obtained using a cryostat microtome and rapidly placed onto glass slides at −30 °C in line with the protocol reported by UNEP/RAMOGE [53]. LF and NL were subsequently assessed according to Donnini et al. [54]. The LF content was assessed by selectively staining the tissue using the Schmorl ferric-ferricyanide reagent (1% $FeCl_3$ and 1% $Fe[KCN]_6$ at a 3 to 1 ratio) for 15 min. For NL, gland sections were exposed for 15 min to a 1% Oil Red O (Merck Life Science, Milan, Italy) solution prepared in 60% triethyl phosphate. After selective staining, both LF and NL slides were analysed in light microscopy at 40× magnification. Four snap photos per each gland were taken using a digital camera (AxioCam MRc, Carl Zeiss, Milan, Italy) and analysed using the image analysis software Scion Image ver. 4.0.2 as described previously [54].

2.5.4. Enzymatic Biomarkers

The activities of the enzymes glutathione S-transferase (GST), catalase (CAT) and acetylcholinesterase (AChE) were assessed in pools of digestive glands and/or gills from four mussels per replicate (vessel). Tissues were homogenised using specific reaction buffers and centrifuged as described in detail in SM (Table S1). GST and CAT were measured in mussel digestive glands and gills as reported previously [19]. For GST activity determination, samples were read for 10 min (at 1 min intervals) to measure the reaction kinetics between GST and the substrate 1-chloro-2,4-dinitrobenzene (CDNB, Merck Life Science, Milan, Italy) in the presence of reduced glutathione (GSH). CAT activity was evaluated by tracking the decrease of sample absorbance at 240 nm for 2 min (at 10 s intervals) in the presence of 55 mM H_2O_2. AChE activity was measured in gills according to Valbonesi et al. [55]. Samples were read at 405 nm for 10 min in the presence of 0.5 mM acetylthiocholine iodide and 0.33 mM 5.5′-dithiobis-2-nitrobenzoic acid (DTNB, Merck Life Science, Milan, Italy).

2.6. Statistical Analysis and Data Integration

Data were analysed using the statistical software SigmaPlot 13 (Systat Software Inc. San Jose, CA, USA). After testing the data for normal distribution and homoscedasticity using the Shapiro-Wilk and Levene's tests, respectively, significant differences ($p < 0.05$) among treatments (including control) were assessed performing a one-way analysis of variance (ANOVA) followed by the Bonferroni's *post-hoc* test for comparisons.

Multivariate principal component analysis (PCA) was performed using the software Primer-e Ver. 6 (Auckland, New Zealand) to investigate the potential relationships among tested endpoints and treatments. The applied battery of biomarkers encompassed parameters showing decreasing (LMS, AChE), increasing (LF) and bell-shaped (NL, GST, CAT) response over toxicant exposure. Therefore, prior to performing the PCA, data from all measured biomarkers were pre-treated to be expressed as increasing percentage of variation with respect to controls to reduce the likelihood of biases due to different basal levels, scale of observation and/or toxicological profile. A maximum of 5 principal components was selected for the analysis; the PCA graphical output was selected to include the spatial distribution of both factors (treatments) and variables (endpoints), the latter being visually displayed based on the eigenvector scores.

Biomarker data were further integrated using the Mussel Expert System [56], which assigns a unique Health Status Index (HSI, in a scale from A to E) to performed treatments based on biomarker modulation(s) and toxicological profiles (more details are reported in SM, Section S3).

3. Results and Discussion

3.1. Gamete Fertilization and Embryotoxicity

The effects of tested MP, CHR-MP and CHR treatments on the mussel gamete fertilization and embryonic development are shown in Figure 1. The percentage of fertilized eggs in control treatments was 88.91%, which is in line with values required for test validation [46]. The results did not highlight differences between the gamete fertilization success attained in control conditions and that measured after exposure to MP, CHR-MP, 0.1 mg/L and 3.78 µg/L CHR (Figure 1A); similarly, the fertilization rate did not change among performed treatments. The impact of plastic debris on gamete fertilization of marine invertebrates may depend on either the size or the degree of functionalization of proffered particles [57,58]. In a recent study, Tallec et al. [59] reported a decreased fertilization success following oyster (*Crassostrea gigas*) gamete exposure to 50-nm aminated PS nanoparticles, while no effect was recorded following exposure to plain (2-µm) polystyrene MP. This may denote a relatively high degree of adaptation of mussels' spermatozoans to the physical disturbance induced by micro-sized debris, as well as a negligible effect of the tested level of MP-sorbed and freely dissolved CHR on their motility/viability. However, the current knowledge on MP or POPs toxicity on mussel gamete fertilization is still limited, and even less is known on the possible cumulative effects induced by their interaction. Further investigations are thus essential to clarify these points.

