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Effects of Bisphenol A analogues and their mixture on the crab *Carcinus aestuarii*: Cytotoxicity, oxidative stress and damage, neurotoxicity, physiological responses, and bioaccumulation

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ABSTRACT

Bisphenol A (BPA) analogues are emerging contaminants, whose ecotoxicological profile for aquatic species, particularly marine ones, is little known. In this study, the effects of an environmentally realistic concentration (300 ng/L) of three BPA analogues (BPAF, BPF, and BPS) - alone or as a mixture (MIX) – were evaluated for the first time on the crab *Carcinus aestuarii*. A multibiomarker approach was adopted to assess the effects of 7 and 14 days of exposure on haemolymph parameters, gill and hepatopancreas biochemical parameters, and physiological responses of crabs. Bioaccumulation of the three bisphenols was also investigated in crabs by UHPLC-HRMS. A significant reduction in total haemocyte counts was recorded in crabs exposed for 7 days to BPAF and MIX and for 14 days to the MIX, whereas an increase was found in crabs treated for 14 days with BPAF. Cell proliferation increased significantly in crabs exposed for 14 days to BPS and MIX. An imbalance of the antioxidant system, as well as oxidative damage, was recorded in gills and hepatopancreas. No neurotoxic effects were observed in crabs. At the physiological level, exposure to MIX increased the respiration rate of crabs. As for bioaccumulation, only bisphenol AF was detected in crabs. Overall, the present study demonstrated that BPA analogues can affect some important cellular parameters, induce oxidative stress and alter physiological responses in crabs.

1. Introduction

Bisphenol A (BPA) is recognized as an endocrine disruptor chemical and the concern regarding its toxicity has led to some limitations in its use. Therefore, since the 2010s several regulations were enforced in the USA, Canada and EU to ban BPA from materials destined for infants and released from food contact materials (FDA, 2012; FDA, 2013 and Government of Canada, 2010). A similar regulation was imposed also in the European Union in 2011, where BPA use in polycarbonate infant feeding bottles was restricted (Michałowicz, 2014), moreover, UE settled a BPA concentration of 2,5 μg/L in drinking water (EU, 2020). Furthermore, the EU limited BPA usage to less than 0.02% by weight in thermal paper since 2020 (EU, 2016), similar to the largest USA thermal receipts manufacturer that removed BPA from its formulation (Konkel, 2013).

Consequently, BPA was replaced by several structurally similar molecules (more than 140 novel synthetic compounds), called BPA analogues. Among these, bisphenol AF (BPAF), bisphenol F (BPF) and bisphenol S (BPS) are the most used. These compounds are commonly used as polycarbonate copolymers, epoxy resins, liners, water pipes, toys, adhesives, food packaging and in thermal paper manufacture (Chen et al., 2016).

Generally, BPA analogues were found in freshwater and marine environments with concentrations ranging from a few ng/L up to tens of ng/L, lower than those experienced for BPA. However, some contamination scenarios of concern have been reported, mainly in Asia. For instance, BPAF was recorded up to 160 ng/L in a wastewater treatment plant (WWTP) in China (Wang et al., 2022), while it reached 84 ng/L in surface water samples from China lakes (Yan et al., 2017). In the case of BPF, it reached a maximum concentration of 1300 ng/L in water samples from the Han River in Korea, and it reached 2850 ng/L in the Tamagawa

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River in Japan (Yamazaki et al., 2015). Furthermore, BPS reached 932 ng/L, in WWTP in China (Huang et al., 2020), while in urban river water, the mean concentration was 3720 ng/L, with a maximum detected concentration of 65600 ng/L (Huang et al., 2018). Regarding seawaters, the three compounds have been commonly detected at concentrations up to tens of ng/L, even if in some cases concentrations reached dozens or hundreds of ng/L, as in the case of BPF in Tokyo Bay where it reached a maximum concentration of 1470 ng/L (Fabrello and Matozzo, 2022; Yamazaki et al., 2015), potentially exceeding the predicted no-effect concentrations (PNEC). Indeed, PNECs predicted by quantitative structure-activity relationship models in freshwater and marine water for the three compounds are 1.02 μg/L and 100 ng/L for BPAF, 5.44 μg/L and 540 ng/L for BPF, and 12,9 μg/L and 27 μg/L for BPS, respectively (NORMAN Ecotoxicology Database, 2024). However, the PNECs were adopted as legislative value only for BPA, which was settled at 1500 ng/L for freshwater and 150 ng/L in marine water by the EU, while the other BPA analogues are still not regulated (EU, 2008).

Despite the scientific interest in the presence of such compounds in the environment is increasing, information about the ecotoxicological effects of BPA analogues on aquatic organisms is scarce. Some studies demonstrated that BPA analogues have an endocrine potential similar to that of BPA (Andújar et al., 2019; Chen et al., 2016), while other studies reported that BPA analogues can cause oxidative stress (Park et al., 2018; Seoane et al., 2021). To fill the information gap on the toxic effects of BPA analogues on aquatic species, in this study, the biological effects and bioaccumulation of BPAF, BPF, BPS and their mixture were evaluated for the first time in crabs. A suite of biomarkers indicative of cytotoxicity, oxidative stress and damage, neurotoxicity, and physiological performance were evaluated in the crab *Carcinus aestuarii*. This crab species was selected because it is considered a model species in ecotoxicological studies (Gürkan, 2018; Qyli et al., 2020; Rodrigues and Pardal, 2014) and for its ecological and economic values in marine coastal areas, such as the Lagoon of Venice. The hypothesis we tested was whether medium (7 days) and long-term (14 days) exposure to BPA analogues can alter important biological responses in target tissues of crabs. Indeed, we expected that exposure to BPA analogues can cause significant alterations in biomarker responses of crabs like those already observed in our previous studies on microalgae (Fabrello et al., 2023a) and bivalves (Fabrello et al., 2023b).

2. Materials and methods

2.1. Crab acclimation and exposure

C. aestuarii specimens were collected by handmade traps in a licensed area for clam culture in the Lagoon of Venice (Italy) and acclimated for 7 days in large aquaria filled with aerated seawater (salinity of 35 ± 1, temperature of 12 ± 0.5 °C) and a photoperiod of 10:14 h light:dark, every day. Mussels (*Mytilus galloprovincialis*) were provided *ad libitum* as food. To avoid the effects of gender on biomarker responses, only male crabs (5 cm mean carapace length) without obvious injuries or infection were used for the experiments.

