Supplementary Material

Assessing the impact of chrysene-sorbed polystyrene micro-plastics on different life stages of the Mediterranean mussel *Mytilus galloprovincialis*

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Section S1. Selected polystyrene microplastics

Polystyrene microbeads (3 ± 0.15 μm size diameter) used in this study were purchased from Polysciences Inc. (Washington, PA, USA). These particles show a density (1.05 g/cm^3) almost identical to that of artificial seawater (about 1.025–1.035 g/cm^3) which allow them to be evenly distributed along the water column. Charge change is unlikely, as the microsphere are neutral and, according to the manufacture's specifications, only covalent interactions should be allowed to bind proteins or nucleic acids. Surfactants were not present or added to the original particle suspension, whose medium was ultrapure water. For 'MP' treatment, stock solutions at the suitable concentrations were prepared in sterile distilled water, aliquoted and stored at 4 °C according to the manufacturer's specification. At each MP administration, 1 aliquot of the stock solution was serially diluted in sterile artificial seawater (ASW) to achieve the final treatment concentration in wells / vessels. Before spiking, lack of aggregation was verified microscopically.

Section S2. CHR calibration curve and recovery

Calibration solutions were prepared by adding a proper volume of working standard into vials filled with 1.5 mL ultrapure water (18.2 Ω·cm, Millipore Direct-Q 5 UV system, Merck KGaA, Darmstadt, Germany). Chrysene (CHR) concentrations of the calibration solutions were 0.05, 0.1, 0.5, 1.5 and 2.5 mg/L to match the concentration range expected in the water sample. 0.1 mL of chrysene-d_{12} at the concentration of 5 mg/L was added into the vials. The recovery of CHR from water samples was determined by SPME analysis of ultrapure water samples spiked with 0.1 mL of chrysene at the concentration of 10 mg L^{-1}. Precision, expressed as relative standard deviation (R.S.D.), was calculated from four extractions and desorptions from calibration solutions.

Calibration solutions subjected to the SPME method produced acceptable linear regression of CHR peak areas *vs*. CHR concentrations when normalized to the peak area and quantity of added internal standard. The $R^2 = 0.9986$ in the 0.05-2.5 mg/L range was comparable to the values reported in other studies on the determination of PAHs in water (King et al., 2004; Santos et al., 2017). The recovery of CHR from the procedure applied to the ultrapure water spiked with CHR standard resulted to be 98 % ± 20 % (N = 4, 1 mg/L expected CHR concentration). The precision was characterized by RSD < 19 % (1 mg/L level, n=4) comparable to the RSD values reported in literature for chrysene determination by SPME analysis (Jabali et al., 2018).

Section S3. Biomarker data integration in the Mussel Expert System (MES)

To synoptically evaluate the impact induced by single leachates based on biomarker modulation, data were integrated using the Mussel Expert System (MES) developed by (Dagnino et al., 2007). The system follows a set of rules for the battery of biomarkers used in the experiment, including the level of biological organization, the biological significance, the mutual inter-relationship and the toxicological trend of each analysed biomarker (Capolupo et al., 2016). Alteration factor (AF) and alteration level (AL) are processed by the system based on the significant variations of different experimental conditions from control samples. AF is the ratio between the means of relevant experimental conditions and the means of control. AL threshold values depend on the biomarker profile. Finally, the system applies a multiple step algorithm to evaluate a Health Status Index (HSI). The most sensitive and representative biomarker among the battery is selected as a guide parameter (GP) and changes in its AL value are primarily considered by the system to interpret the other data. HSI levels are expressed in a scale from A to E (A-healthy; B-low stress; C-medium stress; D-high stress; E-pathological

