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Single and combined potential of polystyrene microparticles and fluoranthene in the induction of DNA damage in haemocytes of Mediterranean mussel (*Mytilus galloprovincialis*)

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Abstract

In this study, the possible 'vector effect' within the exposure of Mediterranean mussels (*Mytilus galloprovincialis*) to polystyrene microplastics with adsorbed fluoranthene was investigated by applying the multibiomarker approach. The major focus was placed on genotoxicological endpoints as to our knowledge there are no literature data on the genotoxicity of polystyrene microparticles alone or with adsorbed fluoranthene in the selected experimental organisms. DNA damage was assessed in haemocytes by comet assay and micronucleus test. For the assessment of neurotoxicity, acetylcholinesterase activity was measured in gills. Glutathione *S*-transferase was assessed in gills and hepatopancreas since these enzymes are induced for biotransformation and excretion of lipophilic compounds such as hydrocarbons. Finally, differences in physiological response within the exposure to polystyrene particles, fluoranthene, or particles with adsorbed fluoranthene were assessed by the variation of heart rate patterns studied by the noninvasive laser fibre-optic method. The uniform response of individual biomarkers within the exposure groups was not recorded. There was no clear pattern in variation of acetylcholinesterase or glutathione *S*-transferase activity which could be attributed to the treatment. Exposure to polystyrene increased DNA damage which was detected by the comet assay but was not confirmed by micronucleus formation. Data of genotoxicity assays indicated differential responses among the groups exposed to fluoranthene alone and fluoranthene adsorbed to polystyrene. Change in the heart rate patterns within the studied groups supports the concept of the Trojan horse effect within the exposure to polystyrene particles with adsorbed fluoranthene.

Keywords: microplastics; fluoranthene; polystyrene; genotoxicity; **Mytilus galloprovincialis**; comet assay

Introduction

The global demand for plastics has consistently increased over recent years and in 2020 reached around 367 million metric tons of plastics manufactured worldwide [1]. Globally, waste management systems are struggling to cope with the resulting influx of waste, and it was estimated that in 2016 alone, up to 23 million tonnes of plastics entered aquatic ecosystems [2]. With time, rather than biodegrading, plastics are crumbled into smaller and smaller chunks, resulting in micro- to nanosized fragments [3]. The particles made of different synthetic polymers such as polyethylene, polystyrene, and polyvinyl chloride, less than 5 mm in diameter are called microplastics and have become a major concern present in ecosystems worldwide [4]. In aquatic environments, these particles are present in the pelagic habitats and slowly deposit in sediments due to transport mechanisms, which are physicochemical or biologically driven via the food web [5]. Particles of microplastics are ingested by zooplankton and then transfer further into the food chain (by ingestion with food or by filtration mechanisms) [6–8]. Microplastics pose serious threats and are treated as a biological hazard at several levels: (i) direct accumulation of particles in tissues of organisms, (ii) release of plasticizers and adsorbed pollutants after the ingestion, and (iii) they are a substrate for the species attachment and serve as a vector of native and alien species [9].

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The effects of direct accumulation of microplastics in organisms have been well documented for the numerous responses triggered in organisms such as inflammation, destabilization of lysosomal membranes, genotoxic effects, impaired reproduction, imbalance in hormone synthesis [10, 11] or at the transcription level differential expression of genes related with lysosomal metabolism, impaired immunological function, antioxidant defence, detoxification, and repair of DNA [12]. In the presence of contaminants, microplastics can take part in several interactive processes such as aggregation, adsorption, and transformation that can lead to synergistic, antagonistic, or potentiating effects [13]. The release and impact of the pollutants adsorbed to microplastics have not been fully investigated, especially in mussels. In this case, microplastics can have a 'vector effect' acting as carriers of hydrophobic pollutants in the water and facilitating their transfer to organisms through the food chain [14]. Many authors described the potential hazards of such a relationship as the Trojan horse effect mechanism [15–17], where the sorption of hydrophobic pollutants to microplastic particles can enhance or multiply their accumulation in organisms. Sorption results in a several-fold increase of concentrations of contaminants on microparticles in comparison with surrounding water [18]. Hereby the particle size, specific surface area, ageing degree, crystallinity, and polarity of particles, and organic pollutant properties (hydrophobicity and dissociated forms) in conjunction with pH, salinity, temperature, and ionic strength are key factors affecting adsorption capacity [19, 20].

Polycyclic aromatic hydrocarbons (PAHs) are one of the major environmental concerns in aquatic ecosystems because of their adverse health effects on organisms. Water Framework Directive 2000/60/EC characterize these substances as priority substances due to their high toxicity, lipophilicity, and persistence in the environment [21]. Members of this group of substances are known for their genotoxic potential, while some of these are classified as cancerogenic. Fluoranthene is one of the priority PAHs. It can be metabolized by aquatic organisms and may generate reactive oxygen species and form adducts, which can exert both acute toxic and genotoxic effects if antioxidant defences are overcome by pro-oxidant forces [22, 23]. The genotoxic potential of fluoranthene in *Mytilus* sp. was demonstrated by Al-Subiai *et al.* [24] by comet assay in haemocytes. Available literature indicates that fluoranthene is harmful either alone or when adsorbed to plastic particles. For instance, studies by Magara *et al.* [25] and Paul-Pont *et al.* [16] indicated that exposures of blue mussels *Mytilus edulis* to fluoranthene in combination with polyethylene or polystyrene microplastics had a significant effect on its accumulation and oxidative stress response in mussels.

Considering all aforementioned, the major goal of this study was to investigate the possible 'vector effect' in the exposure of Mediterranean mussels (*Mytilus galloprovincialis*) to polystyrene microplastics with adsorbed fluoranthene by using the multibiomarker approach. The major focus was placed on genotoxicological endpoints since to our knowledge there are no literature data on the genotoxicity of polystyrene particles alone and with adsorbed fluoranthene in Mediterranean mussels. Neurotoxicity was assessed by acetylcholinesterase (AChE) activity measured in the gills of experimental mussels. Glutathione *S*-transferase (GST) was assessed in gills and hepatopancreas as these enzymes are induced for biotransformation and excretion of lipophilic compounds such as PAHs. Polystyrene particles can enter the circulatory system of *Mytilus* by crossing the gut wall and have the ability to cause damage to vascular tissue and alter cardiac function [26], therefore we investigated whether the Trojan horse mechanism effect can be seen in the change of the heart rate (Hr) patterns within the exposure to polystyrene particles, fluoranthene, or particles with adsorbed fluoranthene. A noninvasive laser fibre-optic method for Hr recording of mussels widely used in bioindication of seawater and freshwater pollution was applied in this study [27–29].