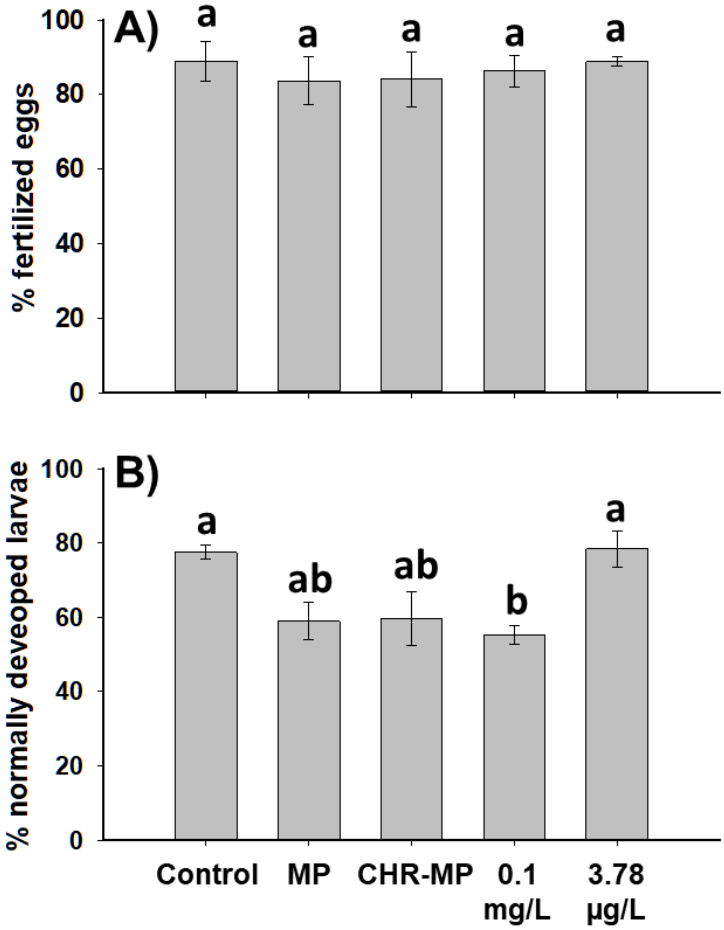

Figure 1. Mean percentage (± SEM, n = 5) of fertilized mussel eggs (**A**) and normally developed D-veliger larvae (**B**) measured after exposure to 25 × 10^3 units/mL FSW of polystyrene microplastics (MP) and chrysene-loaded microplastics (CHR-MP), 0.1 mg/L and 3.78 µg/L of freely dissolved CHR (without MP). Different letters denote statistically significant differences ($p < 0.05$, ANOVA—Bonferroni *post-hoc* comparison) among treatments.

Embryotoxicity data recorded after 48 h of embryo exposure also showed basal levels within the thresholds for test validity (77.50% normally developed D-veligers in the control condition; ASTM [39]) (Figure 1B). The treatment at 0.1 mg/L CHR was the only condition resulting in a significant decrease of normally developed mussel larvae compared to control. The morphology of larvae exposed to this treatment did not differ from that assessed following MP and CHR-MP exposure, but was significantly lower compared to 3.78 µg/L CHR. As highlighted in previous investigations on plastic-associated chemicals [44], the mussel embryonic development generally shows a higher sensitivity to POPs (CHR in this case) compared to gamete fertilization, likely depending on the relatively higher permeability of embryos to hydrophobic substances, as previously speculated [60]. Although further evidence is not reported for CHR, other PAHs such as benzo[a]pyrene, naphthalene, phenanthrene and pyrene are known to elicit embryotoxic effects on marine invertebrates and fish [61–63]. Embryotoxicity data reported here confirm previous evidence of low to absent toxicity of plain polystyrene MP on the fast-evolving processes governing the mussel embryonic development [64] and indicate the lack of cumulative effects of MP-sorbed CHR on the morphology of mussel (D-shaped) veliger larvae. In line with these observations, the co-exposure of MP and 4-nonylphenol performed by Beiras and Tato [65] did not cumulatively increase the effect of polyethylene MP on the embryo-larval development of the sea urchin *Paracentrotus lividus*. Conversely, evidence of increased developmental abnormalities was observed in medaka (*Oryzias latipes*) embryos exposed to benzo[a]pyrene and PFOA-sorbed polyethylene MP compared to virgin particles [66], suggesting that the magnitude of effects induced by POPs -loaded MP may vary depending on specific traits characterizing the ontogeny of marine species.

