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Physiological and biochemical responses to caffeine and microplastics in *Mytilus galloprovincialis*

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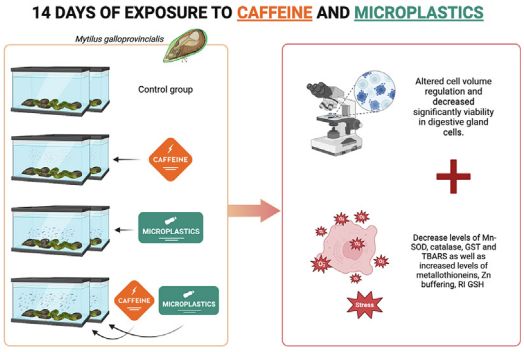
HIGHLIGHTS

- Mussels were exposed to caffeine, microplastics (MP) and their mixture.
- MP and mixture reduce antioxidant activities and increase low-weight thiols levels.
- Caffeine decreases cell viability and caspase-3 activity.
- The mixture causes a multi-stress effect and reduces cell volume regulation.

GRAPHICAL ABSTRACT



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ABSTRACT

Caffeine (Caff) is one of the most widely used substances in the human diet and a well-recognized drug. Its input into surface waters is remarkable, but biological effects on aquatic organisms are unclear, particularly in combination with pollutants of suspected modulatory activity, like microplastics (MP). The aim of this study was to reveal the impact on the marine mussel *Mytilus galloprovincialis* (Lamark, 1819) of Caff (20.0 μg L^{-1}) in the environmentally relevant combination (Mix) with MP 1 mg L^{-1} (size 35–50 μm) after the exposure for 14 days. Untreated and exposed to Caff and MP separately groups were also examined. Cell viability and cell volume regulation in hemocytes and digestive cells, as well as the indexes of oxidative stress, glutathione (GSH/GSSG) and metallothioneins levels, and caspase-3 activity in digestive gland were assessed. MP and Mix reduced Mn-superoxide dismutase, catalase, and glutathione *S*-transferase activities and level of lipid peroxidation, but increased the digestive gland cell viability, GSH/GSSG ratio (by 1.4–1.5-fold), metallothioneins level and their Zn content, while Caff did not affect oxidative stress indexes and metallothionein-related Zn chelation. Protein carbonyls were not targeted in all exposures. The distinguishing feature of the Caff group was the decline (2-fold) in caspase-3 activity and low cell viability. The multi-stress effect of Mix was shown by the worsening of the volume regulation of digestive cells and confirmed by discriminant analysis of biochemical indexes. The special capabilities of *M. galloprovincialis* as a sentinel organism make it an excellent bio-indicator reflecting the multi-stress effects in sub-chronic exposures to potentially harmful substances. The identification of the modulation of individual effects in combined exposure increases the need to base monitoring programs on studies of multi-stress effects in sub-chronic exposures.

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1. Introduction

One of the most common substances in the human diet and a well-known psychoactive drug is caffeine (Caff) (Li et al., 2020). As reported by Vieira et al. (2022), the highest concentration found in seawater was 11.0 mg L^{-1} in Northern Australia, while in Europe the highest Caff concentration was found in the Aegean Sea (3.1 μg L^{-1}). Similar information was provided for the level of Caff in the aquatic environments by Cerveny et al. (2022) with frequent concentrations below 1 μg L^{-1}, a maximum concentration of 39.8 μg L^{-1} in Europe and maximum concentrations up to 1.0 mg L^{-1} in Costa Rica. In the most polluted river sites, the maximum concentration of Caff was 6 μg L^{-1} (Bottoni et al., 2010). Caff has even been recommended as an indicator of anthropogenic inputs of pharmaceutically active compounds into water bodies (Li et al., 2020).

The data concerning the biological effect of Caff are related mostly to the higher vertebrates (Fiani et al., 2021). Additionally to its psychoactive effects, Caff has been of growing scientific interest because it has been shown to have beneficial health effects and to protect against oxidative stress in neurodegenerative diseases, to had anti-cancer effects and impact the metal accumulation (Rossowska and Nakamoto, 1992; Lu et al., 2008; Kachroo et al., 2010; Ku et al., 2011; Liu et al., 2017). Concerning its effect on invertebrate species, Caff is a well-known repellent for slugs and snails that probably causes nervous system damage by acting for a few days in the 1–2 % solutions (Hollingsworth et al., 2002; Barua et al., 2021). Information on its effect on aquatic species is scarce. Caff has been found in the tissues of different aquatic organisms, including fresh wild mussels collected in the Mediterranean Sea, in the range of ng g^{-1} dry weight (López-García et al., 2019). Biochemical signs of toxicity, mainly oxidative stress, reproductive injury and decreased lysosomal membrane stability, have been detected at environmentally relevant concentrations of ng-μg L^{-1} for some marine invertebrates, including the *Mytilus galloprovincialis* (Aguirre-Martínez et al., 2013a, 2013b, 2015a, 2015b, 2016; Cruz et al., 2016; Vieira et al., 2022). The consecutive signs of toxicity in these studies were the decrease in lysosomal membrane stability and neurotoxicity. It has been shown in *Ruditapes philippinarum* after the exposure during 14 days to 0.1 μg $L^{-1}$of Caff (Aguirre-Martínez et al., 2016) and also in hepatocytes of *Carcinus maenas*, exposed during 28 days to 0.1–50 μg L^{-1} of Caff (Aguirre-Martínez et al., 2013b). Basing on their experience, Aguirre-Martínez et al. (2016) recommended the evaluation of lysosomal membrane stability as a sensitive early warning indicator of general stress in the organisms subjected to toxic effects of pharmaceuticals, including Caff. However, despite the Caff has been attested as a high-priority environmentally hazardous emerging pollutant (Vieira et al., 2022), some results did not show its harmful effect on aquatic animals under relevant environmental conditions. In general, further experiments are needed to draw comprehensive conclusions on its environmental hazard.

Filter-fed organisms, such as bivalve molluscs, are considered among the best bioindicators of aquatic ecotoxicity due to their filter-feeder lifestyle, high susceptibility to xenobiotics, the remarkable similarity of crucial metabolic processes with vertebrate model organisms, and also because of their ability to concentrate microorganisms that are pathogenic to human (Bourgeault et al., 2010; Prud'homme et al., 2020; Le Guernic et al., 2022; Aliko et al., 2022a, 2022b; Multisanti et al., 2022). In particular, the marine mussel *M. galloprovincialis* is used in numerous ecotoxicological studies (Maria et al., 2009; Sureda et al., 2018; Curpan et al., 2022; Provenza et al., 2022; Sussarellu et al., 2022).

To reflect the environmental reality, the study of the pharmaceutical effects must take into consideration the possibility of the interaction between different stressful effects, and, consequently, the modulation of the responses to each of them. For this reason, the application of multiple stressors model exposures became particularly popular recently in aquatic ecotoxicology (Li et al., 2012; Piggott et al., 2015; Matozzo et al., 2019; Burgos-Aceves et al., 2021a, 2021b; Filice et al., 2023; Khoma et al., 2022; Mezzelani et al., 2023).

Nowadays, among possible interactions between the biological effects of pharmaceuticals with other contaminants, the focus is on microplastics (MP) (Avio et al., 2015; Menéndez-Pedriza and Jaumot, 2020; Aliko et al., 2022b). The growing plastic industry, and its consumption, are only increasing its discharge into the water in the form of waste products (Banaee et al., 2019; Hodkovicova et al., 2022; Savuca et al., 2022). Consequently, microplastics (MP) are an incredibly devastating problem for ecosystems and, while the effects of these are already widely known (Schirinzi et al., 2020; Impellitteri et al., 2022; Gholamhosseini et al., 2023), it is unclear what the effects are when combined with substances commonly found in water, such as pesticides, herbicides, heavy metals and micropollutants (Gu et al., 2020; Aliko et al., 2022b; Arif et al., 2022; Banaee et al., 2022; Ravi et al., 2023).

Since MP is suspected to interact with the hydrophobic substances and thus become a vector, causing the so-called 'Trojan Horse effect' which may lead to an increased potential risk of contaminant accumulation (de Sá et al., 2018; Menéndez-Pedriza and Jaumot, 2020; Amelia et al., 2021; Burgos-Aceves et al., 2022), it may influence the effect of waterborne Caff. The combined model exposures of bivalve molluscs to MP and some organic substances like triclosan (Syberg et al., 2017), ibuprofen (Martyniuk et al., 2022a, 2022b), salinomycin (Martyniuk et al., 2023) indicated the multistress effects, distinct from the results of single exposures. In the study of dietary chronic exposure to 58 μg L^{-1} of MPs alone and with sorbed benzo[*a*]pyrene on mussels *M. galloprovincialis* during 26 days, the highest toxicity was detected in the combined exposure (González-Soto et al., 2019).

The aim of this study was to reveal the impact of Caff on the marine mussel *Mytilus galloprovincialis* (Lamark, 1819). Due to the possibility of MP adsorb organic substances on the surface and the co-existence of Caff and MP in the water, we also aimed to elucidate possible modulation of Caff impact by MP in the environmentally relevant combination in the case of sub-chronic exposure. Since, the effect of plastic particles can be determined by their size with the higher surface area of small particles (González-Soto et al., 2019; Cole et al., 2020), we selected for our study the MPs of comparatively low size, 35–50 μm, which is relevant for the aquatic environment and can be estimated from histological cuts under polarized light (Moreschi et al., 2020; Sfriso et al., 2020). We utilized both physiological and biochemical markers of effect. Cell viability and cell volume regulation in hemocytes and digestive cells were assessed as sensitive tests of the animal's health status (Impellitteri et al., 2022). Among the biochemical markers studied in the digestive gland, the indexes of oxidative stress, including antioxidant enzymes, redox-active and multi-stress inducible thiols glutathione and metallothioneins (Dondero et al., 2004), and oxidative lesions were selected that can verify the antioxidative effect of Caff on the organism (Vieira et al., 2020). The zinc (Zn) buffering by the metallothioneins was determined to assess the metal sorption as possible target of Caff activity (Rossowska and Nakamoto, 1992; Ratajczak et al., 2021). At last, apoptotic executive enzyme caspase-3 activity was analyzed to assess the severity of impact.

2. Materials and methods

2.1. Chosen concentrations and experimental design

The Caff concentration was chosen based on data reported in the literature for surface water that can reach 20 μg L^{-1} of Caff in raw sewage, rivers, drinking water, groundwater, and lakes, taking into consideration data from the least and most polluted river sites in Europe (0.05 μg L^{-1}–59.5 μg L^{-1}) (Li et al., 2020; Vystavna, 2011; Wilkinson et al., 2022). The size of MP in this study was corresponding to the most frequently revealed in the bivalve (Sfriso et al., 2020). The selected concentration of MP was based mostly on the levels indicated in the fresh waters and explored in our previous exposures (Martyniuk et al., 2022a, 2022b, 2023). The world average microplastic concentration in rivers is 0.3 mg L^{-1} (Schmidt et al., 2017) and reached 9.09 mg·L^{-1} on average in wetlands (Lasee et al., 2017). MP concentrations in seawater are lower than in freshwaters, and in the most polluted waters, such as the Eastern part of the Mediterranean Sea, MP concentrations above 40 items L^{-1} have been detected, according to Beiras and Schönemann (2020). However, it was estimated

that between a maximum of 1500 and 8 items per liter in 16 % of the surface areas of the Mediterranean Sea exceeded the most strict unacceptable level of MP in the seawater (Everaert et al., 2020).

During the experiment, 200 bivalve molluscs of *M. galloprovincialis* of 5.9 ± 0.6 cm in length and 15.3 ± 3.8 g in weight were sampled and purchased from a commercial farm at 'Lago Faro' by the 'Società FARAU SRL, Frutti di Mare' of Messina, Italy. The specimens were randomly divided into four groups (two replicates of 25 animals per group) and acclimatised for about two weeks in eight aquariums with 25 L of water each, equipped with oxygen aerators. Following accommodation, the groups were exposed to Caff (Caff-group, with 20.0 μg L^{-1} of Caff), microplastics (MP-group, 1 mg L^{-1} of microplastics 35–50 μm particle size, **434,272** Polyethylene Ultra-high molecular weight, surface-modified, powder, Sigma-Aldrich, corresponding to 15–70 items of MP L^{-1}), and their combination (Mix group, 1 mg L^{-1} of microplastics of size 35–50 μm and 20.0 μg L^{-1} of Caff), for a period of 14 days. Untreated molluscs (C-group) were also examined after the same period. Every two days, the tank's water was changed, and the chemicals were also prepared and refilled at the same time. The presence of MP in the water of tanks was confirmed (Supplementary Fig. S1). The molluscs were fed with filtered lake water that had been enriched with nutrients and was given by the same company that provided animals during the experiment (salinity: 3.4 0.02 %, pH: 7.6 0.01, T: 16.77 0.09 °C). The experimental room had a light mode of 12:12 light-dark and was 18 °C. After every water exchange, the salinity, pH, and temperature of the water were checked and matched the same standards as initially.

After exposure, the molluscs were immediately anesthetised on ice and dissected. Individual length and soft tissue weight were recorded in each group. Soft tissue condition index (CI) was calculated as the ratio: (drained mass of soft tissue/total body weight) × 100, and condition factor (CF) as the ratio: [total weight/(shell length)3] × 100 (Table 1). The specimens were examined by light microscopy (Carl Zeiss Axioskop 20, Wetzlar, Germany) for the presence of parasites and sex. Only male molluscs determined free of parasites were used for the investigation.