Materials and methods

Preparation of microplastics and microplastics with adsorbed fluoranthene

Stock solutions of fluoranthene (1 mg/ml for genotoxicity/antioxidant defence biomarkers and 10 mg/ml for cardiac activity analyses) were prepared in *n*-hexane. Preparation of microplastics with adsorbed fluoranthene consisted of several steps: (i) 100 mg of polystyrene particles (1.07 kg/dm^3, particle size 14–20 µm) were added to 1 ml of fluoranthene stock in *n*-hexane; (ii) mixtures were stirred in glass tubes by vortex for 2 h and subsequently, *n*-hexane was evaporated until dryness under gentle steam of nitrogen; (iii) dry residue after evaporation (containing 100 mg polystyrene) was dissolved in 5 ml of seawater from aquaria and added to treatment aquaria containing 10 l of seawater (final concentration of polystyrene in aquaria was 10 mg/l). After the particles were removed from the tube, residual fluoranthene was measured by GC to calculate the adsorption of fluoranthene which was above 95%. Analyses were performed using an HP 6890 gas chromatograph equipped with an FI detector and an on-column injector. The HP Ultra 2 column (25 m × 0.32 mm × 0.17 µm) was used for analyses. The carrier gas (helium) flow rate was 1 ml/min.

Exposure conditions and tissue collection for genotoxicity and biochemical markers

Specimen collection

The specimens of *M. galloprovincialis* (300 specimens, shell length 60–70 mm) were collected from the aquaculture farm at the beginning of March 2019 in the Gulf of Trieste at a depth of 2–3 m. Mussels were taken to the laboratory of the Marine Biology Station in Piran; shells were cleaned from algae and marine polychaetes and specimens were maintained at 12°C in tanks containing aerated and daily renewed seawater delivered by a local seawater supply network from 10 m depth and 2 km distance from the coastline with a flow-through system with water grab in front of the Marine Biology Station in Piran. Acclimation lasted for 3 weeks in advance before the exposure experiments.

Treatment

Acclimated mussels with intact byssus threads were divided into 4 groups per 30 specimens and each group was placed in a 20 l glass aquarium containing 10 l of seawater taken in front of the Marine Biology Station in Piran. This area was characterized as a site with a low level of pollution previously [30]. Mussels were left to acclimate to the new aquarium set for 24 h (18°C, 38‰ salinity, and 8.1 mg/l for oxygen). Before the addition of the pollutant, specimens were checked

if they were attached to the aquaria bottom by byssus threads. Treatment groups comprised the following: (i) control aquarium—water spiked with 100 µl of DMSO and 20 µl of Tween 80; (ii) polystyrene—100 mg of polystyrene particles was dissolved in 5 ml of seawater from aquaria spiked with 100 µl of DMSO and 20 µl of Tween 80; (iii) fluoranthene—100 µl of stock solutions of fluoranthene (10 mg/ml) prepared in DMSO was added, additionally water was spiked with 20 µl of Tween 80; (iv) fluoranthene coupled particles—particles prepared as described in 'Preparation of microplastics and microplastics with adsorbed fluoranthene' were resuspended in 5 ml of seawater taken from aquaria. The final concentration of polystyrene particles in aquaria was 3×10^6/l assessed using a haemocytometer.

Each group's treatments were performed in three individual experiments. Specimens were exposed at a constant temperature of 18 ± 1°C in a static system for 96 h and were not fed in order to avoid interaction between tested substances and food. Checkpoints for comet assay and antioxidative enzymes were set at 24, 48, 72, and 96 h of treatment while micronuclei (MN) were analysed after 96 h. At each checkpoint six specimens were taken from each aquarium for analyses and replaced with six new specimens separated by a glass barrier, to make them distinguishable from the starter mussel batch. The animals were treated and housed in accordance with national and international legislation (EUs Directive 2010/63/EU on the protection of animals used for scientific purposes).

Genotoxicity biomarkers

Haemocyte sampling

At each indicated checkpoint, haemolymph was collected separately from six specimens using a 3 ml syringe with a hypodermic 21 G needle containing 200 µl of physiological saline (0.5 M NaCl, 12.5 mM KCl, 5.5 mM EDTA, 20 mM HEPES, pH 7.4) for cell viability assessment, comet assay, and micronucleus test.

Haemocyte viability

Viability was assessed by acridine orange/ethidium bromide differential staining following the protocol given in Kolarević *et al.* [31]. In each mussel, 100 cells were counted and cells stained in red were considered nonviable.

Comet assay

Comet assay was performed by a protocol developed by Singh *et al.* [32] with modifications. Slides for the comet assay were prepared as described in our previous study [33]. Briefly, 30 µl of cell suspension was prepared as indicated above and further mixed with 70 µl of 1% low melting point agarose and placed on the microscope slide coated with 1% normal melting point agarose. Cell lysis was performed for 24 h in a freshly made cold (4°C) lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% Triton X-100, pH 10). After lysis, slides were placed in an electrophoresis chamber containing a cold (4°C) alkaline electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH 13) for 20 min, to allow DNA unwinding. Electrophoresis was performed at 0.75 V/cm and 300 mA for 20 min at (4°C) after which the slides were placed into a freshly made cold (4°C) neutralizing buffer (0.4 M Tris, pH 7.5) for 15 min. Fixation was performed in ice-cold ethanol (15 min at 4°C) and slides were air-dried in darkness. Staining was performed using 20 µl per slide of acridine orange (2 µg/ml) and slides were examined under a fluorescence microscope (Leica, DMLS, Austria, under magnification 400×, excitation filter 450–490 nm, barrier filter 510). For each group, a total of 18 slides were analysed. On each slide, 50 nuclei were scored using Comet IV Computer Software (Perceptive Instruments, UK) and TI% was used as a measure of DNA damage.

Micronucleus test

Slides for the micronucleus test were prepared as described in Bolognesi and Fenech [34]. Briefly, 100 µl of cell suspension per specimen was placed on a slide in a humidity chamber and left for 15 min for haemocytes to attach. Afterwards, slides were washed with saline solution, air-dried for 15 min, and fixed in ice-cold methanol for 20 min. MN were stained with acridine orange 10 µg/ml and examined under 400× magnification with MN validation under dry 1000× magnification (Leica, DMLS, Austria with I3 filter cube with excitation filter 450–490 nm, barrier filter 510). From each slide, 1000 cells were examined. MN were scored by the criteria of Hagger *et al.* [35], the diameter for MN was between 1/3 and 1/10 of the diameter of the main nuclei.

Biochemical markers analyses

Tissue sampling and preservation

At each checkpoint was firstly collected haemolymph and then mussels were dissected; gills and hepatopancreas were excised and stored at −20°C until analyses. Tissues were homogenized using Potter-Elvenhjem homogenizer in 1:2 (w:v) 100 mM KH_2PO_4/K_2HPO_4 (pH 7.4) and homogenates were stored at −80°C until analyses.

Protein determination

Total protein content was measured according to Lowry *et al.* [36] by using the Thermo Fisher BCA protein assay kit for microplates test (Thermo Scientific). Briefly, 2 µl of sample and 8 µl of distilled water were mixed with 190 µl of Pierce reagents A and B (v:v, 50:1) into the well. The mixture was incubated for 30 min at 37°C in dark, absorbance was measured at 562 nm. Samples were measured in duplicates, while standard for calibration curve was BSA (2, 1.5, 1, 0.75, 0.5, 0.25, 0.125, and 0.025 mg/ml).