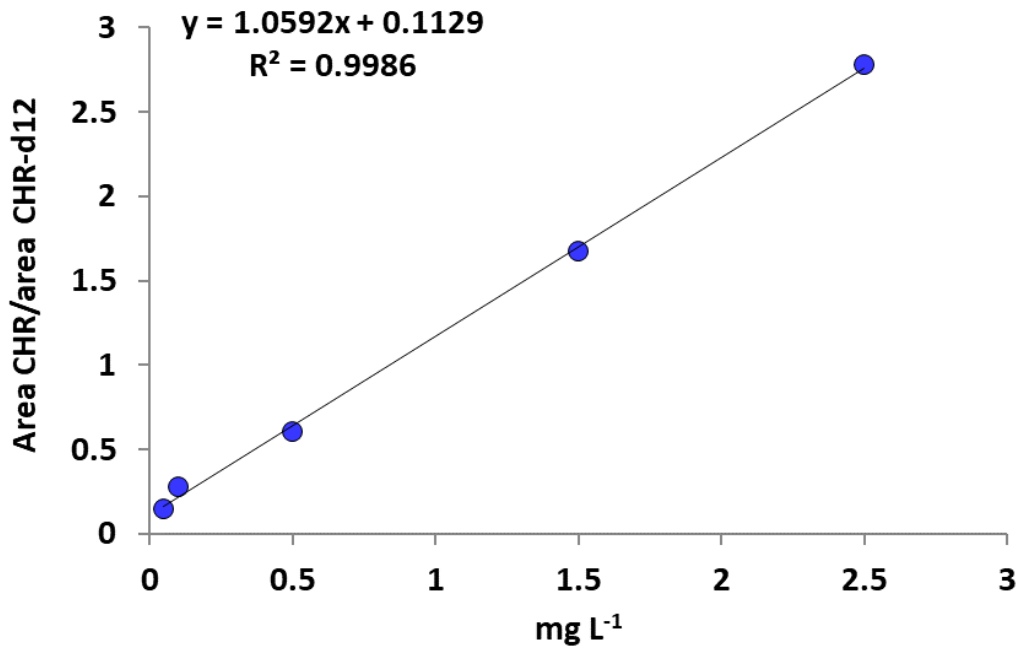


Figure S1. Calibration curve of CHR obtained at constant concentration of internal standard.

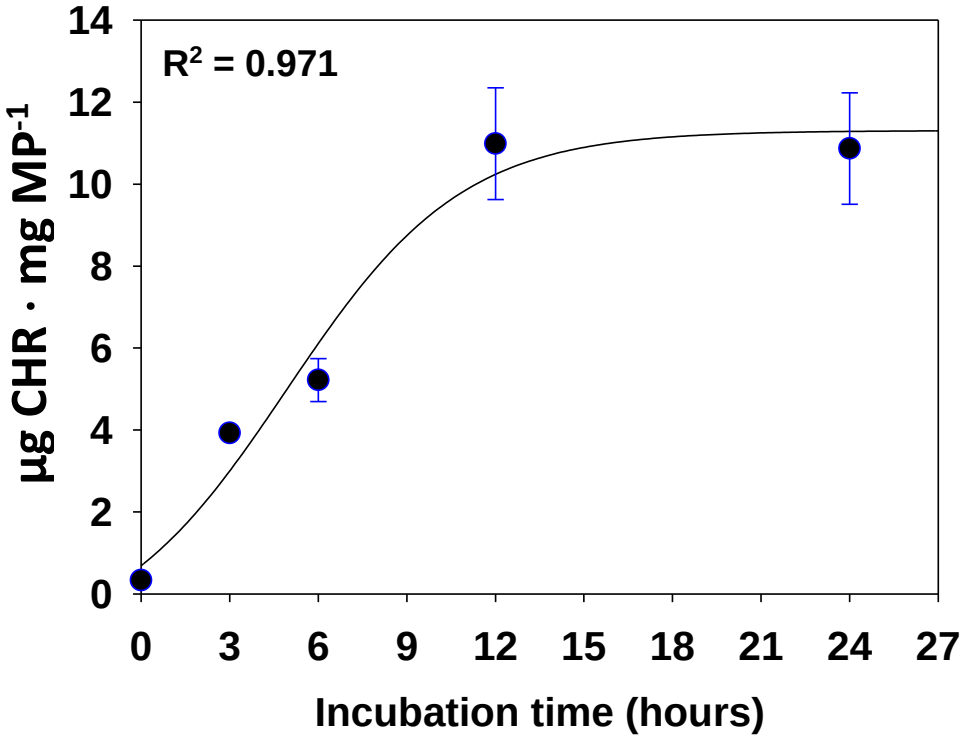


Figure S2. Mean sorption (± SD, N = 3) of chrysene (CHR) on MP after 0, 3, 6, 12 and 24 hours of co-incubation in 20 mL Milli-Q water (0.5 mg MP in 0.5 mg/L CHR). The concentration measured at each time interval was subtracted from that measured in a control solution of nominal 0.5 mg/L CHR without MP added (Table 1 in the main text). Data were finally obtained by normalizing the obtained values per mass (mg) of MP and tested for fitting with a non-linear (three-parameter) logistic regression model.

Biomarker	Tissue homogenation	Centrifugation	Total protein quantification	Reference
GST, CAT	400-600 mg gill/digestive gland in 5 vol. of 50 mM KPB, pH 7.4 (0.5 mM Na_2EDTA and protein inhibitor at 1 µL/100 mg tissue)	15,000 xg for 15 min at 4ºC	Folin–Ciocalteu reaction (Lowry et al., 1951)	(Capolupo et al., 2017)
AChE	500 mg gills in 4 vol. 0.1 M sodium-phosphate buffer, pH 7.4	10,200 rpm for 30 mins at 4ºC		(Valbonesi et al., 2003)

Table S1. Tissue treatment preliminary to the analysis of enzymatic biomarkers. In all cases, tissues were homogenized using a Potter glass homogenizer.

GST, glutathione S-transferase activity; CAT, Catalase activity; AChE, acetylcholinesterase activity; KPB, potassium-phosphate buffer.

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