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The combined effects of polyethylene microplastics and benzoanthracene on Manila clam *Ruditapes philippinarum*

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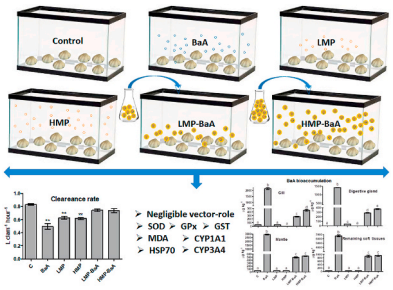
HIGHLIGHTS

- The feeding rate of clams substantially declined by polyethylene MP and Benzo (a)anthracene exposure.
- The exposure of polyethylene MP and BaA influenced SOD, MDA and GPx activities bot not GST.
- The regulation of genes *HSP70*, *CYP1A1* and *CYP3A4* were affected by polyethylene MP and BaA exposure.
- BaA efficiently accumulated in clam tissues.
- Polyethylene MP has a negligible vector role in transporting BaA to clams.

GRAPHICAL ABSTRACT



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ABSTRACT

Microplastic (MP) toxicity has recently been explored in various marine species. Along with the toxicity of plastics polymer itself, additional substances or pollutants that are absorbed onto it may also be harmful. In the present study, we investigated the combined impacts of polyethylene microplastics (PE MPs) and an organic pollutant (Benzo(a)anthracene, BaA) on Manila clam *Ruditapes philippinarum* during a one-week exposure. Two PE MPs concentrations (26 μg L^{-1} and 260 μg L^{-1}) and one BaA concentration (3 μg L^{-1}) were tested. The clams were exposed to BaA and PE MPs either alone or in combination. BaA and PE MPs were incubated before the combined exposure. The biological effects of PE MPs and BaA on the clams were evaluated by considering several assays such as feeding rate, anti-oxidant enzyme activities, and the expression levels of stress-related genes. The feeding rate significantly decreased in individual PE MPs and individual BaA groups while it remained unchanged in combined groups. Superoxide dismutase (SOD) was the most affected among the biochemical parameters. Malondialdehyde (MDA), and glutathione peroxidase (GPx) activities were slightly affected, whereas no changes were observed in glutathione s-transferase (GST) activities. *CYP1A1, CYP3A4,* and *HSP70* gene expressions displayed slightly significant changes. Considering all stressor groups, high PE MPs exposure (260 μg L^{-1} PE MPs) more effectively altered the biological parameters in the clams compared to individual low PE MPs

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and BaA exposure, and their combination. The results also indicated the negligible vector role of PE MPs to transport BaA into the clam tissues.

1. Introduction

Plastics are extensively used in cosmetic, textile, agriculture, electronics, pharmaceutical and automotive industries. The widespread use of plastic is due to its low price and properties such as the ease of fabrication, lightness, waterproofing, and durability (Prokic et al., 2019). Approximately 80% of marine litter is plastic materials (Da Costa et al., 2016). Therefore, plastic pollution in marine environment has been of notable concern in recent years. A plastic particle smaller than 5 mm is referred to as a microplastic (MP) (Frias and Nash, 2019). MPs are ubiquitous in marine ecosystems and cause significant hazards to aquatic life (Eriksen et al., 2014). Over 5 trillion MPs are distributed in the oceans worldwide (Eriksen et al., 2014). Given their small size, MPs can easily accumulate in marine animals such as filter-feeding bivalves and fish (Wang et al., 2020). Some negative biological effects of MPs have been demonstrated on bivalves, such as impaired energy metabolism (Teng et al., 2021), inhibited immune defense (Détréé and Gallardo-Escárate, 2018), induced oxidative stress, genotoxicity, and histological alterations (Li et al., 2022; Hariharan et al., 2021; Sıkdokur et al., 2020). Besides the direct effects of plastic polymer, they can carry plastic additives and any hydrophobic chemicals present in ambient water (Rodrigues et al., 2019; Wang et al., 2018). For instance, marine animals can take the organic contaminants that are adsorbed onto MPs by swallowing them (O'Donovan et al., 2018; Avio et al., 2015; Paul-Pont et al., 2016).

Polycyclic aromatic hydrocarbons (PAHs) are prevalent environmental pollutants and generally enter into the marine environment by rapidly increasing anthropogenic activity over the last several decades. PAHs, which are combustion products of fossil fuels, originate from ship traffic, oil spills, pyrolytic processes, etc. in aquatic environments (Xu et al., 2022). Being lipophilic organic pollutants, PAHs bind to suspended solids and accumulate in sediment owing to their low solubility in water. The hydrophobicity of PAHs facilitates their adsorption to MPs in seawater (Avio et al., 2015). PAHs lead to serious deleterious effects in fish, crustaceans, and mollusks due to their toxic and mutagenic properties (White, 1986). Benzo(a)anthracene (BaA), one of the PAHs, has a high molecular weight and four fused benzene rings. It is recognized as a priority pollutant, and it was reported to have a possible carcinogenic effect on humans (US EPA, 2009). Having a high octanol/water partition coefficient (5.91) (Irwin et al., 1997), BaA tends to bind lipids, such as in cell membranes. The primary sources of BaA are pyrolytic processes like gasoline and waste incineration. A high proportion of the newly formed BaA is released into the atmosphere. BaA adsorbs dust particles in the atmosphere and accumulates in terrestrial and marine environments with wet and dry deposition (Irwin et al., 1997).