AChE activity in gills

AChE activity was measured by the method described by Ellman *et al.* [37] and was adapted for microplates [38]. The reaction mixture was made of 10 µl protein supernatant (gills or hepatopancreas) and 190 µl of 50 mM phosphate buffer (pH 7.0), with 1 mM acetylthiocholine chloride (Sigma-Aldrich, USA) and 0.5 mM 5,5′-dithiobis-2-nitrobenzoic acid (Sigma-Aldrich, USA). The activity was measured for 10 min every 10 s with a microplate reader VIS (TECAN, Switzerland, Austria), at 405 nm at 25°C. A blank reaction was performed by replacing the protein supernatant with 10 µl of 50 mM phosphate buffer. Enzyme activity was expressed as specific activity of AChE in nmol of hydrolysed acetylcholine chloride/min/mg protein (extinction coefficient, $\varepsilon 405 = 13\,600$ mol/l/cm).

GST activity in gills and hepatopancreas

Activity GST was measured by spectrophotometric method [39] on a SPARK microplate reader (TECAN, Switzerland).

The reaction mix was prepared by mixing solution A and solution B in a 1:2 ratio (v:v). Solution A was made by dissolving substrate 1-chloro-2,4-dinitrobenzene (Sigma-Aldrich, ZDA) in the ethanol in a concentration of 50 mM (final concentration of ethanol in the enzymatic reaction was 2% and the activity of GST was not inhibited), and then diluted to final concentration 4 mM with 100 mM potassium phosphate (pH 6.5). Separately 2 mM solution of reduced Glutathione (Sigma-Aldrich, USA) was made in 100 mM potassium phosphate (pH 6.5). In each well, 10 µl of supernatant was added into the well of the microplate and then 190 µl of reaction mix was added. A blank reaction was performed by replacing the protein supernatant with 10 µl of 100 mM potassium phosphate (pH 6.5). The activity was measured for 10 min at 340 nm at 21°C. Specific GST activity was expressed in nmol of conjugated GSH/min/mg protein (extinction coefficient, ε340 = 9600 mol/l/cm).

Exposure conditions for cardiac activity analyses in *M. galloprovincialis*

Specimen collection

For the exposures for cardiac activity analyses, the specimens of *M. galloprovincialis* were collected from the mussel farm of the Institute of Marine Biology at the site Dobrota in the Boka Kotorska Bay (Montenegro, 42°26′ N, 18°45′ E) at the beginning of March 2021 at a depth of 2–3 m. The collection site was used as a reference site for various biomarkers in the previous studies [40, 41]. Mussels were cleaned from algae and marine polychaetes, divided into groups of 10, and placed in an aquarium with 10 l of seawater and constant aeration, temperature 21 ± 1°C, and salinity 32 ± 1‰ for at least 10 days for acclimation.

Exposure for heart rate analyses

Treatment groups comprised the following: (i) control aquarium—water spiked with 1000 µl of DMSO; (ii) polystyrene—100 mg of polystyrene particles was dissolved in 5 ml of seawater from aquaria spiked with 1000 µl of DMSO; (iii) fluoranthene C1—1000 µl of stock solutions of fluoranthene in DMSO (1 mg/ml); (iv) fluoranthene C2—1000 µl of stock solutions of fluoranthene in DMSO (10 mg/ml); (v) fluoranthene coupled particles—particles prepared as described in 'Genotoxicity biomarkers' were resuspended in 5 ml of seawater taken from aquaria.

Hr value recorded in specimens in clean seawater for at least 3 h before the addition of chemicals was used to define the reference point for potential changes of cardiac activity during the exposure (pretreatment period further in text). After the treatment, the substance was washed out with clean seawater to analyse the animal's reactions after test substance depuration from the system, and Hr was additionally recorded for at least 3 h (post-treatment period further in text).

Treatment groups comprised the following: (i) control aquarium—water spiked with 100 µl of DMSO and 20 µl of Tween 80; (ii) polystyrene—100 mg of polystyrene particles was dissolved in 5 ml of seawater from aquaria spiked with 100 µl of DMSO and 20 µl of Tween 80; (iii) fluoranthene—100 µl of stock solutions of fluoranthene (10 mg/ml) prepared in DMSO was added, additionally water was spiked with 20 µl of Tween 80; (iv) fluoranthene coupled particles—particles prepared as described in 'Genotoxicity biomarkers' were resuspended in 4 ml of seawater taken from aquaria.

Heart rate

Cardiac activity recording of mussels was conducted by a noninvasive laser fibre-optic method based on photoplethysmography developed by Fedotov *et al.* [42]. The experimental unit allowed simultaneous recording of the Hr activity of eight mussels. A specific region of the heart was exposed to an IR light beam. Reflected light containing the information about periodical changes in heart volume was detected by small optical sensors glued to the shell above the heart area. Signals were amplified, converted from analogue to digital format, and analysed by VarPulse [43]. dHr parameter which represents the average difference in Hr response relative to the pretreatment period was used as an indication of stress levels. dHr was calculated as $dHr = |Hr_i - \text{mean } Hr_{c1}|$ where Hr_i represents Hr measured in the *i*-period with a 10 s pause between the periods, while Hr_{c1} represents heart rate in the pretreatment period [41]. Considering the extremely high number of values obtained within each treatment (about 35 000 measurements) average value for every 10 measurements was calculated to reduce the size of the dataset. Further, for each treatment, data were divided into six groups as follows: c1—period before the addition of test substance (pretreatment period), T1—the first 24 h of treatment, T2—the period from 24 to 48 h of treatment, T3—the period from 48 to 72 h of treatment, T4—the period from 72 to 96 h of treatment, c2—post-treatment period after washout.

Statistical analysis

Data obtained in three individual experiments were polled together (each group consisted of 18 specimens) and statistically analysed using Statistica 7.0 Software (StatSoft, Inc.). The type of distribution was tested using the Kolmogorov–Smirnov test. The data obtained by the comet assay were not in compliance with the premise of normal distribution, while the MN test, AChE, and GST generated data that followed a normal distribution. In this case, data was analysed using one-way ANOVA and Tukey's *post hoc* test. For data that did not show normal distribution, Kruskal–Wallis one-way ANOVA followed by the Mann–Whitney *U* test was applied. For all comparisons, the level of significance was tested for $P \leq .05$. Considering that the cardiac activity analyses generated large data groups (about 8000 values per group) data were analysed using one-way ANOVA followed by a *t*-test as *post hoc* with Bonferroni correction of the *P* value.