2.2. Haemolymph collection

To perform cell viability tests on hemolymph, approximately 1 mL of hemolymph was taken from the anterior adductor muscle of each mussel using a 1 mL plastic syringe with a 23-gauge needle, following the protocol already established in the literature (Impellitteri et al., 2022; Pagano et al., 2022; Tresnakova et al., 2023).

2.3. Cell viability of digestive gland cells and haemocytes

Cell viability assay was performed on hemolymph, evaluating the viability of hemocytes, and on hepatopancreas, to evaluate the viability of digestive gland cells. The tests performed were Trypan Blue (TB) exclusion method and Neutral Red (NR) retention assay to assess lysosomal membrane stability. In the TB exclusion method, cells were observed after 5 min of incubation, and only unstained cells were considered viable. In the NR test, on the other hand, samples were observed after 15 min of incubation, Cell viability was assessed according to the following formula:

$$\textit{cell viability}\ (\%) = \frac{\textit{number of viable cells}}{\textit{total number of cells}} \times 100$$

2.4. Regulation of volume decrease (RVD) assay

Digestive gland cells were isolated according to the method of Pagano et al. (2017). Once the digestive glands were obtained from 4 randomly selected animals from the aquarium, they were mechanically minced and washed with a calcium and magnesium-free solution (CMFS; 1100 mOsm; pH 7.3). At this point, the resulting mixture is transferred inside a tube containing 6 mL of dissociating solution (0.01 % collagenase) in CMSF. This is stirred slowly for 60 min at a temperature of 18 °C. Then the suspension is filtered and centrifuged (500 rpm/10 min/4 °C). The supernatant was removed, and the cells were resuspended twice with saline. The samples were returned to the thermostatic bath (18 °C) for a further hour. Cell samples were placed on a slide and examined with a 100× immersion objective under a light microscope (Carl Zeiss Axioskop 20, Wetzlar, Germany) which was connected to a Canon 550D camera. Three photos were obtained sequentially after a gentle isotonic solution wash; then the sample was gently washed with a hypotonic solution (800 mOsm) and images were taken. Pictures were captured every minute for the first ten minutes, then every five minutes for the final twenty-five. Using ImageJ software, the cell area of the exposed mussels was compared to that of the control group.

2.5. Biomarkers of oxidative stress

For the biochemical analyses, the samples of digestive gland tissues in single-use aliquots were prepared individually from six molluscs in each experimental assay. For the oxidative stress evaluation, 10 % w/v homogenates in 0.1 M phosphate buffer, pH 7.4, containing 100 mM KCl and 1 mM EDTA, as well as 0.1 mM phenylmethylsulfonyl fluoride (PMSF) for proteolysis inhibition were utilized. Homogenates were centrifuged at 6000 ×*g* for 10 min. The homogenates and the resulting supernatants were kept at −40 °C for measurements. The protein concentration in the supernatant (soluble protein) was measured according to the method of Lowry et al. (1951), using bovine serum albumin as the protein standard. The absorbance values were measured on a spectrophotometer UV/Vis ULAB 102UV (China).

Superoxide dismutase (SOD, EC 1.15.1.1) activity was measured according to the non-enzymatic assay based on aerobic reduction of nitroblue tetrazolium (NBT) in presence of phenazine methosulphate and NADH (Fried, 1975). To assess Mn-SOD activity, the supernatant was preincubated for 60 min at 0 °C in the presence of 5 mM KCN, which produced total inhibition of Cu,Zn-SOD. The latter activity was calculated as the difference between the activities in the absence and the presence of KCN. The reduction of NBT was registered at 560 nm. The results were expressed as SOD units per mg of soluble protein (one unit of SOD is defined as the amount of enzyme that causes 50 % inhibition of NBT reduction).

Catalase (CAT, EC 1.11.1.6) activity was measured by monitoring the decomposition of H_2O_2 according to Aebi (1974). The reaction was measured at 240 nm (ε = 0.04 $mM^{-1}{\cdot}cm^{-1}$) and expressed as μmol $min^{-1}{\cdot}mg^{-1}$ soluble protein.

The glutathione *S*-transferase (GST, EC 2.5.1.18) activity was assayed spectrophotometrically at 25 °C using reduced glutathione (GSH) and 1-chloro-2,4-dinitrobenzene (CDBN) as the substrate in 100 mM tris-HCl, pH 7.4 (Habig et al., 1974). The increase of absorbance at 340 nm was registered for 2 min. The GST activity was calculated utilizing ε = 9.6 $mM^{-1}{\cdot}cm^{-1}$, and expressed as nmol $min^{-1}{\cdot}mg^{-1}$ soluble protein.

Table 1
Morphological indixes and level of protein in the digestive gland of the *Mytilus galloprovincialis* under exposures to caffeine (Caff), microplastics (MP) and their mixture (Mix) during 14 days, M ± SD, N = 12.

Group	Body length (cm)	Total body weight (g)	Shell weight (g)	CF (m t/L^3) (%)	CI soft tissues (m soft/m t) (%)	Protein concentration (mg·g^{-1}) FW
C	5.89 ± 0.48	15.26 ± 3.65	11.09 ± 2.39	7.34 ± 0.74	26.73 ± 5.27	41.19 ± 3.19
Caff	6.45 ± 0.53*	20.57 ± 3.78*	14.50 ± 3.12*	7.73 ± 1.41	29.64 ± 6.23	47.92 ± 8.93
MP	7.24 ± 0.42*	25.94 ± 5.48*	18.40 ± 4.72*	6.81 ± 1.24	29.75 ± 5.43	54.45 ± 2.46*
Mix	6.84 ± 0.66*	21.20 ± 4.66*	9.75 ± 2.98*	6.63 ± 1.07	54.77 ± 6.26*	52.96 ± 2.58*

Note. *, value is significantly different compared to the control value, $p < 0,05$. Data were analyzed using SPSS Statistics for Windows, Version 24.

Total glutathione (reduced plus oxidized, GSH plus GSSG, correspondingly) concentration was quantified by the glutathione reductase recycling assay (Griffith, 1980) in the protein-free extract of 10 % w/v homogenate using DTNB for thiols quantification and expressed as nmol g^{-1} FW. The concentration of GSH was calculated as the difference between the total glutathione and GSSG concentrations. The redox-index of glutathione (RI GSH) as the ratio of concentrations GSH/GSSG was calculated.

The products of lipid peroxidation (LPO) were determined in the 10 % w/v homogenate as the production of thiobarbituric acid-reactive substances (TBARS) after the sedimentation of proteins in sulfosalicylic acid (Ohkawa et al., 1979). The absorbance of the chromogen was determined at 532 nm ($\varepsilon = 1.56 \times 10^5\ M^{-1}\ cm^{-1}$). Concentrations were expressed as nmol·g^{-1} fresh weight (FW).

Protein carbonyls (PC) as an index of protein oxidation were determined in the sediments of proteins from digestive gland tissue in sulfosalicylic acid with 2.4-dinitrophenylhydrazine (DNPH) (Reznick and Packer, 1994). PC concentrations were calculated from the absorbance at 370 nm using a molar absorption coefficient of 22.000 $M^{-1}\ cm^{-1}$ and expressed as μmol PC·g^{-1} FW.

2.6. Assays for the metallothioneins

Metallothionein protein (MTSH) concentration was determined by the method of Viarengo et al. (1997) in the cytosolic fraction of 1:3 w/v homogenate in 20 mM Tris-sucrose buffer pH 8 with 0.01 % ß-mercaptoethanol, 0.5 mM PMSF, 6 μM leupeptin after the ethanol/chloroform extraction and dilution in 5 mM Tris-HCl buffer, contained 1 mM EDTA (pH 7.0), utilizing the 5,5-dithiobis-2-nitrobenzoate (DTNB) for staining of thiols. The absorbance of the samples was read at 412 nm. The concentration of MT was calculated by using a molar extinction coefficient of 5-thio-2-nitrobenzoate (TNB) of 14,150·$M^{-1}\ cm^{-1}$ and expressed in nmol g^{-1} FW taking into account the molecular features of *M. galloprovincialis* MT (https://www.uniprot.org/uniprotkb/Q697L7/entry), for which cysteine residues number is 21, molecular mass - 7080 Da.

For the analysis of Zn concentration, MTs were isolated as the thermostable proteins by size-exclusion chromatography on Sephadex G-50 as it is described in Khoma et al. (2022) with necessary adjustments needed to avoid their oxidation. The concentration of Zn was measured in the digested pooled eluate of MTs-containing chromatographic peak utilizing the reaction of the complexation of Zn(II) with 2-(5-bromo-2-pyridylazo)-5-[N-propyl-N-(3-sulfopropyl) amino]phenol disodium salt dihydrate (5-Br-PAPS) (Karaman and Menek, 2012) and evaluated from the absorbance of the metal-5-Br-PAPS complex at 550 nm. The reliability of the Zn measurements was assessed by analyzing *ERM-CE 278* certified reference material; metal recovery was between 90 % and 110 %. Quality control was performed by the method of Standard Addition. Metal concentration in the tissue and MTs was expressed as μg·g^{-1} FW.

2.7. Apoptotic activity

The apoptosis characteristic was attested from the caspase-3 (EC 3.4.22.56) related activity in the colorimetrical assay based on the cleavage of peptide acetyl-Asp-Glu-Val-Asp p-nitroanilide (Ac-DEVD-pNA) that produces a colored product p-nitroaniline (pNA) (Kaushal et al., 2014). The release of *p*-nitroaniline was recorded at 405 nm ($\varepsilon = 10.5\ mM^{-1}{\cdot}cm^{-1}$) and expressed as pmol pNA $min^{-1}{\cdot}mg^{-1}$ of soluble protein.

2.8. Statistical analysis

Raw data of biochemical traits are represented in the Supplement (Table S1). Zn-MTs analysis was repeated in three replicas for each of the two independent samples in group, resulting in n = 6 for each group. For all other biochemical traits, the sample size was six from six individuals. Data were tested for normality and homogeneity of variance by using the Shapiro-Wilk test and Levene's tests, respectively. Whenever possible, data were normalized by Box-Cox common transforming method. One-way ANOVA was used to test the effect of treatments, followed by post hoc procedures. Pearson correlation analysis was performed to analyze the strength and direction of linear relationship between two continuous variables. The correlation was significant at $p < 0.05$ level. For the data that were not normally distributed, non-parametric tests (Kruskall–Wallis ANOVA and Mann–Whitney *U* test) were performed. Normalized, Box-Cox transformed data were subjected to the principal component analysis (PCA) to assess the relations between measured parameters utilizing the rotation method Varimax with Kaiser Normalization, and Canonical discriminant analysis was utilized for the separation of the exposed groups. The IBM SPSS Statistics version 24 software for Windows was used for calculations. The one-way ANOVA and Tukey's post hoc test were used to compare the viability of digestive gland and haemocyte cells. Results for RVD tests were obtained by employing one-way ANOVA and Tukey's post hoc multiple comparison test. The significance of the p-value was established as $p < 0.05$. The results are expressed as mean ± standard error.

3. Results

3.1. Morphological measurements and protein concentration

Morphological indexes of molluscs indicated that despite the initial values were similar in all specimens distributed randomly among four groups, the exposed during 14 days groups had the highest body length, total and shell weight, particularly in the MP- and Mix groups (Table 1). However, the relative indexes (GF and CI) were correspondent to the control, except for the elevated 106 % CI of soft tissues in the Mix group. The concentration of protein in the digestive gland increased in the exposures to MP and Mix by 1.3 times.

3.2. Cell viability of digestive gland cells and haemocytes

After 14 days of exposure, the hemolymph cells of animals under all conditions tested, i.e., those belonging to the C group, Caff group, MP group, and the Mix group showed high lysosomal membrane stability and high viability (>90 %) as shown in Table 2. The same trend was observed when hemolymph cells were evaluated using the Trypan Blue dye exclusion assay.

On the other hand, as for the viability tests performed on digestive gland cells, while they remained viable, as the percentage of cells found alive again settled above 90 %, there were still significant differences. As shown in Table 3, the Caff group had significantly lower viability than the C group. This alteration was found in the NR test. In addition, the MP group (1 mg L^{-1}) and the Mix group increased the percentage of viability compared with the Caff group in the TB test. Trend confirmed, with higher significance ("aa" $p < 0.01$) in the NR test.

3.3. RVD assay

M. galloprovincialis is an osmoconform organism; consequently, hemolymph cells can adjust their cell volume based on the presence or absence

Table 2
Percentage viability of *Mytilus galloprovincialis* haemocytes exposed to caffeine (Caff), microplastics (MP) and their mixture (Mix) during 14 days. The tests conducted were Trypan Blue (TB) exclusion method and Neutral Red (NR) retention assay. Values are presented as mean ± SE (n = 14).

Method	Tested group			
	Control	Caff (20 μg/L^{-1})	MP (1 mg/L^{-1})	Mix
TB	98.84 ± 0.85	98.03 ± 0.74	97.54 ± 0.44	98.01 ± 0.60
NR	99.33 ± 0.36	97.4 ± 0.81	97.63 ± 0.50	97.78 ± 0.77

One-way ANOVA was used to assert the difference between the control group and was used for comparing the treats to each other. The * represents the differences compared to the control group: *$p < 0.05$, **$p < 0.0$, ***$p < 0.0001$. The letter "a" represents the significant differences of the treated groups versus caffeine: "a" $p < 0.05$, "aa" $p < 0.01$, "aaa" $p < 0.0001$.

