Multilevel toxicity assessment of polypropylene microplastics and pyrene on mussels: DNA damage, oxidative stress, and physiological effects

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Abstract

Despite extensive research on microplastic pollution, combined biological effects of microplastics and associated pollutants on marine invertebrates remain unclear. We present an integrative assessment of polypropylene (PP) and pyrene, individually and in co-exposure, in mussel *Mytilus galloprovincialis*. Mussels were exposed to 1 mg L^{-1} PP (~40 µm) and 50 µg L^{-1} of pyrene for 7 and 14 days, representing a scenario relevant to highly polluted coastal areas. DNA damage increased significantly in mussels exposed to pyrene or PP alone, but remained at control levels under combined exposure, suggesting an interaction that may reduce genotoxic potential. Lipid peroxidation remained stable across treatments, despite significant changes in antioxidant enzymes. Catalase activity increased in pyrene and pyrene + polypropylene treatments, with tissue-specific trends, indicating enhanced antioxidant protection. Glutathione *S*-transferase activity was stable in digestive glands but significantly inhibited in gills after seven days under PP exposure. ETS activity increased in pyrene-containing treatments after 14 days, reflecting elevated metabolic demand after prolonged exposure. Respiration rate declined under PP exposure. Heart rate recovery time after the hyposalinity test was the slowest in the pyrene + polypropylene group, indicating compromised physiological resilience. These findings reveal interactive, tissue- and biomarker-specific effects of PP and pyrene. Their combination suggested attenuation of genotoxicity but enhanced physiological stress responses, highlighting the complexity of pollutant interactions and importance of evaluating multiple biomarkers, tissues and pollutants. Presented data provide the first ever biomarker-based evaluation of PP and pyrene co-exposure, offering novel insights into microplastic-pollutant interactions and potential ecological consequences for marine invertebrates.

Keywords: microplastics, PAHs, multi-biomarker approach, co-exposure, *Mytilus galloprovincialis*

1. Introduction

The marine environment is increasingly polluted by the complex mixtures of anthropogenic pollutants, among which microplastics (MPs) and polycyclic aromatic hydrocarbons (PAHs) are of particular concern (Sun et al., 2021). Although most polymers are chemically stable and degrade very slowly, the leaching of additives and weathering-induced surface oxidation can alter their reactivity and interactions with other pollutants. Consequently, plastic litter accumulates in the environment and poses a particular problem for filter-feeding organisms in aquatic ecosystems and facilitates its transfer through the food web. Microplastics, particles from 1 to 1000 μm in size (ISO, 2020), are a persistent and bioavailable pollutant, especially in coastal ecosystems where their sources are diverse and continuous. PAHs are a large group of hydrophobic organic compounds, many of which are toxic, mutagenic, and carcinogenic. They are widely present in marine sediments and in the water column due to natural and anthropogenic sources (Vijayanand et al., 2023). Due to their high lipophilicity, toxicity and persistence in the environment, several PAHs are classified as priority substances under the EU Water Framework Directive (2000/60/EC, European Commission, 2000), so that their monitoring and remediation in the event of oil spills is listed under Descriptor 8 in the Marine Strategy Framework Directive (2008/56/EC, European Commission, 2008). Due to the high risks to marine organisms, litter and microplastics are also of a high concern (Galgani et al., 2024).

Among plastic polymers, polypropylene (PP) is one of the most widely produced in the world and is often used for food packaging, textiles, electronics and consumer goods due to its low density, mechanical strength and chemical resistance (Milošža et al., 2021). Despite its dominance in marine plastic litter, especially in its microplastic form, PP has only recently received very little attention in the in vitro experimental studies mostly on fish (Bobori et al., 2022; Jeyavani et al., 2023; Daniel et al., 2024). Bobori et al. (2022) investigated cytotoxicity of PP in two freshwater fish (*Danio rerio* and *Perca fluviatilis*) and concluded that PP affected the cellular function of the gills and hepatic cells by lipid peroxidation, DNA damage, protein ubiquitination, apoptosis, autophagy, and changes in metabolite concentration. Similar findings were confirmed by Jeyavani et al. (2023) who reported on the increase in reactive oxygen species levels, an alteration in antioxidant parameters, including superoxide dismutase (SOD), catalase (CAT), glutathione-S–transferase (GST), and glutathione peroxidase (GPx) disturbances in the neurotransmitter enzyme acetylcholinesterase (AChE) in *Oreochromis mossambicus* after exposure to PP for 96 h and 14 days. Daniel et al. (2024) provided the first

results of PP exposure on mussels with consequences on decreased feeding rates and lower condition index of the mussels, especially at the high dosage of 50 mg L^{-1}. No alterations occurred in metabolic enzymes, while CAT activities decreased after four days of acute exposure, but significantly increased after the chronic exposure (28 days). Accordingly, several reviews have emphasized the need to shift focus from widely studied polystyrene (PS) and polyethylene (PE) to less-investigated, but environmentally dominant, polymers like PP (de Sá et al., 2018; Baroja et al., 2021). Another key concern with PP and other MPs is their potential to adsorb hydrophobic pollutants such as PAHs from the surrounding water, which raises concerns about their role as vectors for chemical pollutants. Due to their large surface area-to-volume ratio, it is known that MP particles can sometimes serve as vectors for other pollutants, referred to as a "Trojan horse effect" (Sendra et al., 2017; Kolarević et al., 2023). Such interactions can result in triggering either additive, synergistic or antagonistic effects on organisms (Sendra et al., 2021), highlighting the need to study MPs not only as pollutants *per se*, but also as vectors.

While several studies in recent years have investigated the combined effects of different types of microplastics and PAHs on mussels using more commonly studied PAHs as benzo[a]pyrene (Pittura et al., 2018; González-Soto et al., 2019; Wang et al., 2023), or fluoranthene (Paul-Pont et al., 2016; Magara et al., 2018; Kolarević et al., 2023), considerably less attention has been given to interactions with pyrene —a four ring PAH frequently detected in urban and industrial marine areas. Although less studied, pyrene is still environmentally relevant and exhibits genotoxic and oxidative stress-inducing properties (Oliviera et al., 2012) and is also on the United States Environmental Protection Agency's list of priority pollutants.

As already mentioned, only one study has examined the combined effects of pyrene with PE and PS microplastics in mussels (Avio et al., 2015), and, there is only one study to date on the exposure of mussels to PP (Daniel et al., 2024). However, Daniel et al. (2024) used high PP concentrations and only the digestive gland as a tissue of interest. Most importantly, there is no data available on the combined effects of PP and pyrene. Additionally, there is a significant lack of data using environmentally relevant concentrations of PAHs and MPs. Since there is an obvious lack of *in vivo* studies on the effects of PP and pyrene on biota, in this study we selected pyrene as a model PAH and PP particles as a relevant MP type to investigate their individual and combined toxicological effects on mussels at environmentally relevant concentrations. This approach allowed us to evaluate not only the direct biological responses to each stressor, but also the interactive effects of PP and pyrene under realistic co-exposure conditions. Due to their ecological relevance, wide distribution and established use in pollution monitoring, we used the

Mediterranean mussel (*Mytilus galloprovincialis*) as a bioindicator. As filter feeders, mussels efficiently accumulate pollutants, including microplastics (Regoli, 1998; Sendra et al., 2021). Gills and digestive glands were chosen for analysis as primary sites of uptake and detoxification (Geffard et al., 2001; Sendra et al., 2021). Further, several *Mytilus* species have already been used in MPs studies (*cf* de Sá et al., 2018; Baroja et al., 2021), allowing a comparative analysis of closely related species. We applied a multi-biomarker approach including condition indices, assessment of genotoxicity (comet assay), enzymatic and oxidative stress responses (catalase (CAT), glutathione *S*-transferase (GST), malondialdehyde (MDA)), neurotoxicity (acetylcholinesterase, AChE), cellular metabolic activity (electron transport system, ETS) and whole organism physiological parameters (respiration rate, RR and heart rate, HR) to comprehensively assess the biological responses of mussels to exposure to PP particles, pyrene and their co-exposure. By integrating these endpoints in different tissues and changes at different levels of biological organization, we aimed to (i) determine the detrimental effects of PP, pyrene and PP with adsorbed pyrene; (ii) evaluate the role of PP as a modulator of pyrene toxicity; (iii) compare biomarker responses in different bioindicator tissues; (iv) assess ecotoxicological responses at multiple levels of biological organization and (v) contribute to the understanding of the effects of microplastic-chemical mixtures on marine organisms. Our research fills a critical knowledge gap regarding PP toxicity, the ecological relevance of less studied PAHs such as pyrene, and the interactive effects of MPs and adsorbed pollutants, which can alter pollutant bioavailability, toxicity, and accumulation dynamics in marine organisms. These findings have direct implications for environmental risk assessment and the development of regulatory frameworks for marine pollution.

2. Materials and methods

2.1. Mussels sampling and acclimation

The mussels (about 600 individuals, shell length 50–70 mm) were collected from the mussel farm in Piran Bay, Slovenia, located in a pristine area, at the end of October 2024 at a depth of three meters. Mussels were immediately transferred to Marine Biology Station Piran, National Institute of Biology, where they were counted, separated into live and dead mussels, cleaned of algae and epiphytes, examined for the presence of flatworms and then placed in the tanks for the acclimation. The mussels were kept in four tanks with natural seawater filtered through a 38 µm fine mesh in a chamber at 21 ºC. The seawater was aerated with air pumps and

replaced twice a week. Mussels were fed daily with commercial food Aquaforest Phyto Mix (5 µL L^{-1} seawater). Acclimation period lasted 2.5 weeks prior to the exposure experiment.

2.2. Preparation of polypropylene microplastics and adsorption of pyrene

2.2.1. Milling

Pellets of polypropylene (isotactic, Mw = 12,000 g mol^{-1}, Mn = 5,000 g mol^{-1}; Sigma-Aldrich, Germany) were used as the plastic material for the experiment. Pellets were milled using the ultra-centrifugal mill ZM 300 (Retsch, Germany) at 8000 rpm under cryogenic conditions. The produced microparticles were sieved through series of sieves with the smallest mesh size being 80 µm. That way, a fraction <80 µm was obtained for the experiment and part of the sample was analysed for particle size distribution.

2.2.2. Morphology of particles and particle size distribution

The shapes and morphology of the obtained PP particles were assessed by scanning electron microscopy. Microplastics were coated with a 5 nm thick conductive Au-Pd layer using the Rotary Pumped Coater Q150R Plus (USA) and subjected to SEM using the Tescan Mira (Brno, Czech Republic). The analysis was performed at various magnifications with an SEM acceleration voltage of 2 kV.

Particle size distribution by number was measured using the laser diffraction analyser Bluewave S3500 (Microtrac, Germany) in a dry unit. Measurements were performed three times with around 0.5 g of the sample (Rozman et al., 2021). The results were presented as the mean diameter and number particle size distribution.