The Manila clam, *R. philippinarum*, has a high potential to uptake tiny particles, including MPs, and then deposit them in its tissues (Nie et al., 2017; Sezer et al., 2018; Wang et al., 2018). It is one of the most consumed bivalves worldwide with a production of 4.27 million tons in 2020 (FAO, 2022). Due to its widespread distribution, ease of laboratory acclimation, high tolerance to stress conditions, relatively long life (about 8 years), and feeding through filtration, it is a useful bioindicator species for assessing coastal pollution and is recognized as a model species in ecotoxicology studies (Jiang et al., 2021). Studies on the combined effects of MPs and organic pollutants in *R. philippinarum* are scarce. On the other hand, our understanding is still poor for the vector roles of MPs in the translocation and bioaccumulation of POPs (persistent organic pollutants) in marine organisms. Therefore, we aimed to assess the individual and combined effects of MPs and BaA on Manila clam and the vector role of MPs on the translocation of BaA. After the exposure was completed, we examined a number of biological endpoints, including filtration rate, the level of biochemical biomarkers, and expression of the stress-related genes. Furthermore, we used the BaA and MPs combined treatment in an incubated state to better understand that how the adsorption process affects the vector function of MPs.

2. Materials and methods

2.1. Experimental setup

Polyethylene microbeads (10 − 45 μm) were used as a model MP (Cospheric Inc., USA). Polyethylene is mainly used in packaging, cosmetics, agricultural and cleaning sectors and most abundantly produced plastic polymer worldwide (PlasticsEurope, 2021). Two distinct MP concentrations (26 μg L^{-1} and 260 μg L^{-1}) were tested in the study. The Tween-80 (0.00001% in seawater, vol/vol) was used to prevent the aggregation of polyethylene particles. Tween-80 in this concentration was shown to be non-toxic to the organisms (Olbrich et al., 2004).

BaA ($C_{12}H_{18}$, 228.294 g mol^{-1}) was dissolved in an acetone solution in an amber bottle. The final concentration of acetone was 0.001% (vol) which has no toxic effect on clams (Giannapas et al., 2012).The BaA treatment concentration was 3 μg L^{-1} in the study. BaA concentrations in four tissues (gill, digestive gland, mantle, and remaining soft tissues) were measured by using a GC/MS (Agilent Technologies, 7890A GC System, 5975C inert XL MSD with Triple-Axis Detector, USA).

In the experimental processes, 360 clams, were purchased at Yenikapı fish market. They were transferred immediately to Radioecology Laboratory at Istanbul University. They were acclimated to the laboratory in 15 L glass aquariums filled with 10 L artificial seawater, at 20 ± 2 °C, and salinity of 22 PSU for 10 days. Sea salt (Instant Ocean®) and de-ionized water were mixed to prepare artificial seawater. 15 clams were put into each aquarium. The experimental setup included 6 groups as follows: control (C), 26 μg L^{-1} of MP (LMP), 260 μg L^{-1} of MP (HMP), 3 μg L^{-1} of BaA (BaA), 3 μg L^{-1} BaA incubated with 26 μg L^{-1} of MP (LMP-BaA) and 3 μg L^{-1} BaA incubated with 260 μg L^{-1} of MP (HMP-BaA). During the experiment, seawater was changed daily in each aquarium. To achieve the target concentrations, BaA and MP were added to the aquariums following each seawater renewal. To distribute the MPs and BaA homogeneously in seawater, two aquarium pumps were simultaneously used in each aquarium. During the acclimation and exposure periods, the clams were fed with microalgae *Isochrysis aff. galbana* at the concentration of 5×10^4 cell mL^{-1} every other day. For the feeding, the clams were transferred to 5 L feeding chambers containing microalgae but not MPs and BaA. Therefore, microalgae, BaA and MPs did not coexist during the feeding process. Exposure to the stressors began after the acclimation period and continued for one week. During the experiment, the clams were kept in a 12-h light/12-h dark period. No mortality was observed throughout the experiment. The experiment was performed in three consecutive replications.

2.2. Clearance rate

The clearance rate was measured using the method given by Riisgård et al. (2013). It was calculated by decreasing rate of the prey (*I. galbana*) concentration in seawater during the 1-h feeding of clams. Seawater was taken from the aquariums repeatedly at t = 0 and t = 60 min during the feeding and then fixed with 0.5% Lugol solution for counting thereafter.

The clearance rate was calculated using the equation below.

$$CR = V \times \ln(C_0 / C_t)/N \times t \quad (1)$$

CR: Clearance rate; V: Water volume (L); N: Number of clams in the aquarium (15); t: Time; C_0: Microalgae concentration at t = 0; C_{60}: Microalgae concentration at time t = 60 min.

2.3. Biochemical analysis

At the end of the one-week experiment, the gill and the digestive gland were dissected from 9 clams. Biochemical analysis was carried out in both tissues using 9 clams from 3 replicates. Total protein concentrations were measured by the colorimetric method (Bradford, 1976). Protein concentrations (mg mL^{-1}) were determined to present lipid peroxidation (LPO) levels and enzyme activities in a standard unit, i.e. the activity per mg protein. The activities of SOD, GPx, GST and the concentrations of total protein were measured in the gill and the digestive gland of each organism. First, the tissue samples were homogenized in sodium phosphate buffer (0.1 M, pH:8.0) by using a homogenization instrument (MagNA Lyser, Roche). Then, centrifugation of the homogenates was done at 9000 g at 4 °C for 5 min. The homogenates were separated into 4 aliquots in Eppendorf tubes which were frozen at −80 °C for determination of SOD, GPx, MDA, and GST activities. SOD activity was determined according to the method given by McCord and Fridovich (1969). This method is based on the spectrophotometric measurement of superoxide anions at 550 nm. The SOD results were expressed as U SOD/mg protein. GPx activities are based on the reduction in NADPH at the existence of reduced glutathione (GSH) and glutathione reductase (GR) using the cumene hydroperoxide probe (Lawrence and Burk, 1978). Decreasing NADPH at 340 nm is considered a direct proportion of the activity of GPx. The results were given as U GPx/mg protein. GST activity was determined according to Habig and Jakoby (1981). The method is based on measuring the product formed as a result of the conjugation of GSH and 1-chloro-2,4-dinitrobenzene (CDNB) at 340 nm. GST activities were given as U GST/mg protein. LPO was determined based on thiobarbituric acid (TBA) at 532 nm (Ohkawa et al., 1979). LPO levels were given as nmol MDA/mg protein.