3.2. Lysosomal Parameters

Lysosomal parameters measured in adult mussels after 7 days of in vivo exposure to selected treatments are shown in Figure 2. A significant LMS reduction with respect to control was observed in haemocytes of mussels exposed to MP, CHR-MP and 0.1 mg/L CHR (Figure 2A). The latter treatment also induced a significant LMS decrease compared to MP, CHR-MP and 0. 73 ng/L CHR. Similarly, the NL content measured in mussel digestive gland showed a significant increase compared to control following mussel exposure to MP, CHR-MP and 0.1 mg/L CHR (Figure 2B). No significant change was identified between CHR-MP and MP, both showing levels significantly lower compared to 0.1 mg/L CHR. NL content measured at 0.73 ng/L CHR was significantly lower compared to those measured in organisms exposed to MP, CHR-MP and 0.1 mg/L CHR.

The intra-lysosomal content of LF increased significantly compared to control in digestive cells of mussel exposed to CHR-MP and 0.1 mg/L CHR (Figure 2C). Mussels exposed to MP showed increased though not significantly different LF levels compared to control and CHR-MP-treated mussels. LF levels measured at 0.73 ng/L CHR were similar to control and significantly lower compared to CHR-MP and 0.1 mg/L CHR.

In bivalves, lysosomes play an active role in food intracellular uptake and cell turnover, and modulate the overall organisms' response to external stressors by regulating toxicant inactivation and cell homeostasis [67]. Toxicant-induced lysosomal dysfunctions are thus generally recognized as early-warning signals of possible alterations to higher functions, such as growth and/or reproduction [67,68]. LMS and NL alterations have been widely documented in mussels exposed to both PAHs [69,70] and MP [18,19] and describe the onset of a stress syndrome associated to an increased susceptibility toward physicochemical/microbiological agents and fatty acid metabolism disorders, respectively. LF are final products of the peroxidation of biological membrane lipid layers, which may result in cell injuries and altered organ functions in the long-term. Overall, LMS, NL and LF data from this study indicate that the mussel exposure to 0.1 mg/L CHR have higher deleterious consequences on lysosomal integrity and functionality than micro-sized particle exposure, and that, at the tested experimental conditions, the sorption of CHR did not increase the toxicity of plain MP on most lysosomal endpoints. Similar evidence was obtained by González-Soto

et al. [31], who did not observe changes in LMS variations between mussels exposed for 26 days compared to plain and benzo[a]pyrene-loaded (0.5 to 4.5 um) polystyrene MP. The lack of effects observed at 0.73 ng/L CHR allows us to hypothesize that the amount of CHR sorbed on MP was not sufficient to trigger additional effects to those elicited by plain MP on lysosomal endpoints. However, though levels of all lysosomal parameters did not significantly differ between MP and CHR-MP, an increased LF content compared to control values was observed in CHR-MP-exposed mussel, possibly reflecting a cumulative action of MP and sorbed PAH on lipid peroxidation pathways.