2.2.3. Preparation and analysis of polypropylene microplastics with adsorbed pyrene

Stock solutions of pyrene (Sigma Aldrich, USA) were prepared by dissolving 1 g pyrene in 20 mL of HPLC grade hexane. An aliquot of 2 mL of this solution was added to 2 g of grinded PP in a glass tube. This mixture was stirred by vortex for 2 hours at room temperature and subsequently hexane was evaporated until dryness under gentle stream of nitrogen. The PP MP with adsorbed pyrene were removed from the tube and the proportion of adsorbed pyrene was determined by analysing the residual pyrene. The residual pyrene was dissolved in methanol and analysed using gas chromatography. An HP 6890 gas chromatograph equipped with an FI detector and an on-column injector was used for the analysis. The column used was a HP Ultra 2 (25 m × 0.32 mm × 0.17 µm) and the carrier gas (helium) flow rate was 1 mL min^{-1}. The adsorption efficiency was calculated based on the mass difference between the original

pyrene and the residual pyrene recovered in methanol. The calculated adsorption of pyrene on PP MPs was almost 95%.

2.3. Pyrene preparation

Pyrene stock solution (2 mg mL^{-1}) was prepared by dissolving 200 mg of pyrene (Sigma Aldrich, USA) in 100 mL dimethyl sulfoxide (DMSO) (Sigma Aldrich, USA) and then adequate volume of stock solution was added into the tank depending on the volume of water at the moment of changing water to maintain the constant concentration of 50 µg L^{-1}. Final concentration of DMSO in the tank was <0.005% throughout the whole experiment.

2.4. Exposure experiment

Mussels with intact byssus threads after acclimation were randomly divided into 4 groups of 120 individuals and each group was placed in an 80-L glass tank containing 0.35 L of seawater per mussel taken in front of the Marine Biology Station in Piran. They were left over the night in the tanks at the constant temperature of 21 ºC. Before the addition of the pollutant the next day, we checked if mussels were attached to the tank bottom by byssus threads. During the experiment, the mussels were kept at 21 ºC and 10/14 h light/dark regime.

Treatment groups were: (1) control tank – mussels in seawater without pollutants (2) pyrene – mussels exposed to 50 µg L^{-1} pyrene (prepared from the stock solution); (3) polypropylene particles (PP) – mussels exposed to 1 mg L^{-1} PP; (4) pyrene adsorbed to PP particles – mussels exposed to pyrene + polypropylene with the corresponding concentrations in treatments 2 and 3.

The exposure was conducted for 14 days under the stable conditions in a static system, in which the natural seawater was changed twice a week and filtered through a 38 µm net, while the mussels were also fed with Aquaforest Phyto Mix (10 µL L^{-1} water). Stratification of particles on the surface of the tank was prevented by circulation with air bubbling pumps. After each water change and cleaning of pseudofeces from the bottom, pollutants were added again to maintain constant concentrations. Checkpoints for comet assay, cell viability, enzymatic activities and respiration rate were set at the 7^{th} and 14^{th} day, while condition indices and heart rate (HR) were analysed after 14 days. At each sampling, five specimens were taken from each tank for comet assay and cell viability, another five for respiration rate (same mussels used on 7^{th} and 14^{th} day), 20 mussels for biochemical markers, and on 14^{th} day, an additional 10 for condition indices and 8 for HR measurements. Digestive glands and gills were rapidly dissected and frozen in liquid nitrogen and maintained at −80 ºC until biochemical analysis. Animals

were treated and housed in accordance with national and international legislation (EU Directive 2010/63/EU on the protection of animals used for scientific purposes, European Commission, 2010).

2.5. Biometry and condition index

In the dominant size class ten mussels were selected from each tank and the length, width and height of the shells were measured, and the entire viscera of each mussel were weighed and then lyophilised for 48 hours. Condition index was calculated according to formula: CI= SO/LE x WI x HE, where SO is dry mass of viscera (mg), LE is length (cm), WI is width (cm) and HE is height of the mussel (cm) (Lobel et al., 1991).

2.6. Assessment of genotoxicity

2.6.1. Haemolymph collection and cell viability assessment

Haemolymph was collected from five animals in each experimental group after seven and 14 days of exposure. The collection was performed using a 5 mL syringe equipped with a 21 G hypodermic needle. To prevent haemocyte clogging, each syringe was pre-filled with 200 µL of physiological saline (0.5 M NaCl, 12.5 mM KCl, 5.5 mM EDTA, 20 mM HEPES; pH 7.4). Cell suspensions were subsequently used for cell viability and genotoxicity analyses. 20 µL of cell suspension was mixed with 2 µL acridine orange/ethidium bromide staining (Squier and Cohen, 2001). Viability was assessed by examining 100 haemocytes per sample, green-stained cells classified as viable and red-stained cells as nonviable.

2.6.2. Comet assay

The DNA damages were evaluated using the comet assay in the experimental groups. Alkaline comet assay on minigels was performed according to the protocol of Singh et al. (1988) and adapted for mussels by Kolarević et al. (2023). Microscope slides were precoated with 1% normal melting point agarose (NMP), while the minigels were prepared by mixing the cell suspension with 1% low melting point agarose (LMP). Each minigel consisted of 12 µL of a mixture made from 30 µL of cell suspension and 70 µL of LMP. Minigels from five specimens were placed in triplicate on each slide, resulting in a total of 15 minigels per slide. After gel solidification, slides were incubated in a cold lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% Triton X-100, pH 10) for 2.5 hours. Further, slides were transferred to an electrophoresis chamber containing electrophoretic buffer (300 mM NaOH, 1 mM EDTA, pH 13). Denaturation in this buffer was performed for 20 minutes, followed by electrophoresis at

0.75 V/cm and 200 mA for 20 minutes. After electrophoresis slides were immersed in cold neutralization buffer (0.4 M Tris, pH 7.5) for 15 minutes, fixed in cold ethanol for 15 minutes, and left to dry overnight in the dark. Staining was performed using 20 µL of nucleic acid gel stain (GelGreen, Biotium) at a concentration of 2 µL mL^{-1}. Slides were analyzed under a fluorescence microscope (Leica DM4 B, Austria) at 400× magnification, using an excitation filter of 450–490 nm and a barrier filter of 510 nm. On each minigel, 50 nuclei were scored with Comet IV software (Instem, UK), and the percentage of DNA in the tail (TI %) was recorded as an indicator of DNA damage.

2.7. Biochemical markers

Homogenization and biochemical biomarkers measurements mostly followed the procedures already described in Mijošek et al. (2019, 2021) and Kiralj et al. (2025), but some protocols were adjusted for the mussels, in particular dilutions and volumes of homogenates suitable for a smaller tissue mass. Proteins were measured with BCA Protein Assay Kit (Thermo Scientific, USA). Spectrophotometric measurements of biomarkers were conducted on Spark microplate reader (Tecan, Switzerland).

2.7.1. Homogenization procedures

Digestive glands and gills of mussels taken from -80 °C were homogenized in a cold 50 mM potassium phosphate buffer, pH 8.1 (mass:volume 1:2 to 1:4 for digestive glands and 1:2 or 1:3 for the gills depending on the tissue mass) for biochemical biomarkers (AChE, CAT, GST, MDA). Homogenization was performed in a cooled tube kept on ice using the Potter-Elvehjem homogenizer (Glas-Col, USA), and homogenates were kept continuously on ice during the whole procedure. Prepared homogenates were centrifuged at 10000×g for 30 min at 4 °C. The supernatants were aliquoted for different analyses and kept at -80 ºC until specific analysis.

Homogenates of digestive glands for ETS measurements were prepared in homogenization buffer (0.1 M Na_2HPO_4, 0.1M KH_2PO_4, 0.05 M Tris base, 75 µM $MgSO_4 \cdot 7H_2O$, 1.5 mg/mL PVP, 0.2% Triton-X, pH 8.5). Briefly, around 50 mg of the tissue was homogenized in 2 mL of the buffer, using a Potter-Elvehjem homogenizer, and diluted up to 40 times. Supernatants were centrifuged at 3000×g for 10 min at 4 °C and aliquots were stored at -80 ºC.

2.7.2. Acetylcholinesterase activity - AChE

AChE in gills was analysed following the method described by Ellman et al. (1961), adapted for microplates. Reaction mixture consisting of 5 µL of sample and 250 µL of 0.1 M potassium phosphate buffer (pH 7.4 at 25 °C) containing 0.1 mM DTNB (Sigma Aldrich, USA) was incubated in the dark for 15 minutes. Reaction was started by adding 25 µL of 10 mM acetylthiocholine iodide (Sigma Aldrich, USA), and increasing of absorbance was immediately monitored at 412 nm in 1-minute intervals for five minutes. Homogenization buffer was used as a blank. The specific enzymatic activity was expressed as nmol of acetylthiocholine hydrolysed per min per mg of proteins, using the ε= 13.6 mM^{-1} cm^{-1} of acetylthiocholine.

2.7.3. Catalase activity - CAT

Specific activity of CAT was performed at 240 nm and 25 °C following the method by Claiborne (1985) with some modifications for microplates from Mijošek et al. (2019). Sodium phosphate buffer (50 mM, pH 7.0) and 30% H_2O_2 were used to prepare 10.34 mM H_2O_2, of which 290 µL was added to 10 µL of five times diluted samples (digestive gland or gills). Homogenization buffer was used as a blank and treated the same as samples. Absorbance was measured every 10 seconds for one minute. Specific activity was calculated using ε= 43.6 M^{-1} cm^{-1} of H_2O_2 and expressed as µmol of H_2O_2 degraded per minute per mg of proteins.

2.7.4. Glutathione *S*-transferase activity - GST

Measurements of GST activity were based on the method of Habig et al. (1974), which was modified for microtiter plates by Kiralj et al. (2025). Samples of gills and digestive glands were diluted five times in 0.1 M sodium phosphate buffer (pH 6.5), and 5 µL of the diluted sample was added to the plate. Following, 10 µL of a 20 mM GSH solution (final concentration 1 mM; Sigma Aldrich, USA) and 185 µL of a 1 mM 1-chloro-2,4-dinitrobenzene solution (CDNB; Sigma Aldrich, USA) were added. Homogenization buffer was used as a blank. Measurements were performed at 340 nm in 15-second intervals for 3 minutes. Enzyme activity was expressed as µmol per minute per mg of proteins, and ε=9.6 mM^{-1} cm^{-1} of CDNB was used for calculations.