2.4. RNA isolation and quantitative real-time PCR (qPCR)

The expression levels of CYP1A1, CYP3A4 and HSP70 genes were measured by quantitative real-time PCR (qPCR) method. For total RNA extraction, gill and digestive gland tissues (50 mg) of 6 clams were flash-frozen with liquid nitrogen, pulverized to a powder with a mortar, and homogenized in 1 mL TRIzol Reagent (Invitrogen, USA) according to instructions of the manufacturer. The concentration of RNA and purity were measured using the NanoDrop 2000 UV/Vis spectrophotometer (Thermo Scientific, USA), while RNA integrity was checked by agarose (1%) gel electrophoresis. DNase-I-treated RNA (1 μg) was used for cDNA synthesis using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) following the manufacturer's protocol. Quantitative real-time PCR (qPCR) was performed using SYBR Green Master Mix (Hibrigen, Türkiye), and the reaction was carried out by CFX96 Touch Real-Time PCR Detection System (BioRad, USA). The qPCR cycling conditions were 95 °C for 2 min; 40 cycles of 95 °C for 1 min, 60 °C for 1 min and 72 °C for 2 min; and 72 °C for 5 min. 18S ribosomal RNA (18S rRNA) was used as a reference gene to quantify mRNA expressions of target genes. Primer sequences of the reference gene and target genes are presented in Table 1. The calculation of relative expression levels was based on the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2021).

2.5. Measurement of BaA concentrations

At the end of the one-week experiment, the clams were dissected into gill, digestive gland, mantle and remaining soft tissues. Two clams were pooled for each replication (six clams in total from three replicates). BaA concentrations were measured in each tissue. The extraction and cleaning procedures are based on a Quechuers kit protocol with some modifications. Lyophilized tissues were weighed 1.5 g and transferred to a 50 mL centrifuge tube. The tissues were vortexed using 6 mL ultrapure water, 8 mL acetonitrile and two ceramic bars (Part No: 5982-9313) for 2 min. Then, 4 g $MgSO_4$ was added and mixed in a hand homogenizer at 1500 rpm for 2 min. After centrifugation at 4000 rpm for 5 min, the acetonitrile phase was transferred to Agilent AOAC fatty sample dispersive SPE 15 mL tube (Part No: 5982-5158), vortexed for 1 min and centrifuged again at 4000 rpm for 5 min. The supernatant was stored at +4 °C until GC-MS analysis.

GC-MS analysis of the tissue samples was carried out using an Agilent GC-MS system (GC 7890A- MS 5975C) equipped with an auto-sampler. Analysis was performed using a DB-5MS UI capillary column (20 m × 0.18 mm i.d., 0.18 μm). As the carrier gas (constant flow 1.7 mL/min) helium was used and gas saver 30 mL/min at 2 min. The column temperature was initially programmed for 0.4 min at 50 °C and increased to 195 °C at 25 °C/min and held for 1.5 min, then to 265 °C at 8 °C/min and held for 1 min and increased to 315 °C with 20 °C/min and was kept at 315 °C for 1.25 min. Injector and detector temperatures were 320 °C. To increase sensitivity, all GC-MS measurements were done in single ion monitoring (SIM) mode using the *m/z* ratio characteristic for BaA. Quantification of BaA was done using solvent mixture solutions ($CHCl_3$: hexane, 1:2, v/v) at concentrations of 0.2, 0.5, 1, 2, 4, 8, 15 and 20 ng per 200 μL and conjunction with the preparation of calibration curves, procedural blanks (solvents, reagents, glassware) were analyzed to test for background contamination.

2.6. Statistical analyses

Statistical analysis and formation of figures were achieved using SPSS22 and Prism 5, respectively. The data of each assay was firstly evaluated in terms of the normal distribution and homogeneity of variance using Kolmogorov-Smirnov and Levene's tests, respectively. When those tests verified that the data has a normal distribution and homoscedasticity, a One-Way ANOVA was used for comparing the groups. Then, Tukey HSD or Bonferroni post hoc tests were employed for pairwise comparison for equal and different sample sizes, respectively. When normality and homoscedasticity assumptions were violated, the nonparametric Kruskal-Wallis test and the associated post hoc test were used for the comparison.

3. Results

3.1. Clearance rate

The clearance rates are depicted in Fig. 1. The filtration rates were significantly lower in BaA and both MP (LMP and HMP) groups ($p < 0.01$) compared to the control group. The clearance rate was mostly affected by BaA among the stressors applications. However, when BaA

Table 1
Primer sequences of the reference gene and target genes.

Gene	Forward Primer	Reverse Primer
18S rRNA	5′-GTACAAAGGGCAGGGACGTA-3′	5′-CTCCTTCGTGCTAGGGATTG-3′
CYP1A1	5′-ATTATCCAGGTCAAGGTTGATTGG-3′	5′-AATGGCCCACTGGAGTGATGTA-3′
CYP3A4	5′-GTGTGTGGTTTGGACCATTTAC-3′	5′-CATTTGGACCCTCCTGCTATTA-3′
HSP70	5′-CGGAGGCAAGCCAAAACTAC-3′	5′-AGCCTCGGCAGTTTCTTTCA-3′

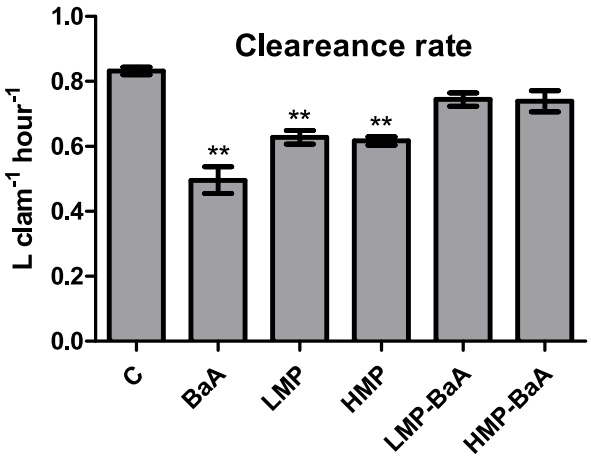

Fig. 1. Clearance rates (L clam^{-1} h^{-1}) (ANOVA and Tukey HSD test post-hoc test, **p < 0.01, replicate = 3, n = 15).

and MP were combined, the clearance rate did not differ from the control ($p > 0.05$).