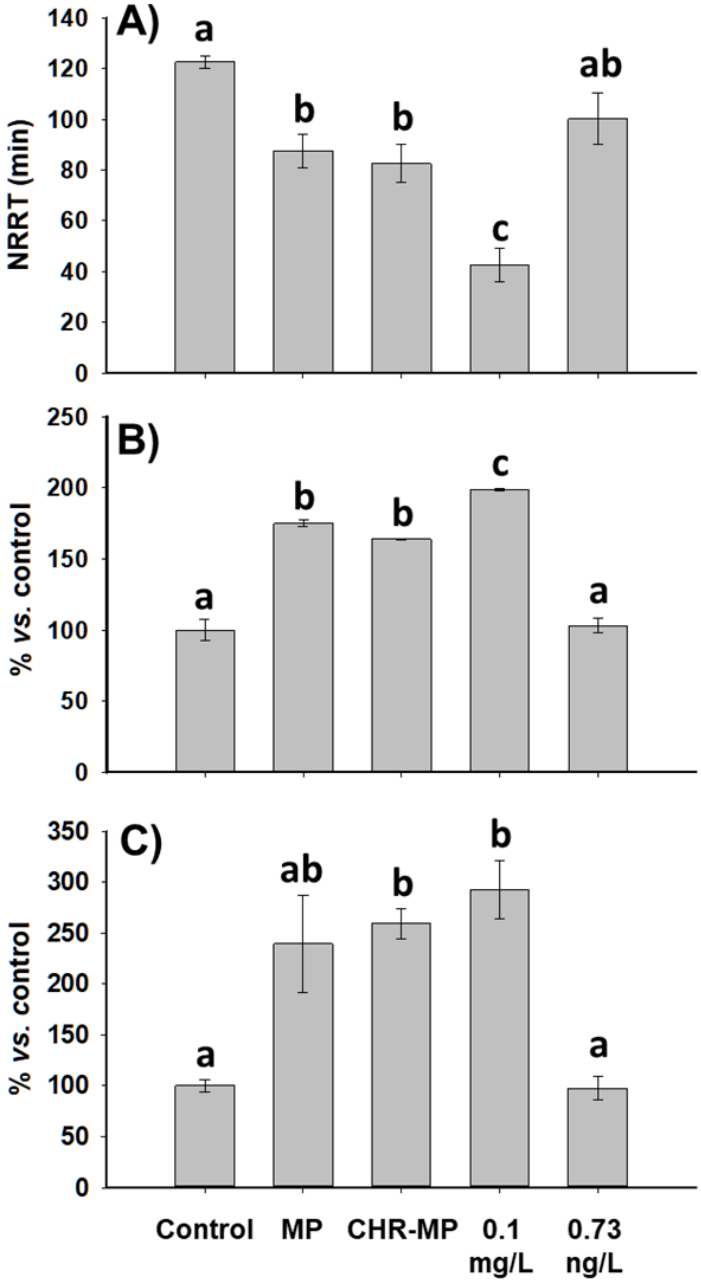

Figure 2. Lysosomal parameters (mean ± SEM, $n = 3$) analysed in mussels after 7 days of exposure to 5×10^3 units/L of polystyrene microplastics (MP) and chrysene-loaded microplastics (CHR-MP), 0.1 mg/L and 0.73 ng/L of freely dissolved CHR (without MP); (**A**), LMS assessed in mussel haemocytes; (**B**,**C**), lysosomal content of NL (**B**) and LF (**C**) in mussel digestive gland. Different letters denote statistically significant differences ($p < 0.05$, ANOVA—Bonferroni *post-hoc* comparison) among treatments.

3.3. Antioxidant and Detoxification Pathways: CAT and GST Activities

The mechanisms involved in the counteraction of toxicant- and reactive oxygen species (ROS)-mediated injuries include the activation of enzymes catalysing phase-II reactions and ROS-scavenging processes, such as GST and CAT [71]. As can be observed in Figure 3, selected treatments had an overall slighter impact on CAT and GST activities compared to lysosomal responses. GST levels measured in the digestive gland of mussels did not vary among treatments (Figure 3A); in gills, a significantly higher GST activity compared to either control or all other treatments was noted in mussels exposed to 0.1 mg/L CHR (Figure 3B). In digestive glands, the CAT activity decreased significantly compared to control only in mussels exposed to MP (Figure 3C); CAT levels measured at this treatment, however, did not change significantly compared to the other tested conditions. In gills, the exposure to 0.1 mg/L CHR induced significantly lower CAT activity compared to all the other treatments, including control (Figure 3D).