2.7.5. Malondialdehyde concentration - MDA

MDA concentrations were measured according to the procedure described in Mijošek et al. (2019, 2021). Solution containing 1% butylated hydroxytoluene (BHT, Sigma-Aldrich, USA) dissolved in ethanol (Honeywell, USA) and 10% trichloroacetic acid (TCA, Sigma-Aldrich, USA) dissolved in Milli-Q water (BHT/ TCA = 1:100) was added to samples. Samples were vigorously mixed and kept on ice, centrifuged at 4000×g for 15 minutes at 4 °C. Supernatants were transferred into 1.5-mL eppendorf tubes and 2-thiobarbituric acid (TBA,

Sigma-Aldrich, USA) was added. After that, samples were heated at 100 ºC for 30 minutes and left to cool before transferring to microplates. Homogenization buffer was used as a blank and absorbance of reaction was measured at 535 nm. A calibration curve was made of eight concentrations from 2 to 100 µM MDA (Sigma-Aldrich, USA) and final product of reaction was expressed as nmol of MDA per gram of wet tissue mass,

2.7.6. Electron transport system activity – ETS

The electron transport system (ETS) activity was measured based on the method of King and Packard (1975) and modifications by Erk et al. (2011). Activity was measured after mixing 180 µL of buffered substrate solution (1.7 mM NADH and 0.25 mM NADPH dissolved in 0.1 M Na_2HPO_4, 0.1 M KH_2PO_4, 0.05 M Tris Base, 0.2% Triton X-100, pH 8.5) with 60 µL of homogenate from digestive gland. Reaction started by the addition of 60 µL of iodonitrotetrazolium chloride (INT) into the wells. Homogenization buffer was used as a blank and treated the same as the samples of the digestive glands. Absorbance was immediately measured at 490 nm in intervals of 25 s for five minutes. The ε=15.9 mM^{-1} cm^{-1} of INT was used to calculate the amount of formazan formed and activity was expressed in mJ mg^{-1} w.w. h^{-1} as described in Verslycke and Janssen (2002) by applying the oxyenthalpic equivalents for an average lipid, protein and carbohydrate mixture (484 kJ mol^{-1} O_2) (Gnaiger, 1983).

2.7.7. Protein determination

The total protein content in the samples was measured spectrophotometrically using BCA Protein Assay Kit (Thermo Scientific, USA). Pierce reagent was made by mixing reagent A and B (50:1) and then 12.5 µL of the sample was mixed with 87.5 µL of the prepared reagent. Plate was incubated for 30 min at 37 ºC in the dark, and absorbance was measured at 562 nm. Calibration curve was made with different BSA concentrations from 0.025 mg mL^{-1} to 2 mg mL^{-1}.

2.8. Physiological endpoints

2.8.1. Respiration rate

The respiration rate (RR) was estimated by the closed bottle method using a fiber optic oxygen meter (PreSens OXY-4, Germany) (Simčič and Sket, 2019). The 500-mL ground glass stoppered bottles were filled with filtered and aerated seawater from the same well-mixed container. Experimental bottles contained animals, while two bottles without animals served as controls. All bottles were sealed and kept in the dark at the experimental temperature. We measured the oxygen concentration in the experimental and control bottles with an optical fiber

and a sensor spot glued to the inner wall of the bottle after 0.5 hour to give the animals time to recover from handling stress (initial measurement) and after 1 hour (final measurement). The oxygen consumption of the animals was determined by calculating the difference in oxygen concentration in each experimental bottle between the start and end of incubation and then subtracting the average difference observed in the control bottles. Respiration rate was expressed as mg O_2 per individual per h as we wanted to use the same mussel individuals on both sampling days.

2.8.2. Heart rate

2.8.2.1. Recording of cardiac activity

The heart rate (HR) recording of the mussels was carried out by a non-invasive laser fiber-optic method based on photoplethysmography (PPG; Fedotov et al., 2000). The method relies on changes in light intensity reflected from or transmitted through the tissue, providing information on the heartbeats (Allen, 2007). The experimental unit includes eight PPG devices (Photoplethismograph, RIC "Eco-Contour"; Russia) for the simultaneous recording of the cardiac activity of eight mussels. The detailed procedure for the cardiac activity registration of benthic invertebrates was explained by Kholodkevich et al. (2017).

2.8.2.2. Hyposalinity test

Eight mussels from each experimental group were subjected to hyposalinity test conducted according to Kholodkevich et al. (2017). The hyposalinity test started by a gentle addition of distilled water to the seawater in the tank to reduce the salinity by 50%, as the ranges of the physiological tolerance limits for different species were defined previously. Salinity was measured by WTW Multi 3620 IDS probe (WTW GmbH; Germany). The artificial sea salt (Aquaforest, Poland) solution was prepared based on calculation and added to the tank after 1-1.5 hour, to restore the background salinity values determined before the test. HR was recorded in real time in 10 second intervals during the experiment. Three important recording periods were: 1) before hyposalinity test (at least of 2 h of HR recording in clean seawater to establish HR resting response as the HR baseline level), 2) during hyposalinity test (at least 1-1.5 h of HR recording in seawater with 50% lower salinity) and 3) after hyposalinity test (at least 2 h of recording to follow HR recovery after salinity restoration).

2.8.2.3. Calculation of HR recovery time

The calculation of HR recovery time (T_{rec}) was based on the result graphs of HR pattern in MS Excel for each specimen separately. T_{rec} was defined as the time–distance between two

points (end of salinity restoration and re-achieving the stable HR values recorded before the test (Kholodkevich et al., 2017).

2.9. Statistics

SigmaPlot 11.0 was used for statistical analyses, graph drawing, and basic calculations were done in MS Excel. To assess the variability of majority of biomarkers in digestive gland and gills of mussels from four different treatments after seven and 14 days, Kruskal-Wallis one-way analysis of variance on ranks, followed by Dunn's or Tukey's tests was used. Mann-Whitney U test was applied to test the data variability between the two sampling points for each biomarker, all considering that the assumptions of normality and homogeneity of variance were not consistently met. Due to the normal distribution of data on condition indices, cell viability, and heart and respiration rates, differences between treatments on these sets were tested using one-way ANOVA and Tukey's test, and differences between the two sampling days with t-test. Significance level was always set at $p < 0.05$. Statistically significant differences between treatments are indicated by different numbers (1-4) in the graphs: significant difference from treatment indicated by a specific number: 1 - control; 2 – pyrene; 3 – PP; 4 – pyrene + PP. Difference between two sampling points for each treatment are indicated with asterisk *.

3. Results and discussion

Despite widespread presence in marine environment, PP microplastics remain underexplored in ecotoxicological research, particularly regarding environmental concentrations and interactions with hydrophobic pollutants such as pyrene. The fate, behavior, and biological effects of PP, especially as a vector of adsorbed pollutants are poorly understood, with, to our knowledge, only one *in vivo* study on its impacts in mussels. This recently published study used higher PP concentrations (5 and 50 mg L^{-1}) than in our study, assessing different endpoints and only digestive gland as a bioindicator tissue (Daniel et al., 2024). Therefore, we chose concentrations of both pollutants (PP and pyrene) that can be reached in the marine environment and are expected to have genotoxic potential and trigger biomarker responses in marine organisms according to the previous knowledge (Oliveira et al., 2012, 2013; Almeida et al., 2012; Avio et al., 2015). Pyrene levels between 17 and 20 µg L^{-1} can be considered as indicative of unpolluted conditions (Oliveira et al., 2013), whereas levels higher than 40 µg L^{-1} have been used as an indication of PAHs pollution (Dissanayake et al., 2010). In this study, a pyrene concentration of 50 µg L^{-1} was used, which represents plausible and relevant

concentrations in some highly polluted coastal areas. At sites with intensive anthropogenic activities and heavy oil spills, even higher concentrations than 200 µg L^{-1} can be found (Dissanayake et al., 2010). Additionally, pyrene concentration of 50 µg L^{-1} was also previously used in assessing pyrene bioavailability and toxicological risk from polystyrene and polyethylene microplastics in marine mussels (Avio et al., 2015). The concentration of 1 mg L^{-1} PP is considerably higher than currently found in the Mediterranean Sea (Suaria et al., 2016), but is within the range of concentrations observed in highly polluted estuaries (Liong et al., 2021). Moreover, we need to consider also many times overlooked evidence, that field data mostly considered only bigger MP particles, while smaller particles (10-40 µm) are often missed. In this respect, this study aims to represent a realistic scenario from the marine environment and may be indicative of environmentally relevant concentrations. As these smaller, biologically relevant sizes are preferred by filter-feeding organisms, the used microplastic size range can be considered as a relevant range to evaluate long-term exposure to small MPs (Pittura et al., 2018). Used concentration is also lower than those used in previous exposures of mussels to MPs (Avio et al., 2015; Pittura et al., 2018; Kolarević et al., 2023; Daniel et al. 2024, Park et al., 2024). The combination of PP and pyrene exposure in our study enabled us to assess individual detrimental/toxicological impacts and the interactive effects of microplastic-bound PAHs on marine organisms.

3.1. Morphology of microplastic particles and particle size distribution

The milled PP microplastics were in the shape of variable fragments with cracks and sharp edges (Fig. 1A), and demonstrated a broad size distribution of 38.13 ± 18.21 µm (average particle size ± S.D.). The majority of particles in the sieved fraction were in the range of 20 to 50 µm (Fig. 1B), as seen after the number particle size distribution analysis.

These results are very important and relevant from an ecotoxicological point of view. Namely, it is known that smaller microplastic particles have higher bioavailability, as they have a higher surface-to-volume ratio and are more likely to be internalized by cells (Galloway et al., 2017). Further, the use of polydisperse fragments as we obtained by milling (Fig. 1A) represents environmentally relevant microplastics compared to the commercially available spheres (Phuong et al., 2016), which are most commonly used in research.

3.2. General status of mussels

Condition indices (CI), as a general indicator of organismal health and energy status (Lobel et al., 1991), were measured at the end of the experiment after 14 days and did not differ

significantly between the treatments (Table 1). The lowest condition was observed in mussels exposed to PP, but none of the exposures led to significant feeding impairment, or severe energetic imbalance over the 14-day period. Condition index was also assessed by Daniel et al. (2024) who reported on CI decrease after chronic exposure to really high PP concentration of 50 mg L^{-1}, while lower concentration of 5 mg L^{-1} did not affect general condition of mussels. The overall stability of CI indicates the limited sensitivity of this parameter for assessing early sublethal effects. Similar findings have been reported in several studies (Ribeiro et al., 2017; Santana et al., 2018; González-Soto et al.,2019) after exposure of *Scrobicularia plana* for 14 days to 20 μm PS, *Perna perna* for 90 days to 0.1–1 μm PVC particles, and *Mytilus galloprovincialis* for 26 days to PS alone and with adsorbed benzo[a]pyrene (BaP), respectively.

Table 1. Condition indices (CI) for *Mytilus galloprovincialis* after 14-day exposure to pyrene, polypropylene and pyrene adsorbed to polypropylene particles. Results are presented as average ± S.D. (standard deviation).