After the acclimation, crabs were exposed for 7 and 14 days to 300 ng/L of BPAF, BPF, BPS and to a mixture (MIX) composed of 100 ng/L of each BPA analogue in 35 L glass tanks (two tanks per concentration, with 30 crabs per tank) under the same laboratory conditions adopted during crab acclimation phase. Bisphenol concentrations were chosen based on literature data on the presence of these compounds in aquatic ecosystems, including coastal marine ones (see Introduction). Stocks solution of BPAF and BPF were prepared in methanol, while BPS was solubilised in ultrapure water, at a concentration of 100 mg/L. All the reagents were purchased from Merck (Milan, Italy) and bisphenols were used at the analytical standard grade. The concentration of methanol used in BPAF and BPF exposure tanks corresponded to 0.0003%. A solvent control was not performed, since it is known that methanol can exert acute and chronic effects at higher concentrations (tens and hundreds of mg/L) in aquatic species, including crustaceans (Hutchinson et al., 2006; Kaviraj et al., 2004). Seawater, BPA analogues, and food supply (one mussel per crab) were renewed every 48 h.

In this study, bioaccumulation in crabs was evaluated and not the actual concentrations of the contaminants in seawater from exposure tanks because bisphenol actual concentrations were previously evaluated in an experiment like the present testing the same concentrations for the same exposure duration (7 and 14 days). In that study, the actual concentrations were very similar to the nominal ones (Fabrello et al., 2023b).

2.2. Tissue collection

Haemolymph was collected from the abdominal segment by a 1-mL plastic syringe and stored in tubes on ice. At each sampling time (7 and 14 days), 5 pools of haemolymph (from four crabs each) for each experimental condition were prepared. After sampling, total haemocyte count (THC), haemocyte diameter and volume, lactate dehydrogenase (LDH) activity and haemocyte proliferation (XTT assay) were measured. The remaining sample of pooled haemolymph was then centrifuged at 780×*g* for 10 min and the pellets (=haemocytes) were resuspended in distilled water to obtain haemocyte lysate (HL), frozen in liquid nitrogen and stored at −80 °C until analyses. Gills and hepatopancreas were excided from crabs and pooled to obtain five different pools of four crabs each, divided into aliquots, frozen in liquid nitrogen, and stored at −80 °C until analyses.

2.3. Haemolymph and haemocyte biomarkers

THC, haemocyte diameter and volume, were determined using a Scepter™ 2.0 Automated Cell Counter (Millipore, FL, USA). In detail, 20 μL of haemolymph were diluted into 2 mL of Coulter Isoton II diluent. The THC was expressed as the number of haemocytes (10^6)/mL of haemolymph, while haemocyte diameter and volume were expressed in μm and picolitres (pL), respectively.

LDH activity in cell-free haemolymph (CFH) was measured using a commercial kit (*Cytotoxicity Detection* Kit, Roche). Briefly, haemolymph was centrifuged at 780×*g* for 10 min, and 500 μL of supernatant (=CFH) was then collected for the assay following the manufacturer's instructions. The results were expressed as optical density (OD) at 490 nm.

Haemocyte proliferation was evaluated using the *Cell proliferation* Kit II. In detail, a volume of 200 μL of the reagent mixture provided by the kit was added to 400 μL of pooled haemolymph and incubated for 4 h. The absorbance at 450 nm was then recorded and results were normalized to THC values of each experimental group and expressed as optical density (OD) at 450 nm.

The lysozyme activity was measured in haemocyte lysate (HL) derived from pooled haemolymph. Briefly, 50 μL of HL was added to 950 μL of a 0.15% suspension of *Micrococcus lysodeikticus* (Sigma) in 66 mM phosphate buffer (pH 6.2), and the decrease in absorbance was continuously recorded at 450 nm for 3 min at room temperature. The results were expressed as μg lysozyme/mg of protein.

The arylsulfatase activity was measured in HL samples according to Zucker-Franklin et al. (1983). The amount of produced p-nitrocatechol was quantified after 1 h at 515 nm using a microplate reader and then calculated using the formula proposed by Baum et al. (1959). Results are expressed as μg of p-nitrocatechol produced per hour/mg of protein.

The acid phosphatase, alkaline phosphatase and phenoloxidase (PO) activity were measured both in HL and CFH. The acid phosphatase hydrolyses the substrate 4-nitrophenyl phosphate during the incubation at 37 °C and the absorbance was read at 405 nm using a microplate reader. Results were expressed as U/mg of protein. Similarly, the alkaline phosphatase hydrolyses the same substrate in an alkaline buffer and after the incubation at 30 °C the absorbance was recorded at 405 nm (Ross et al., 2000). PO activity was quantified using l-DOPA (3,4-dihydroxy-l-phenyl-alanine, Sigma) as substrate in a colorimetric assay. In

detail, one hundred μL of HL and CFH were added to 900 μL of 1 mg L-DOPA/mL of phosphate buffered saline, pH 7.2 and incubated for 30 min at 37 °C. Absorbance at 490 nm was then recorded and results were expressed as U/mg of proteins (Matozzo et al., 2011).

The glucose level in haemolymph was measured using a coupled colorimetric reaction. Indeed, the glucose was oxidized to gluconic acid originating hydrogen peroxide that reacts with o-dianisidine giving a colored compound quantified at 430 nm (Odebunmi and Owalude, 2007). Results were expressed as mg glucose/mL of haemolymph.

2.4. Antioxidant, oxidative damage, and neurotoxicity biomarkers

Gills and hepatopancreas samples were homogenized for 5 min and 50 oscillations per second at 4 °C using TissueLyser LT (Quiagen) in four volumes of 10 mM Tris-HCl buffer, pH 7.5, containing 0.15 M KCl, 0.5 M sucrose, 1 mM EDTA and protease inhibitor cocktail (1:10 v/v) (Sigma-Aldrich). After the centrifugation at 12,000 g for 30 min at 4 °C, supernatants (SN) were collected for analyses.

The total antioxidant capacity in both gills and hepatopancreas was measured according to the CUPRAC method (Apak et al., 2004). In detail, the reaction produced cupric ions that reacted with a specific colorimetric indicator, creating a complex that was measured at 450 nm using a microplate reader. The results are expressed as mM of Trolox equivalents/mg proteins.

Total superoxide dismutase (SOD) activity was measured in both gills and hepatopancreas SN in triplicate using the xanthine oxidase/cytochrome *c* method proposed by Crapo et al. (1978). Enzyme activity was expressed as U/mg proteins, one unit of SOD has been defined as the amount of sample causing 50% inhibition under the assay conditions.