Table 3
Percentage of the viability of digestive cells in *Mytilus galloprovincialis* exposed to caffeine (Caff), microplastics (MP) and their mixture (Mix) during 14 days. The tests conducted were Trypan Blue (TB) exclusion method and Neutral Red (NR) retention assay. Values are presented as mean ± SE (n = 4).

Method	Tested group			
	Control	Caff (20 μg L^{-1-1})	MP (1 mg L^{-1-1})	Mix
TB	99.12 ± 0.89	98.27 ± 0.30	98.40 ± 0.22[a]	99.29 ± 0.10[a]
NR	98.51 ± 0.41	97.44 ± 0.38*	99.48 ± 0.08[aa]	99.47 ± 0.09[aa]

One-way ANOVA was used to assert the difference between the control group and was used for comparing the treats to each other. The * represents the differences compared to the control group: *p < 0.05, **p < 0.0, ***p < 0.0001. The letter "a" represents the significant differences of the treated groups versus caffeine: "a" p < 0.05, "aa" p < 0.01.

of an environment characterized by a hypotonic solution. In a healthy organism, hemolymph cells subjected to a wash of hypotonic solution swell in volume and then gradually return to their initial volume. In the experiment conducted, cells in the CAF group swelled by approximately 18 % when subjected to the hypotonic solution, similar to the control group, in which cells swelled by 17 % under the same conditions. Once they reach the peak, they gradually return to their initial volume, remaining unaffected in their RVD-related physiological functions (Fig. 1).

For the MP group, upon swelling, the cells undergo more shrinkage than is necessary to return to their initial volume. However, the cells in the Mix group, while retaining the ability to swell in the presence of hypotonic solution, failed to recover their initial volume, showing significant differences compared to the control group.

3.4. Oxidative stress indexes

The evaluation of the antioxidants manifestations detected similar regularities for all studied enzymes in exposed groups (Fig. 2A, B, C, D). They decreased under the effect of MP (Mn-SOD, Catalase, GST) and Mix (Mn-SOD, Cu, Zn-SOD, Catalase). Any of the exposures did not activate antioxidant enzymes.

Similarly, the generation of lipid peroxidation (TBARS) and protein oxidation (Protein carbonyls) products was not enhanced in the applied exposures (Fig. 2E, F). Their level was even decreased by MP (TBARS). The exposure to Caff did not affect any of the studied markers of oxidative stress.

3.5. Low-weight thiols and apoptotic activity

The exposure to Caff did not affect the level of GSH, GSSG, whereas MP increased the GSH concentration, and MP and Mix decreased the concentration of GSSG in the digestive gland of molluscs (Fig. 3A, B). Correspondingly, RI GSH was not changed compared to control in the Caff group but increased by 1.5 and 1.4 times in the MP- and Mix-groups (Fig. 3C). The indication of MTs-contained peak obtained by the by size-exclusion chromatography was confirmed by the thermostability, low weight and particular absorption spectrum of these proteins in all studied groups (Fig. S2). Analysis of the metallothionein concentration indicated the simultaneous regularity for the total MT protein, detected from thiols, and the level of Zn eluted with the MTs by the size-exclusion chromatography (Zn-MT). It was revealed that only the exposure to MP caused the increase of both MTs parameters (Fig. 3D, E). All other exposures did not influence them. The most prominent and particular effect was shown for the caspase-3, which activity dropped by 2 times by Caff and did not change compared to control in other exposures (Fig. 3F).

Principal Component Analysis was applied to the dataset in order to identify the relations between measured biochemical parameters. Fig. 4A illustrates the score plot (component 1 vs. component 2). The first principal component (Factor 1) included all antioxidant enzymes (Mn- and Cu, Zn-SODs, catalase (Cat), GST), and the metabolites resulted in oxidative lesions (protein carbonyls (PC) and GSSG) with significance >0.5 and TBARS with significance = 0.44, all in the positive part of the axis, indicating the balanced manifestations of antioxidants and oxidative lesions. The second principal component (Factor 2) included the metallothionein characteristics MT-SH and Zn-MT in the positive part of the axis opposite of Mn-SOD and GSSG with a significance |>0.5|. Caspase-3 and GSH had no significant relations to the Factors 1 and 2.

Fig. 4B shows that all groups are well inter-separated in the space of discriminant variables with the group centroid mean values 6.29 (Caff) and −6.45 (Mix) relating to Function 1, and 3.18 (C) and 5.76 (MP) relating Function 2. The predicted group membership was confirmed at a level of 83.3 % for control, and 100 % for all exposed groups.

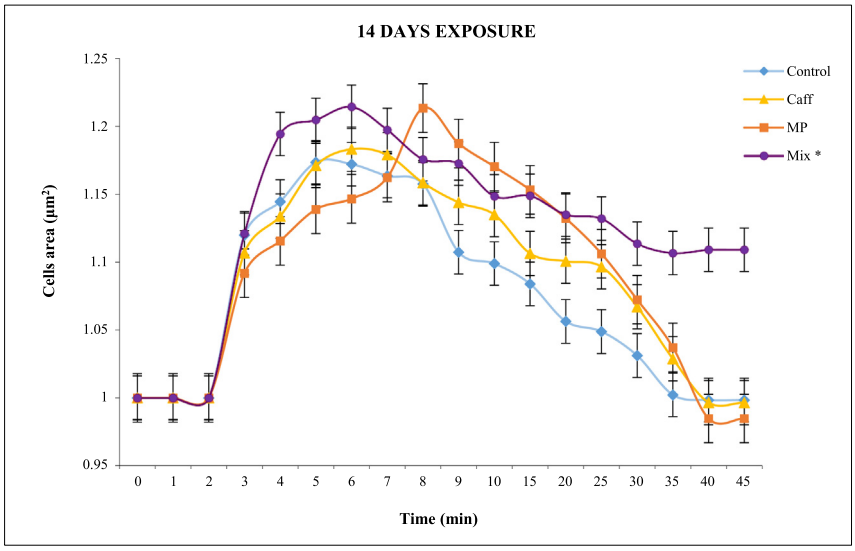

Fig. 1. Regulation volume decrease (RVD) of digestive gland cells of *Mytilus galloprovincialis* after 14 days of exposure to the pollutants. Rhombuses (◆) represent control (0 mg L^{-1}); triangles (▲) represent the Caff group (20.0 μg $L^{-1-1}$of Caffeine); squares (■□) represent the MP group (1.0 mg $L^{-1-1}$of microplastics); circles (□) represent the Mix group (20.0 μg $L^{-1-1}$of Caffeine + 1.0 mg $L^{-1-1}$of microplastics). Data are presented as mean ± SE (n = 4). Significant differences in the Mix group compared with values in the Control group (p < 0.05) are indicated by * (two-way ANOVA test).

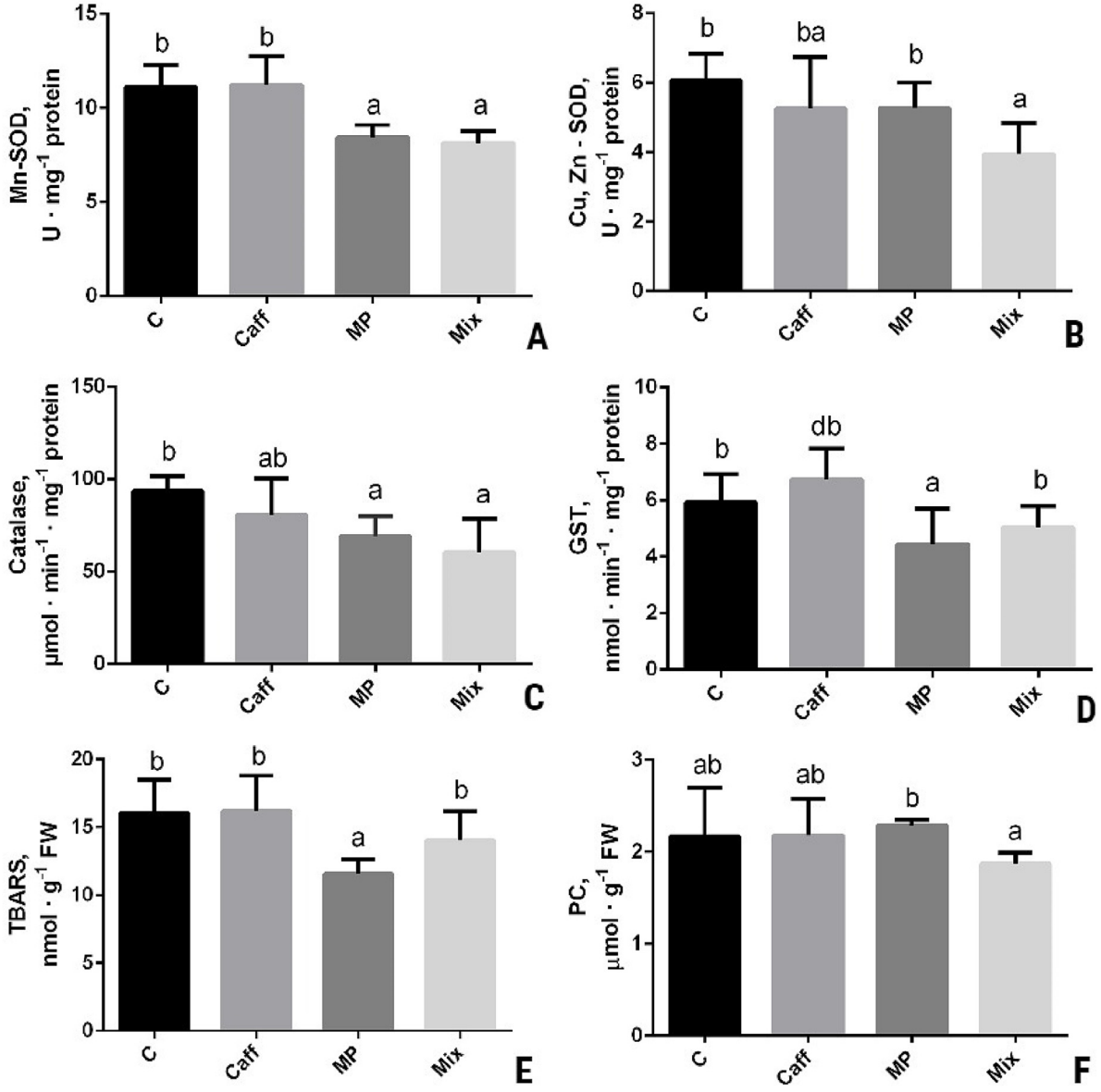

Fig. 2. The antioxidant enzyme activities and oxidative lesions in the digestive gland of bivalve molluscs *Mytilus galloprovincialis* exposed to caffeine (Caff), microplastics (MP) and their mixture (Mix) during 14 days, M ± SD, N = 6. A, Mn-SOD; B, Cu,Zn-SOD; C, Catalase; D, GST; E, TBARS; F, Protein carbonyls. Different letters above the columns indicate significant differences between groups, $P < 0.05$. Data were analyzed by using SPSS Statistics for Windows, Version 24.

4. Discussion

Marine mussel, *M. galloprovincialis* is one of the most successful bioindicators of aquatic pollution (Beiras, 2018; Curpan et al., 2022). Nowadays, its implementation is discussed in the framework of MP bioindication within the Mussel watch program (Provenza et al., 2022). *M. galloprovoncialis* is also a verified indicator for the waterborne pharmaceuticals' toxicity assessment (Gonzalez-Rey et al., 2014). However, the particular responses and sensitivity of this species to different types of pollutants have not been thoroughly assayed. Just 11 research focused on mussels under the influence of MP have recently been published in the scientific literature, mostly by assessing their oxidative stress indicators (Provenza et al., 2022).

Among the most frequent micropollutants in wastewater, Caff certainly stands out (Kim and Zoh, 2016; Warner et al., 2019). With a view to a study that aims to reflect as closely as possible on the reality of the environment in which marine organisms are found, this work was developed. The purpose was to evaluate the toxic effects given by the combination of microplastics and Caff on one of the most studied model organisms, as well as one of the molluscs that most usually enter the human food chain: *M. galloprovincialis* (Curpan et al., 2022).