Results

Genotoxicity biomarkers

Comet assay

The viability of haemocytes was above 90% in all treatment groups. Comet assay results are summarized in Fig. 1. After 24 h of exposure, there was no significant increase in DNA damage in any of the treated groups in comparison with the control group. However, the level of DNA damage in the group exposed to fluoranthene was significantly higher in comparison with the group exposed to polystyrene and polystyrene with adsorbed fluoranthene. After 48 h of exposure, a significant increase of DNA damage in comparison with the control group was observed in animals exposed to fluoranthene and polystyrene with adsorbed fluoranthene. After 72 and 96 h of exposure, a significant increase of DNA damage in comparison with control was observed in all three

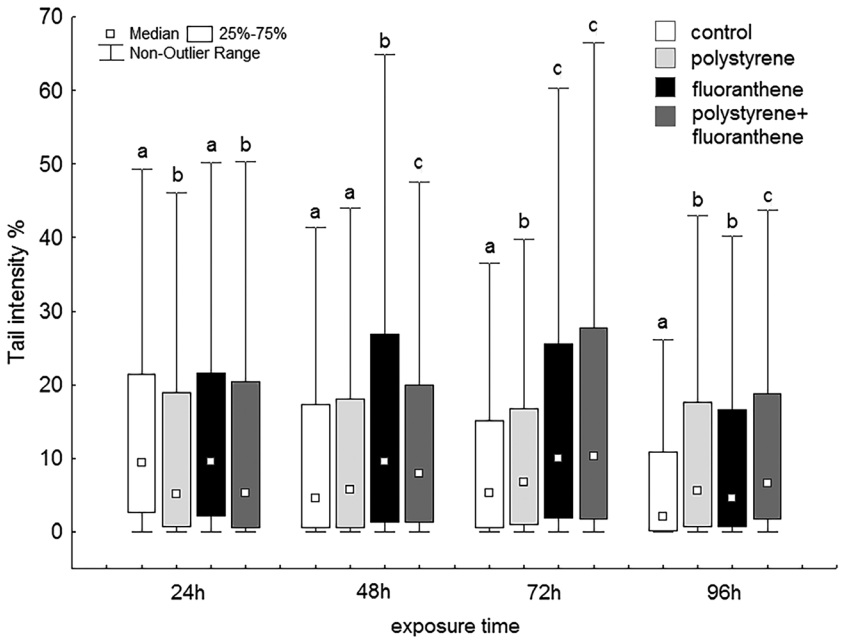

Figure 1. The effects of polystyrene particles, fluoranthene, and polystyrene particles with adsorbed fluoranthene on the level of DNA damage in haemocytes of *M. galloprovincialis* during 96 h of exposure assessed by comet assay; same letters shared by different groups (a, b, c) indicate lack of statistically significant difference $P < .05$.

treated groups. Response in groups treated with fluoranthene and polystyrene with adsorbed fluoranthene was significantly higher in comparison with the group treated with polystyrene only after 48 and 72 h of treatment.

Micronucleus test

After 96 h of exposure, the level of DNA damage was assessed by a micronucleus test (Fig. 2). A significant increase in MN frequency was recorded in the group exposed to fluoranthene and polystyrene particles with adsorbed fluoranthene. The frequency of DNA damage in the group exposed to polystyrene with adsorbed fluoranthene was lower in comparison with the group exposed to fluoranthene alone. Polystyrene particles did not induce a significant increase in DNA damage in comparison with the control group.

AChE activity

During the first 72 h of exposure, there was no significant difference among the studied group. The gradual decrease in AChE activity was observed in all groups in comparison with control after 96 h of treatment, however, the difference was significant only in the case of the group exposed to polystyrene with adsorbed fluoranthene (Fig. 3).

GST activity

Within the exposure, a significant increase of GST activity in comparison with the control, was detected only after 24 h in the hepatopancreas of mussels exposed to polystyrene with adsorbed fluoranthene. Results are shown in Fig. 4.

Cardiac activity

The results for the cardiac activity recording expressed as dHr relative to the baseline value in pretreatment, during the exposure, and in the post-treatment period are summarized in Fig. 5. In clean seawater, in comparison with the pretreatment period, a significant increase of dHr occurred within the first 24 h which remained at a stable level within 96 h of the experiment and following 3 h after washout. Similar trends were observed in the group treated with polystyrene particles. An altered level of dHr which occurred in the first 24 h of treatment was present throughout the exposure period and remained present in the following 3 h of post-treatment. Mussels were exposed to two concentrations of fluoranthene. Concentration fluoranthene C1 (0.1 mg/l) was also used in experiments for genotoxicity/antioxidant defence biomarkers. Although a significant increase dHr occurred after the first 24 h of exposure, the response was not so prominent and was in the range recorded for the

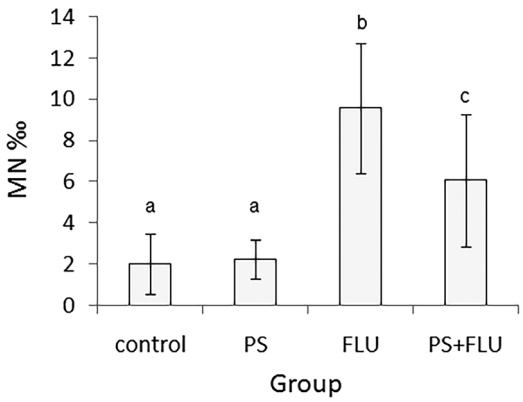

Figure 2. The effects of polystyrene particles (PS), fluoranthene (FLU), and polystyrene particles with adsorbed fluoranthene (PS + FLU) on the level of DNA damage in haemocytes of *M. galloprovincialis* after 96 h of exposure assessed by micronucleus frequency (mean ± SD); same letters shared by different groups (a, b, c) indicate lack of statistically significant difference among the groups $P < .05$.

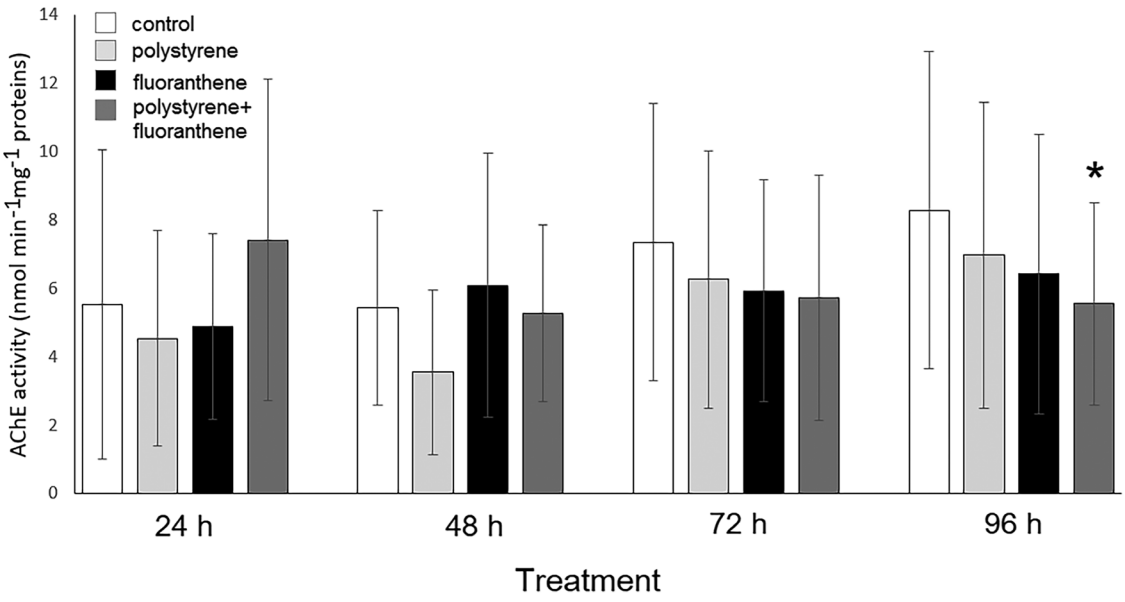

Figure 3. The effects of polystyrene particles, fluoranthene, and polystyrene particles with adsorbed fluoranthene on the AChE activity in gills of *M. galloprovincialis* during 96 h of exposure; values are expressed as mean ± SD; *significant difference in comparison with control in corresponding treatment period $P < .05$.

control and polystyrene group. Therefore additional, 10× higher concentration was selected (1 mg/l) for exposure, marked as fluoranthene C2. In this group, the highest response was recorded within the first 24 h of exposure. The value of dHr decreased in the T2 period and remained constant until the end of the exposure period, while washout resulted in a slight increase. In the group exposed to polystyrene particles with adsorbed fluoranthene, a constant increase of dHr was observed during the exposure period. The highest variation was observed in the T4 period of exposure. Interestingly, a significant decrease in dHr was observed in the post-treatment period.