Catalase (CAT) activity was measured in gills and hepatopancreas SN in triplicate following the method proposed by Aebi (1984). The enzyme activity was measured at 240 nm and expressed as U/mg proteins. One unit of CAT was defined as the amount of enzyme that catalysed the dismutation of 1 μmol of H_2O_2/min.

Glutathione reductase (GR) activity was measured in both gills and hepatopancreas SN according to Smith et al. (1988), by measuring the (5-thio (2-nitrobenzoic acid)) TNB production at 412 nm. The enzyme activity was expressed as U/mg proteins.

Glutathione S-transferase (GST) activity was measured in hepatopancreas SN according to the method described in Habig et al. (1974) using 1-chloro-2,4-dinitrobenzene (CDNB) and reduced glutathione (GSH) as substrates. GST activity was expressed as nmol/min/mg proteins.

Selenium-dependent glutathione peroxidase (GPX) activity was measured according to Livingstone et al. (1992), following the oxidation of NADPH at 340 nm by glutathione reductase for the reduction of GSSG. Results are expressed as U/mg proteins. One unit of activity is defined as the amount of enzyme that leads to the oxidation of 1 μmol of NADPH per minute.

Amylolytic enzymes activity was measured in hepatopancreas SN following the method proposed by Oliveira et al. (2019). Briefly, the α-amylase and amyloglucosidase enzymes hydrolysed the starch leading to the disappearance of the blue colour of the iodine-starch complex that was quantified at 580 nm. Results are expressed as U of amylolytic enzymes/mg proteins.

Oxidative damage in both gills and digestive gland was measured through protein carbonyl content (PCC) and lipid peroxidation (LPO) measurement. Briefly, PCC was measured in duplicate using the method of Mecocci et al. (1999) following the reaction with 2,4-dinitrophenylhydrazide (DNPH). Results were expressed as nmol carbonyl group/mg proteins. The LPO was quantified using the malondialdehyde (MDA) assay, according to the method of Buege and Aust (1978). Absorbance was read spectrophotometrically at 532 nm and the results were expressed as nmoles of thiobarbituric reactive substances (TBARS)/mg proteins. TBARS, considered as "MDA-like peroxide products'', were quantified by reference to MDA absorbance ($\varepsilon = 156 \times 10^3\ M^{-1}\ cm^{-1}$) (Damiens et al., 2007).

Acetylcholinesterase (AChE) activity was measured in gills following the colorimetric reaction between acetylthiocholine and the reagent dithiobisnitrobenzoate (Ellman et al., 1961). Absorbance was recorded at 405 nm for 5 min on a microplate reader at room temperature. The results were expressed as nmol/min/mg proteins. Butyrylcholinesterase (BChE) activity was measured according to Escartín and Porte (1997) using butyrylthiocholine as substrate. Results are expressed as nmol/min/mg proteins.

In all assays, total protein concentrations were quantified according to Bradford (1976).

2.5. Physiological biomarkers

The oxygen consumption of six crabs per experimental condition was measured using glass respirometry chambers equipped with an O_2 sensor spot (OXSP5, Pyro Science GmbH, Aachen, Germany). Before starting measurements, individual crabs were placed in chambers filled with 0.45 μm filtered seawater and sealed with a screw cap. One chamber without animals was used as blank. Then the Fiber-Optic Oxygen Meter Piccolo2 (Pyro Science GmbH, Aachen, Germany) was used to detect the O_2 concentration after 30 min. Oxygen saturation never fell below 70%. Values are reported as the O_2 concentration difference between 0 min and 30 min and expressed as the oxygen consumption rate in mg/g/h, after correcting the values with the blank.

Ammonia excretion was measured in 10 mL samples of seawater collected from each respirometry chamber after 30 min. The concentration of ammonia was measured according to Solórzano (1969). Ammonia excretion values were expressed as μmol NH_4/g/h.

2.6. Bioaccumulation

Methanol, acetonitrile, ammonium acetate, BPAF, BPF, BPS and bisphenol A d-16, used as internal standard, were purchased from Merck (Milan, Italy). Ultrapure-grade water (resistivity <18.2 MΩxcm) was produced with a Pure-Lab Option Q apparatus (Elga Lab Water, High Wycombe, UK). Bioaccumulation of bisphenols was evaluated in five single organisms collected at the 7 and 14 days of exposure. Each crab tissue sample was accurately weighed and homogenized (Homogeniser SHM1, Avantor, VWR International Srl, Milano, Italia) after the addition of 1 mL of ultrapure water. The homogenate was treated with 7 mL of cold acetonitrile containing the internal standard at 500 μg/L, vortexed for 3 min, and centrifuged for 5 min at 3000×*g*. After a further centrifugation step (11000×*g*, 10 min, 4 °C), 20 μL of the supernatant were analysed by UHPLC-HRMS. The system was equipped with an Agilent 1260 Infinity II LC chromatographer coupled to an Agilent 6545 LC-Q-TOF mass analyser (Agilent Technologies, Palo Alto, Santa Clara, CA, USA). The analytical column was a Kinetex 2.6 μm C18 Polar, 100 A, 100 × 2.1 mm (Phenomenex, Bologna, Italy), at 25 °C. Mobile phases A and B were water and acetonitrile, respectively, both containing 10 mM ammonium acetate and the eluent flow rate was 0.30 mL/min. The mobile phase gradient profile was as follows (t in min): $t_{0–4}$ 0% B; $t_{4–22}$ 0–100% B, $t_{22–25}$ 100% B; $t_{25–32}$ 0% B. The MS conditions were: electrospray (ESI) ionization in negative mode, gas temperature 320 °C, drying gas 12 L/min, nebulizer 35 psi, sheath gas temperature 350 °C, sheath gas flow 11 L/min, VCap 5000 V, nozzle voltage 0 V, fragmentor 150 V. Centroid full scan mass spectra were recorded in the range 100–1000 *m/z* with a scan rate of 2 spectra/s. The QTOF calibration was performed daily with the manufacturer's solution in this mass range. The MS results were analysed by the Mass Hunter Qualitative Analysis 10.0 software (Agilent Technologies, Palo Alto, Santa Clara,CA, USA).

Homogenates from no-treated organisms were used to build a matrix-matched seven-point external calibration curve, in the range 0.1–100 μg/L (corresponding to 0.8–800 ng/g in the initial animal tissues). Linearity was evaluated by the least squares regression and $R^2 >$ 0.998 was obtained for all the analytes. LODs, assessed from the lowest

calibration point as concentration corresponding to S/N = 3, were 40 ng/g for BPF, 4 ng/g for BPS and 1 ng/g for BPAF. The precision of the method, assessed by the relative standard deviation of the IS chromatographic peak area in samples (N = 40), was lower than 10%. As already reported, each treated organism was analysed separately, and results are reported as mean and standard deviation (N = 5).