CONDITION INDEX (mg cm^{-3})	CONTROL	PYRENE	POLYPROPYLENE	PYRENE + POLYPROPYLENE
day 14	11.96±1.78	11.22±1.78	10.58±2.00	11.13±2.32

3.3. Genotoxicity assessment – cell viability and comet assay

The viability of the haemocytes was above 80% in all treatments at 7th and 14th day of sampling (Table 2), confirming the suitability of the samples for the comet assay. Cell viability in the selected bioindicator tissue must be over 70–80% to conduct the comet assay (Tice et al., 2000; Azqueta and Collins, 2013). Although different responses of haemocytes to exposure to MPs and/or PAHs have been observed in the literature, there were no significant differences in cell viability between treatments or between sampling days in the present study (Table 2). The cell viability between treatments or between sampling days in the present study was in range from 90.5±3.70 to 94. 0±3.00 % in control, while in treatments range is 84.2±11.3 to 94.0±4.24 (Table 2). Nevertheless, a slightly lower cell viability was observed in the co-exposure treatment after 14 days, suggesting that PP particles might influence the uptake of pyrene via food and its bioavailability to mussels, possibly causing additional toxicity (Table 2). The higher variability in this treatment likely reflects individual differences in response to simultaneous exposure to pyrene and PP particles, leading to more heterogeneous haemocyte

responses compared to the single-stressor conditions. The high cell viability observed in our results indicates that the biochemical and genotoxic effects described later in the manuscript occurred under sublethal exposure conditions. This suggests that these responses are not caused by general cytotoxicity, cell death or serious organismal damage, but rather reflect early biological disturbances as a response to stress. Therefore, the selected biomarkers demonstrate sensitivity to sublethal stress and are validated as valuable early warning indicators of potential adverse effects.

Table 2. Cell viability (expressed as percentage of live haemocytes) of *Mytilus galloprovincialis* during 14-day exposure to pyrene, polypropylene and pyrene adsorbed to polypropylene particles. Results are presented as average ± S.D. (standard deviation).

CELL VIABILITY (%)	CONTROL	PYRENE	POLYPROPYLENE	PYRENE + POLYPROPYLENE
day 7	90.5±3.70	88.8±4.55	88.0±8.49	94.0±4.24
day 14	94.0±3.00	92.8±3.42	93.4±6.35	84.2±11.3

The results of the comet assay, shown in Fig. 2, demonstrated a significant genotoxic effect following exposure to pyrene and PP. After seven days, a significant increase in DNA damage in haemocytes was observed in mussels exposed to both pollutants compared to the control, with a significantly stronger response to PP than to pyrene (Fig. 2). The genotoxicity of MPs may be due to several mechanisms, including ROS production and oxidative stress due to the surface chemistry of the particles, physical disruption of membranes or cellular processes, or leaching of additives or in association with the polymer matrix (Ribeiro et al., 2017). The damage could be caused by covalent binding to DNA (Jung et al., 2009; Umamaheswari et al., 2021) or by inhibition of DNA synthesis (Hidalgo and Dominguez, 1998; Umamaheswari et al., 2021). In addition, MPs can enter cells via endocytosis or adhesion and cause physical disruption of membranes and organelles, potentially reaching the nucleus and exacerbating genotoxic stress. After 14 days, DNA damage was still significantly increased in the pyrene and PP groups, but the difference between them was no longer significant. Importantly, mussels co-exposed to pyrene and PP showed DNA damage comparable to the control group at both time points. This is consistent with the results of Avio et al. (2015), who exposed mussels to polyethylene, polystyrene and pyrene adsorbed to both types of polymers and found enhanced effects of both types of MPs alone compared to MPs with adsorbed pyrene in terms of DNA

damage. González-Soto et al. (2019) also conducted an exposure to polystyrene microplastics of different sizes alone and with adsorbed B(a)P and found reduced DNA damage after 26 days in mussels exposed to MPs and B(a)P together. According to the authors, such response could be explained by the initiation of additional DNA repair pathways triggered by exposure to B(a)P (Bihari et al., 1990; González-Soto et al., 2019). It is hypothesized that such a mechanism for initiating DNA strand break repair pathways exists in mussels exposed to B(a)P (Bihari et al., 1990), so a similar pathway could be activated during the exposure to pyrene. Further, binding of pyrene to particles can reduce bioavailability of pyrene, limiting its diffusion through gill epithelia, as in case of only waterborne uptake, or endocytosis by immune cells. Alternatively, the presence of pyrene on the plastic surface can also modify its interaction with biological membranes, altering its uptake kinetics or metabolism in mussels (Xie et al., 2017). Mussels are also known to be selective filters and various physicochemical parameters of the particles, including the binding of other chemicals, may influence their particle selection (Zhao et al., 2019; Li et al., 2021), but the specific mechanisms behind this behaviour are still unknown. In this sense, the mussels may also have selectively ingested fewer PP particles with adsorbed pyrene than PP particles alone. These results support the findings that microplastics can act as a vector or reservoir for hydrophobic pollutants, but that such interactions can attenuate or modify toxicity depending on binding strength, desorption potential and particle properties (Bakir et al., 2014). Such potentially antagonistic pattern during co-exposure to pyrene and PP should be interpreted with caution. While the reduction in DNA strand breaks compared to pyrene alone may suggest a mitigating effect of PP, several alternative explanations described above are possible. However, because pyrene bioaccumulation, feeding behavior and DNA repair activity were not directly measured in this study, these interpretations remain hypothetical and should be verified in future research. Acknowledging these uncertainties, our findings indicate that co-exposure to PP and pyrene have lower genotoxicity compared to the single exposures, emphasizing the importance of studying combined pollutant interactions.

After 14 days, a significant increase in DNA damage compared to day 7 was observed only in the pyrene treatment, while the extent of DNA damage in mussels from other treatments remained similar (Fig. 2). This suggests that pyrene treatment may additionally cause a delayed genotoxic effect, with the highest DNA damage occurring after long-term exposure, when the initially activated DNA repair mechanisms may be too severely impaired (Bihari et al., 1990). On the other hand, DNA damage remained at the same level after PP exposure, as mussels may have a reduced filtration rate as part of the adaptive response and upregulate base excision repair, extending the damage until day 14.

3.4. Biochemical markers

3.4.1. Acetylcholinesterase activity

In our study, the average AChE activity after seven days of exposure ranged from 12.07 to 13.46 nmol min^{-1} mg^{-1} proteins and after 14 days from 10.29 to 14.19 nmol min^{-1} mg^{-1} proteins with the lowest activity in mussels exposed to PP and the highest in the control group. Nevertheless, no significant changes in AChE activity in gills were observed between the different groups at any sampling time (Fig. 3). We did not detect neurotoxicity effects in the experimental groups within a 14-day period. In mussels exposed to pyrene and PP, a non-significant decrease in AChE activity over time was observed, with lower activity after 14 days than after seven days (Fig. 3). Similarly, AChE activity was not affected in the haemocytes of mussels exposed to polyethylene, polystyrene and pyrene adsorbed to both types of polymers (1.5 g L^{-1}), but was significantly reduced in the gills after treatment with both polymers (Avio et al., 2015), as such high concentration of MP can damage the gill's function and cause inhibition of AChE activity. Oliveira et al. (2013) described a decrease in AChE activity in juvenile fish common goby *Pomatoschistus microps* induced by MPs and pyrene as single substances or their combination. Although the activities in these tested fish groups were lower than in the control group, there were no differences between the individual pollutants and their combination, as in our study (Fig. 3). There are many factors that influence the potential neurotoxicity of MP particles, including exposure level, exposure duration, particle type, shape or size (Prüst et al., 2020). Therefore, any effect of MPs on AChE activity should be taken with caution, as this stressor is widespread and this enzyme plays a crucial role in many physiological and behavioural processes (Oliveira et al., 2012). Although no significant neurotoxic effect was detected under the tested conditions, this does not necessarily imply the general absence of neurophysiological disturbance in relation to PP or pyrene exposure. AChE activity is often inhibited only at higher pollutant concentrations or after longer exposure periods, and its response can also depend on tissue type and enzymatic turnover rate. Therefore, subtle neurophysiological alterations cannot be excluded and should be explored under chronic or environmentally variable exposures.

3.4.2. Biomarkers of oxidative stress – malondialdehyde, catalase, glutathione *S*-transferase

Oxidative stress was assessed by the lipid peroxidation levels (MDA) and the activities of two major antioxidant enzymes, catalase (CAT) and glutathione *S*-transferase (GST), in the digestive glands and gills. MDA levels in both tissues did not differ significantly between

583 treatments or between sampling days (Fig. 4a), suggesting that no measurable lipid damage
584 occurred under the exposure conditions tested or that the antioxidant system was effective in
585 preventing oxidative damage. Similar trend was observed by Lan et al. (2025) investigating
586 impact of phthalates (PAEs) and microplastics on mussels. No significant change in MDA
587 levels was observed, indicating that under environmental concentrations, the coordinated
588 response between antioxidant enzymes effectively neutralizes reactive oxygen species, thereby
589 preventing lipid peroxidation of cells. Average MDA levels after seven days in our research
590 ranged from 18.6 to 31.7 nmol g^{-1} w.w. in digestive glands, and from 39.5 to 62.5 nmol g^{-1} w.w.
591 in gills, depending on treatment. In the digestive glands, average values of 13.4 to 29.8 nmol g^{-1}
592 w.w. and in the gills of 32.1 to 41.6 nmol g^{-1} w.w. were observed after 14 days, but large
593 individual variations were observed within and between all groups (Fig. 4a). The high inter-
594 individual variability, as also already reported for the caged mussels (Turja et al., 2014),
595 probably contributed to the lack of statistically significant differences. MDA values in our study
596 remained within the ranges previously reported for this species (Turja et al., 2014; Paul-Pont et
597 al., 2016). The absence of significant lipid peroxidation may also reflect either effective
598 antioxidant protection or the limited temporal sensitivity of MDA, as lipid peroxidation
599 products can be rapidly metabolized or redistributed within tissues.
600 Although the MDA values were stable, the responses of the antioxidant enzymes
601 indicated a mobilization of the defence mechanisms. Looking at the CAT activity in the
602 digestive glands, the average values varied between 14.7 and 19.4 µmol min^{-1} mg^{-1} proteins
603 after seven days and 13.0 to 21.9 µmol min^{-1} mg^{-1} proteins after 14 days. CAT activity increased
604 significantly in the digestive glands of mussels exposed to pyrene and pyrene adsorbed to PP
605 compared to controls at both time points (Fig. 4b). This indicates an increased production of
606 reactive oxygen species (ROS) triggered by pyrene metabolism, probably via cytochrome
607 P450-mediated pathways, necessitating increased degradation of hydrogen peroxide by CAT
608 activation (Livingstone, 2001). CAT activity was the highest in the pyrene-only group after 14
609 days (Fig. 4b) and increased significantly over time due to persistent pollution with pyrene
610 causing oxidative stress. An increase of CAT activity in digestive glands of mussels was also
611 observed by Daniel et al. (2024) after 28-day exposure to 50 mg L^{-1} PP, as well as by Chen et
612 al. (2024) following PS exposure. The same trend could be seen in exposure of mussels to PVC
613 for 14 days (Chen et al., 2025), supporting the consistency of enzymatic antioxidant responses
614 across different polymer types. In addition, CAT activity in the digestive glands of mussels
615 exposed to pyrene in our research was higher after 14 days than in those exposed to PP (Fig.
616 4b) due to the biotransformation of pyrene. Co-exposure to both pollutants also caused