3.2. Enzyme activities and LPO concentrations

SOD activity was significantly increased only in the LMP group in gill tissue, while significant increases were observed in the all groups in the digestive gland ($p < 0.05$) (Fig. 2). GPx activities did not change significantly except for the significant decrease in the digestive gland in HMP group compared to the control ($p < 0.05$) (Fig. 2). No significant difference was observed regarding the GST activities in both the gill and the digestive gland ($p > 0.05$) (Fig. 2).

In the MDA concentrations, a significant increase was determined only in the gill tissue in the HMP group ($p < 0.05$) (Fig. 2). In the digestive gland, a prominent increase was determined only in the HMP group compared to the control ($p < 0.05$) (Fig. 2).

3.3. Gene expression

CYP1A1 expression changed only in the HMP group ($p < 0.05$), however, no significant differences were determined in *CYP1A1* expression between the other stressor groups and the control group ($p > 0.05$).

The expression of *CYP3A4* was significantly higher in the gill in the HMP group compared to the control group. Its expression is markedly lower in the digestive gland in HMP-BaA group compared to the control group.

HSP70 expression was markedly higher in LMP, HMP, and HMP-BaA groups in the gill compared to the control. In the digestive gland, the expression significantly increased only in the HMP-BaA group compared to the control ($p < 0.05$) (Fig. 3).

3.4. BaA concentrations

In all clam tissues, far more BaA accumulated in individual BaA group compared to combined MP-BaA groups. The highest BaA concentration was detected in the remaining soft tissue among the body parts and BaA concentration ranked as remaining soft tissues > mantle > gill > digestive gland (Fig. 4). Not surprisingly, BaA concentration was as low as 50 μg kg^{-1} in the BaA-free groups and not comparable with other groups (Fig. 4). This concentration represents the BaA accumulation in natural environment. BaA concentration was several-fold higher in the BaA group compared with the LMP-BaA and HMP-BaA groups in both tissues (Fig. 4).

4. Discussion

4.1. Clearance rate

The clearance rate is considered a critical parameter in terms of the metabolic status of the filter-feeders (Oliveira et al., 2018; Pinheiro et al., 2019; Belivermiş et al., 2020; Zhang et al., 2022; Laubscher et al., 2023). A decrease in filtration rate in bivalves as a result of exposure to MPs and/or various stressors may be related to valve-closure for preventing contact with the stressors (Sıkdokur et al., 2020; Esperanza et al., 2020). The increased frequency and duration of shell valve-closure suppress feeding (Ortmann and Grıeshaber, 2003; Oliveira et al., 2018; Belivermiş et al., 2020), therefore, the number of microparticles to be taken also proportionally reduce. This may lead to potential starvation and a decrease in the condition factor in long-term exposure to pollutants (Oliveira et al., 2018; Yin et al., 2018). In the current study, the clams were exposed to BaA (3 μg L^{-1}), low and high concentrations of MP (26 μg L^{-1} and 260 μg L^{-1}), as well as BaA (3 μg L^{-1}) adsorbed to MP at two different concentrations (26 μg L^{-1} and 260 μg L^{-1}). Significant decreases in filtration rates were observed in the BaA, LMP and HMP groups compared to the control ($p < 0.01$) (Fig. 1). This is probably due to the induced valve closure as an adaptive strategy in clam to prevent contact with the stressors. The other reason for decreasing clearance rate might be switching to low metabolic status in the stressful condition (Belivermiş et al., 2020). On the other hand, the filtration rates did not change significantly in the groups of LMP-BaA and HMP-BaA compared to the control. Therefore, no pivotal variation was found between both groups and the control ($p > 0.05$) (Fig. 1). This might be owing to the antagonistic effect of both stressors.

4.2. SOD, GPx, GST activities and LPO concentrations

Antioxidant enzymes are widely used to evaluate the biochemical effects of MPs, organic xenobiotics, and other stressors in bivalves (Dellali et al., 2004; Avio et al., 2015; Ribeiro et al., 2017; Sıkdokur et al., 2020). PAH exposure gives rise to the production of reactive oxygen species (ROS), which lead to an imbalance between oxidants and antioxidants in favor of oxidants, in aquatic species (Breitwieser et al., 2018). Pro-oxidant effects of ROS due to superoxide and hydroxyl radicals are neutralized by the cell's enzymatic and non-enzymatic antioxidants. ROS are usually associated with lipid peroxides in the cell membrane. Thus, ROS induce free radical chain reactions in the intracellular environment. Biomarker responses are investigated in the gills and digestive gland which are known to accumulate relatively higher concentrations of pollutants in bivalves (Dellali et al., 2021; Pittura et al., 2018; Sıkdokur et al., 2020). In our study, SOD, GPx, and GST activities and MDA concentrations were investigated in the gill and digestive glands. The results of the enzymes indicated that individual exposure to PE MPs and BaA in Manila clam was partly more effective than their combination (Fig. 2). This outcome might partially be ascribed to the antagonistic effect of combined stressors. The responses of SOD, GPx, and GST to stressors were tissue-specific (Fig. 2). This tissue-specific response can be elucidated by tissues' contrasting structure and physiology. In addition to being the only organ involved in filtration, gills are the first tissue to interact with the stressors such as MPs and PAH compounds which have substantial properties in terms of uptake processes and oxygen metabolism (Ribeiro et al., 2017). The digestive gland is the tissue where the detoxification and biotransformation of xenobiotics occur. Therefore, the contrasting anti-oxidant capacity in these tissues arises from their different metabolic roles.