2.7. Statistical analysis

The normal distribution of data (Shapiro-Wilk's test) and the homogeneity of the variances (Bartlett's test) were assessed. The two-way ANOVA analysis was performed to highlight the effects of the independent factors "exposure time", "treatment" and "exposure time-treatment interaction" on biomarkers. Pairwise comparisons among experimental conditions were performed using Fisher's LSD post-hoc test. The significant difference was set at $p < 0.05$. All results are reported as boxplots showing the median and the interquartile range (N = 5 for all biomarkers, except for physiological biomarkers that had N = 6). In addition, a Principal Component Analysis (PCA) was performed using cellular and biochemical biomarkers. Lastly, the IBRv2 values for each sampled tissue (haemolymph, gills and hepatopancreas) were calculated using "IBRtools" (Resende and Pereira, 2023) and according to the method proposed by Sanchez et al. (2013). The software packages OriginPro 2023, R and Rstudio software version 4.2.3 (OriginPro, 2023; R Core Team, 2022; RStudio Team, 2023) were used for the statistical analyses.

3. Results

A table summarising all the results of the two-way ANOVA analysis is reported in Supplementary material (SM1).

3.1. Haemolymph and haemocyte biomarkers

Statistical analysis revealed that the independent factors "treatment" ($p < 0.05$), "exposure time" ($p < 0.01$), and their "interaction" ($p < 0.01$) significantly affected THC in crabs.

The pairwise comparison revealed that BPAF and MIX caused a significant decrease in THC after 7 days of exposure when compared to the related control. On the contrary, after 14 days, significantly increased THC values were recorded in BPAF-treated crabs, compared to controls (Fig. 1A).

No significant effects of the experimental conditions tested on haemocyte volume and diameter were recorded (data not shown).

Similarly, no cytotoxic effects of BPA analogues were observed (data not shown). Indeed, LDH activity was affected only by the factor "exposure time" ($p < 0.001$).

Cellular proliferation was influenced by all the independent factors, including their "interaction" ($p < 0.001$). The post-hoc test revealed that there was an increased haemocyte proliferation in crabs exposed for 14 days to BPS and MIX (Fig. 1B).

HL lysozyme activity, as well as arylsulfatase and acid phosphatase activity, were not affected in both HL and CFH by exposure to bisphenols (data not shown), whereas alkaline phosphatase activity was influenced only by the factor "exposure time" in both HL ($p < 0.05$) and CFH ($p < 0.01$) (data not shown).

Interestingly, no significant effects of independent factors were observed in HL PO activity, whereas the factor "treatment" significantly affected PO enzyme activity in CFH ($p < 0.05$). In detail, the post-hoc test revealed an increased activity in CFH of crabs exposed for 14 days to BPAF (Fig. 1C).

As for haemolymph glucose level, only the factor "exposure time" influenced such parameter ($p < 0.01$).

3.2. Antioxidant, oxidative damage, and neurotoxicity biomarkers

Gill total antioxidant capacity measured according to the CUPRAC method was altered by the factors "exposure time", "treatment" ($p < 0.001$), and their "interaction" ($p < 0.01$), while in hepatopancreas the CUPRAC levels were altered only by the factor "treatment" ($p < 0.05$). The post-hoc test showed that BPS and MIX caused a significant reduction in gill CUPRAC levels after 7 days of exposure, with respect to the related control, while increased CUPRAC levels were found after 14 days of exposure of crabs to BPF (Fig. 2A). As for hepatopancreas, exposure

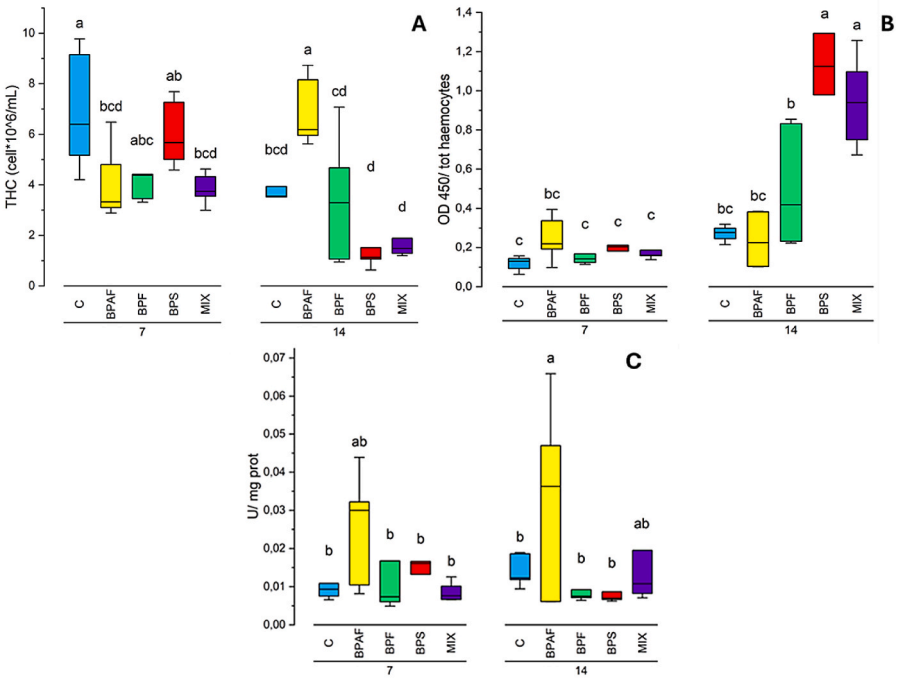

Fig. 1. Boxplots of total haemocyte count (THC), expressed as n° haemocytes (10^6)/mL haemolymph (A), haemocyte proliferation, expressed as OD_{450} (B), and phenoloxidase activity in CFH, expressed as U/mg proteins (C) results. Different letters indicate significant differences among experimental conditions. N = 5.