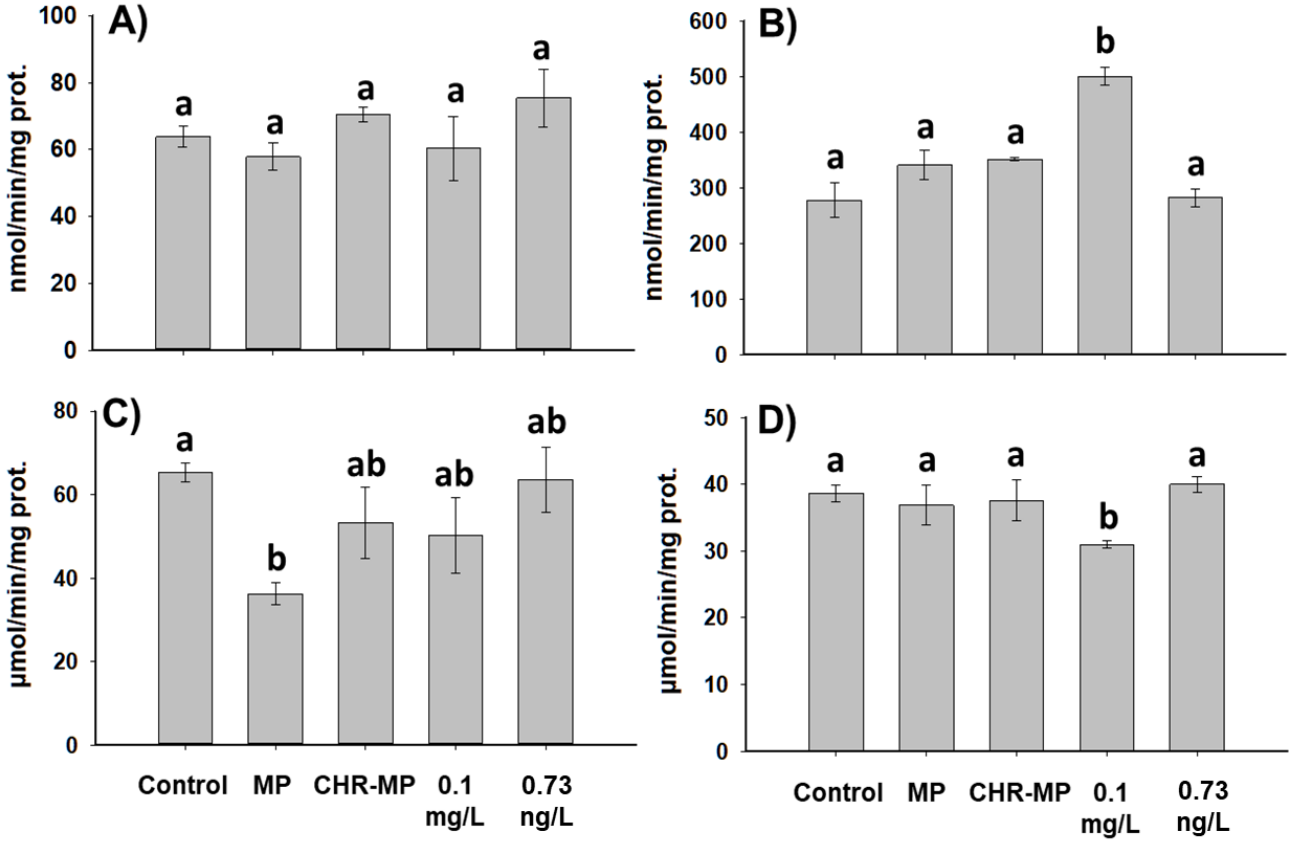

Figure 3. Specific activity of the enzymes GST and CAT (mean ± SEM, n = 3) measured in mussels after 7 days of exposure to 5×10^3 units/L of polystyrene microplastics (MP) and chrysene-loaded microplastics (CHR-MP), 0.1 mg/L and 0.73 ng/L of freely dissolved CHR (without MP); (**A**,**B**), GST activity in mussel digestive gland (**A**) and gills (**B**); (**C**,**D**), CAT activity in mussel digestive gland (**C**) and gills (**D**). Different letters denote statistically significant differences ($p < 0.05$, ANOVA—Bonferroni *post-hoc* comparison) among treatments.

GST and CAT levels measured in this study suggest that MP, CHR-MP and 0.73 ng/L of freely dissolved CHR had an absent or limited impact on the processes governing antioxidant and detoxification pathways in mussels. The lack of GST modulation in mussel digestive gland is in line with previous observations obtained in mussels exposed for 7 days to virgin and pyrene-contaminated MP [18] and may be related to either the low stimulation of biotransformation processes in response to physical factors or a limited ROS-scavenging role played by the enzyme in this tissue, as previously postulated [19]. Accordingly, in response to higher CHR concentrations, GST was upregulated in gills,

where it shows generally higher basal levels, likely reflecting a greater involvement in ROS-scavenging activity and/or phase-II metabolism of waterborne toxicants.

Interestingly, CAT activity in the digestive gland was significantly inhibited in the presence of plain MP, while it did not show changes in response to CHR-loaded particles. Previous studies highlighted a sensitively greater CAT inhibition and ROS production in mussels exposed to virgin MP compared to fluoranthene-incubated MP [72,73]. These findings may suggest that below specific toxicity thresholds, the sorption of organic chemicals may act as a compensating, rather than cumulative, factor with respect to some MP-related alterations of mussel antioxidant defences.

3.4. Neurological Mechanisms: AChE Activity

AChE is the serine hydrolase involved in the regulation of the acetylcholine-mediated neuronal impulse and is one of the most effective biomarkers of neurologic alterations induced by toxicants in aquatic species [55,74,75]. As shown in Figure 4 a significant decrease of AChE activity was observed in mussels exposed to 0.1 mg/L CHR compared to mussels exposed to control conditions and 0.73 ng/L CHR; mussels exposed to MP and CHR-MP showed no change compared to each other and to any other performed treatment.