Discussion

In preparation for the experimental design, we choose concentrations of fluoranthene and polystyrene which are expected to induce a genotoxic response according to the available literature [24, 44]. Another criterion was that concentrations should be nontoxic to avoid possible interference of apoptosis in comet assay [45]. The viability of haemocytes was above 90% in all treatment groups which is in compliance with the results of Paul-Pont *et al.* [16] where fluoranthene and polystyrene particles reduced the viability in treated groups only for a few percentages in comparison with the control group. Considering that DNA damage detected with comet assay does not necessarily have to be permanent and could have relatively short longevity, especially in the case of oxidative damage [46], samples were taken at checkpoints set at each 24 h of treatment. A similar strategy was employed in our previous study in an assessment of the impact of tributyltin chloride on *M. galloprovincialis* [41]. For our current study, we have used the same concentration of fluoranthene which gave significant induction of DNA damage as in the study of Al-Subai *et al.* [24] so the impact of fluoranthene was detected after 48, 72, and 96 h of exposure was expected. A slight increase in the level of DNA damage was observed in mussels exposed to polystyrene particles after 72 and 96 h. Available literature data on genotoxicity of polystyrene are ambiguous. Most of it indicates that neither polystyrene microplastics nor nanoplastics pose genotoxic potential [47, 48]. Moreover, the study of Nakai *et al.* [49] indicated that the risk of the genotoxicity of styrene oligomers leached from polystyrene is likely very low. Conversely, the study of Nugnes *et al.* [44] demonstrated that polystyrene microparticles can induce alterations in the genetic material of *Ceriodaphnia dubia* neonates while the study by Brandts *et al.* [50] shows that polystyrene nanoparticles induce genotoxicity in haemocytes of *M. galloprovincialis* alone and in combination with carbamazepine. Microplastics appear capable of eliciting direct effects on the oxidative stress system and the mode of action is distinct from chemical exposures, tears, and abrasions in tissue during uptake of particulates may thus result in the alteration of antioxidant responses [25] and we can speculate that this could be reflected on the level of DNA damage. It is important to emphasize that there was no significant increase in MN frequency in the group exposed to polystyrene indicating short longevity of DNA damage assessed by comet assay in this case. The concentration of polystyrene particles used in the current study was in the range of reported genotoxic concentrations of other authors Nugnes *et al.* [44], but still, it should be considered that these are very far from environmental concentrations.

In the group exposed to polystyrene particles with adsorbed fluoranthene, a gradual increase in DNA damage was detected with a maximum reached after 72 h of exposure. When comparing data obtained in the group treated with fluoranthene and the group exposed to polystyrene with adsorbed fluoranthene, a lag in DNA damage induction of 1 day can be observed. Similar observations were found in the study of Paul-Pont *et al.* [16] which explained that this might be due to lag in the kinetics of fluoranthene desorption/assimilation from polystyrene particles. In the same study, this resulted in prolonged depuration of fluoranthene from tissues of *M.*