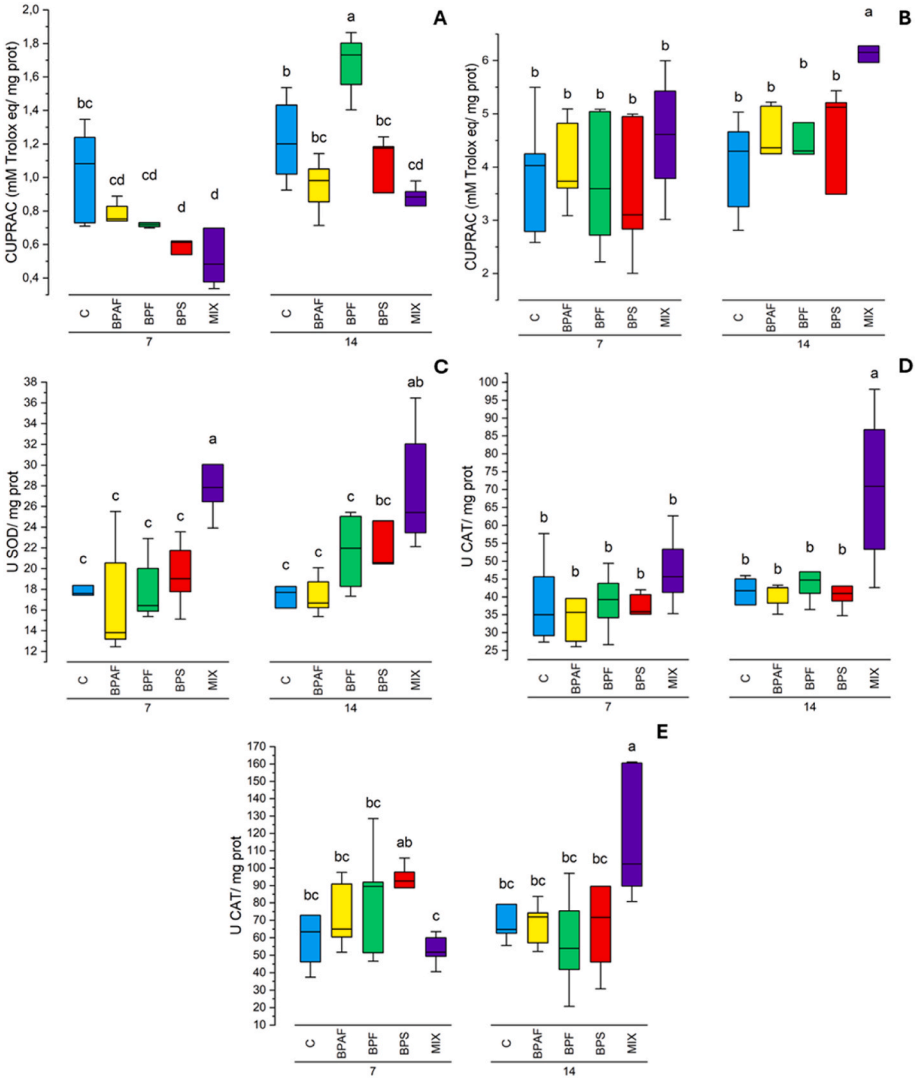

Fig. 2. Boxplots of the results of CUPRAC in gills (A) and hepatopancreas (B), expressed as mM of Trolox equivalents/mg proteins, SOD activity in hepatopancreas (C), expressed as U/mg proteins, and CAT activity in gills (D) and hepatopancreas (E), expressed as U/mg proteins. Different letters indicate significant differences among experimental conditions. N = 5.

for 14 days to MIX caused an increase in CUPRAC levels, when compared to the related control (Fig. 2B).

Hepatopancreas SOD activity was significantly affected by the factor "treatment" ($p < 0.001$). According to the post-hoc results, exposure to MIX significantly increased SOD activity after both 7 and 14 days (Fig. 2C), when compared to the related controls. On the contrary, gill SOD activity was altered only by the factor "exposure time" ($p < 0.001$) (data not shown).

CAT activity was altered in gills by the factors "treatment" ($p < 0.001$) and "exposure time" ($p < 0.05$), while in hepatopancreas the factor "treatment-time interaction" significantly influenced enzyme activity ($p < 0.01$). The post-hoc test revealed a significant increase in CAT activity in the gills of MIX-treated crabs after 14 days of exposure (Fig. 2D). Similarly, in hepatopancreas, exposure to MIX significantly increased enzyme activity after 14 days, with respect to the related control (Fig. 2E).

As for enzymes involved in the glutathione cycle, all those tested in this study were significantly affected by exposure to bisphenols. In gills, the factors "treatment" ($p < 0.01$) and "treatment-exposure time interaction" significantly affected ($p < 0.05$) GR activity, with a significant increase after 7 days of exposure in BPF-treated crabs, when compared to the related control (Fig. 3A). Similarly, significant effects of the factors "exposure time" ($p < 0.001$), "treatment" ($p < 0.05$) and their interaction ($p < 0.001$) were recorded in hepatopancreas, with a significant increase in GR activity in crabs exposed for 7 days to BPF and for 14 days to MIX (Fig. 3B).

Gill GPX activity was affected significantly by the factors "exposure time", "treatment" ($p < 0.01$) and their "interaction" ($p < 0.05$), while there was a significant effect of the factors "treatment" and "exposure time-treatment interaction" ($p < 0.01$) on hepatopancreas enzyme activity. In detail, there was an increase in enzyme activity in gills from MIX-treated crabs after both 7 and 14 days of exposure, with respect to the related controls (Fig. 3C). In hepatopancreas, BPF and MIX caused a statistically significant increase in GPX activity after 7 days, while BPS increased enzyme activity after 14 days of exposure when compared to the related controls (Fig. 3D).

GST activity was affected significantly only by the factor "exposure time-treatment interaction" ($p < 0.05$), with a significant reduction in enzyme activity after 14 days of exposure of crabs to MIX (Fig. 3E).

As for oxidative damage biomarkers, PCC was significantly affected in gills by both the factors "exposure time" ($p < 0.05$) and "treatment" ($p < 0.01$). The post-hoc test revealed a statistically significant increase in PCC values in crabs exposed for 7 days to BPAF, and a significant reduction in animals exposed for 14 days to BPS and MIX (Fig. 4A). In