significantly higher CAT activity in digestive glands in our study than in the control (Fig. 4b), but different responses to simultaneous exposure to MPs and PAHs have been observed in the literature. A non-significant decrease in CAT activity was observed in the digestive glands of *M. galloprovincialis* after seven days of simultaneous exposure to MPs and pyrene (Avio et al., 2015). On the opposite, the response to co-exposure to LDPE and B(a)P resulted in higher CAT activity in the digestive glands after 14 and 28 days of exposure (Pittura et al., 2018). In the gills, CAT activity also responded strongly to pyrene exposure, but with slightly different dynamics and patterns than in the digestive glands (Fig. 4b). According to Lan et al. (2025), mussels might primarily rely on CAT to mitigate oxidative stress under the stressful conditions. In the gills, CAT activity ranged from 15.5 to 20.2 µmol min^{-1} mg^{-1} proteins after seven days and from 14.7 to 20.5 µmol min^{-1} mg^{-1} proteins after 14 days, depending on exposure. After seven days, pyrene-exposed mussels showed an increased CAT activity compared to the controls, while after 14 days the highest CAT activity was measured in the pyrene+PP group, which was significantly higher than that of the PP-only group (Fig. 4b). This pattern may represent an interactive (possibly cumulative or additive) oxidative stress response, reflecting the combined metabolic cost of pyrene detoxification and particle-induced ROS production rather than a strictly additive effect. During the exposure, a significant increase in CAT activity was observed in the gills of the control mussels and the mussels exposed to pyrene and PP (Fig. 4b). In the present study, CAT activity of the mussels was found to be in similar ranges in both investigated tissues in all treatment groups (Fig. 4b). This result is consistent with the role of CAT as a constitutive antioxidant enzyme expressed in many tissues to counteract oxidative stress. Activity of CAT in both tissues is consistent with studies on congener species *M. trossolus* (Turja et al., 2014) and *M. galloprovincialis* (Ferreira et al., 2023). Nevertheless, some tissue-specific responses related to the different patterns indicate differences in their roles and sensitivities. The gills are the first organ in contact with the environment and a site of intense oxygen exchange. They are constantly exposed to reactive oxygen species (ROS) produced during respiration and by pollutants in the water. The digestive gland, the main organ for xenobiotic metabolism, produces ROS as a result of enzymatic detoxification processes. The lack of significant changes in lipid peroxidation (MDA) despite the CAT responses may indicate that antioxidant defence was largely effective against oxidative damage under current exposure conditions and durations (Fig. 4b).

GST activity showed more tissue-specific responses and remained largely unchanged in the digestive glands throughout the experiment (Fig. 4c). Precisely, the average GST activity after seven days was between 101 and 124 nmol min^{-1} mg^{-1} proteins and from 104 to 120 nmol

651 min^{-1} mg^{-1} proteins, depending on the day and treatment. A similar response without significant
652 differences has already been observed in the digestive glands of mussels exposed to PP (Daniel
653 et al. 2024), PS and PE alone and in combination with pyrene (Avio et al., 2015). The same
654 patterns were observed in evaluating the effects of chrysene-adsorbed PS (Capolupo et al.,
655 2021). Similar values of GST activity were also observed in all these studies (Fig. 4c, Avio et
656 al., 2015; Capolupo et al., 2021). Avio et al. (2015) and Daniel et al. (2024) attributed those
657 lack of alterations to MPs (or the compounds in their matrix) not being conjugated via GSTs.
658 In contrast, significant differences in our research were observed in gills where the average
659 values of GST activity are between 187 and 246 nmol min^{-1} mg^{-1} proteins after seven days and
660 between 184 and 240 nmol min^{-1} mg^{-1} proteins after 14 days. GST activity in the gills was
661 significantly reduced by PP exposure after seven days, suggesting a possible suppression or
662 inhibition of GST-related detoxification processes by microplastics (Fig. 4c). There were no
663 significant differences in GST activity in the gills after 14 days, although lower activity was
664 observed in mussels exposed to pyrene and those exposed to polypropylene (Fig. 4c). Notably,
665 a significant decrease in GST activity over time was observed in pyrene-exposed mussels (Fig.
666 4c), indicating a time-dependent down-regulation of this enzyme in the gills, possibly due to
667 the prolonged stress or enzyme depletion. Consistently observed higher GST activity in gills
668 compared to digestive glands, as observed in our study (Fig. 4c) supports their role as a frontline
669 detoxification site for waterborne pollutants (Power and Sheehan, 1996; Capolupo et al., 2021;
670 Kolarević et al., 2023).

671 These results suggest that pyrene and, to a lesser extent, PP triggered significant
672 enzymatic antioxidant responses in relation to environmental stress, although no measurable
673 lipid damage occurred. The pattern of enzyme activation without concomitant lipid
674 peroxidation indicates a functionally effective antioxidant system under these exposure
675 conditions. However, the tissue-specific and compound-specific nature of these responses
676 highlights the importance of using multiple biomarkers and target tissues to fully capture the
677 complexity of oxidative stress in response to various pollutants. It should also be noted that
678 oxidative stress was assessed only at two exposure time points; therefore, transient or early
679 oxidative imbalances could have been missed. Future studies with higher temporal resolution
680 are needed to clarify the kinetics of redox responses in mussels.

681 3.4.3. Electron transport system activity

682 Electron transport system (ETS) activity is a widely recognized biomarker of the
683 maximum potential rate of mitochondrial respiration and overall metabolic capacity in aquatic

invertebrates, including mussels (Verslycke and Janssen, 2002; Erk et al., 2012). ETS activity was measured only in the digestive glands, a tissue previously suggested to be most suitable for assessment of energetic status in mussels (Erk et al., 2011). ETS was measured by using the reduction of INT in the presence of NADH and NADPH as substrates, estimating the maximum enzymatic capacity for electron transfer in mitochondria under substrate-saturated conditions, reflecting the organism's ability to generate ATP to meet all metabolic demands (Verslycke and Janssen, 2002).

Average ETS activity in our study ranged from 19.1 to 23.9 mJ mg^{-1} w.w. h^{-1}, depending on the treatment and sampling point (Fig. 5). Such values were in similar ranges as for *M. galloprovincialis*, and *M. trossulus* obtained by Erk et al. (2011) and Turja et al. (2014), respectively. In this study, neither pyrene nor PP particles caused significant changes in ETS activity after seven days of exposure. A significant induction of ETS activity was observed with both pyrene-containing treatments compared to controls after 14 days, while PP alone did not cause a significant effect (Fig. 5). The observed increase in ETS activity after prolonged pyrene exposure indicates a delayed upregulation of mitochondrial metabolism, likely reflecting increased energy requirements for biotransformation, cellular repair and antioxidant defence (Erk et al., 2011). Pyrene is metabolized via phase I and II detoxification pathways, processes that are energetically costly and generate reactive oxygen species (ROS) that require further ATP investment in antioxidant responses (Livingstone, 1991). Increased ETS activity can therefore be interpreted as a sign of increased metabolic demand and stress, with potential consequences for energy allocation at the organismal level (Sokolova et al., 2012). Since ETS is not often used as a biomarker in the assessment of MP exposure, we could only compare our data with those of Shang et al. (2021), in which high MP concentrations (10^6 particles L^{-1}) induced ETS activity in the mussel *M. coruscus*, while exposure to low MP concentrations had no effect after 14 days. This suggests that PP alone, at the more moderate concentrations we used, may not be a sufficient metabolic stress requiring upregulation of the mitochondrial capacity. At lower, more ecologically realistic concentrations, mussels can compensate for exposure to MPs without major shifts in energy metabolism. Combined exposure to pyrene and PP resulted in ETS activity comparable to that of pyrene alone. This suggests that the presence of PP does not further attenuate the metabolic response to pyrene, possibly due to the high adsorption affinity of pyrene to PP, which may reduce the bioavailability of free pyrene but does not preclude the need for energy-intensive detoxification processes.

3.5. Physiological endpoints

718 Physiological endpoints such as respiration rate and heart rate are widely recognized as
719 sensitive and integrative indicators of mussel health and stress. Respiration rate reflects the
720 metabolic activity and oxygen uptake capacity of the organism and provides insight into the
721 energy distribution and overall metabolic effects of environmental stressors (Simčič and Sket,
722 2019). Changes in respiration can signal disturbances in gas exchange, shifts in metabolic
723 priorities or compensatory responses to toxins and physical challenges, making it an important
724 parameter in ecotoxicology studies. Heart rate provides a real-time insight into the
725 cardiovascular and neurophysiological status of mussels (Kholodkevich et al., 2017). It is
726 highly responsive to acute and chronic environmental changes including hypoxia, temperature,
727 salinity and contaminant exposure, and can reveal sublethal stress effects before overt signs of
728 toxicity or mortality occur (Braby and Somero, 2006). Heart rate recovery time from stress also
729 reflects the animal's physiological resilience and ability to maintain homeostasis (Martinović
730 et al., 2022).
731 Taken together, these endpoints provide a holistic assessment of organismal function and
732 ecological fitness by linking cellular and biochemical responses to whole animal responses.
733 Their measurement is crucial for understanding the ecological relevance of pollutant effects,
734 such as those caused by MPs and PAHs, and for assessing the potential impact on population
735 dynamics and ecosystem services of mussels. As far as we know, these are the first ever data
736 reported for M. *galloprovincialis* for both these parameters under the exposure to either PP,
737 pyrene or their combination.

738 3.5.1. Respiration rate

739 Exposure to pyrene and PP did not significantly affect the respiration rate of mussels after
740 seven days. However, after 14 days, significantly higher respiration rates were observed in the
741 control mussels and the mussels exposed only to pyrene compared to the mussels exposed only
742 to PP (Fig. 6). In mussels exposed to PP, the respiration rate was significantly reduced on day
743 14, indicating a cumulative effect of this stressor. This decrease may result from valve closure
744 or reduced filtration activity caused by particle accumulation, as suggested in previous studies
745 (Riisgård et al., 2003), although additional physiological factors such as reduced feeding
746 efficiency or mucus secretion could also contribute. Our results are consistent with a study by
747 Rist et al. (2016) who reported a decline in *Perna viridis* oxygen respiration rate and clearance
748 rate in response to microplastics following a 44 - day exposure. Same authors found no
749 additional effect of exposure to adsorbed fluoranthene as compared to PVC alone, similar to
750 our results where co-exposure to PP and pyrene did not further reduce respiration rates

compared to PP alone (Fig. 6). In another study, González-Soto et al. (2019) found no effect of PS, either alone or with adsorbed B(a)P, on the respiration of mussels, aligning with the unchanged rates we observed in pyrene treatments. Further, Yap et al. (2020), as well as Pedersen et al. (2020) did not observe any significant effect of MPs exposure on the respiration rate of *M. galloprovincialis* and *Dreissena bugensis*, respectively. In oysters *Ostrea edulis* the respiration rate was not significantly affected by exposures to different polymers of microplastics for 60 days, despite some variability among different microplastics treatments (Green, 2016). Furthermore, Camus et al. (2002) found no significant effect of PAH exposure on respiration in the Arctic spider crab (*Hyas araneus*), supporting our observation that pyrene alone did not significantly suppress respiration in mussels (Fig. 6). The slight, but not significant, increase in respiration rate observed in pyrene-exposed mussels compared to control group after 14 days may reflect a compensatory metabolic response aimed at maintaining homeostasis, consistent with enhanced ETS activity (Figs. 5, 6) observed under the same treatment (Van Cauwenberghe et al., 2015). The absence of strong respiration changes across treatments may therefore indicate physiological compensation, although longer exposures or fluctuating environmental conditions could yield more pronounced effects.