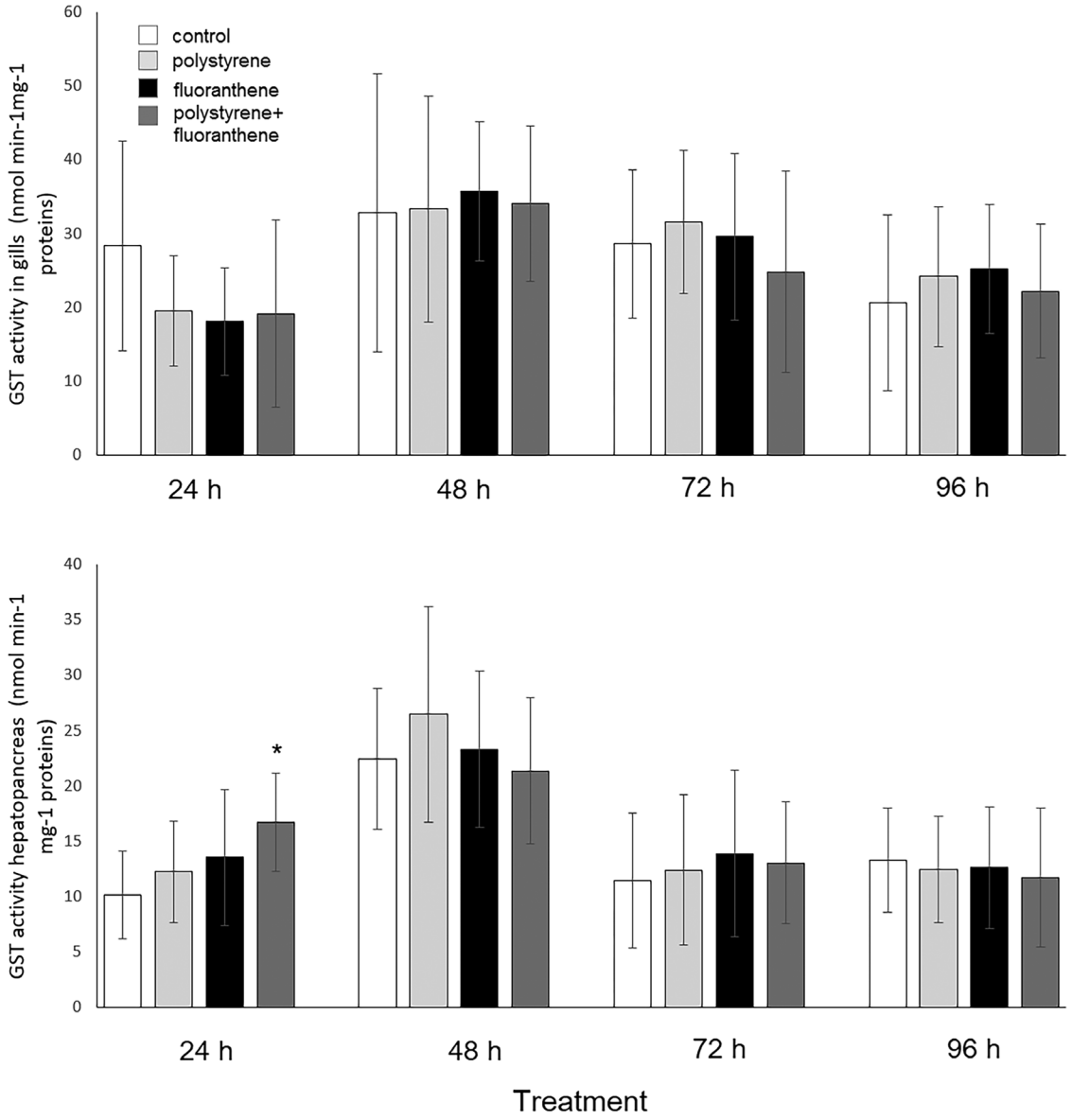

Figure 4. The effects of polystyrene particles, fluoranthene, and polystyrene particles with adsorbed fluoranthene on the GST activity in gills (upper chart) and hepatopancreas (lower chart) of *M. galloprovincialis* during 96 h of exposure, values are expressed as mean ± SD; *significant difference in comparison with control in corresponding treatment period $P < .05$.

galloprovincialis. Similarly, Oliveira *et al.* [51] indicated toxicologically relevant interactions between microplastics and pyrene by the delay of the lethal time observed in fish (common goby *Pomatoschistus microps*) exposed to the mixture treatment relative to the pyrene single exposure.

Genotoxicity was also assessed by micronucleus test but considering the time needed for the formation of MN, samples for analyses were collected only at the end of exposure (after 96 h). A significant increase in micronucleus frequency in comparison with the control group was observed in groups exposed to fluoranthene and polystyrene with adsorbed fluoranthene. To our knowledge, in available literature, there are no reports on the induction of MN by fluoranthene in aquatic invertebrates. The genotoxic potential of fluoranthene is discussed through the generation of reactive oxygen species and adducts formation [24, 52]. According to the abovementioned data, we can conclude that a portion of DNA breaks detected by comet assay was translated into permanent damage consequently recorded by micronucleus assay. Same as for the comet assay (in the first 48 h of exposure), mitigation of response was recorded in the group exposed to fluoranthene adsorbed to polystyrene in comparison with the group exposed to fluoranthene alone.

The physiological aspect of microplastics' influence on the early stages of fish was studied by Yang *et al.* [53], where they observed Hr increase of goldfish larvae due to oxidative stress induced by 1 mg/l of polystyrene, while 20 µg/l of polystyrene decreased Hr of marine medaka embryos [54]. Also, lower concentrations of polystyrene can mitigate toxicity by cadmium adsorption leading to normalization of Hr and reduced mortality of zebrafish embryos [55]. In our study, we did not observe any Hr changes of *M. galloprovincialis* during the treatment with microplastics, except subsequent Hr variations in specific days, which is consistent with the recent study of Shen and Nugegoda [56], showing similar Hr variations of Mediterranean mussel in control and during

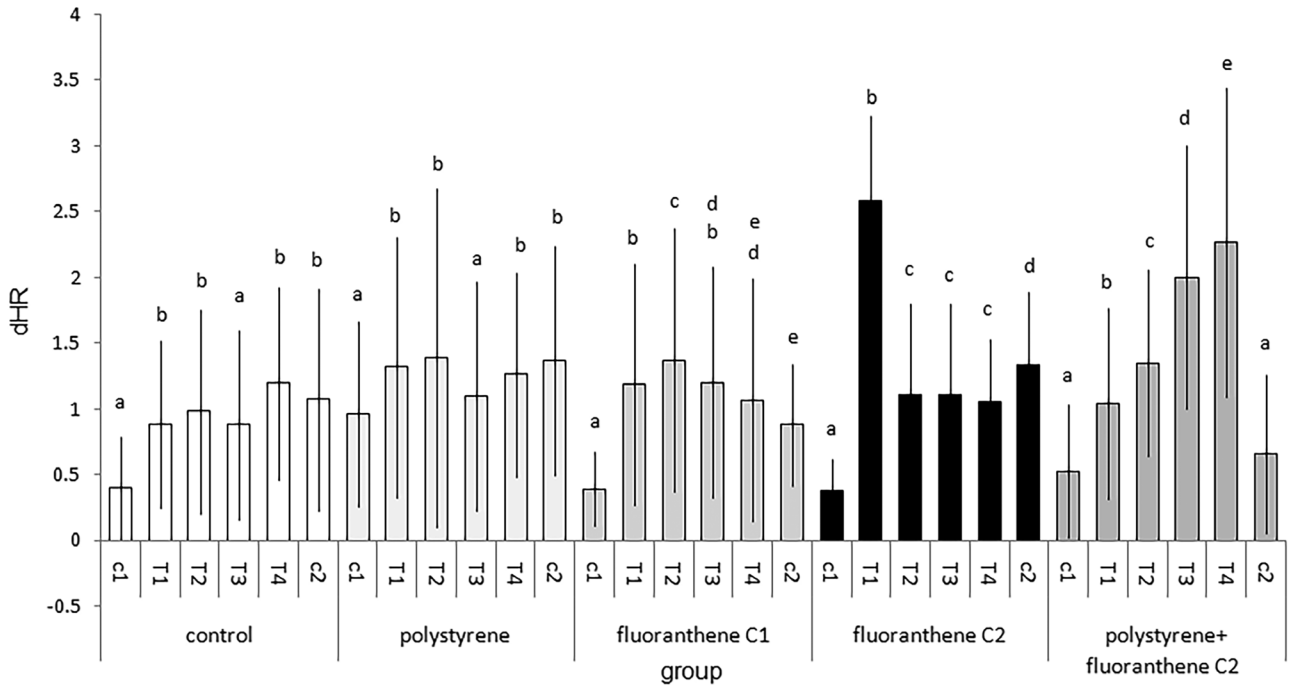

Figure 5. The effects of polystyrene particles, fluoranthene (C1—0.1 mg/l; C2—1 mg/l), and polystyrene particles with adsorbed fluoranthene (with corresponding higher concentration of fluoranthene C2) on the heart rate of *M. galloprovincialis* during 96 h of exposure assessed by dHr (mean ± SD). c1—period before the addition of test substance (pretreatment period), T1—the first 24 h of treatment, T2—the period from 24 to 48 h of treatment, T3—the period from 48 to 72 h of treatment, T4—the period from 72 to 96 h of treatment, c2—post-treatment period after washout; same letters shared by different groups (a, b, c, d, e) indicate lack of statistically significant difference among the groups $P < .05$.

microplastics exposure. If we compare control experiments of mussel's Hr pattern from our previous papers [33, 41] with those in control and under microplastic treatment in this study, observed Hr variations are more likely the consequence of static system conditions in aquarium tanks rather than the influence of microplastics. A similar range of Hr variations was observed under the exposure of microplastics with adsorbed fluoranthene, in a contrast to a sharp increase of cardiac activity, clearly visible by dHr shortly after the onset of fluoranthene C2 treatment. Gradual increase of dHr to the end of exposure with polystyrene + fluoranthene C2 and lack of any response within the first hours indicate possible mitigation of fluoranthene effect by microplastics adsorption, which was shown in the aforementioned study with cadmium.