SOD catalyzes the conversion of superoxide ions to H_2O_2 (Li et al., 2020). Dellali et al. (2021) reported that low BaP concentration (100 μg L^{-1}) stimulates SOD activity in the digestive gland of *M. galloprovincialis*, while high concentration (300 μg L^{-1}) inhibits SOD activity. Revel et al. (2019) indicated that SOD activity increases in the gills and digestive glands as a result of MP exposure in mussel *M. edulis*. The result

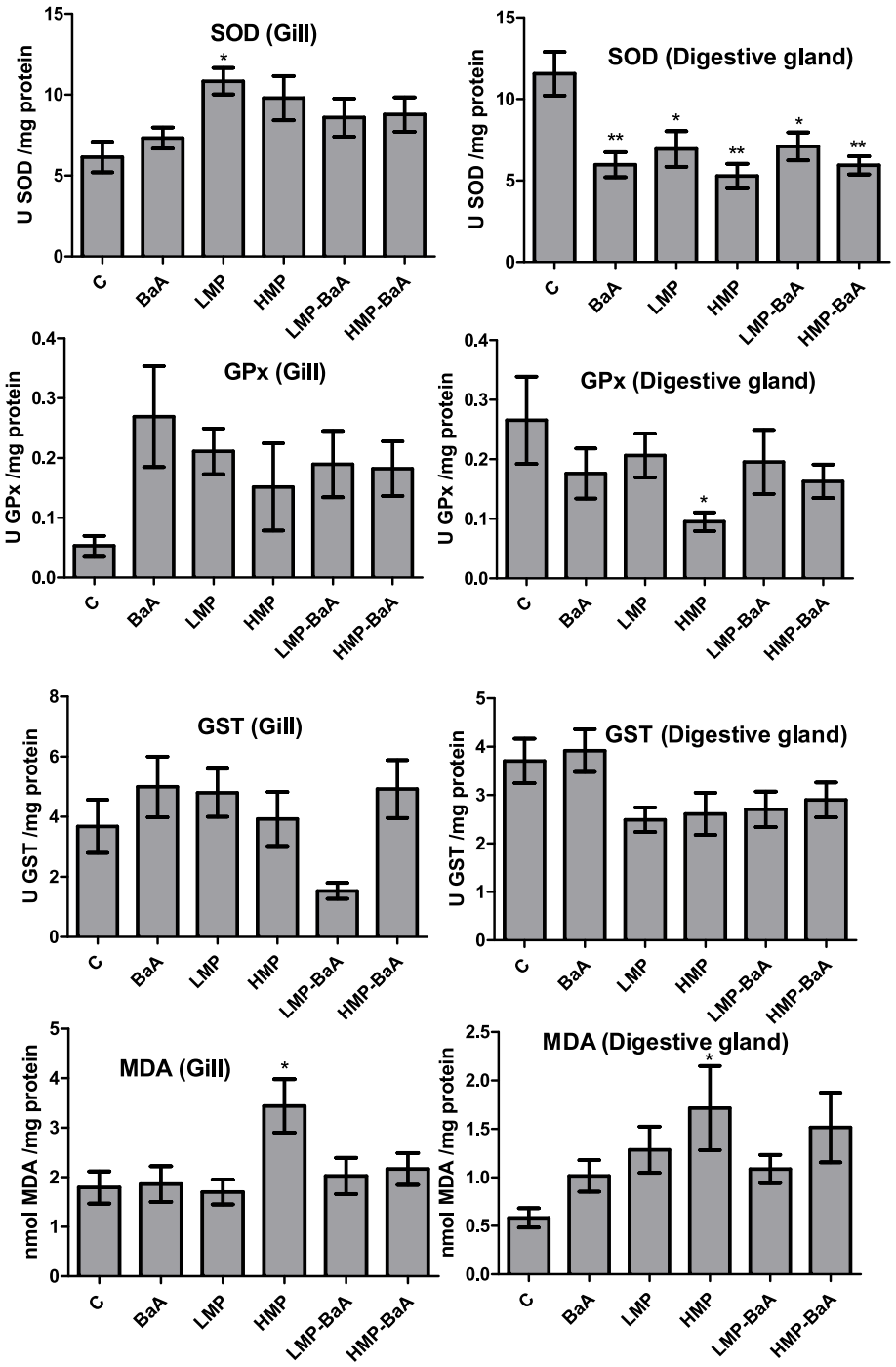

Fig. 2. SOD, GPx, GST activities and MDA concentrations in gill and digestive gland (One Way ANOVA Bonferroni was used for SOD-Gill, and MDA-Gill-Digestive gland, and Kruskal Wallis Pairwise was used for SOD-Digestive gland, GPx-Gill-Digestive gland, GST-Gill-Digestive gland *$p < 0.05$, **$p < 0.01$, replicate = 3, n = 9).

addressed the excessive superoxide radical ($O_2^{\bullet-}$) after exposure to PE MP and polypropylene microplastic (PP MP) to prevent cellular oxidative damage. For the gill, we determined an increase in SOD activities only in the low MP group (LMP) ($p < 0.05$) (Fig. 2). This increase most likely was related to the increase in ROS (Dellali et al., 2021). In the digestive gland, on the contrary, significant decreases in SOD activities were determined in all stressors groups compared to the control ($p < 0.05$) (Fig. 2). The reduced SOD activity under stress may be due to an inhibitory effect and/or a negative feedback mechanism initiated by the cytosolic H_2O_2 derived from the SOD reaction (Vlahogianni and Valavanidis, 2007). The decline of SOD in the digestive gland might be attributed to the inability for balancing oxidative stress (Rodrigues et al., 2022).