3.5.2. Heart rate

Changes in cardiac activity can be considered as an indication of the physiological fitness of organisms (Vereycken and Aldridge, 2023). So far, only two studies (Shen and Nugegoda, 2022; Kolarević et al., 2023) have directly investigated the effects of MPs on HR of *M. galloprovincialis* and no data are available on the impact on cardiac activity of other bivalve species in response to MPs. In both studies, no significant changes in HR patterns were observed under MPs exposure. Accordingly, in this study we modified the experimental approach to investigate the adaptive capacity of mussels after stress, induced by the short-term hyposalinity test in clean seawater after 14 days of exposure to PP, pyrene and pyrene adsorbed to PP. A tendency for the slower recovery of individual heart rates to their baseline values is considered an indicator of reduced physiological resilience and impaired homeostatic capacity following environmental stress (Kholodkevich et al., 2017). The group of mussels previously exposed to pyrene adsorbed to PP showed significantly longer T_{rec} than those previously exposed only to PP (Fig. 7). The reason could be a lower adaptive capacity or ability of mussels to withstand environmental stress, leading to impaired physiological/health condition. The group of mussels previously exposed to pyrene alone showed a trend towards longer T_{rec} as well, although it was not statistically significant (Fig. 7). These results suggest that previous

784 treatment with pyrene, either alone or in combination with PP, had more prominent effect on
785 T_{rec} after stress than PP alone. Overall, the average T_{rec} in our study was between 41 and 84
786 minutes, depending on the treatment (Fig. 7). According to Kholodkevich et al. (2017), fast
787 recovery times of up to 60 minutes indicate good health, pristine conditions and/or good
788 adaptive potential of the mussels. In our experiment, mean T_{rec} below or close to this threshold
789 were observed in all groups, except for the group previously exposed to PP with adsorbed
790 pyrene where the mean T_{rec} exceeded 80 minutes. Similar HR recovery times have been
791 reported in several marine and freshwater mussels at the reference or clean sites (Turja et al.,
792 2014; Kholodkevich et al., 2017; Nikolić et al., 2019; Martinović et al., 2022), further
793 confirming our results. Obtained results in our study point toward an interactive effect of pyrene
794 and PP mixture on the cardiac regulation of *M. galloprovincialis* (Fig. 7). Still, the possible
795 "additive effect" observed in heart rate should be interpreted with caution, as it may represent
796 an interactive physiological response rather than a strictly additive toxic effect. Such changes
797 could reflect compensatory metabolic adjustments or stress adaptation mechanisms in mussels
798 rather than direct synergistic toxicity of pyrene and PP. Uncertainty is further supported by the
799 lack of significant differences compared to the control group, but longer T_{rec} within the control
800 group is probably attributed to mussel spawning induced by the addition of distilled water in
801 tank, which was observed only in the control mussels in the repeated experiment as well. As
802 this was the first application of the above-mentioned experimental approach with MPs, the
803 results obtained are indicative of further research into the effects of MPs on the overall health
804 of the mussels based on HR recordings.
805 Interestingly, response of HR is largely in contrast to the respiration rate, where a
806 significant reduction was observed only in the PP-exposed group after 14 days. This indicates
807 that respiration and HR respond differently to chemical and particle-induced stressors: PP may
808 impair gas exchange by physically disrupting it (e.g. by closing valves or irritating the gills),
809 whereas pyrene appears to disrupt cardiac regulation more strongly, probably through
810 circulatory effects. The apparent differences in these responses highlight the importance of
811 measuring multiple physiological functions, as reliance on a single parameter may
812 underestimate the broader physiological effects of complex mixtures of pollutants.
813
814 Taken together, the biochemical, genotoxic, and physiological responses observed in this
815 study reveal that *M. galloprovincialis* employs multiple, interconnected defence and
816 compensation mechanisms when exposed to microplastics and pyrene. The activation of
817 antioxidant enzymes and the presence of DNA strand breaks suggest that oxidative stress plays

a central role in mediating these effects, while the relative stability of MDA and AChE responses points to an efficient short-term regulation of redox balance and neural activity. The physiological parameters further support the interpretation of adaptive responses, reflecting both the energy costs and the resilience of mussels under combined chemical and particulate stress.

Overall, the integrated results emphasize the importance of combining genotoxic, biochemical, and physiological biomarkers to obtain a comprehensive picture of organismal stress and to better understand the complexity of interactions between microplastics and hydrophobic organic contaminants.

4. Conclusions

This study provides one of the first comprehensive evaluations of the sublethal effects of polypropylene (PP) and pyrene, in co-exposure experiments and individually on *M. galloprovincialis* with assessment of bichemical biomarkers and physiological response under environmentally relevant concentrations of PP and pyrene. To our knowledge, this is the first study to assess the combined effects of PP particles and adsorbed pyrene, thereby providing novel data on the wide set of biological responses to this environmentally present pollutant mixture.

Results demonstrate that environmentally relevant concentrations of selected pollutants can significantly impact multiple biological endpoints. Independent exposure to PP or pyrene led to distinct toxic effects, with PP primarily affecting respiration and gill-related functions, and pyrene inducing genotoxicity, antioxidative defence, and metabolic disturbance. Co-exposure revealed a clear alteration in effect, particularly a reduction in pyrene-induced DNA damage when adsorbed to PP, suggesting that microplastics can modulate the bioavailability and toxicity of co-contaminants. Taken together, the results underscore the importance of assessing multiple levels of biological organization to understand pollutant impacts. Among the tested biomarkers, DNA damage and CAT activity appeared to be more sensitive and responsive indicators, likely reflecting early molecular defense and genotoxic responses to microplastic and pyrene exposure. DNA strand breaks can occur rapidly as a result of reactive oxygen species or reactive PAH metabolites, while CAT activity promptly adjusts to fluctuations in hydrogen peroxide levels. In contrast, MDA accumulation represents a more cumulative outcome of lipid peroxidation, and AChE inhibition generally manifests only under higher or prolonged toxicant exposure, explaining their comparatively lower sensitivity under

the experimental conditions. This highlights the importance of selecting battery of biomarkers based on the mode of action and expected toxicity pathways. Most importantly, the discrepancy between the effects of single and combined exposures reflects the complexity of responses to environmental mixtures. Co-exposure effects are not merely additive, but appeared interactive or modulating, as reflected in the altered genotoxic or physiological responses. While this study provides valuable first evidence of PP–pyrene interactions in marine mussels, the proposed mechanisms remain hypothetical given the absence of direct measurements of pyrene bioaccumulation, adsorption–desorption dynamics, or DNA repair activity. Given the scarcity of data on PP–pyrene interactions, this study provides the first evidence of their biological effects in marine organisms. Future research should investigate longer-term exposures, different particle sizes, shapes and concentrations as well as aging conditions and ecological relevance in different species.

In conclusion, this integrative assessment emphasizes that MPs are not only passive vectors of pollutants but can actively influence the toxicity of chemical mixtures. The findings contribute to the growing evidence that the risk assessment of MPs must consider not only their intrinsic properties but also their ability to adsorb and modulate biological effects together with other common environmental pollutants.

5. References

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Author contributions

Tatjana Mijošek Pavin: Conceptualization; Methodology; Validation; Formal analysis; Investigation; Writing – original draft; Writing – review and editing, Visualization; Project administration. **Margareta Kračun Kolarević**: Methodology; Validation; Investigation; Writing – review and editing. **Stoimir Kolarević**: Methodology; Validation; Investigation; Writing – review and editing. **Tatjana Simčič**: Methodology; Validation; Investigation; Writing – review and editing. **Rajko Martinović**: Methodology; Validation; Investigation; Writing – review and editing. **Oliver Bajt**: Investigation; Writing – review and editing. **Gabriela Kalčíková**: Investigation; Writing – review and editing. **Andreja Ramšak**: Conceptualization; Methodology; Validation; Investigation; Resources; Writing – review and editing; Supervision; Project administration; Funding acquisition.

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Data availability

The data from this study have not yet been made publicly available. Data can be obtained from the corresponding authors upon request.

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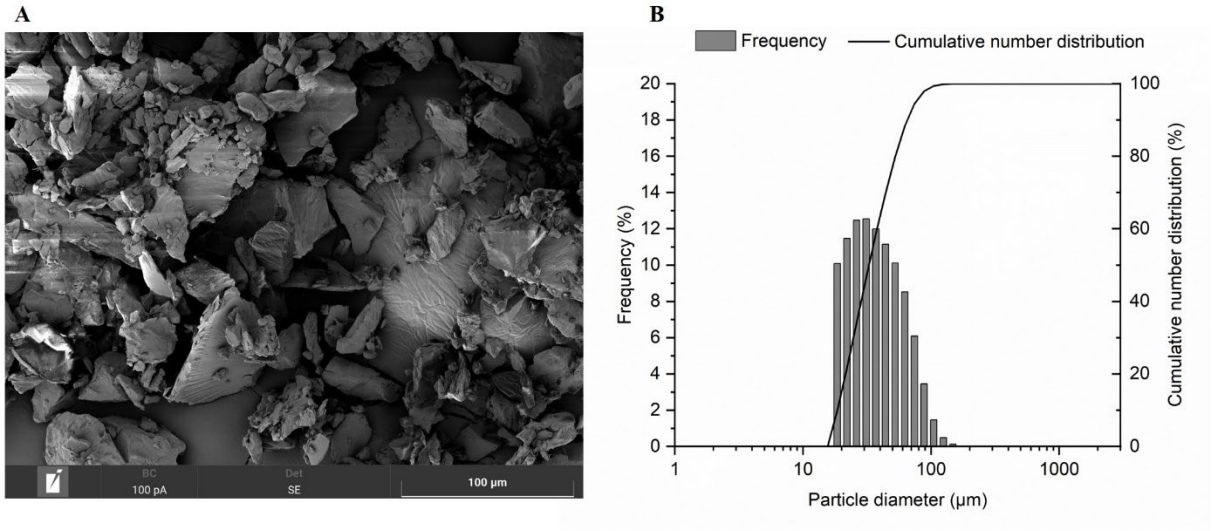


Figure 1. A Morphology of milled polypropylene particles; B Number particle size distribution of the polypropylene microplastics.