Previous studies have reported AChE activity inhibition induced by polystyrene [57] and fluoranthene [58]. In our experiments at all checkpoints, there was a slight (but insignificant) inhibition in AChE activity in the group exposed to polystyrene in comparison with the control group. Also, within the 96 h of exposure to fluoranthene alone, an insignificant decrease in AChE activity was noticed. Oliveira *et al.* [51] found that polyethylene microspheres exposed in combination with PAH pyrene to juvenile goby fish, appeared to inhibit the activity of the neurotransmitter AChE, but no marked effect on GST. We observed a similar effect after 96 h where exposure to polystyrene with adsorbed fluoranthene resulted in significant inhibition of AChE activity. On the other hand, with exception of the pancreas after 24 h of exposure, there was no significant difference in GST activity in the group exposed to polystyrene with adsorbed fluoranthene and control in any of the checkpoints. There was no observable difference when comparing groups exposed to fluoranthene and fluoranthene adsorbed to polystyrene in regards to AChE and GST activity.

Conclusions

In the present study, a multibiomarker approach was used to assess the response of Mediterranean mussels to polystyrene, fluoranthene, and polystyrene with adsorbed fluoranthene with a focus on genotoxicological endpoints. The uniform response of individual biomarkers within the exposure groups was not recorded. There was no clear pattern in variation of AChE or GST activity which could be attributed to the treatment. Exposure to polystyrene increased DNA damage which was detected by the comet assay but was not confirmed by micronucleus formation. Data of genotoxicity assays indicated differential responses among the groups exposed to fluoranthene alone and fluoranthene adsorbed to polystyrene. There was no clear synergistic effect between polystyrene and fluoranthene. Change in the heart rate patterns within the studied groups supports the concept of the Trojan horse effect within the exposure to polystyrene particles with adsorbed fluoranthene.

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Data availability

Data are available on request. The data underlying this article will be shared upon reasonable request to the corresponding author.

References

1. *Plastics—The Facts*. 2022. https://plasticseurope.org/wp-content/uploads/2021/12/Plastics-the-Facts-2021-web-final.pdf. Accessed 5 September 2022.
2. Borrelle SB, Ringma J, Law KL et al. Predicted growth in plastic waste exceeds efforts to mitigate plastic pollution. *Science* 2020;369:1515–8.
3. Lamichhane G, Acharya A, Marahatha R et al. Microplastics in environment: global concern, challenges, and controlling measures. *Int J Environ Sci Technol* 2022:1–22.
4. Do Sul JAI, Costa MF. The present and future of microplastic pollution in the marine environment. *Environ Pollut* 2014;185:352–64.
5. Van Cauwenberghe L, Devriese L, Galgani F et al. Microplastics in sediments: a review of techniques, occurrence and effects. *Mar Environ Res* 2015;111:5–17.
6. Cole M, Lindeque P, Fileman E et al. Microplastic ingestion by zooplankton. *Environ Sci Technol* 2013;47:6646–55.
7. Desforges JPW, Galbraith M, Ross PS. Ingestion of microplastics by zooplankton in the Northeast Pacific Ocean. *Arch Environ Contam Toxicol* 2015;69:320–30.
8. Botterell ZL, Beaumont N, Dorrington T et al. Bioavailability and effects of microplastics on marine zooplankton: a review. *Environ Pollut* 2019;245:98–110.
9. Teuten EL, Saquing JM, Knappe DR et al. Transport and release of chemicals from plastics to the environment and to wildlife. *Philos Trans R Soc B Biol Sci* 2009;364:2027–45.
10. Cole M, Lindeque P, Halsband C et al. Microplastics as contaminants in the marine environment: a review. *Mar Pollut Bull* 2011;62:2588–97.
11. Sussarellu R, Suquet M, Thomas Y et al. Oyster reproduction is affected by exposure to polystyrene microplastics. *Proc Natl Acad Sci USA* 2016;113:2430–5.
12. Avio CG, Gorbi S, Regoli F. Plastics and microplastics in the oceans: from emerging pollutants to emerged threat. *Mar Environ Res* 2017;128:2–11.
13. Bhagat J, Nishimura N, Shimada Y. Toxicological interactions of microplastics/nanoplastics and environmental contaminants: current knowledge and future perspectives. *J Hazard Mater* 2021;405:123913.
14. González-Soto N, Hatfield J, Katsumiti A et al. Impacts of dietary exposure to different sized polystyrene microplastics alone and with sorbed benzo[*a*]pyrene on biomarkers and whole organism responses in mussels *Mytilus galloprovincialis*. *Sci Total Environ* 2019;684:548–66.
15. Syberg K, Khan FR, Selck H et al. Microplastics: addressing ecological risk through lessons learned. *Environ Toxicol Chem* 2015;34:945–53.
16. Paul-Pont I, Lacroix C, Fernández CG et al. Exposure of marine mussels *Mytilus* spp. to polystyrene microplastics: toxicity and influence on fluoranthene bioaccumulation. *Environ Pollut* 2016;216:724–37.
17. Monikh FA, Vijver MG, Guo Z et al. Metal sorption onto nanoscale plastic debris and Trojan horse effects in *Daphnia magna*: role of dissolved organic matter. *Water Res* 2020;186:116410.
18. Ziccardi LM, Edgington A, Hentz K et al. Microplastics as vectors for bioaccumulation of hydrophobic organic chemicals in the marine environment: a state-of-the-science review. *Environ Toxicol Chem* 2016;35:1667–76.
19. Fu L, Li J, Wang G et al. Adsorption behavior of organic pollutants on microplastics. *Ecotoxicol Environ Saf* 2021;217:112207.
20. Sørensen L, Rogers E, Altin D et al. Sorption of PAHs to microplastic and their bioavailability and toxicity to marine copepods under co-exposure conditions. *Environ Pollut* 2020;258:113844.
21. Nikolaou A, Kostopoulou M, Lofrano G et al. Determination of PAHs in marine sediments: analytical methods and environmental concerns. *Glob NEST J* 2009;11:391–405.
22. Palmqvist A, Rasmussen LJ, Forbes VE. Influence of biotransformation on trophic transfer of the PAH, fluoranthene. *Aquat Toxicol* 2006;80:309–19.
23. Qiao R, Sheng C, Lu Y et al. Microplastics induce intestinal inflammation, oxidative stress, and disorders of metabolome and microbiome in zebrafish. *Sci Total Environ* 2019;662:246–53.
24. Al-Subiai SN, Arlt VM, Frickers PE et al. Merging nano-genotoxicology with eco-genotoxicology: an integrated approach to determine interactive genotoxic and sub-lethal toxic effects of C60 fullerenes and fluoranthene in marine mussels, *Mytilus* sp. *Mutat Res/Genet Toxicol Environ Mutagen* 2012;745:92–103.
25. Magara G, Elia AC, Syberg K et al. Single contaminant and combined exposures of polyethylene microplastics and fluoranthene: accumulation and oxidative stress response in the blue mussel, *Mytilus edulis*. *J Toxicol Environ Health Part A* 2018;81:761–73.
26. Browne MA, Dissanayake A, Galloway TS et al. Ingested microscopic plastic translocates to the circulatory system of the mussel, *Mytilus edulis* (L.). *Environ Sci Technol* 2008;42:5026–31.
27. Turja R, Höher N, Snoeijs P et al. A multibiomarker approach to the assessment of pollution impacts in two Baltic Sea coastal areas in Sweden using caged mussels (*Mytilus trossulus*). *Sci Total Environ* 2014;473:398–409.
28. Berezina NA, Verbitsky VB, Sharov AN et al. Biomarkers in bivalve mollusks and amphipods for assessment of effects linked to cyanobacteria and elodea: mesocosm study. *Ecotoxicol Environ Saf* 2020;203:110994.
29. Bakhmet IN, Kusnetsova T, Kholodkevich S. Heart rhythm of the ocean quahog *Arctica islandica*. *Knowl Soc Sci* 2022:1–5.
30. Bajt O, Ramšak A, Milun V et al. Assessing chemical contamination in the coastal waters of the Adriatic Sea using active mussel biomonitoring with *Mytilus galloprovincialis*. *Mar Pollut Bull* 2019;141:283–98.
31. Kolarević S, Aborgiba M, Kračun-Kolarević M et al. Evaluation of genotoxic pressure along the Sava River. *PLoS One* 2016;11:e0162450.
32. Singh NP, McCoy MT, Tice RR et al. A simple technique for quantitation of low levels of DNA damage in individual cells. *Exp Cell Res* 1988;175:184–91.
33. Martinović R, Kolarević S, Kračun-Kolarević M et al. Genotoxic potential and heart rate disorders in the Mediterranean mussel *Mytilus galloprovincialis* exposed to Superdispersant-25 and dispersed diesel oil. *Mar Environ Res* 2015;108:83–90.
34. Bolognesi C, Fenech M. Mussel micronucleus cytome assay. *Nat Protoc* 2012;7:1125–37.
35. Hagger JA, Depledge MH, Galloway TS. Toxicity of tributyltin in the marine mollusc *Mytilus edulis*. *Mar Pollut Bull* 2005;51:811–6.
36. Lowry O, Rosebrough N, Farr A et al. Protein measurement with the Folin phenol reagent. *J Biol Chem* 1951;193:265–75.
37. Ellman G, Courtney KD Jr, Andres V et al. A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem Pharmacol* 1961;7:88–95.
38. Mancini I, Sicurelli A, Guella G et al. Synthesis and bioactivity of linear oligomers related to polymeric alkylpyridinium metabolites