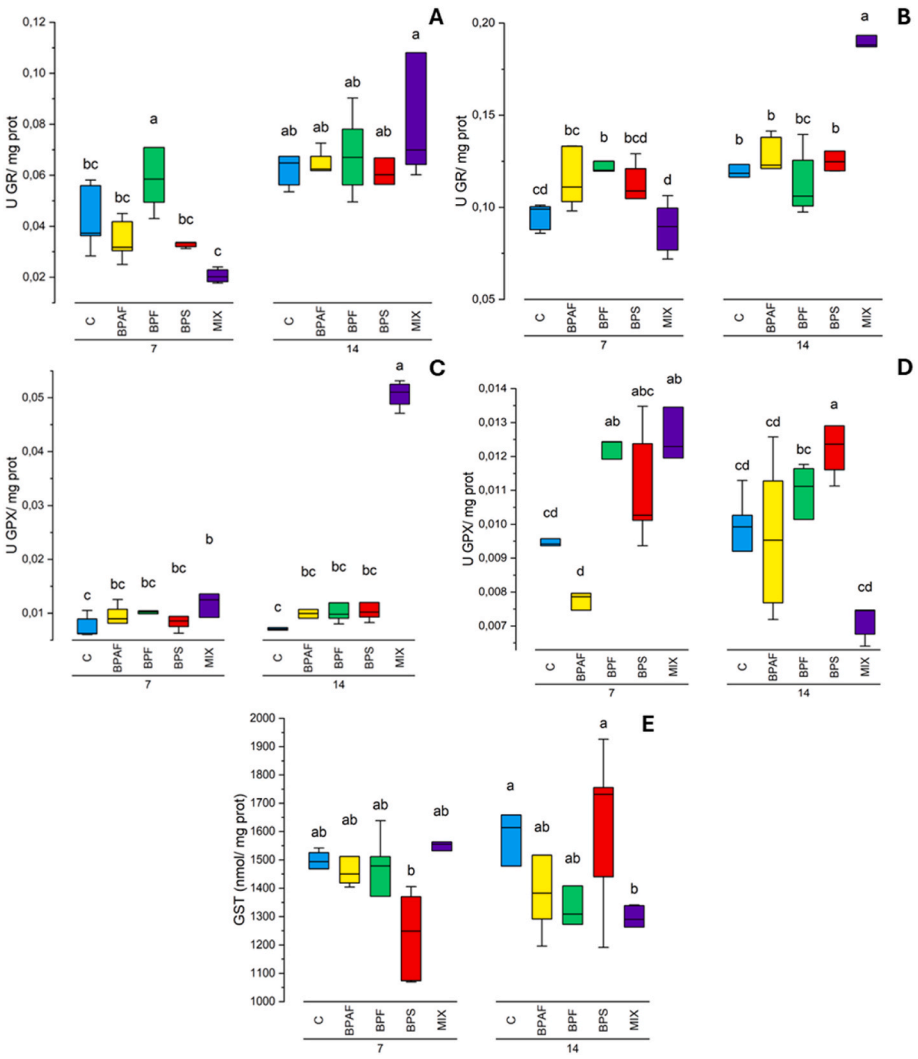

Fig. 3. Boxplots of the results of GR activity in gills (A) and hepatopancreas (B), expressed as U/mg proteins, GPX activity in gills (C) and hepatopancreas (D), expressed as U/mg proteins, and GST activity in hepatopancreas (E), expressed as nmol/mg proteins. Different letters indicate significant differences among experimental conditions. N = 5.

hepatopancreas, the PCC values were affected by all the independent factors ($p < 0.001$), and the post-hoc test revealed a significant increase of PCC values after 14 days of exposure of crabs to BPF and MIX, when compared to the related control (Fig. 4B).

Regarding LPO levels, they were influenced in crab gills only by the factor "exposure time-treatment interaction" ($p < 0.05$). The post-hoc test revealed a significant increase in lipid peroxidation in crabs exposed for 7 days to BPF and a significant reduction in crabs exposed for 14 days to the MIX (Fig. 4C).

The possible neurotoxic effects of BPA analogues were assessed by measuring the activity of both AChE and BChE. As for AChE, we observed that the factors "exposure time" ($p < 0.01$) and "treatment" ($p < 0.05$) had a significant effect on enzyme activity. In detail, the post-hoc test revealed that BPF induced a significant increase in enzyme activity after both 7 and 14 days of exposure (Fig. 5). On the contrary, BChE activity was affected by the factor "exposure time" ($p < 0.01$) (graph not shown).

The PCA performed using cellular and biochemical biomarkers (Fig. 6) revealed that the two components explained 18,27% and 11,57% of the variance, respectively. A clear separation between the two exposure times was observed. Regarding data clusterization, there was a clear cluster of biomarker results of the BPF group at 7 days and of the MIX group at 14 days. Moreover, to better rank the general toxicity of BPA analogues the IBRv2 index was calculated for each tested compound and for each exposure time. Due to the high number of biomarkers measured in this study, it was not possible to calculate a single comprehensive index (R Core Team, 2022; RStudio Team, 2023). For this reason, the IBRv2 values were calculated for each tissue analysed. A table reporting the IBRv2 values and radar charts is provided in supplementary materials (SM2 and SM3-SM8). Based on the IBRv2 results, BPAF seems to cause marked alterations in crab haemolymph after 7 days of exposure, with a value of 27.732, while BPS altered markedly haemolymph parameters after 14 days of exposure, with an IBRv2 value of 21.259. As for biomarkers measured in gills and hepatopancreas, exposure to the MIX caused the most important alterations after both 7 and 14 days. Indeed, MIX-associated IBRv2 values in gills were 13.329 and 15.519, respectively, while in the hepatopancreas were 9.218 and 14.486. These results suggest that BPA analogues can exert synergic interaction if present in a mixture.

3.3. Physiological biomarkers

The respiration rate of crabs was affected by the factor "treatment" ($p < 0.05$), while the excretion rate was affected by the factors "exposure time" ($p < 0.01$) and "treatment" ($p < 0.05$). The post-hoc test revealed that exposure to the MIX significantly increased crab respiration rate