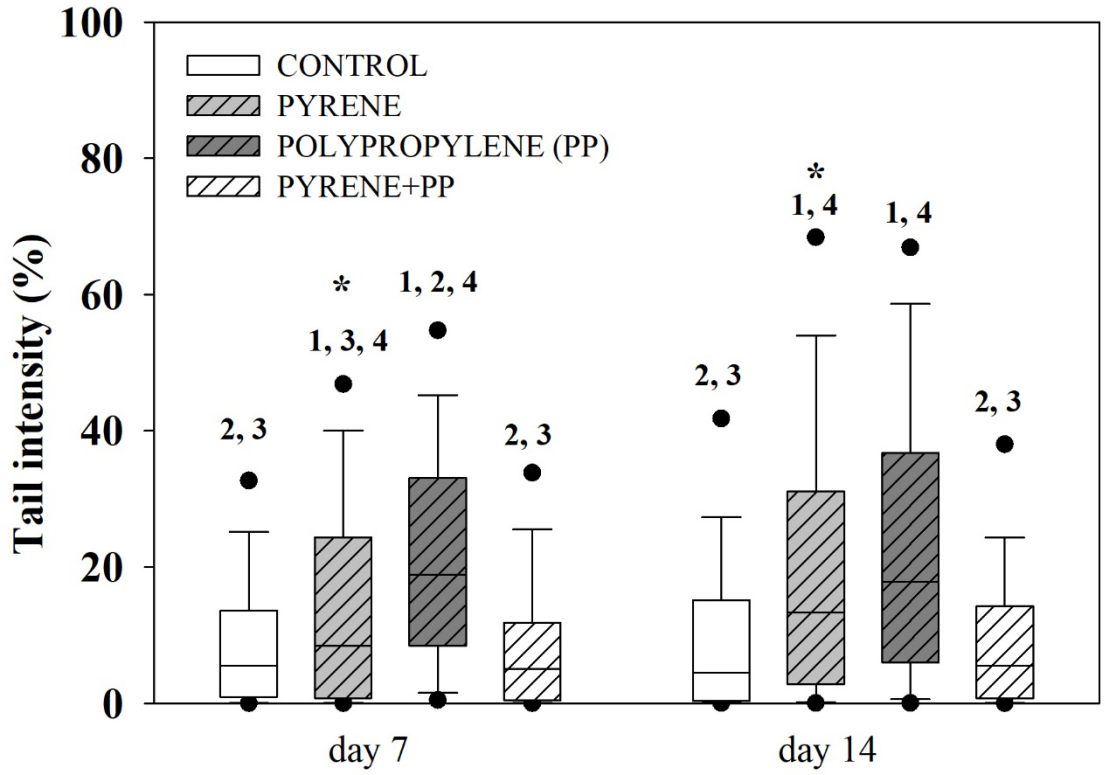


Figure 2. The effects of pyrene, polypropylene particles, and pyrene adsorbed to polypropylene particles on the level of DNA damage in haemocytes of *Mytilus galloprovincialis* during 14 days of exposure. Statistically significant differences between treatments are indicated with

numbers in the graphs: significant difference from treatment indicated by a specific number: 1 - control; 2 – pyrene; 3 – PP; 4 – pyrene + PP. The difference between two sampling points for each treatment are indicated with asterisk *. Significance level was always set at $p < 0.05$.

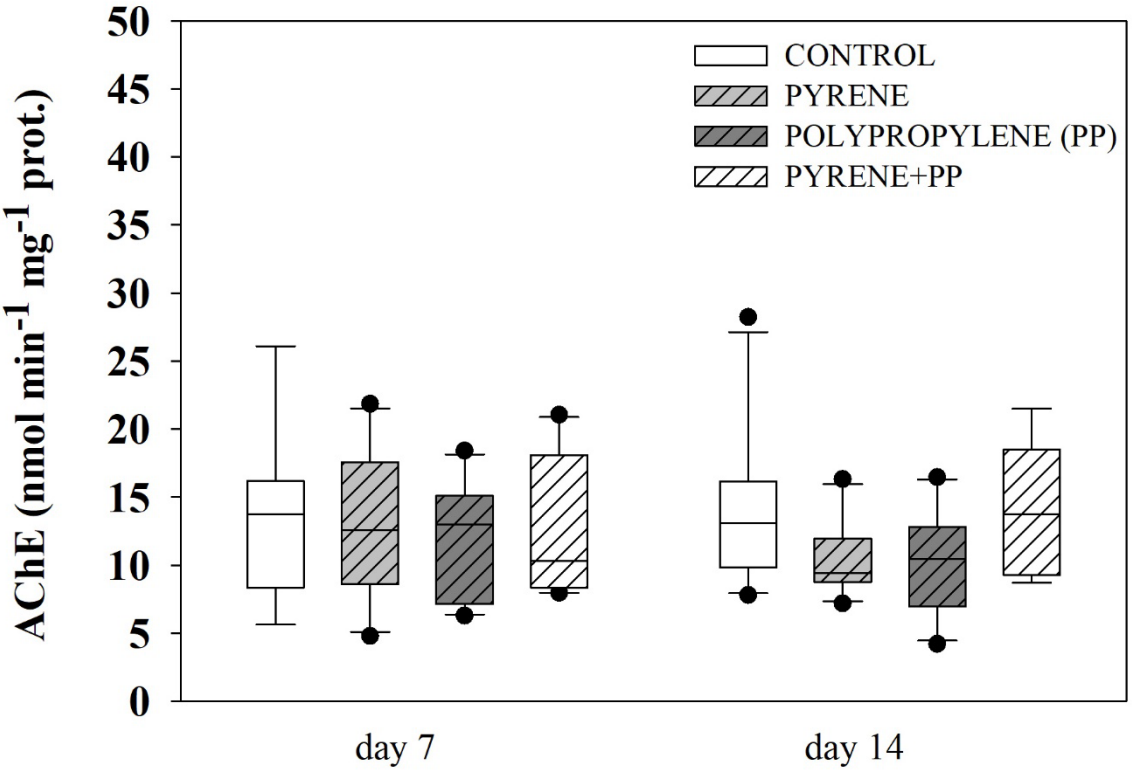


Figure 3. The effects of pyrene, polypropylene particles, and pyrene adsorbed to polypropylene particles on the AChE activity in gills of *Mytilus galloprovincialis* during 14 days of exposure.

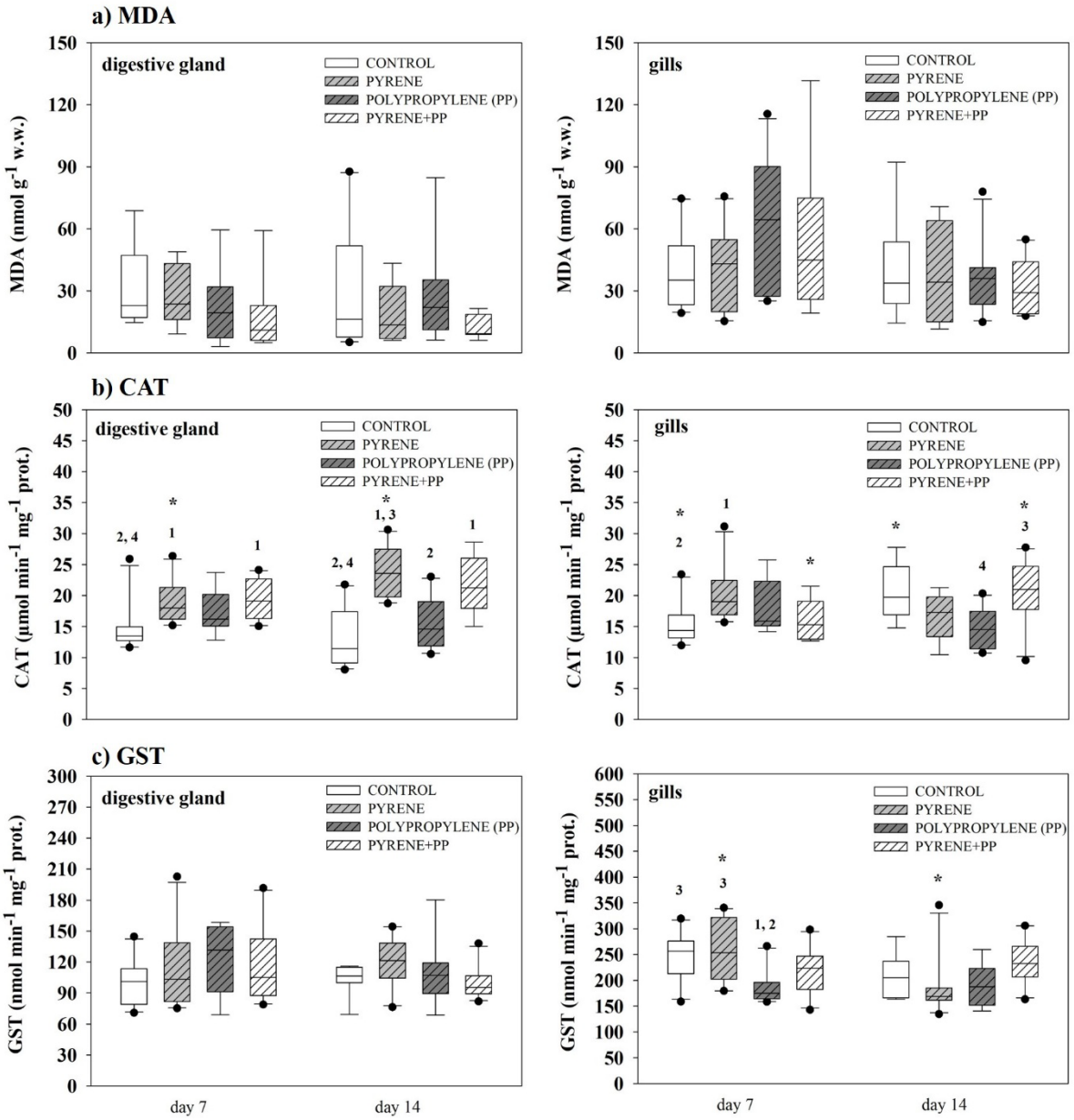


Figure 4. The effects of pyrene, polypropylene particles, and pyrene adsorbed to polypropylene particles on the biomarkers of oxidative stress in digestive glands and gills of *Mytilus galloprovincialis* during 14 days of exposure: a) MDA; b) CAT activity; c) GST activity. Statistically significant differences between treatments are indicated with numbers in the graphs: significant difference from treatment indicated by a specific number: 1 - control; 2 – pyrene; 3 – PP; 4 – pyrene + PP. The difference between two sampling points for each treatment are indicated with asterisk *. Significance level was always set at $p < 0.05$. Note: Y-axis scales for GST activity differ between digestive gland and gills to reflect tissue-specific activity ranges; values are therefore not directly comparable between tissues.