4.1. Morphological characteristics

In the present study, the effect of exposures was evident even throw the indication of morphological parameters, particularly for MP and Mix groups where the increase of weight and length of molluscs was particularly prominent and combined with the increased concentration of protein (Table 1). A similar effect of increasing in size and weight under the exposure to MP was shown in our previous study on the freshwater bivalve *Unio tumidus* (Martyniuk et al., 2023). These molluscs, exposed to the same concentration of MPs (1 mg L^{-1}) but smaller in size (2 μm), increased their length and weight, whereas in the case of application of MP with larger size (0.1–0.5 mm), the effect was not prominent (Martyniuk et al., 2022a). Thomas et al. (2019) also reported a similar regularity for juvenile *Crassostrea gigas* oysters exposed to polystyrene MP (6 μm in size) during the growth phase for a period of 80 days. It was hypothesised that MP causes an increase in filtration rate. The high protein concentration in the

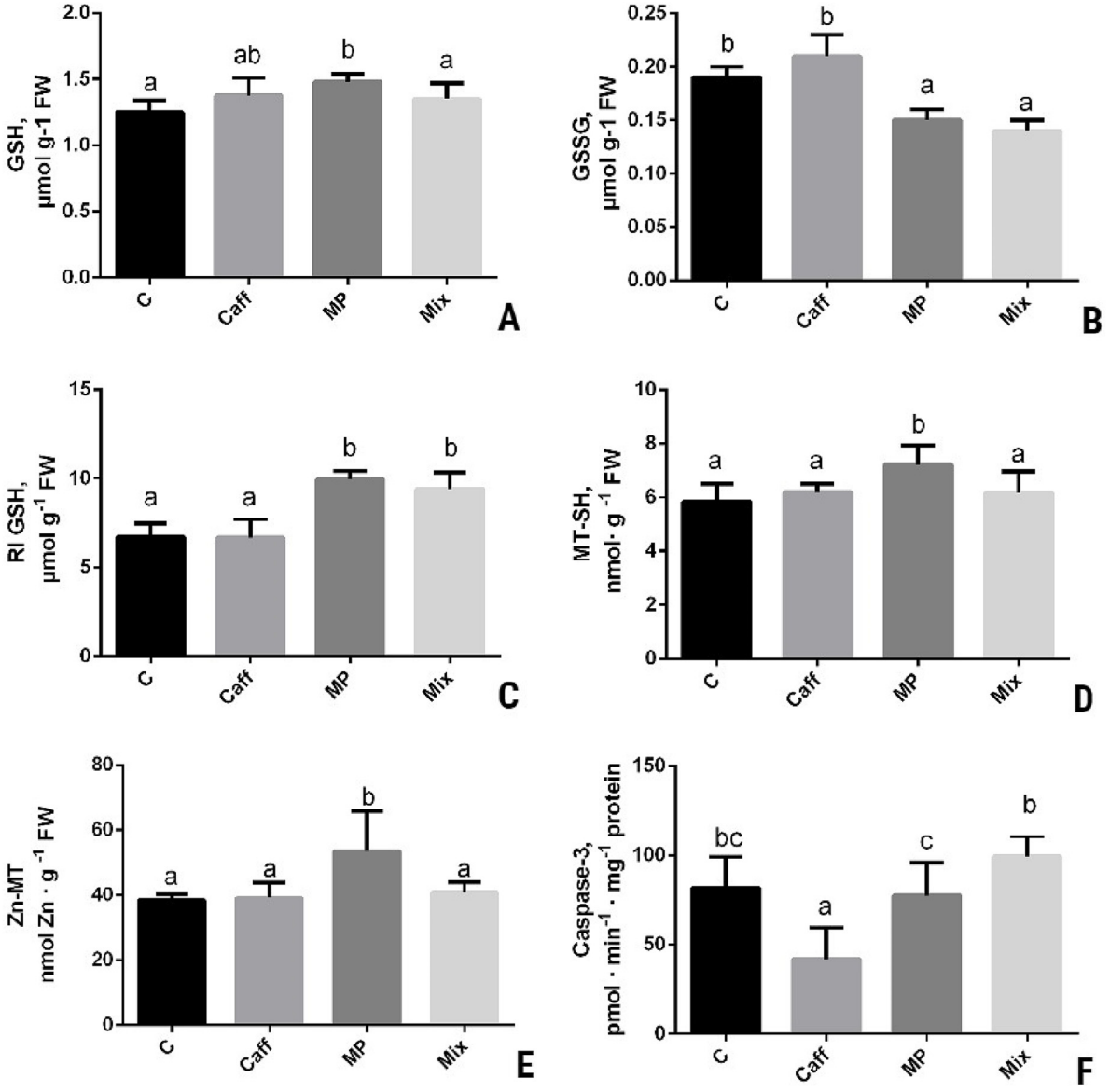

Fig. 3. The concentrations of GSH (A), GSSG (B), metallothionein protein (MT-SH), Zn in metallothioneins (Zn_MT), and caspase-3 activity in the digestive gland of bivalve molluscs *Mytilus galloprovincialis* exposed to caffeine (Caf), microplastic (MP) and their mixture (Mix) during 14 days, M ± SD, N = 6. Different letters above the columns indicate significant differences between groups, $P < 0.05$. Data were analyzed by using SPSS Statistics for Windows, Version 24.

MP and Mix groups is consistent with this assumption (Table 1). Such effect in the present study can be explained from a 'false satiation' effect in the sub-chronic exposure, when indigestible anthropogenic particles replace volumetric mass of digestible matter, whereas the long-term exposure (94 days) decreases growth rate, respiration rate and clearance rate of *Mytilus* spp. (Walkinshaw et al., 2023).

4.2. Cell viability of digestive gland cells and haemocytes

From a physiological perspective, the viability of haemolymphatic and digestive gland cells was assessed. The hemolymph is crucial in assessing the health status of the bivalve mollusc *M. galloprovincialis*; this is because circulating hemocytes act as the organism's defence cell line, being, for example, endowed with phagocytosis. The data examined by the Trypan Blue and Neutral Red tests, however, showed no significant alteration in cell viability in any of the groups tested. These results are in line with recent toxicity studies done on the same species, including on tebuconazole, thiacloprid (Freitas et al., 2021; Pagano et al., 2020; Stara et al., 2020, 2021; Tresnakova et al., 2023) and on MP specifically, where the hemocyte viability was found to be above 90 %, as in this work (Kolarević et al., 2022; Roman et al., 2023).

Different results have been obtained in the study of cell viability at the level of the digestive gland. In the Caff group, a decrease in cell viability was observed in the NR assay compared to the C group. By means of this assay, lower cell viability was also observed in the MP and Mix groups. The same assay also loss in cell viability in the MP and Mix groups compared to the Caff group. As the cells of the digestive gland are implicated in digestion and metabolism, they absorb compounds from the environment, including xenobiotics, making this organ unquestionably essential to the study of animal health (Zhang et al., 2017a; Zhang et al., 2017b). Therefore, a reduction in the viability of these cells may be a symptom of damage to the hepatopancreatic cell membrane and cells caused by the interaction of the cells with xenobiotics.

4.3. Apoptotic assessment from RVD assay and caspase-3 activity

Molluscs are known to be osmoconformers, that is, the osmotic concentration of their internal medium follows changes in the external

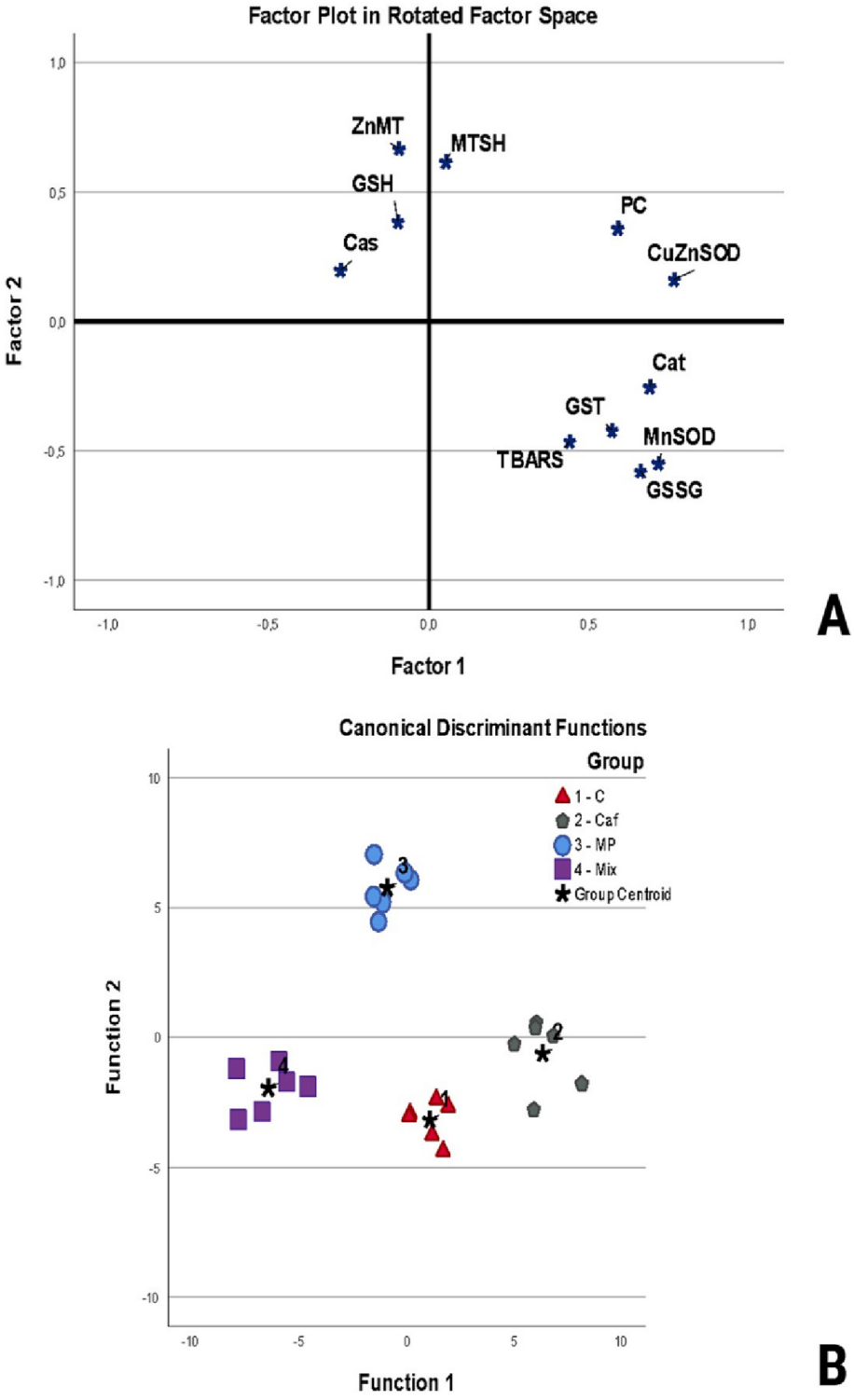

Fig. 4. The results of Principal Component Analysis (A) and scatter plots of the canonical values on the first and second canonical discriminant axes to discriminate the groups of molluscs (B). See the text for abbreviations.

environment. Therefore, following a brief osmotic contraction or swelling, cells may alter their volume in a non-linear way when exposed to anisotonic environments. However, even in normotonic physiological settings, cells undergoing programmed cell death (apoptosis) exhibit prolonged cell contraction. Cells frequently experience protracted swelling or shrinking under pathophysiological circumstances without displaying volume regulation (Neufeld and Wright, 1996; Tresnakova et al., 2023). Apoptotic and necrotic cell death's early phases are linked to this impaired volume regulation. During RVD analysis, there was damage to the cell's functional ability to regulate its cell volume. Indeed, in the Mix group, while we can observe the swelling of digestive cells in the presence of a hypotonic environment, we can also determine how these cells are no longer able to return to their initial volume, remaining in a swelling state until the end of the analysis. This tendency has been confirmed by numerous previous findings on the effects of xenobiotics on *M. galloprovincialis* (Pagano et al., 2022; Tresnakova et al., 2023), and this failure could have detrimental effects

on the activity of some or all the enzymes involved in the metabolism and digestion of nutrients and could ultimately lead to whole organ dysfunction.

However, the biochemical analysis did not reveal activated apoptosis in the Mix-group. On the contrary, only under the exposure to Caff, the decline of the caspase-3 activity was reported. Generally, the apoptotic activities under the effect of Caff in the aquatic invertebrates have not been studied. The same unusual response, suppression of caspase-3, was indicated in the bivalve *U. tumidus* under the effect of pharmaceutic ibuprofen for 14 days (Martyniuk et al., 2022b). The activities of caspases in bivalve molluscs are highly recognized, but the understanding of the diversity and function of bivalve caspases is currently very limited (Romero et al., 2015; Vogeler et al., 2021). Caspase-3 up-regulation is primarily associated with degenerative processes (Motta et al., 2013), and the decrease in its activity can indicate anti-inflammatory changes (Smith et al., 2017). It is worth noting that the indicated in this study effect of Caff is opposite to its known influences on the higher vertebrates. It is proved that Caff activated apoptosis in human osteoblasts via a mitochondria-dependent pathway decreasing the cell viability in the treated osteoblasts in a dose-dependent manner (Lu et al., 2008). Caff treatment significantly suppressed gastric cancer cell growth and viability and induced apoptosis by activating the caspase-9/−3 pathway producing anticancer effects (Liu et al., 2017) and had anticancer effects on gliomas, both in vitro and in vivo (Ku et al., 2011). Nevertheless, we can expect differences in the stimulation of apoptotic processes in human cancer cells and bivalve molluscs. Generally, the results of the cell death detected by two approaches in this study can indicate the different stages of the viability of the cells. Their combination in one study allowed to deeply understand the regularities of pharmaceuticals and combine exposures' impact on molluscs.

4.4. Oxidative stress manifestations

Oxidative stress is the most proven damage as a result of the impact of contaminants, both in field studies and model exposures to pharmaceuticals and MPs of the aquatic species, (Stoliar and Lushchak, 2012; Aliko et al., 2019; Benedetti et al., 2022). The manifestations of oxidative stress may differ depending on the substance, organism and exposure conditions. Caff exposure has been reported to cause pro-oxidative manifestations in aquatic invertebrates (Cruz et al., 2016; Vieira et al., 2022) and fish (Li et al., 2012). For example, in the polychaete species *Diopatra neapolitana* and *Arenicola marina*, low concentrations of Caff (0.5, 3.0, and 18.0 μg L^{-1}) acting for 28 days caused increased SOD activity and lipid peroxidation and decreased GSH/GSSG ratio, but did not affect catalase activity (Pires et al., 2016). A 28-day exposure of *C. maenas* to Caff (0.1, 5, 15, 50 μg L^{-1}) induced lipid peroxidation, DNA damage in hepatopancreas tissues and decreased lysosomal membrane stability starting from 5 μg L^{-1} (Aguirre-Martínez et al., 2013b). In the clam *R. philippinarum*, after exposure for 28 days to Caff (0.5, 3.0 and 18.0 μg L^{-1}) antioxidant defence activity and a simultaneous increase in lipid peroxidation with the increasing Caff concentration was found (Cruz et al., 2016). For the fish (*Carassius auratus*), it was shown that high Caff concentrations (≥0.4 mg L^{-1} and higher) increased SOD activity after 1, 2, 4, and 7 days of exposure (Li et al., 2012). The antioxidant activity of Caff was reported in humans (Liang and Kitts, 2014). Nevertheless, in the present study, we did not confirm the sensitivity of the oxidative stress response in the mussel to Caff.