GPx, a scavenger of H_2O_2, is produced by metabolic processes after exposure to stressors such as trace elements, organic pollutants and MPs (Parolini et al., 2020; Magara et al., 2018; Xu et al., 2018; Perić and Burić, 2019). In the current study, no significant change was found among the groups regarding GPx ($p > 0.05$) (Fig. 2), except for a significant decline in the HMP group ($p < 0.05$) (Fig. 2). Parolini et al. (2020) reported that exposure to a high concentration of PET MP led to a pivotal increase in CAT activity and inhibition of GPx activity, resulting in a modulation of the oxidative state. CAT activity was not investigated in the current study, however, the decline in GPx activity in the digestive gland compared to control in all groups may also be related to increased

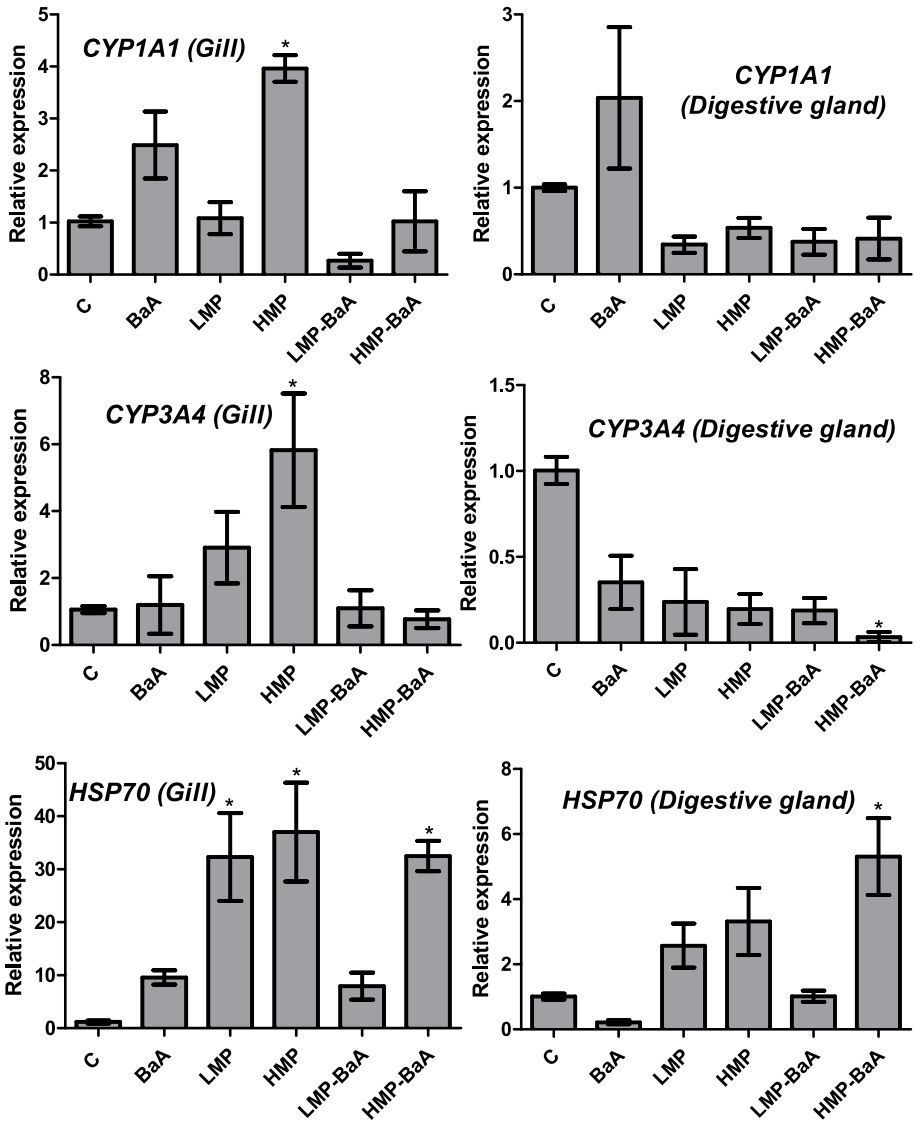

Fig. 3. *CYP1A1, CYP3A4* and *HSP70* expressions with standard deviations in gill and digestive gland. Asterisk represents significant differences between control and other treatments (Kruskal Wallis Pairwise, *p < 0.05, replicate = 3, n = 6).

CAT activity. In addition, Regoli and Giuliani (2014) stated that the differences in the activities of both these enzymes, which play a simultaneous role in the removal of H_2O_2, are probably regarding the production of the pro-oxidant molecule.

GST plays a crucial role in the antioxidant defense, biotransformation, and detoxification processes (Guilhermino et al., 2018). A previous study reported a decrease in GST activity in the gills and digestive glands of *M. edulis* exposed to 32 μg L^{-1} polystyrene microplastics for 7 days, however, they indicated that an increase in GST activity for 14 days (Paul-Pont et al., 2016). Magara et al. (2019) showed in *M. edulis* that fluoranthene-incubated PE MPs exposure decreased GST activity in the gill but not in the digestive gland. Revel et al. (2019) stated that one possible explanation for the decrease in GST activity is its degradation. Here, oxidation can cause the loss of the thiol groups on the surface of the enzyme, which results in the malfunction of the enzyme (Letelier et al., 2006). Li et al. (2020) reported that a low concentration of PAH induces GST activity, while a high concentration of PAH inhibits GST activity. Jiang et al. (2021) reported a down-regulation in GST transcript in *R. philippinarum* exposed to a mixture of Hg^{2+} and BaP. However, it was stated that there was a significant upregulation of GST in Antarctic bivalves after exposure to a PCB (polychlorinated biphenyl) mixture for 48 h (Park et al., 2009). These inconsistent results can be attributed to various pollutants' types and concentrations, as well as exposure time. GST responses are specifically related to the biotransformation and/or detoxification of organic xenobiotics such as BaA. However, in the current study, GST activity was not altered by stressors, since those concentrations or exposure periods might have not been sufficient to induce observable effects in GST (Fig. 2).