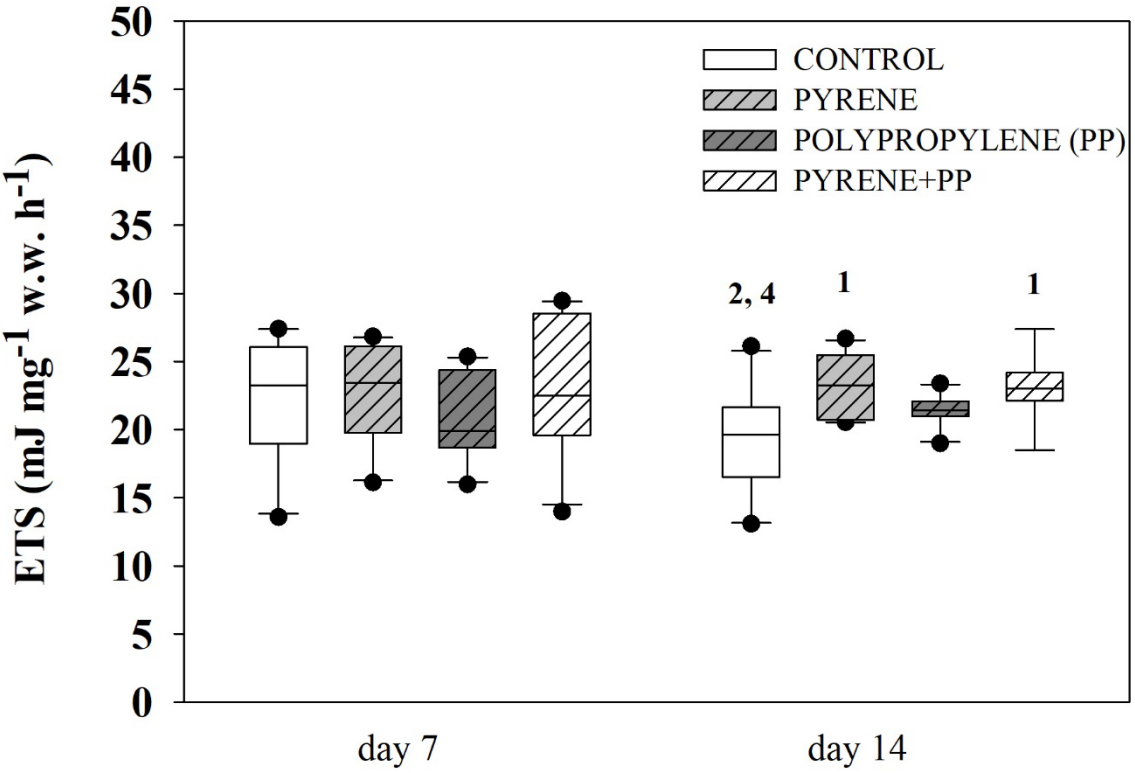
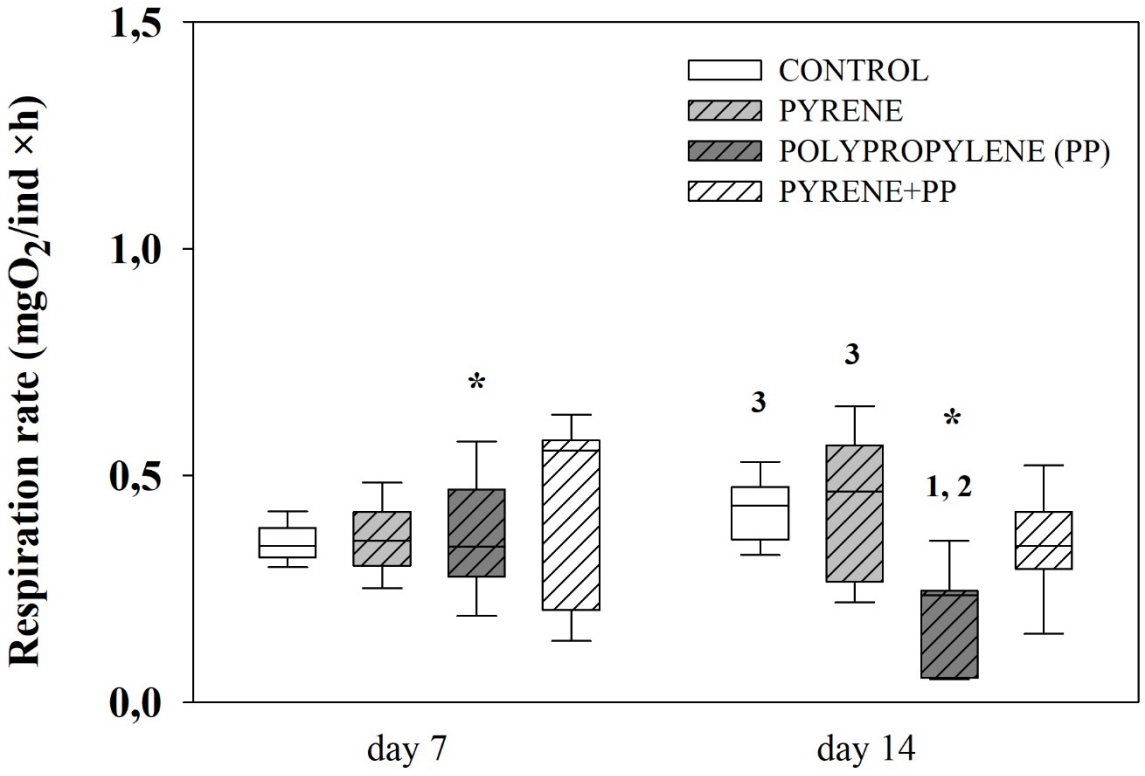


Figure 5. The effects of pyrene, polypropylene particles, and pyrene adsorbed to polypropylene particles on ETS activity in digestive glands of *Mytilus galloprovincialis* during 14 days of exposure. Statistically significant differences between treatments are indicated with numbers in the graphs: significant difference from treatment indicated by a specific number: 1 - control; 2 – pyrene; 3 – PP; 4 – pyrene + PP. The difference between two sampling points for each treatment are indicated with asterisk *. Significance level was always set at $p < 0.05$.



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1311 **Figure 6**. The effects of pyrene, polypropylene particles, and pyrene adsorbed to polypropylene
1312 particles on the respiration rate of *Mytilus galloprovincialis* during 14 days of exposure.
1313 Statistically significant differences between treatments are indicated with numbers in the
1314 graphs: significant difference from treatment indicated by a specific number: 1 - control; 2 –
1315 pyrene; 3 – PP; 4 – pyrene + PP. The difference between two sampling points for each treatment
1316 are indicated with asterisk *. Significance level was always set at $p < 0.05$.

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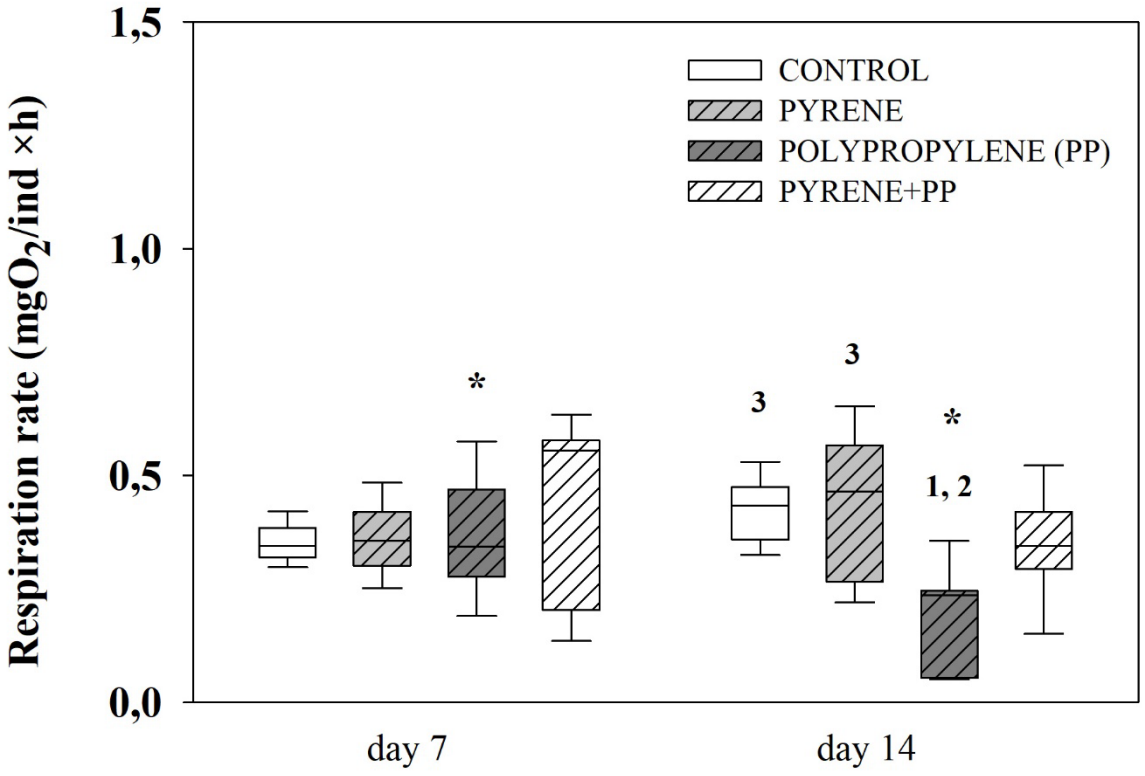
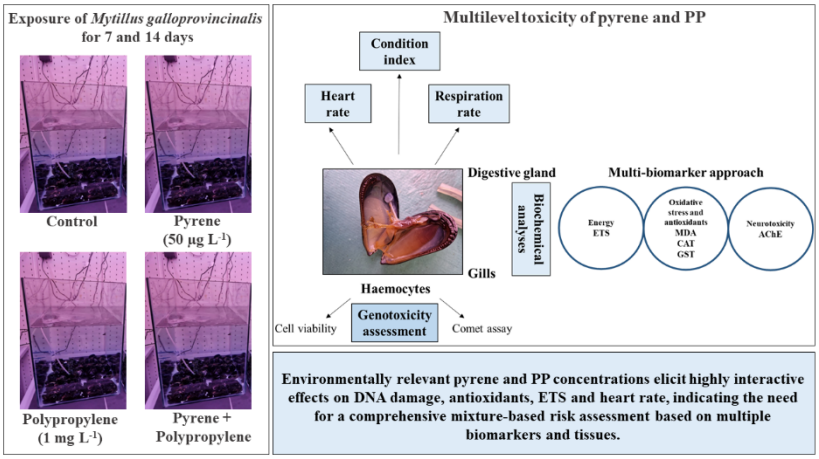


Figure 7. The effects of pyrene, polypropylene particles, and pyrene adsorbed to polypropylene particles on the heart rate of *Mytilus galloprovincialis* during 14 days of exposure. Statistically significant differences between treatments are indicated with numbers in the graphs: significant difference from treatment indicated by a specific number: 1 - control; 2 – pyrene; 3 – PP; 4 – pyrene + PP. The difference between two sampling points for each treatment are indicated with asterisk *. Significance level was always set at $p < 0.05$.



Graphical abstract