Conversely, in the exposures to MP and Mix, the manifestations of oxidative stress were prominent. In the present study, the results indicate the balanced reduction of the levels of antioxidant enzymes and lipid peroxidation by MP and, with an exception, by Mix. This can be explained by the weakening of the defence mechanisms and energetic reserves with the increase of cellular energy demands during sub-chronic exposure (Shang et al., 2021). Meanwhile, the freshwater mollusc *U. tumidus* demonstrated different oxidative stress responses to MP (1 mg L^{-1}, 14 days) depending on the size. There were no changes following exposure to particles of 2 μm in size (Martyniuk et al., 2023) or increased levels of Mn-SOD and TBARS from MP of 0.1–0.5 mm in size (Martyniuk et al., 2022a). Similarly, in the *M. galloprovincialis* caged in the area polluted by MP, the balanced increase of antioxidant activities and oxidative destructions was indicated. Newertheless, overall, among the variable reported oxidative stress responses to MP in *Mytilus* spp. and other marine invertebrates, the most common manifestations were down-regulation of antioxidant enzymes and lipid peroxidation levels (refereed by Trestrail et al., 2020). For example, long-term exposures to MPs of mussels *Mytilus* spp. followed by depuration provoked the increases in SOD and catalase activities in the digestive gland in the low (8 ng L^{-1}) and medium (10 μg L^{-1}) concentrations of MP, whereas 100 μg L^{-1} caused the decrease in their activities (Revel et al., 2019). However, in the same modelling exposure to MP, any biological effects were not indicated in the Pacific oyster *Crassostrea gigas* (Revel et al., 2020). In the pearl oyster *Pinctada margaritifera*, MP at 0.25, 2.5, and 25 μg L^{-1} in the long-term (2-month) exposure induced a dose-dependent decrease of antioxidant response (Gardon et al., 2020). In *Mytilus edulis* exposed to MP for four days, SOD activity in the gills and digestive gland did not change significantly; however, catalase activity increased or decrease depending on the exposure (Magara et al., 2018, 2019). The short-term (96 h) exposure to MP led to decreased activity levels of CAT and GST in gills, and SOD in the digestive gland of *M. edulis* (Magara et al., 2018). Thus, our results are well coordinated with the data of other authors and add to the understanding of the specificity of the responses regards species, concentration and size of particles.

4.5. Low-weight thiols response

In our study, the absence of oxidative damage despite low SOD, catalase and GST activities, can be explained by the functioning of non-enzymatic antioxidants, namely GSH and MTSH. The remarkable increase of the RI GSH in the MP- and Mix- groups indicates that elevated redox state in these groups can promote the successful elimination of radicals. As Trestrail et al. (2020) mentioned, the main studied indexes of oxidative stress are antioxidant enzyme activities and products of lipid peroxidation, whereas little attention has been paid to the responses of the redox-buffers, like GSH. Moreover, almost anything was reported about MTSH as part of cellular thiolome and redox-buffer. The values of GSH indicated in our study are corresponding to the results of Regoli and Principato (1995) and Cappello et al. (2021) for *M. galloprovincialis* exposed in the field and laboratory conditions. Similar effect of MP on the GSH system in the digestive gland under the effect of MP (1 mg L^{-1}, 14 days) with the increase of the level of GSH, GSSG and RI GSH was reported for freshwater bivalve *U. tumidus* (Martyniuk et al., 2022b).

MTSH represent the valuable store of redox-active thiols for antioxidant defence. Each molecule of MTSH in *M. galloprovonciallys* contains 21 cysteine residues, therefore detected level of MTSH in the tissue can provide 100–150 nmol g^{-1} FW of redox-active thiols, which can add substantially to the antioxidant potential of ~1 μmol thiols g^{-1} FW derived from GSH. Surprisingly, the values reported by Perić et al. (2017) for MTs are extremely lower (in the limits of 100–200 ng g^{-1} FW that is corresponding to 15–30 pmol g^{-1} FW). But despite this discordance, MTs in bivalves in most cases reflect the environmental impact. The antioxidant potential of MTs in bivalve molluscs in the environmental and experimental exposures was proved in plural experiments (Mourgaud et al., 2002; Zorita et al., 2005; Gagné et al., 2007; Gnatyshyna et al., 2020; Khoma et al., 2020a, 2020b, 2021, 2022; Santovito et al., 2021; Martyniuk et al., 2022b), whereas, in contrast, in the aquatic gastropod *Lymnaea stagnalis* this source of thiols was insignificant even under the exposure to cadmium (Gnatyshyna et al., 2022). In any case, MT of *M. galloprovincialis* demonstrates high radical scavenging activity, which was higher than that in fish, and substantially higher than that of rabbit MT (Buico et al., 2008). MT content was increased in the *M. galloprovincialis* under exposure to chlorpyrifos (0.03–100 μg L^{-1}) during 4 days, which might indicate its requirement for scavenging of radicals generated by this inducer (Perić et al., 2017). In the present study, the correspondence of MTSH and Zn-MT levels indicated the successful MT functioning that did not distort the metal

binding in the thiolate clusters. Moreover, we did not find the decrease of Zn-MT that was supposed to be a possible target of Caff activity (Rossowska and Nakamoto, 1992; Ratajczak et al., 2021).

4.6. Does the interaction between caffeine and microplastics effects occur?

MP and Caff co-occur in wastewater due to their massive use in everyday life. Moreover, the hydrophobicity of MP enriches its ability to interact with Caff in water, but studies on MP-bound Caff vector transport are still in their infancy (Seidensticker et al., 2018). We cannot prove the sorption of substance on the particles of MP (Sleight et al., 2016), basing on our results. However, the interaction between their effects after 14 days of exposure was evident. Despite the ability of bivalves to excrete significant amounts of particles in the form of pseudofaeces in the short-term experiments (de Sá et al., 2018), in the chronic exposures they displayed the high microplastic contamination in the field (Sfriso et al., 2020) and experimental (Martyniuk et al., 2022a, 2022b) exposures. In the present study, the discriminant analysis of biochemical indexes indicated the specific responses to each exposure (Fig. 4B). The indication of the viability in the digestive cells also confirms distinct manifestations caused by Mix. It looks like the combination of Caff and MP distorts the responses to every single exposure and magnifies the injury, due to the inability of the digestive cell to return to their initial volume in the hypotonic solution. The most distinct differences between the single and combined exposures were the responses of caspase-3 in the Caff group and metallothioneins, GSH, GST and TBARS in the MP group, that were alleviated in the combined exposure.

The modulatory role of Caff in combined exposures was represented in several studies. For example, in the mice, Caff altered dopaminergic neuron loss induced by exposure to environmentally relevant pesticides (paraquat and maneb) over eight weeks (Kachroo et al., 2010). The protection from arsenic and mercury was demonstrated for Caff in the freshwater bivalve *Lamellidens corrianus* (Dhondiram and Popatrao, 2014). MP have also been shown to modulate the effect of different substances. Specifically, in the study of dietary exposure to polystyrene MPs of 0.5 and 4.5 μm (0.058 mg L^{-1}) alone and with sorbed benzo[*a*]pyrene (BaP) on mussels *M. galloprovincialis* during 26 days, MP with sorbed BaP was most toxic (González-Soto et al., 2019). Combined exposure to MP and some organic substances like triclosan (Syberg et al., 2017), ibuprofen (Martyniuk et al., 2022a, 2022b), salinomycin (Martyniuk et al., 2023) also indicated modulation of the effect of the individual substances, both for substance and MP. In the present study, the increased toxicity under the combine exposure comparing to the effect of single substances was best indicated by the worsening of the volume regulation of digestive cells.

5. Conclusion

The present study aimed to show for the first time the results of multi-stress exposure of the marine mussel *M. galloprovincialis* to Caff and MP. For this study, we selected a commercially important species. In the Mediterranean Sea, which is highly polluted by MP and pharmaceuticals, this mussel is particularly important for the development of biomonitoring programs. Results from sub-chronic exposure indicated that the viability markers in digestive gland tissue are more sensitive to exposure than haemocytes. The results of the physiological and biochemical assays confirmed the differences and even contradictions between the responses to Caff and MP. They also organically proved the modulation by microplastic of the response to Caff in the combined exposure. The distinguishing feature of the effect of Caff was the decline in caspase-3 activity, which has been previously detected for other pharmaceuticals in molluscs. MP caused a balanced decrease in antioxidant enzymes and TBARS levels, in accordance with reported data for this mussel. For the first time, the responses of low-weight cellular thiols, GSH and metallothioneins and the increase of their redox power in accordance with the increase of vitality signs were shown in microplastics exposures. The assessment of the cell viability detected most clearly that the combination of two substances was more toxic than the single exposures. The identification of the modulation of individual effects in combined exposure increases the need to base monitoring programs on studies of multi-stress effects in sub-chronic exposures.

CRediT authorship contribution statement

F. Impellitteri: Writing – review & editing, Formal analysis, Methodology. **K. Yunko:** Investigation, Visualization, Validation, Software. **V. Martyniuk:** Investigation, Visualization, Validation. **T. Matskiv:** Investigation, Validation. **S. Lechachenko:** Investigation, Validation. **V. Khoma:** Software, Investigation, Formal analysis. **A. Mudra:** Methodology, Visualization, Validation. **G. Piccione:** Supervision, Validation. **O. Stoliar:** Conceptualization, Data curation, Writing – original draft, Writing – review & editing, Project administration. **C. Faggio:** Writing – original draft, Writing – review & editing, Project administration, Supervision.

Data availability

The authors do not have permission to share data.

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2023.164075.

References

Aebi, H., 1974. Catalase. In: Bergmeyer, H.U. (Ed.)Meth. Enzym. Anal.. Academic Press, London, pp. 671–684

Aguirre-Martínez, G.V., Buratti, S., Fabbr, E., DelValls, A.T., Martín-Díaz, M.L., 2013a. Using lysosomal membrane stability of haemocytes in *Ruditapes philippinarum* as a biomarker of cellular stress to assess contamination by caffeine, ibuprofen, carbamazepine and novobiocin. J. Environ. Sci. 25 (7), 1408–1418. https://doi.org/10.1016/s1001-0742(12)60207-1.

Aguirre-Martínez, G.V., Del Valls, T.A., Martín-Díaz, M.L., 2013b. Identification of biomarkers responsive to chronic exposure to pharmaceuticals in target tissues of *Carcinus maenas*. Mar. Environ. Res. 87–88, 1–11. https://doi.org/10.1016/j.marenvres.2013.02.011.

Aguirre-Martínez, G.V., DelValls, A.T., Laura Martín-Díaz, M., 2015a. Yes, caffeine, ibuprofen, carbamazepine, novobiocin and tamoxifen have an effect on *Corbicula fluminea* (Müller, 1774). Ecotoxicol. Environ. Saf. 120, 142–154. https://doi.org/10.1016/j.ecoenv.2015.05.036.

Aguirre-Martínez, G.V., Owuor, M.A., Garrido-Pérez, C., Salamanca, M.J., Del Valls, T.A., Martín-Díaz, M.L., 2015b. Are standard tests sensitive enough to evaluate effects of human pharmaceuticals in aquatic biota? Facing changes in research approaches when performing risk assessment of drugs. Chemosphere 120, 75–85. https://doi.org/10.1016/j.chemosphere.2014.05.087.

Aguirre-Martínez, G.V., DelValls, T.A., Martín-Díaz, M.L., 2016. General stress, detoxification pathways, neurotoxicity and genotoxicity evaluated in *Ruditapes philippinarum* exposed to human pharmaceuticals. Ecotoxicol. Environ. Saf. 124, 18–31. https://doi.org/10.1016/j.ecoenv.2015.09.031.

Aliko, V., Mehmeti, E., Qirjo, M., Faggio, C., 2019. 'Drink and sleep like a fish': goldfish as a behavior model to study pharmaceutical effects in freshwater ecosystems. Journal of Biological Research - Bollettino Della Società Italiana Di Biologia. Sperimentale 92 (1). https://doi.org/10.4081/jbr.2019.7939.

Aliko, V., Multisanti, C.R., Turani, B., Faggio, C., 2022a. Get rid of marine pollution: bioremediation an innovative, attractive, and successful cleaning strategy. Sustainability 14 (18), 11784.

Aliko, V., Goga Beqiraj, E., Qirjo, M., Cani, M., Rama, A., Bego, K., Reka, A., Faggio, C., 2022b. Plastic invasion tolling: first evaluation of microplastics in water and two crab species from the nature reserve lagoonary complex of Kune-vain, Albania. Sci. Total Environ. 849, 157799.

Amelia, T.S.M., Khalik, W.M.A.W.M., Ong, M.C., Shao, Y.T., Pan, H.-J., Bhubalan, K., 2021. Marine microplastics as vectors of major ocean pollutants and its hazards to the marine ecosystem and humans. Prog. Earth Planet. Sci. 8, 12. https://doi.org/10.1186/s40645-020-00405-4.