from the Mediterranean sponge *Reniera sarai*. *Org Biomol Chem* 2004;2:1368–75.
39. Habig WH, Jakoby WB. Assays for differentiation of glutathione *S*-transferases. *Methods Enzymol* 1981;77:398–405.
40. Da Ros L, Moschino V, Macic V et al. An ecotoxicological approach for the Boka Kotorska Bay (south-eastern Adriatic Sea): first evaluation of lysosomal responses and metallothionein induction in mussels. *Mar Pollut Bull* 2011;63:326–33.
41. Martinović R, Kolarević S, Kračun-Kolarević M et al. Comparative assessment of cardiac activity and DNA damage in haemocytes of the Mediterranean mussel *Mytilus galloprovincialis* in exposure to tributyltin chloride. *Environ Toxicol Pharmacol* 2016;47:165–74.
42. Fedotov VP, Kholodkevich SV, Strochilo AG. Study of contractile activity of the crayfish heart with the aid of a new non-invasive technique. *J Evol Biochem Physiol* 2000;36:288–93.
43. Kholodkevich SV, Sharov AN, Chuiko GM et al. Quality assessment of freshwater ecosystems by the functional state of bivalved Mollusks. *Water Resour* 2019;46:249–57.
44. Nugnes R, Lavorgna M, Orlo E et al. Toxic impact of polystyrene microplastic particles in freshwater organisms. *Chemosphere* 2022;299:134373.
45. Choucroun P, Gillet D, Dorange G et al. Comet assay and early apoptosis. *Mutat Res/Fund Mol Mech Mutagen* 2001;478:89–96.
46. Štambuk A, Pavlica M, Malović L et al. Persistence of DNA damage in the freshwater mussel *Unio pictorum* upon exposure to ethyl methanesulphonate and hydrogen peroxide. *Environ Mol Mutagen* 2008;49:217–25.
47. Magni S, Gagné F, André C et al. Evaluation of uptake and chronic toxicity of virgin polystyrene microbeads in freshwater zebra mussel *Dreissena polymorpha* (Mollusca: Bivalvia). *Sci Total Environ* 2018;631:778–88.
48. Paget V, Dekali S, Kortulewski T et al. Specific uptake and genotoxicity induced by polystyrene nanobeads with distinct surface chemistry on human lung epithelial cells and macrophages. *PLoS One* 2015;10:e0123297.
49. Nakai M, Tsubokura M, Suzuki M et al. Genotoxicity of styrene oligomers extracted from polystyrene intended for use in contact with food. *Toxicol Rep* 2014;1:1175–80.
50. Brandts I, Teles M, Gonçalves AP et al. Effects of nanoplastics on *Mytilus galloprovincialis* after individual and combined exposure with carbamazepine. *Sci Total Environ* 2018;643:775–84.
51. Oliveira M, Ribeiro A, Hylland K et al. Single and combined effects of microplastics and pyrene on juveniles (0+ group) of the common goby *Pomatoschistus microps* (Teleostei, Gobiidae). *Ecol Indic* 2013;34:641–7.
52. Wessel N, Ménard D, Pichavant-Rafini K et al. Genotoxic and enzymatic effects of fluoranthene in microsomes and freshly isolated hepatocytes from sole (*Solea solea*). *Aquat Toxicol* 2012;108:33–41.
53. Yang H, Xiong H, Mi K et al. Toxicity comparison of nano-sized and micron-sized microplastics to goldfish *Carassius auratus* larvae. *J Hazard Mater* 2020;388:122058.
54. Wang J, Li Y, Lu L et al. Polystyrene microplastics cause tissue damages, sex-specific reproductive disruption and transgenerational effects in marine medaka (*Oryzias melastigma*). *Environ Pollut* 2019;254:113024.
55. Zhang R, Wang M, Chen X et al. Combined toxicity of microplastics and cadmium on the zebrafish embryos (*Danio rerio*). *Sci Total Environ* 2020;743:140638.
56. Shen H, Nugegoda D. Real-time automated behavioural monitoring of mussels during contaminant exposures using an improved microcontroller-based device. *Sci Total Environ* 2022;806:150567.
57. Fu L, Xi M, Nicholaus R et al. Behaviors and biochemical responses of macroinvertebrate *Corbicula fluminea* to polystyrene microplastics. *Sci Total Environ* 2022;813:152617.
58. Vidal-Liñán L, Bellas J, Etxebarria N et al. Glutathione *S*-transferase, glutathione peroxidase and acetylcholinesterase activities in mussels transplanted to harbour areas. *Sci Total Environ* 2014;470:107–16.