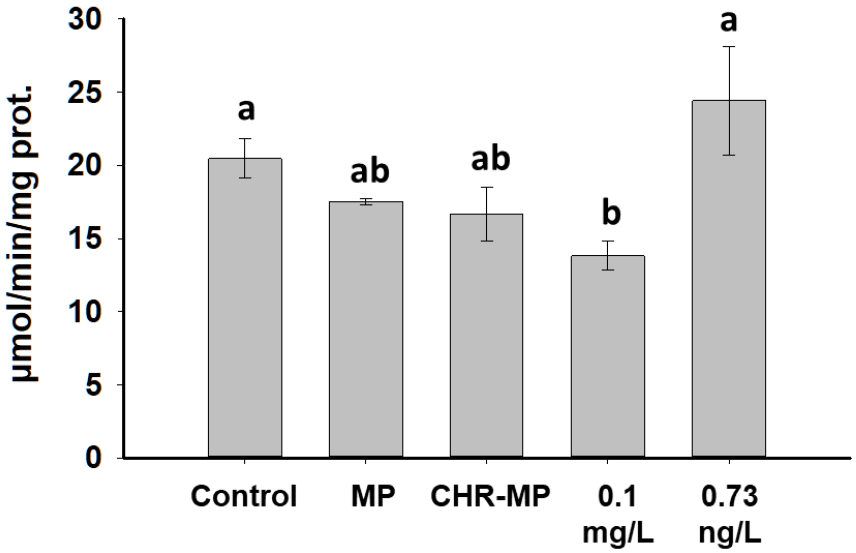

Figure 4. AChE specific activity (mean ± SEM, $n = 3$) measured in mussel gills after 7 days of exposure to 5×10^3 units/L of polystyrene microplastics (MP) and chrysene-loaded microplastics (CHR-MP), 0.1 mg/L and 0.73 ng/L of freely dissolved CHR (without MP). Different letters denote statistically significant differences ($p < 0.05$, ANOVA—Bonferroni *post-hoc* comparison) among treatments.

Overall, these data show that mussel AChE activity was not influenced by either the exposure to virgin or CHR-loaded MP at our experimental conditions. As to the former, this was quite expectable based on our recent findings obtained comparing the effects of both 3-µm and 50 nm PS particles in *M. galloprovincialis* [19]; notably, we found only nano-sized plastics were able to inhibit AChE activity after 21 days of exposure, corroborating evidence comparatively obtained in other aquatic organisms (reviewed by Prüst et al. [76]). Further evidence suggests that, once ingested, POPs sorbed on MP might undergo metabolic processes or gut biota interactions, thus eventually hampering their toxic action toward neurological targets. Accordingly, previous studies using mussels showed that MP loaded with other PAHs, such as benzo[a]pyrene and pyrene, do not exacerbate the neurotoxic disorders singularly induced by plain micro/nanoplastics or tested chemicals [18,32]; therefore, there is some consistency to suggest that regardless of POPs sorption, the structural and physical properties of ingested MP are the main factors characterizing the associated risks for neurological effects. The AChE inhibition observed after mussel exposure to 0.1 mg/L CHR indicates adverse effects of relatively

high concentrations of CHR on cholinergic neurotransmission, which might translate into nervous and neuromuscular dysfunctions. To our knowledge, there are no previous data about CHR toxicity on AChE in mussels and marine invertebrates in general. Field investigations using deployed mussels suggest that the exposure to PAH-contaminated environments may be responsible for AChE inhibition [51], although the mechanisms underlying these changes remain largely unknown. A possible explanation may be the involvement of the CHR-mediated oxidative stress, highlighted by both LF and CAT modulations observed in CHR (0.1 mg/L)-exposed mussels. Evidence supporting this hypothesis was obtained by Garcimartín et al. [77], who found H_2O_2 to act as a potent AChE allosteric inhibitor, confirming that cholinergic pathways may effectively be altered in conditions of ROS overproduction.

3.5. PCA and Mussel Expert System (MES) Data Integration

The output of the PCA performed on biomarker data measured in adult mussels exposed to MP, CHR-MP, 0.1 mg/L and 0.73 ng/L CHR is shown in Figure 5.