Arif, I., Shang, Y., Zhang, C., Khan, F.U., Tan, K.A., Waiho, K., Wang, Y., Kwan, K.Y., Hu, M., 2022. Combined effects of nanoplastics and heavy metal on antioxidant parameters of juvenile tri-spine horseshoe crabs. Front. Mar. Sci. 9. https://doi.org/10.3389/fmars.2022.1005820.

Avio, C.G., Gorbi, S., Milan, M., Benedetti, M., Fattorini, D., d'Errico, G., Pauletto, M., Bargelloni, L., Regoli, F., 2015. Pollutants bioavailability and toxicological risk from microplastics to marine mussels. Environ. Pollut. 198, 211–222. https://doi.org/10.1016/j.envpol.2014.12.021.

Banaee, M., Soltanian, S., Sureda, A., Gholamhosseini, A., Haghi, B.N., Akhlaghi, M., Derikvandy, A., 2019. Evaluation of single and combined effects of cadmium and micro-plastic particles on biochemical and immunological parameters of common carp (*Cyprinus carpio*). Chemosphere 236, 124335. https://doi.org/10.1016/j.chemosphere.2019.07.066.

Banaee, M., Impellitteri, F., Evaz-Zadeh Samani, H., Piccione, G., Faggio, C., 2022. Dietary arthrospira platensis in rainbow trout (*Oncorhynchus mykiss*): a means to reduce threats caused by CdCl2 exposure? Toxics 10 (12), 731. https://doi.org/10.3390/toxics10120731.

Barua, A., Williams, C.D., Ross, J.L., 2021. A literature review of biological and bio-rational control strategies for slugs: current research and future prospects. Insects 12 (6), 541. https://doi.org/10.3390/insects12060541.

Beiras, R., 2018. Marine pollution monitoring programs. Marine Pollution. Sources, Fate and Effects of Pollutants in Coastal Ecosystems, pp. 293–311.

Beiras, R., Schönemann, A.M., 2020. Currently monitored microplastics pose negligible ecological risk to the global ocean. Sci. Rep. 10 (1), 22281. https://doi.org/10.1038/s41598-020-79304-z.

Benedetti, M., Giuliani, M.E., Mezzelani, M., Nardi, A., Pittura, L., Gorbi, S., Regoli, F., 2022. Emerging environmental stressors and oxidative pathways in marine organisms: current knowledge on regulation mechanisms and functional effects. Biocell. 46, 37–49. https://doi.org/10.32604/biocell.2022.017507.

Bottoni, P., Caroli, S., Barra Caracciolo, A., 2010. Pharmaceuticals as priority water contaminants. Toxicol. Environ. Chem. 92 (3), 549–565. https://doi.org/10.1080/02772241003614320.

Bourgeault, A., Gourlay-Francé, C., Vincent-Hubert, F., Palais, F., Geffard, A., Biagianti-Risbourg, S., Pain-Devin, S., Tusseau-Vuillemin, M.H., 2010. Lessons from a transplantation of zebra mussels into a small urban river: an integrated ecotoxicological assessment. Environ. Toxicol. 25 (5), 468–478. https://doi.org/10.1002/tox.20591.

Buico, A., Cassino, C., Dondero, F., Vergani, L., Osella, D., 2008. Radical scavenging abilities of fish MT-A and mussel MT-10 metallothionein isoforms: an ESR study. J. Inorg. Biochem. 102 (4), 921–927. https://doi.org/10.1016/j.jinorgbio.2007.12.012.

Burgos-Aceves, M.A., Abo-Al-Ela, H.G., Faggio, C., 2021a. Physiological and metabolic approach of plastic additives effects: immune cells responses. J. Hazard. Mater. 404 (Pt A), 124114.

Burgos-Aceves, M.A., Abo-Al-Ela, H.G., Faggio, C., 2021b. Impact of phthalates and bisphenols plasticizers on haemocyte immune function of aquatic invertebrates: a review on physiological, biochemical, and genomic aspects. J. Hazard. Mater. 419 (1), 126426.

Burgos-Aceves, M.A., Faggio, C., Betancourt-Lozanoc, M., González-Milled, D.J., Ilizaliturri-Hernándeza, C.A., 2022. Ecotoxicological perspectives of microplastic pollution in amphibians. J. Toxicol. Environ. Health B 25 (8), 405–421.

Cappello, T., De Marco, G., Oliveri Conti, G., Giannetto, A., Ferrante, M., Mauceri, A., Maisano, M., 2021. Time-dependent metabolic disorders induced by short-term exposure to polystyrene microplastics in the Mediterranean mussel *Mytilus galloprovincialis*. Ecotoxicol. Environ. Saf. 209, 111780. https://doi.org/10.1016/j.ecoenv.2020.111780.

Cerveny, D., Cisar, P., Brodin, T., McCallum, E.S., Fick, J., 2022. Environmentally relevant concentration of caffeine-effect on activity and circadian rhythm in wild perch. Environ. Sci. Pollut. Res. Int. 29 (36), 54264–54272. https://doi.org/10.1007/s11356-022-19583-3.

Cole, M., Liddle, C., Consolandi, G., Drago, C., Hird, C., Lindeque, P.K., Galloway, T.S., 2020. Microplastics, microfibres and nanoplastics cause variable sub-lethal responses in mussels (*Mytilus* spp.). Mar. Pollut. Bull. 160, 111552. https://doi.org/10.1016/j.marpolbul.2020.111552.

Cruz, D., Almeida, Â., Calisto, V., Esteves, V.I., Schneider, R.J., Wrona, F.J., Soares, A.M., Figueira, E., Freitas, R., 2016. Caffeine impacts in the clam *Ruditapes philippinarum*: alterations on energy reserves, metabolic activity and oxidative stress biomarkers. Chemosphere 160, 95–103. https://doi.org/10.1016/j.chemosphere.2016.06.068.

Curpan, A.S., Impellitteri, F., Plavan, G., Ciobica, A., Faggio, C., 2022. Review: *Mytilus galloprovincialis*: an essential, low-cost model organism for the impact of xenobiotics on oxidative stress and public health. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 256, 109302. https://doi.org/10.1016/j.cbpc.2022.109302.

de Sá, L.C., Oliveira, M., Ribeiro, F., Rocha, T.L., Futter, M.N., 2018. Studies of the effects of microplastics on aquatic organisms: what do we know and where should we focus our efforts in the future? Sci. Total Environ. 645, 1029–1039. https://doi.org/10.1016/j.scitotenv.2018.07.207.

Dhondiram, G.S., Popatrao, Z.S., 2014. Caffeine (1,3,7-trimethylxanthine) as a modulator of Arsenic bioaccumulation in the experimental Pelecypod model, *Lamellidens corrianus*. Int. J. Env. Health Eng. 3, 10.. https://www.ijehe.org/text.asp?2014/3/1/10/132682.

Dondero, F., Cavaletto, M., Ghezzi, A.R., La Terza, A., Banni, M., Viarengo, A., 2004. Biochemical characterization and quantitative gene expression analysis of the multi-stress inducible metallothionein from *Tetrahymena thermophila*. Protist 155, 157–168. https://doi.org/10.1078/143446104774199565.

Everaert, G., De Rijcke, M., Lonneville, B., Janssen, C.R., Backhaus, T., Mees, J., van Sebille, E., Koelmans, A.A., Catarino, A.I., Vandegehuchte, M.B., 2020. Risks of floating microplastic in the global ocean. Environ. Pollut. 267, 115499. https://doi.org/10.1016/j.envpol.2020.115499.

Fiani, B., Zhu, L., Musch, B.L., Briceno, S., Andel, R., Sadeq, N., Ansari, A.Z., 2021. The neurophysiology of caffeine as a central nervous system stimulant and the resultant effects on cognitive function. Cureus 13, e15032. https://doi.org/10.7759/cureus.15032.

Filice, M., Reinero, F.R., Cerra, M.C., Faggio, C., Leonetti, F.L., Micarelli, P., Giglio, G., Sperone, E., Barca, D., Imbrogno, S., 2023. Contamination by trace elements and oxidative stress in the skeletal muscle of *Scyliorhinus canicula* from the Central Tyrrhenian Sea. Antioxidants 12 (2), 524. https://doi.org/10.3390/antiox12020524.

Freitas, R., Coppola, F., Meucci, V., Battaglia, F., Soares, A.M.V.M., Pretti, C., Faggio, C., 2021. The influence of salinity on sodium lauryl sulfate toxicity in *Mytilus galloprovincialis*. Environ. Toxicol. Pharmacol. 87, 103715. https://doi.org/10.1016/j.etap.2021.103715.

Fried, R., 1975. Enzymatic and non-enzymatic assay of superoxide dismutase. Biochimie 57 (5), 657–660. https://doi.org/10.1016/s0300-9084(75)80147-7.

Gagné, F., Gagnon, C., Turcotte, P., Blaise, C., 2007. Changes in metallothionein levels in freshwater mussels exposed to urban wastewaters: effects from exposure to heavy metals? Biomark. Insights 2, 107–116. https://doi.org/10.1177/117727190700200012.

Gardon, T., Morvan, L., Huvet, A., Quillien, V., Soyez, C., Le Moullac, G., Le Luyer, J., 2020. Microplastics induce dose-specific transcriptomic disruptions in energy metabolism and immunity of the pearl oyster *Pinctada margaritifera*. Environ. Pollut. 266 (Pt 3), 115180. https://doi.org/10.1016/j.envpol.2020.115180.

Gholamhosseini, A., Banaee, M., Sureda, A., Timar, N., Zeidi, A., Faggio, C., 2023. Physiological response of freshwater crayfish, *Astacus leptodactylus* exposed to polyethylene microplastics at different temperature. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 109581. https://doi.org/10.1016/j.cbpc.2023.109581.

Gnatyshyna, L., Khoma, V., Mishchuk, O., Martinyuk, V., Spriņģe, G., Stoliar, O., 2020. Multimarker study of the responses of the *Unio tumidus* from the areas of small and micro hydropower plants at the Dniester River Basin, Ukraine. Environ. Sci. Pollut. Res. Int. 27 (10), 11038–11049. https://doi.org/10.1007/s11356-020-07698-4.

Gnatyshyna, L., Khoma, V., Martinyuk, V., Matskiv, T., Pedrini-Martha, V., Niederwanger, M., Stoliar, O., Dallinger, R., 2022. Sublethal cadmium exposure in the freshwater snail *Lymnaea stagnalis* meets a deficient, poorly responsive metallothionein system while evoking oxidative and cellular stress. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 263, 109490. https://doi.org/10.1016/j.cbpc.2022.109490.

Gonzalez-Rey, M., Mattos, J.J., Piazza, C.E., Bainy, A.C., Bebianno, M.J., 2014. Effects of active pharmaceutical ingredients mixtures in mussel *Mytilus galloprovincialis*. Aquat. Toxicol. 153, 12–26. https://doi.org/10.1016/j.aquatox.2014.02.006.

González-Soto, N., Hatfield, J., Katsumiti, A., Duroudier, N., Lacave, J.M., Bilbao, E., Orbea, A., Navarro, E., Cajaraville, M.P., 2019. Impacts of dietary exposure to different sized polystyrene microplastics alone and with sorbed benzo[a]pyrene on biomarkers and whole organism responses in mussels *Mytilus galloprovincialis*. Sci. Total Environ. 684, 548–566. https://doi.org/10.1016/j.scitotenv.2019.05.161.

Griffith, O.W., 1980. Determination of glutathione and glutathione disulfide using glutathione reductase and 2-vinylpyridine. Anal. Biochem. 106 (1), 207–212. https://doi.org/10.1016/0003-2697(80)90139-6.

Gu, H., Wei, S., Hu, M., Wei, H., Wang, X., Shang, Y., Li, L., Shi, H., Wang, Y., 2020. Microplastics aggravate the adverse effects of BDE-47 on physiological and defense performance in mussels. J. Hazard. Mater. 398, 122909. https://doi.org/10.1016/j.jhazmat.2020.122909.

Habig, W.H., Pabst, M.J., Jakoby, W.B., 1974. Glutathione S-transferases. The first enzymatic step in mercapturic acid formation. J. Biol. Chem. 249 (22), 7130–7139. https://doi.org/10.1016/S0021-9258(19)42083-8.

Hodkovicova, N., Hollerova, A., Svobodova, Z., Faldyna, M., Faggio, C., 2022. Effects of plastic particles on aquatic invertebrates and fish – a review. Environ. Toxicol. Pharmacol. 96, 104013.

Hollingsworth, R.G., Armstrong, J.W., Campbell, E., 2002. Caffeine as a repellent for slugs and snails. Nature. 417 (6892), 915–916. https://doi.org/10.1038/417915a.

Impellitteri, F., Curpăn, A.S., Plăvan, G., Ciobica, A., Faggio, C., 2022. Hemocytes: a useful tool for assessing the toxicity of microplastics, heavy metals, and pesticides on aquatic invertebrates. Int. J. Environ. Res. Public Health 19 (24), 16830. https://doi.org/10.3390/ijerph192416830.

Kachroo, A., Irizarry, M.C., Schwarzschild, M.A., 2010. Caffeine protects against combined paraquat and maneb-induced dopaminergic neuron degeneration. Exp. Neurol. 223 (2), 657–661. https://doi.org/10.1016/j.expneurol.2010.02.007.