MDA is a biomarker of lipid peroxidation and represents a substantial prooxidative response bringing about ROS production and the induction of related antioxidant enzymes in a short period (Pytharopoulou et al., 2011). Lipid peroxidation leads to oxidative damage to cell structures. Islam et al. (2021) determined that MP-induced ROS caused a significant reduction in LPO levels as a result of appropriate antioxidant defenses to cope with the attack on membrane lipids in the exposure of polystyrene MPs (20 μm) in the clam *Scrobicularia plana*. Oliveira et al. (2018) stated that LPO enhanced because of oxidative stress caused by MP, Hg, and their combination. In another study, it was stated that oxidative damage to lipids may occur as a result of exposure to PET-MPs and may lead to cellular changes, including disruption of the cell membrane (Parolini et al., 2020). A substantial increase in lipid peroxidation levels was reported in clam gills as a result of exposure to high concentrations of MPs (Parolini et al., 2020). O'Donovan et al. (2018) determined that 14-day exposure to the combination of polyethylene MP (11–13 μm in size) and

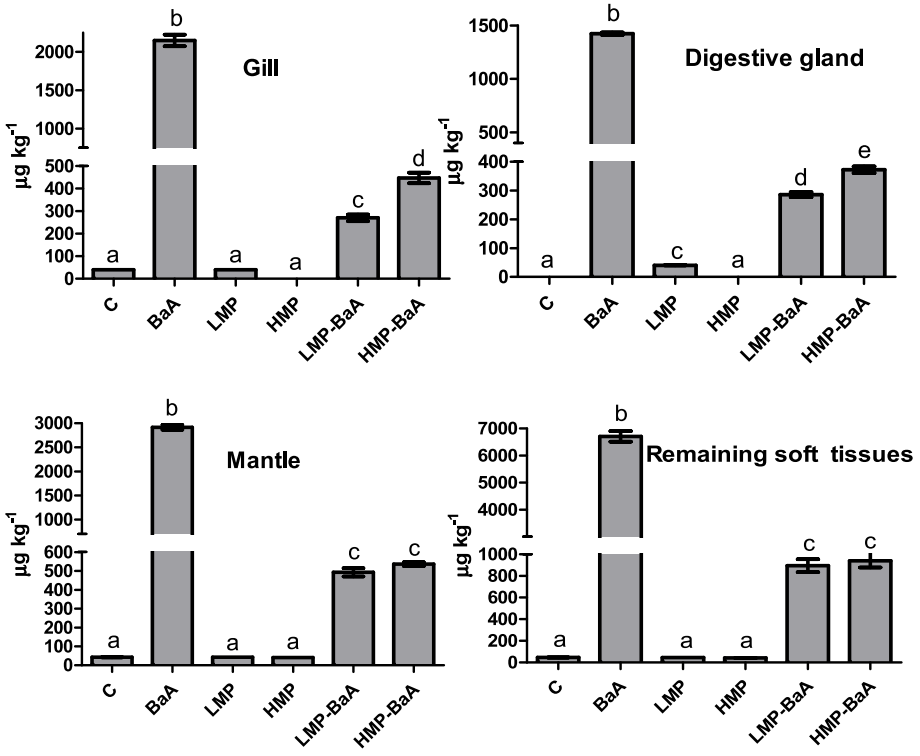

Fig. 4. BaA concentrations (µg kg^{-1}) with standard deviations in different tissues. Different letters represent significant differences between groups. (ANOVA and Tukey HSD test post-hoc test, $p < 0.05$, replicate = 3, n = 6).

BaP caused a significant increase in LPO levels in the gill and digestive gland tissues of *S. plana*. In this study, LPO levels substantially increased in both tissues only in the HMP group ($p < 0.05$) (Fig. 2). Similar to the other studies, exposure to high concentrations of MPs might have caused oxidative damage to lipids and the disruption of the cell membrane (Parolini et al., 2020). Thus, a high concentration of MP might trigger oxidative stress and lipid peroxidation in both tissues.

4.3. Gene expression

Cytochrome P450s (*CYPs*) contain one of the greatest and multi-pronged protein families that can catalyze the oxidation of a broad variety of exogenous compounds or xenobiotics including drugs, toxicants, and chemical carcinogens (Gonzalez, 2005; Uno et al., 2012). *CYP1A1* gene encodes a member of the *CYPs* family that functions as terminal oxidase enzymes in the electron transfer chain. Chi et al. (2019) stated that the expressions of *CYP1A1* were significantly increased in the gills of bivalve *Argopecten irradians* by exposure to 0.68 µM thiazolidinedione 49. Wang et al. (2010) demonstrated a significant *CYP1A1* upregulation in killifish *Fundulus heteroclitus* exposed to 5 mg L^{-1} BaP. In our study, *CYP1A1* expression significantly changed only in the HMP group in gill ($p < 0.05$) (Fig. 3). A slight upregulation was determined in the BaA group of both tissues ($p > 0.05$). These increased *CYP1A1* transcript levels are probably due to the targeting and metabolizing of MP and BaA. Among the *CYPs, CYP3A4* is responsible for the degradation and biotransformation of xenobiotics such as pyrene, and benzopyrene (BaP) (Sharifian et al., 2020). In this study, *CYP3A4* significantly increased only in the HMP group in gill, however, it decreased only in the HMP-BaA group in the digestive gland ($p < 0.05$) (Fig. 3). The outcomes displayed that the only group triggering *CYP3A4* expression was the high MP group (260 µg L^{-1} PE MP). However, the concentrations of stressors in the other group are presumably not adequate to induce the *CYP3A4* expression. Moreover, *CYP3A4* expression was significantly downregulated in the HMP-BaA.