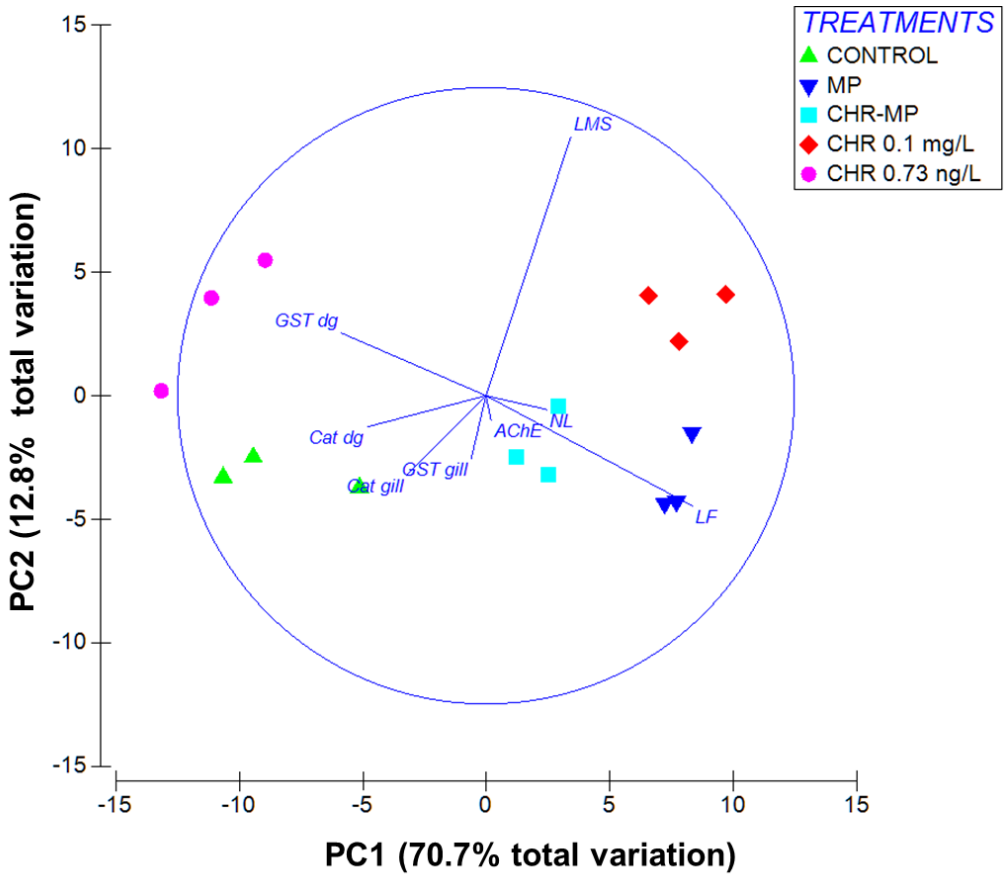

Figure 5. Biplot showing the output of the Principal Component Analysis (PCA) on biomarker data obtained in adult mussels exposed to polystyrene microplastics (MP), chrysene-loaded microplastics (CHR-MP), 0.1 mg/L and 0.73 ng/L of freely dissolved CHR.

The first two axes accounted for the 83.5% of the total variance among samples/endpoints. Data from control mussels tended to cluster together in the PC1 < 0/PC2 < 0 domain. Data from MP and CHR-MP scored for PC1 > 0/PC2 < 0. Data from 0.1 mg/L CHR were scaled at PC1 > 0/PC2 > 0, while those from 0.73 ng/L CHR at PC1 < 0/PC2 > 0. Vectors for CAT activity (in both gills and digestive gland) and GST activity measured in gills show coordinates in the PC1 < 0/PC2 < 0 domain; the GST activity in digestive gland was scaled at PC1 < 0/PC2 > 0; AChE, NL and LF clustered in the PC1 > 0/PC2 < 0 domain, while LMS was the only variable clustering at PC1 > 0/PC2 > 0. The spatial distribution of

data from treatments and variables identifies three main clusters. Data from control and 0.73 ng/L CHR tended to cluster together in line with the substantial lack of differences between biomarker levels measured at these conditions. A second cluster (PC1 > 0/PC2 < 0) includes data collected in organisms exposed to MP and CHR-MP; this association seems to be mainly explained by AChE, NL and LF data, although similar levels between these two conditions were also observed for most selected endpoints. Finally, data from mussels exposed to 0.1 mg/L CHR tended to cluster differently from the other treatments, in line with the generally higher impact induced on most selected endpoints, notably on LMS. The relationships identified by the PCA output seem to be confirmed by the MES data integration (Table 2). Given its sensitivity and known responsiveness to stress factors [56], the LMS was selected as a guide parameter to perform the data integration. The system did not identify health alterations in control and at 0.73 ng/L CHR (HSI = A); the exposure to MP and CHR-MP resulted in the onset of a low stress condition (HSI = B) while a moderate stress level (HSI = C) was identified for mussels exposed to 0.1 mg/L CHR.

Table 2. Output of the Mussel Expert System (MES) integrating biomarker responses of adult mussels exposed to MP, CHR-MP and CHR treatments in a unique health status index (HSI).