Karaman, Y., Menek, N., 2012. Investigation of electrochemical behavior of 2-(5-bromo-2-pyridylazo)-5-[N-propyl-N-(3-sulfopropyl)amino]phenol disodium salt dihydrate. J. Electrochem. Soc. 159 (10), H805–H810. https://doi.org/10.1149/2.031210jes.

Kaushal, V., Herzog, C., Haun, R.S., Kaushal, G.P., 2014. Caspase protocols in mice. Methods Mol. Biol. 1133, 141–154. https://doi.org/10.1007/978-1-4939-0357-3_9.

Khoma, V., Gnatyshyna, L., Martinyuk, V., Rarok, Y., Mudra, A., Stoliar, O., 2020a. Biochemical responses of the bivalve mollusk *Unio tumidus* inhabiting a small power plant reservoir on the Dniester River Basin, Ukraine. Bull. Environ. Contam. Toxicol. 105 (1), 67–75. https://doi.org/10.1007/s00128-020-02873-2.

Khoma, V.V., Gnatyshyna, L.L., Martinyuk, V.V., Mackiv, T.R., Mishchuk, N.Y., Stoliar, O.B., 2020b. Metallothioneins contribution to the response of bivalve mollusk to xenobiotics. Ukr. Biochem. J. 92 (5), 87–96. https://doi.org/10.15407/ubj92.05.087.

Khoma, V., Gnatyshyna, L., Martinyuk, V., Mackiv, T., Mishchenko, L., Manusadžianas, L., Stoliar, O., 2021. Common and particular biochemical responses of *Unio tumidus* to herbicide, pharmaceuticals and their combined exposure with heating. Ecotoxicol. Environ. Saf. 208, 111695. https://doi.org/10.1016/j.ecoenv.2020.111695.

Khoma, V., Martinyuk, V., Matskiv, T., Gnatyshyna, L., Baranovsky, V., Gladiuk, M., Gylytė, B., Manusadžianas, L., Stoliar, O., 2022. Environmental concentrations of Roundup in combination with chlorpromazine or heating causes biochemical disturbances in the bivalve mollusc *Unio tumidus*. Environ. Sci. Pollut. Res. Int. 29 (10), 14131–14142. https://doi.org/10.1007/s11356-021-16775-1.

Kim, M.-K., Zoh, K.-D., 2016. Occurrence and removals of micropollutants in water environment. Environ. Eng. Res. 21, 319–332. https://doi.org/10.4491/eer.2016.115.

Kolarević, S., Kračun-Kolarević, M., Marić, J.J., Djordjević, J., Vuković-Gačić, B., Joksimović, D., Martinović, R., Bajt, O., Ramšak, A., 2022. Single and combined potential of polystyrene microparticles and fluoranthene in the induction of DNA damage in haemocytes of Mediterranean mussel (*Mytilus galloprovincialis*). Mutagenesis 38 (1), 3–12. https://doi.org/10.1093/mutage/geac017.

Ku, B.M., Lee, Y.K., Jeong, J.Y., Ryu, J., Choi, J., Kim, J.S., Cho, Y.W., Roh, G.S., Kim, H.J., Cho, G.J., Choi, W.S., Kang, S.S., 2011. Caffeine inhibits cell proliferation and regulates PKA/GSK3β pathways in U87MG human glioma cells. Mol. Cell 31 (3), 275–279. https://doi.org/10.1007/s10059-011-0027-5.

Lasee, S., Mauricio, J., Thompson, W.A., Karnjanapiboonwong, A., Kasumba, J., Subbiah, S., Morse, A.N., Anderson, T.A., 2017. Microplastics in a freshwater environment receiving treated wastewater effluent. Integr. Environ. Assess. Manag. 13 (3), 528–532. https://doi.org/10.1002/ieam.1915.

Le Guernic, A., Palos Ladeiro, M., Boudaud, N., Do Nascimento, J., Gantzer, C., Inglard, J.C., Mouchel, J.M., Pochet, C., Moulin, L., Rocher, V., Waldman, P., Wurtzer, S., Geffard, A., 2022. First evidence of SARS-CoV-2 genome detection in zebra mussel (*Dreissena polymorpha*). J. Environ. Manag. 301, 113866. https://doi.org/10.1016/j.jenvman.2021.113866.

Li, Z., Lu, G., Yang, X., Wang, C., 2012. Single and combined effects of selected pharmaceuticals at sublethal concentrations on multiple biomarkers in *Carassius auratus*. Ecotoxicology, 353–361 https://doi.org/10.1007/s10646-011-0796-9.

Li, S., He, B., Wang, J., Liu, J., Hu, X., 2020. Risks of caffeine residues in the environment: necessity for a targeted ecopharmacovigilance program. Chemosphere 243, 125343. https://doi.org/10.1016/j.chemosphere.2019.125343.

Liang, N., Kitts, D.D., 2014. Antioxidant property of coffee components: assessment of methods that define mechanisms of action. Molecules 19 (11), 19180–19208. https://doi.org/10.3390/molecules191119180.

Liu, H., Zhou, Y., Tang, L., 2017. Caffeine induces sustained apoptosis of human gastric cancer cells by activating the caspase-9/caspase-3 signalling pathway. Mol. Med. Rep. 16 (3), 2445–2454. https://doi.org/10.3892/mmr.2017.6894.

López-García, E., Postigo, C., López de Alda, M., 2019. Psychoactive substances in mussels: analysis and occurrence assessment. Mar. Pollut. Bull. 146, 985–992. https://doi.org/10.1016/j.marpolbul.2019.07.042.

Lowry, O.H., Rosebroungh, H.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193 (1), 265–275. https://doi.org/10.1016/S0021-9258(19)52451-6.

Lu, P.Z., Lai, C.Y., Chan, W.H., 2008. Caffeine induces cell death via activation of apoptotic signal and inactivation of survival signal in human osteoblasts. Int. J. Mol. Sci. 9 (5), 698–718. https://doi.org/10.3390/ijms9050698.

Magara, G., Elia, A.C., Syberg, K., Khan, F.R., 2018. Single contaminant and combined exposures of polyethylene microplastics and fluoranthene: accumulation and oxidative stress response in the blue mussel, *Mytilus edulis*. J. Toxicol. Environ. Health A 81 (16), 761–773. https://doi.org/10.1080/15287394.2018.1488639.

Magara, G., Khan, F.R., Pinti, M., Syberg, K., Inzirillo, A., Elia, A.C., 2019. Effects of combined exposures of fluoranthene and polyethylene or polyhydroxybutyrate microplastics on oxidative stress biomarkers in the blue mussel (*Mytilus edulis*). J. Toxicol. Environ. Health A 82 (10), 616–625. https://doi.org/10.1080/15287394.2019.1633451.

Maria, V.L., Santos, M.A., Bebianno, M.J., 2009. Biomarkers of damage and protection in *Mytilus galloprovincialis* cross transplanted in Ria Formosa Lagoon (Portugal). Ecotoxicology 18 (8), 1018–1028. https://doi.org/10.1007/s10646-009-0361-y.

Martyniuk, V., Gylytė, B., Matskiv, T., Khoma, V., Tulaidan, H., Gnatyshyna, L., Orlova-Hudim, K., Manusadžianas, L., Stoliar, O., 2022a. Stress responses of bivalve mollusc *Unio tumidus* from two areas to ibuprofen, microplastic and their mixture. Ecotoxicology 31 (9), 1369–1381. https://doi.org/10.1007/s10646-022-02594-8.

Martyniuk, V., Khoma, V., Matskiv, T., Baranovsky, V., Orlova-Hudim, K., Gylytė, B., Symchak, R., Matciuk, O., Gnatyshyna, L., Manusadžianas, L., Stoliar, O., 2022b. Indication of the impact of environmental stress on the responses of the bivalve mollusk *Unio tumidus* to ibuprofen and microplastics based on biomarkers of reductive stress and apoptosis. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 261, 109425. https://doi.org/10.1016/j.cbpc.2022.109425.

Martyniuk, V., Khoma, V., Matskiv, T., Yunko, K., Gnatyshyna, L., Stoliar, O., Faggio, C., 2023. Combined effect of microplastic, salinomycin and heating on *Unio tumidus*. Environ. Toxicol. Pharmacol. 98, 104068. https://doi.org/10.1016/j.etap.2023.104068 (Advance online publication).

Matozzo, V., Munari, M., Masiero, L., Finos, L., Marin, M.G., 2019. Ecotoxicological hazard of a mixture of glyphosate and aminomethylphosphonic acid to the mussel *Mytilus galloprovincialis* (Lamarck 1819). Sci. Rep. 9 (1), 14302. https://doi.org/10.1038/s41598-019-50607-0.

Menéndez-Pedriza, A., Jaumot, J., 2020. Interaction of environmental pollutants with microplastics: a critical review of sorption factors, bioaccumulation and ecotoxicological effects. Toxics 8 (2), 40. https://doi.org/10.3390/toxics8020040.

Mezzelani, M., Peruzza, L., d'Errico, G., Milan, M., Gorbi, S., Regoli, F., 2023. Mixtures of environmental pharmaceuticals in marine organisms: mechanistic evidence of carbamazepine and valsartan effects on *Mytilus galloprovincialis*. Sci. Total Environ. 860, 160465. https://doi.org/10.1016/j.scitotenv.2022.160465.

Moreschi, A.C., Callil, C.T., Christo, S.W., Junior, A.L.F., Nardes, C., de Faria, É., Girard, P., 2020. Filtration, assimilation and elimination of microplastics by freshwater bivalves. Case Stud. Chem. Environ. Eng. 2, 100053. https://doi.org/10.1016/j.cscee.2020.100053.

Motta, C.M., Frezza, V., Simoniello, P., 2013. Caspase 3 in molluscan tissues: localization and possible function. J. Exp. Zool. A Ecol. Genet. Physiol. 319 (10), 548–559. https://doi.org/10.1002/jez.1817.

Mourgaud, Y., Martinez, E., Geffard, A., Andral, B., Stanisiere, J.Y., Amiard, J.C., 2002. Metallothionein concentration in the mussel *Mytilus galloprovincialis* as a biomarker of response to metal contamination: validation in the field. Biomarkers 7 (6), 479–490. https://doi.org/10.1080/1354750021000034528.

Multisanti, C.R., Merola, C., Perugini, M., Aliko, V., Faggio, C., 2022. Sentinel species selection for monitoring microplastic pollution: a review on One health approach. Ecol. Indic. 145, 109587.

Neufeld, D.S., Wright, S.H., 1996. Response of cell volume in *Mytilus gill* to acute salinity change. J. Exp. Biol. 199 (Pt 2), 473–484. https://doi.org/10.1242/jeb.199.2.473.

Ohkawa, H., Onishi, N., Yagi, K., 1979. Assay for lipid peroxidation in animal tissue by thiobarbituric acid reaction. Anal. Biochem. 95, 351–358.

Pagano, M., Porcino, C., Briglia, M., Fiorino, E., Vazzana, M., Silvestro, S., Faggio, C., 2017. The influence of exposure of cadmium chloride and zinc chloride on haemolymph and digestive gland cells from *Mytilus galloprovincialis*. Int. J. Environ. Res. 11, 207–216. https://doi.org/10.1007/s41742-017-0020-8.

Pagano, M., Stara, A., Aliko, V., Faggio, C., 2020. Impact of neonicotinoids to aquatic invertebrates—in vitro studies on *Mytilus galloprovincialis*: a review. J. Mar. Sci. Eng. 8 (10), 801. https://doi.org/10.3390/jmse8100801.

Pagano, M., Savoca, S., Impellitteri, F., Albano, M., Capillo, G., Faggio, C., 2022. Toxicological evaluation of acetylsalicylic acid in non-target organisms: chronic exposure on *Mytilus galloprovincialis* (Lamarck, 1819). Front. Physiol. 13, 920952. https://doi.org/10.3389/fphys.2022.920952.

Perić, L., Nerlović, V., Žurga, P., Žilić, L., Ramšak, A., 2017. Variations of biomarkers response in mussels *Mytilus galloprovincialis* to low, moderate and high concentrations of organic chemicals and metals. Chemosphere 174, 554–562. https://doi.org/10.1016/j.chemosphere.2017.01.138.

Piggott, J.J., Townsend, C.R., Matthaei, C.D., 2015. Reconceptualizing synergism and antagonism among multiple stressors. Ecol. Evol. 5 (7), 1538–1547. https://doi.org/10.1002/ece3.1465.

Pires, A., Almeida, Â., Calisto, V., Schneider, R.J., Esteves, V.I., Wrona, F.J., Soares, A.M.V.M., Figueira, E., Freitas, R., 2016. Long-term exposure of polychaetes to caffeine: biochemical alterations induced in *Diopatra neapolitana* and *Arenicola marina*. Environ. Pollut. 214, 456–463. https://doi.org/10.1016/j.envpol.2016.04.031.

Provenza, F., Rampih, D., Pignattelli, S., Pastorino, P., Barceló, D., Prearo, M., Specchiulli, A., Renzi, M., 2022. Mussel watch program for microplastics in the Mediterranean sea: identification of biomarkers of exposure using *Mytilus galloprovincialis*. Ecol. Indic. 142, 109212. https://doi.org/10.1016/j.ecolind.2022.109212.

Prud'homme, S.M., Hani, Y.M.I., Cox, N., Lippens, G., Nuzillard, J.M., Geffard, A., 2020. The zebra mussel (*Dreissena polymorpha*) as a model organism for ecotoxicological studies: a prior 1H NMR spectrum interpretation of a whole body extract for metabolism monitoring. Metabolites 10 (6), 256. https://doi.org/10.3390/metabo10060256.