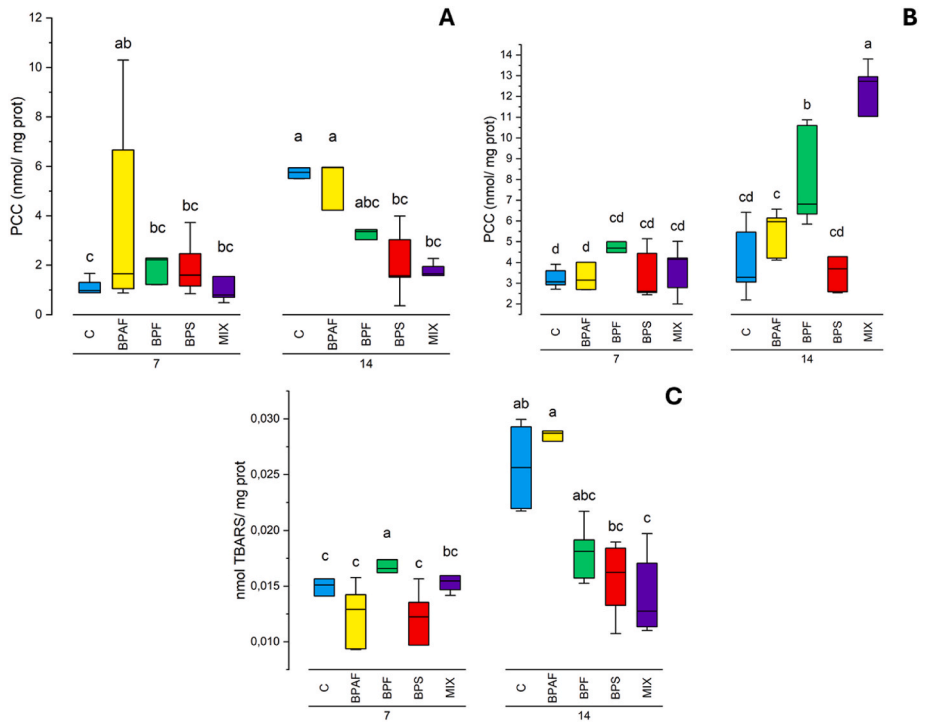

Fig. 4. Boxplots of PCC in gills (A) and hepatopancreas (B), expressed as nmol/mg proteins, and LPO in gills (C), expressed as nmol TBARS/mg proteins. Different letters indicate significant differences among experimental conditions. N = 5.

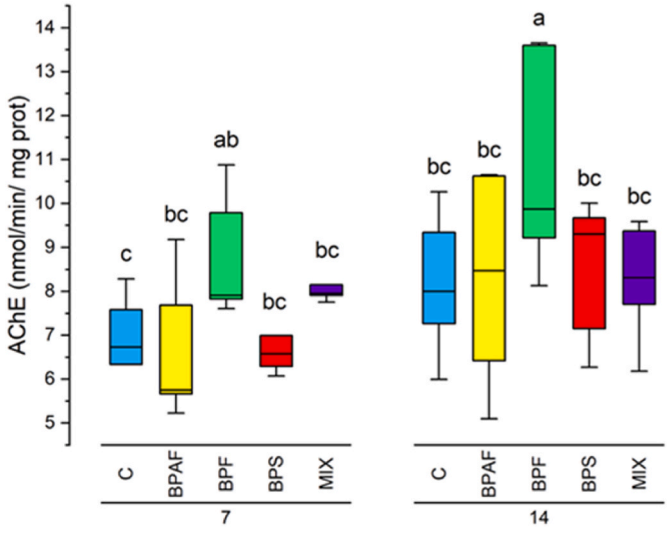

Fig. 5. Boxplots of the results of AChE activity in gills, expressed as nmol/min/mg proteins. Different letters indicate significant differences among experimental conditions. N = 5.

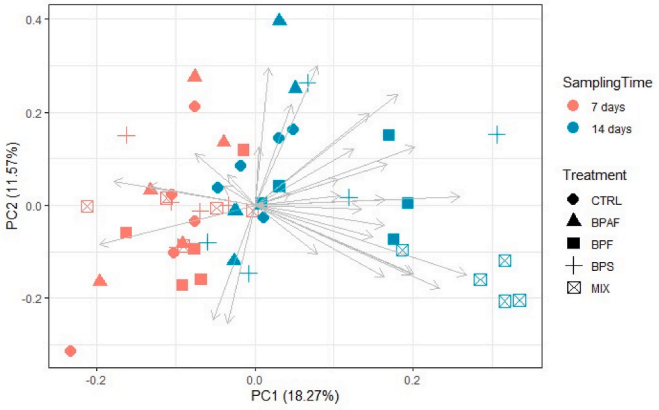

Fig. 6. PCA ordinations of the whole data set, including biomarkers measures in crabs and BPA analogues concentrations.

after 14 days of exposure (Fig. 7A). On the contrary, pairwise comparison did not highlight significant differences in excretion rate among experimental conditions (Fig. 7B).

3.4. Bioaccumulation

Chemical analysis indicated that only BPAF was bioaccumulated in crabs (Table 1), with concentrations depending on the exposure time. In particular, after 7 days of exposure the concentration in animal tissues was 3.8 ng/g f.w., increasing to 6 ng/g f.w. after 14 days of exposure. The biological variability was negligible, as the relative standard deviation among different organisms was always lower than 20%. Concentrations of BPF and BPS were under the LOD.

4. Discussion

Since there is not much data available in the literature on the effects of BPA analogues in crabs or other crustacean species, the discussion of the results of the present study was mainly made based on information available for other aquatic invertebrate species and for the most studied compound, namely BPA.

4.1. Haemolymph and haemocyte biomarkers

In this study, we evaluated the effects of an environmentally realistic concentration of three BPA analogues on the crab *C. aestuarii* following 7 and 14 days of exposure. Overall, the tested compounds were able to alter most of the haemolymph/haemocyte parameters. Indeed, THC

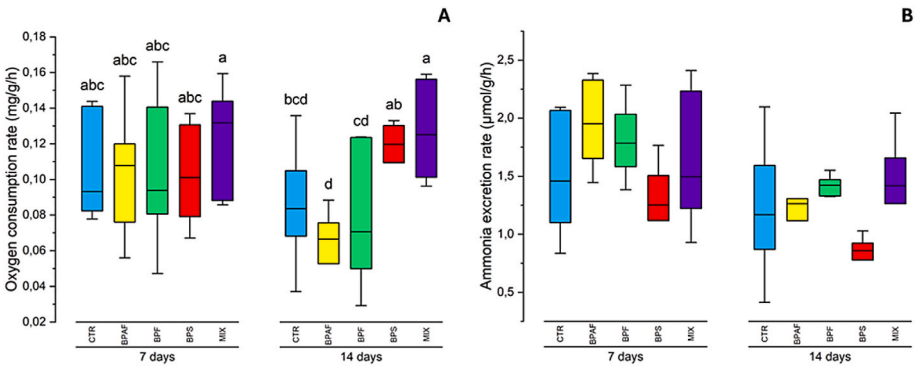

Fig. 7. Boxplots of respiration rate (A), expressed as mg/g/h, and excretion rate (B), expressed as µmol/g/h, results. Different letters indicate significant differences in comparison with the related control. N = 6.

Table 1
Concentrations of BPA analogues in whole soft tissues of crabs. Results are reported as mean ± standard deviation (N = 5) and expressed as ng/g of fresh weight (ng/g f.w.). LODs were 1 ng/g for BPAF, 40 ng/g for BPF and 4 ng/g for BPS.