Biomarkers	Toxicological Profile		MP	CHR-MP	CHR 0.1 mg/L	CHR 0.73 ng/L
Cell level						
LMS^{GP}	decreasing	AF	0.71 *	0.67 *	0.35 *	0.82
		AL	-	-	–	NV
NL	bell-shaped	AF	1.75 *	1.64 *	1.99 *	1.03
		AL	+	+	+	NV
LF	increasing	AF	2.40	2.59 *	2.93 *	0.98
		AL	NV	++	++	NV
GST_{dg}	bell-shaped	AF	0.91	1.10	0.95	1.18
		AL	NV	NV	NV	NV
GST_g	bell-shaped	AF	1.23	1.26	1.80 *	1.01
		AL	NV	NV	+	NV
CAT_{dg}	bell-shaped	AF	0.56 *	0.82	0.77 *	0.97
		AL	NV	NV	NV	NV
CAT_g	bell-shaped	AF	0.95	0.92	0.80	1.03
		AL	NV	NV	NV	NV
AChE	decreasing	AF	0.86	0.82	0.68 *	1.19
		AL	NV	NV	-	NV
Organism level						
Survival	decreasing	AF	1.00	1.00	1.00	1.00
		AL	NV	NV	NV	NV
HSI			B (Low stress)	B (Low stress)	C (Moderate stress)	A (Healthy)

LMS—lysosomal membrane stability; NL—neutral lipid content; LF—lipofuscin content; GST_{dg}—glutathione S-transferase activity in digestive gland; GST_g—glutathione S-transferase activity in gills; CAT_{dg}—catalase activity in digestive gland; CAT_g—catalase activity in gills; AChE—acetylcholinesterase activity; GP—guide parameter; HSI—health status index; AF—alteration factor; AL—alteration level. AL thresholds for increasing/bell-shaped biomarkers: "NV" (no variation) = AF < 1.2; "+" = AF > 1.2; "++" = AF > 2.00; AF thresholds for decreasing biomarkers: "NV" = AF > 0.8; "-" = AF < 0.8;"–" = AF < 0.5. "*", $p < 0.05$ vs. control.

4. Conclusions

Overall, data from this study show that the exposure to virgin and CHR-loaded polystyrene MP did not alter the *M. galloprovincialis* gamete fertilization and embryonic development, and generally induced qualitatively and quantitatively similar sub-lethal alterations in adult mussels. Single exposure to CHR were effective only at 0.1 mg/L CHR on embryological, lysosomal, antioxidant and neurological pathways; on the other hand, when exposing organisms to levels of freely dissolved CHR comparable to those sorbed on proffered MP, none of the abovementioned alterations were observed. Altogether, these results let us hypothesize that, at our experimental conditions, the levels of CHR sorbed on MP was not sufficient to induce cumulative and measurable effects on the tested species. Another possible explanation may be that sorbed CHR might not be readily bioavailable to

mussels, which presuppose the involvement of adaptation or biotransformation processes of MP-sorbed POPs still not adequately characterized in marine organisms. Lysosomal endpoints were generally more consistently altered in response to selected treatments, confirming the role played by lysosomes as a main target of physical/chemical stressors in marine invertebrates. This investigation was conducted in controlled laboratory conditions using spherical PS MP to alleviate the likelihood of biases due to the co-occurring variables of physical and chemical nature. On the other hand, many factors may influence the sorption of POPs on plastic debris in a natural environment, including the presence of complex chemical mixtures, the chemical structure of dissolved chemicals and the size, structure and polymer type of MP. In this respect, data from this study may serve as baseline knowledge for future investigations using a more realistic environmental setting, including the sorption of chemicals in mixtures on micro/nano-sized particles of diverse nature. Moreover, to gain a more reliable estimation of the risks posed on exposed ecosystems efforts need to be expanded to ascertain the influence of physicochemical factors (e.g., temperature or pH) on the pathways modulating the sorption of waterborne pollutants on MP and the associated effects on marine organisms.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/app11198924/s1, Section S1: Selected polystyrene microplastics, Section S2: CHR calibration curve and recovery, Section S3: Biomarker data integration in the Mussel Expert System (MES), Figure S1: Calibration curve of CHR obtained at constant concentration of internal standard, Figure S2: Mean sorption (± SD, n = 3) of chrysene (CHR) on MP after 0, 3, 6, 12 and 24 h of co-incubation in 20 mL Milli-Q water (0.5 mg MP in 0.5 mg/L CHR). Table S1: Tissue treatment preliminary to the analysis of enzymatic biomarkers.

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Institutional Review Board Statement: No specific authorization is required for the use of *Mytilus galloprovincialis* in experimental applications according to the EC Directive 2010/63/EU.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data is contained within the article and Supplementary Material.

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