Heat shock protein 70 (*HSP70*) plays a key role in protecting the cells against damages caused by environmental stressors and in dealing with detoxification metabolism (Gao et al., 2007; Liu et al., 2015; Gu et al., 2020). Huang et al. (2021) stated that *HSP70* was significantly upregulated in mussel (*Mytilus coruscus*) after a two-week exposure to polystyrene MPs. Détréé and Gallardo-Escárate (2018) showed that *HSP70* was upregulated in the recovery period following PE-MPs exposure in *M. galloprovincialis*. On the other side, Gu et al. (2020) found that *HSP70* significantly decreased in *Mytilus coruscus* exposed to BDE-47 (Tetrabromodiphenyl ether) and PS-MPs. In another study, it was reported that *HSP70* was upregulated in silver carp (*Hypophthalmichthys molitrix*) when exposed to 80 µg L^{-1} MPs for 48 h (Zhang et al., 2021). In the current study, *HSP70* was significantly upregulated in the groups of LMP, HMP and HMP-BaA in gill ($p < 0.05$) (Fig. 3). In the digestive gland, *HSP70* is significantly upregulated only in the HMP-BaA group ($p < 0.05$), which is probably due to the synergistic effect of MP and BaA. However, BaA alone did not significantly alter *HSP70* expression. Overall, the high MP exposure led to significant upregulation in all select genes in the gill tissue.

4.4. Bioaccumulation of BaA and its transport by MP

The BaA was efficiently accumulated in all clam tissues. Therefore, *R. philippinarum* can be considered as a reliable bioaccumulator for BaA. This is likely because mollusks have high uptake rates of PAHs in acute exposure, whereas the biotransformation of PAHs is poor (Livingstone, 1998). As far as different tissues were concerned, the remaining soft tissue was determined as the main site for BaA accumulation ($p < 0.05$) (Fig. 4). This might be because of the tendency of BaA with a high log Kow (5.91) to accumulate in remaining soft tissues such as the digestive tract, intestine and gonads which have a comparatively higher lipid (Yu et al., 2019).

The adsorption of PAH compounds (benzo(a)pyrene (BaP), pyrene, and fluoranthene) via microplastics (PE, PS, and LDPE) have been investigated in bivalves such as *Mytilus galloprovincialis*, *Scrobicularia plana* and *Mytilus edulis* in previous studies (Avio et al., 2015;

O'Donovan et al., 2018; Pittura et al. et al., 2018). Those studies reported that MPs play a role in the transfer of PAH compounds into the bivalve body. In two of these studies, PAH + MP group was compared only with the control, thus, MP was expressed as an effective carrier for the contaminants (Avio et al., 2015; O'Donovan et al., 2018). In the other study, PAH + MP group was compared with control (no stressor) and PAH group, and the concentration was determined higher in individual PAH group compared with combined MP + PAH group in the species being studied (Pittura et al., 2018). In our previous study, we detected a negligible vector role of PE MP in Hg transfer in *R. phillippinarum* (Sıkdokur et al., 2020). In the current study, BaA and MPs were incubated first and then exposed to the clams to investigate the potential vector role of PE MP on the uptake of BaA (Fig. 4). Here there are only two routes for organisms to uptake BaA: 1) uptake of BaA directly from water and 2) uptake of BaA desorbed from MPs during digestive processes. However, the accumulation of BaA in the BaA MPs incubation group was lower than in the individual BaA group (Fig. 4). Hence, the vector role of PE MPs is unsubstantial on the transport of BaA through the organisms. Here, the smooth surfaces and spherical shape of polyethylene particles which might limit contact between BaA and polyethylene particles, resulting in a poor vector role. Previous studies indicated that aging (weathering) of MPs, which expands the surface, increases the adsorption affinity of contaminants to the MPs (Abaroa-Pérez et al., 2022; Bhagat et al., 2022; Chen et al., 2019). Biofilm formation on MPs also facilitates contaminant adsorption (Wang et al., 2020; Qiongjie et al., 2022; Zhang et al., 2022). Compared with Hg transport by PE MP in *Ruditapes philippinarum* in our previous study (Hg transfer: 0.36–1.05%) (Sıkdokur et al., 2020), the transport of BaA with PE MP was found to be relatively higher in the current study. This could be ascribed to the different properties of Hg and BaA (surface charge, molecular weight, etc.) since both the model organism and microplastic were identical in these studies. BaA is more easily adsorbed to PE MPs (Amelia et al., 2021).

5. Conclusion

In the present study, filtration rate, anti-oxidant activity (SOD, GPx, and GST), LPO, gene expression (*CYP1A1, CYP3A4,* and *HSP70*), and the vector role of PE MP were investigated in *R. philippinarum* after being exposed to individual PE MP (26 and 260 μg L^{-1}) and BaA (3 μg L^{-1}) treatments, and their combination. The results demonstrated that individual PE MP and BaA influenced the filtration rate; however, the combined exposure had no similar effect. While there was no change in GST, slight changes were observed in MDA and GPx in general. Furthermore, SOD was determined to be more affected by exposure to the stressors compared to the other biochemical biomarkers. As far as gene expressions are concerned, *CYP1A1, CYP3A4* and *HSP70* expressions were observed to be partially affected by the stressors. One of the other remarkable results is that the HMP exposure had the highest impact on clam's biological parameters. The obtained results demonstrated that PE MP has a negligible vector role to transfer BaA through the organisms.

Overall, this is the first study on the effects of individual BaA and combined BaA and PE MP exposure in Manila clam. Thus, we believe that the obtained results contribute substantial information and could be a reference for similar researches. In addition, further studies are necessary to better understand chronic exposures, the influence of various MP shapes, and especially the effect of multiple simultaneous stressors. Moreover, it is crucial to investigate the vector role of MP using non-virgin and/or biofilm-covered MP in more detail, using toxic organic pollutants in the context of the transport of these pollutants through the organisms. Future research must compare the role of MPs as a vector for incorporating BaA with other marine debris, such as glass and metal, as well as biogenic particles, such as microalgae.

Author statement

Önder Kılıç: Conceptualization, Methodology, Investigation, Writing-Original Draft, Writing – Review & Editing, Funding acquisition. **Murat Belivermiş**: Validation, Methodology, Formal analysis, Writing – Review & Editing. **Ercan Sıkdokur**: Investigation, Methodology. **Narin Sezer**: Investigation, Methodology. **Yunus Aksüt**: Methodology. **Murat Pekmez**: Methodology. **Taylan Kösesakal**: Methodology. **Yusuf Can Gerçek**: Methodology.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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