Ratajczak, A.E., Szymczak-Tomczak, A., Zawada, A., Rychter, A.M., Dobrowolska, A., Krela-Kaźmierczak, I., 2021. Does drinking coffee and tea affect bone metabolism in patients with inflammatory bowel diseases? Nutrients 13, 216. https://doi.org/10.3390/nu13010216.

Ravi, R., Athisuyambulingam, M., Kanagaraj, S., Tresnakova, N., Impellitteri, F., Viswambaran, G., Faggio, C., 2023. Impact of chlorpyrifos on cytopathological indices in mangrove crab, *Episesarma tetragonum* (Fabricius). Vet. Sci. 10 (1), 53. https://doi.org/10.3390/vetsci10010053.

Regoli, F., Principato, G., 1995. Glutathione, glutathione-dependant and antioxidant enzymes in mussel, *Mytilus Galloprovincialis* exposed to metals in different field and laboratory condition. implications for a proper use of biochemical markers. Aquat. Toxicol. 31, 143–164. https://doi.org/10.1016/0166-445X(94)00064-W.

Revel, M., Lagarde, F., Perrein-Ettajani, H., Bruneau, M., Akcha, F., Sussarellu, R., Rouxel, J., Costil, K., Decottignies, P., Cognie, B., Châtel, A., Mouneyrac, C., 2019. Tissue-specific biomarker responses in the blue mussel *Mytilus* spp. exposed to a mixture of microplastics at environmentally relevant concentrations. Front. Environ. Sci. https://doi.org/10.3389/fenvs.2019.00033 21 March 2019 Sec. Environmental Toxicology. 7.

Revel, M., Châtel, A., Perrein-Ettajani, H., Bruneau, M., Akcha, F., Sussarellu, R., Rouxel, J., Costil, K., Decottignies, P., Cognie, B., Lagarde, F., Mouneyrac, C., 2020. Realistic environmental exposure to microplastics does not induce biological effects in the Pacific oyster *Crassostrea gigas*. Mar. Pollut. Bull. 150, 110627. https://doi.org/10.1016/j.marpolbul.2019.110627.

Reznick, A.Z., Packer, L., 1994. Oxidative damage to proteins: spectrophotometric method for carbonyl assay. Methods Enzymol. 233, 357–363. https://doi.org/10.1016/s0076-6879(94)33041-7.

Roman, C., Mahé, P., Latchere, O., Catrouillet, C., Gigault, J., Métais, I., Châtel, A., 2023. Effect of size continuum from nanoplastics to microplastics on marine mussel *Mytilus edulis*: comparison in vitro/in vivo exposure scenarios. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 264, 109512. https://doi.org/10.1016/j.cbpc.2022.109512.

Romero, A., Novoa, B., Figueras, A., 2015. The complexity of apoptotic cell death in mollusks: an update. Fish Shellfish Immunol. 46 (1), 79–87. https://doi.org/10.1016/j.fsi.2015.03.038.

Rossowska, M., Nakamoto, T., 1992. Caffeine decreases zinc and metallothionein levels in heart of newborn and adult rats. Pediatr. Res. 32, 330–332. https://doi.org/10.1203/00006450-199209000-00017.

Santovito, G., Trentin, E., Gobbi, I., Bisaccia, P., Tallandini, L., Irato, P., 2021. Non-enzymatic antioxidant responses of *Mytilus galloprovincialis*: insights into the physiological role against metal-induced oxidative stress. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 240, 108909. https://doi.org/10.1016/j.cbpc.2020.108909.

Savuca, A., Nicoara, M.N., Faggio, C., 2022. Comprehensive review regarding the profile of the microplastic pollution in the Black Sea. Sustainability 14 (21), 14376.

Schirinzi, G.F., Pedà, C., Battaglia, P., Laface, F., Galli, M., Baini, M., Consoli, P., Scotti, G., Esposito, V., Faggio, C., Farré, M., Barceló, D., Fossi, M.C., Andaloro, F., Romeo, T., 2020. A new digestion approach for the extraction of microplastics from gastrointestinal tracts (GITs) of the common dolphinfish (*Coryphaena hippurus*) from the western Mediterranean Sea. J. Hazard. Mater. 397, 122794. https://doi.org/10.1016/j.jhazmat.2020.122794.

Schmidt, C., Krauth, T., Wagner, S., 2017. Export of plastic debris by rivers into the sea. Sea. Environ. Sci. Technol. 51 (21), 12246–12253. https://doi.org/10.1021/acs.est.7b02368.

Seidensticker, S., Grathwohl, P., Lamprecht, J., Zarfl, C., 2018. A combined experimental and modeling study to evaluate pH-dependent sorption of polar and non-polar compounds to polyethylene and polystyrene microplastics. Environ. Sci. Eur. 30 (1), 30. https://doi.org/10.1186/s12302-018-0155-z.

Sfriso, A.A., Tomio, Y., Rosso, B., Gambaro, A., Sfriso, A., Corami, F., Rastelli, E., Corinaldesi, C., Mistri, M., Munari, C., 2020. Microplastic accumulation in benthic invertebrates in Terra Nova Bay (Ross Sea, Antarctica). Environ. Int. 137, 105587. https://doi.org/10.1016/j.envint.2020.105587.

Shang, Y., Wang, X., Chang, X., Sokolova, I.M., Wei, S., Liu, W., Fang, J.K.H., Hu, M., Huang, W., Wang, Y., 2021. The effect of microplastics on the bioenergetics of the mussel *Mytilus coruscus* assessed by cellular energy allocation approach. Front. Mar. Sci. 8, 754789. https://doi.org/10.3389/fmars.2021.754789.

Sleight, V.A., Bakir, A., Thompson, R.C., Henry, T.B., 2016. Assessment of microplastic-sorbed contaminant bioavailability through analysis of biomarker gene expression in larval zebrafish. Mar. Pollut. Bull. 116 (1-2), 291–297. https://doi.org/10.1016/j.marpolbul.2016.12.055.

Smith, C.E., Soti, S., Jones, T.A., Nakagawa, A., Xue, D., Yin, H., 2017. Non-steroidal anti-inflammatory drugs are caspase inhibitors. Cell Chem. Biol. 24 (3), 281–292. https://doi.org/10.1016/j.chembiol.2017.02.003.

Stara, A., Pagano, M., Capillo, G., Fabrello, J., Sandova, M., Albano, M., Zuskova, E., Velisek, J., Matozzo, V., Faggio, C., 2020. Acute effects of neonicotinoid insecticides on *Mytilus galloprovincialis*: a case study with the active compound thiacloprid and the commercial formulation calypso 480 SC. Ecotoxicol. Environ. Saf. 203, 110980. https://doi.org/10.1016/j.ecoenv.2020.110980.

Stara, A., Pagano, M., Albano, M., Savoca, S., Di Bella, G., Albergamo, A., Koutkova, Z., Sandova, M., Velisek, J., Fabrello, J., Matozzo, V., Faggio, C., 2021. Effects of long-term exposure of *Mytilus galloprovincialis* to thiacloprid: a multibiomarker approach. Environ. Pollut. 289, 117892. https://doi.org/10.1016/j.envpol.2021.117892.

Stoliar, O., Lushchak, V.I., 2012. Environmental pollution and oxidative stress in fish. In: Lushchak, V. (Ed.), Oxidative Stress - Environmental Induction and Dietary Antioxidants , pp. 131–166.. http://www.intechopen.com/books/oxidative-stress-environmental-induction-and-dietary-antioxidants/environmental-pollution-and-oxidative-stress-in-fish.

Sureda, A., Capó, X., Busquets-Cortés, C., Tejada, S., 2018. Acute exposure to sunscreen containing titanium induces an adaptive response and oxidative stress in *Mytillus galloprovincialis*. Ecotoxicol. Environ. Saf. 49, 58–63. https://doi.org/10.1016/j.ecoenv.2017.11.014.

Sussarellu, R., Chouvelon, T., Aminot, Y., Couteau, J., Loppion, G., Dégremont, L., Lamy, J.B., Akcha, F., Rouxel, J., Berthelin, C., Briaudeau, T., Izagirre, U., Mauffret, A., Grouhel, A., Burgeot, T., 2022. Differences in chemical contaminants bioaccumulation and ecotoxicology biomarkers in *Mytilus edulis* and *Mytilus galloprovincialis* and their hybrids. Environ. Pollut. 292 (Pt A), 118328. https://doi.org/10.1016/j.envpol.2021.118328.

Syberg, K., Nielsen, A., Khan, F.R., Banta, G.T., Palmqvist, A., Jepsen, P.M., 2017. Microplastic potentiates triclosan toxicity to the marine copepod *Acartia tonsa* (Dana). J. Toxicol. Environ. Health A 80 (23–24), 1369–1371. https://doi.org/10.1080/15287394.2017.1385046.

Thomas, M., Jon, B., Craig, S., Edward, R., Ruth, H., John, B., Dick, V.A., Heather, L.A., Matthew, S., 2019. The world is your oyster: low-dose, long-term microplastic exposure of juvenile oysters. Heliyon 6 (1), e03103. https://doi.org/10.1016/j.heliyon.2019.e03103.

Tresnakova, N., Famulari, S., Zicarelli, G., Impellitteri, F., Pagano, M., Presti, G., Filice, M., Caferro, A., Gulotta, E., Salvatore, G., Sandova, M., Vazzana, I., Imbrogno, S., Capillo, G., Savoca, S., Velisek, J., Faggio, C., 2023. Multi-characteristic toxicity of enantioselective chiral fungicide tebuconazole to a model organism Mediterranean mussel *Mytilus galloprovincialis* Lamarck, 1819 (Bivalve: *Mytilidae*). Sci. Total Environ. 862, 160874. https://doi.org/10.1016/j.scitotenv.2022.160874.

Trestrail, C., Nugegoda, D., Shimeta, J., 2020. Invertebrate responses to microplastic ingestion: reviewing the role of the antioxidant system. Sci. Total Environ. 734, 138559. https://doi.org/10.1016/j.scitotenv.2020.138559.

Viarengo, A., Ponzano, E., Dondero, F., Fabbri, R., 1997. A simple spectrophotometric method for metallothionein evaluation in marine organisms: an application to Mediterranean and Antarctic Molluscs. Mar. Environ. Res. 44, 69–84. https://doi.org/10.1016/S0141-1136(96)00103-1.

Vieira, A.J., Gaspar, E.M., Santos, P.M., 2020. Mechanisms of potential antioxidant activity of caffeine. Radiat. Phys. Chem. 174, 108968. https://doi.org/10.1016/j.radphyschem.2020.108968.

Vieira, L.R., Soares, A.M.V.M., Freitas, R., 2022. Caffeine as a contaminant of concern: a review on concentrations and impacts in marine coastal systems. Chemosphere 286 (Pt 2), 131675. https://doi.org/10.1016/j.chemosphere.2021.131675.

Vogeler, S., Carboni, S., Li, X., Joyce, A., 2021. Phylogenetic analysis of the caspase family in bivalves: implications for programmed cell death, immune response and development. BMC Genomics 22 (1), 80. https://doi.org/10.1186/s12864-021-07380-0.

Vystavna, Y., 2011. Study of pharmaceuticals in a model urban river as potential markers of wastewater effluents, their source and socio-economic correlates (the city Kharkiv, Ukraine). Proceedings of a Polish-Swedish-Ukrainian Seminar, Krakow Poland, October 17–19, 2011 . https://www.researchgate.net/publication/262419537_Study_of_pharmaceuticals_in_a_model_urban_river_as_potential_markers_of_wastewater_effluents_their_source_and_socio-economic_correlates_the_city_Kharkiv_Ukraine.

Walkinshaw, C., Tolhurst, T.J., Lindeque, P.K., Thompson, R.C., Cole, M., 2023. Impact of polyester and cotton microfibers on growth and sublethal biomarkers in juvenile mussels. Micropl.&Nanopl. 3. https://doi.org/10.1186/s43591-023-00052-8.

Warner, W., Licha, T., Nödler, K., 2019. Qualitative and quantitative use of micropollutants as source and process indicators. A review. Sci. Total Environ. 686, 75–89. https://doi.org/10.1016/j.scitotenv.2019.05.385.

Wilkinson, J.L., Boxall, A.B.A., Kolpin, D.W., Teta, C., 2022. Pharmaceutical pollution of the world's rivers. PNAS. 119 (8), e2113947119. https://doi.org/10.1073/pnas.2113947119.

Zhang, Y.F., Chen, S.Y., Qu, M.J., Adeleye, A.O., Di, Y.N., 2017a. Utilization of isolated marine mussel cells as an in vitro model to assess xenobiotics induced genotoxicity. Toxicol. in Vitro 44, 219–229. https://doi.org/10.1016/j.tiv.2017.05.018.

Zhang, Z., Zhang, Q., Gao, B., Gou, G., Li, L., Shi, H., Wang, M., 2017b. Simultaneous enantioselective determination of the chiral fungicide prothioconazole and its major chiral metabolite prothioconazole-desthio in food and environmental samples by ultraperformance liquid chromatography-tandem mass spectrometry. J. Agric. Food Chem. 65 (37), 8241–8247. https://doi.org/10.1021/acs.jafc.7b02903.

Zorita, I., Strogyloudi, E., Buxens, A., Mazón, L.I., Papathanassiou, E., Soto, M., Cajaraville, M.P., 2005. Application of two SH-based methods for metallothionein determination in mussels and intercalibration of the spectrophotometric method: laboratory and field studies in the Mediterranean Sea. Biomarkers 10 (5), 342–359. https://doi.org/10.1080/13547500500264645.