		T = 7 days	T = 14 days
BPF [ng/g]		<40	<40
BPS [ng/g]		<4	<4
BPAF [ng/g]		3.8 (0.3)	6.0 (1.2)
MIX	BPF [ng/g]	<40	<40
	BPS [ng/g]	<4	<4
	BPAF [ng/g]	<1	<1

assay results indicated that BPAF and the mixture of bisphenols reduced the number of circulating haemocytes after 7 days of exposure. Similarly, Juhel et al. (2017) reported a reduction in haemocyte number in the green mussel *Perna viridis* exposed to 11 ng/L of BPA and a reduction of the extracellular lysozyme activity after 7 days. However, we observed a recovery of the THC value after two weeks of exposure in BPAF-treated crabs, with a significantly increased value with respect to the control. In a study on the clam *Tegillarca granosa*, exposure for two weeks to 10 and 100 ng/L of BPA caused a significant decrease in THC value. In the same study, cell type fractions changed in clams exposed to 100 ng/L of BPA and the phagocytic activity was suppressed in a dose-dependent manner, as well as the expression of four immune-related genes (Tang et al., 2020). Furthermore, a significant reduction of THC values was found in the shrimp *Charybdis japonica* exposed to BPA (Peng et al., 2018).

As for haemocyte features, we did not observe significant alterations in the mean haemocyte volume and diameter in crabs. Conversely, in a previous study performed by exposing the clam *Ruditapes philippinarum* to the same experimental conditions of the present survey (the same BPA analogue concentrations and exposure duration), we observed that both haemocyte diameter and volume were reduced by BPF, BPS and MIX after one week (Fabrello et al., 2023b). Furthermore, in the present study, BPA analogues did not exert cytotoxic effects on crab haemocytes, whereas caused an increase in haemocyte proliferation, mainly after 14 days of exposure to BPS and MIX. Similarly, in clams (*R. philippinarum*) exposed to BPA analogues, no cytotoxic effects (LDH assay) were detected, whereas cell proliferation increased after exposure to the MIX (Fabrello et al., 2023b). The increased cell proliferation observed in 14 days-exposed crabs suggested that animals were trying to compensate for the initial reduction in the number of circulating haemocytes recorded during exposure.

Regarding the immune response-related enzymes, slight alterations were recorded in crabs. In particular, an increase in the activity of PO was observed in CFH of crabs exposed for 14 days to BPAF. PO activity also increased in haemolymph of *Charibdys japonica* exposed to BPA (Peng et al., 2018). In addition, in that study lysozyme activity increased significantly after exposure to 0.25 mg/L of BPA, while it reduced to the two highest concentrations tested. On the contrary, in the narrow-clawed crayfish *Pontastacus leptodactylus*, lysozyme activity did not change after exposure to high concentrations of BPA (Diler et al., 2022). An alteration of the PO activity and the expression of immune-related genes was also observed in both gills and hepatopancreas of the intertidal mud crab *Macrophthalmus japonicus* exposed to BPA (Park et al., 2019). Similarly, altered immune-related enzymes were observed in *R. philippinarum* exposed to BPAF, BPF BPS and their mixture. Indeed, the acid phosphatase activity in CFH dropped after exposure to BPAF, BPF, BPS and MIX (Fabrello et al., 2023b). Moreover, the arylsulfatase activity increased in clams exposed to BPF and BPS (Fabrello et al., 2023b). Lastly, in the hepatopancreas of the crayfish *Procambarus clarkii* both acid and alkaline phosphates activity were significantly decreased in the animals exposed to 1, 10 and 100 µg/L of BPS for 14 days (Pu et al., 2024).

Overall, the results of the present study highlight that exposure to BPA analogues can alter some important haemocyte parameters in crabs.

4.2. Antioxidant and oxidative damage biomarkers

In this study, the total antioxidant capacity (CUPRAC levels) of crab gills was significantly reduced after 7 days of exposure to BPS and MIX, whereas increased CUPRAC levels were recorded after 14 days of exposure to BPF. In crab hepatopancreas, a similar pattern of variation was observed after 14 days of exposure to MIX.

As for antioxidant enzyme activity, exposure for 7 and 14 days to MIX significantly increased SOD activity in crab hepatopancreas. Similarly, CAT activity increased significantly in both gills and hepatopancreas of MIX-treated crabs after 14 days. These results suggested that BPA analogues were able to cause oxidative stress, the activity of these two important antioxidants increasing probably to counteract oxidative stress, mainly in MIX-treated crabs. Interestingly, in the present study, all the enzymes involved in the glutathione cycle were significantly affected by exposure of crabs to BPA analogues. As for GR activity, BPF significantly increased gill enzyme activity after 7 days of exposure, while GR activity increased in hepatopancreas after 7 days of exposure to BPF and after 14 days in the MIX-treated crabs. In the case of GPX, enzyme activity increased in the gills of crabs exposed for 7 and 14 days to the MIX. In hepatopancreas, exposure for 7 days of crabs to BPF and MIX caused a statistically significant increase in GPX activity, while BPS increased enzyme activity after 14 days of exposure. Lastly, GST activity was significantly reduced by MIX after two weeks of exposure. The variation pattern of GPX activity is in accordance with that of the other antioxidant enzymes, the activity of which resulted significantly increased after BPA analogue exposure. In addition, the GR activity increased, as observed in both gills and hepatopancreas of the exposed

crabs, suggesting that the animals have to reconstitute the GSH level, which was probably affected by bisphenols, as revealed by the CUPRAC results.

An impairment of the antioxidant system in crustaceans was also recorded in BPA-exposed animals, even if higher concentrations were generally tested in previous studies with respect to those tested in the present survey. For instance, SOD, CAT and GPX activity were significantly increased in the hepatopancreas of the crab *Charybdis japonica* exposed to BPA (Peng et al., 2018). Increased production of reactive oxygen species (ROS) was observed in the hepatopancreas of *P. clarkii* after one week of exposure to BPA, followed by a decrease in SOD, CAT and GPX activities (Zhang et al., 2020). In the same species, exposure to BPS caused a significant reduction of SOD and CAT activity as well as GSH levels (Pu et al., 2024). Furthermore, SOD, GST, and GR activities were significantly decreased in the narrow-clawed crayfish *Pontastacus leptodactylus* exposed to 10–100 μg/L of BPA (Diler et al., 2022). Uçkun (2022) reported a significant increment of SOD, GPX and GST activities in *Astacus leptodactylus* exposed to BPA, while GR activity reduced significantly.

In a long-term exposure to BPA (28 days), alterations in SOD activity and GSH levels were observed in the ganglia of the mussel *Lithophaga lithophaga* (Abd Elkader and Al-Shami, 2023). In the marine rotifer *Brachionus koreanus*, exposure for 24 h to BPS and BPF increased both ROS and GST levels (Park et al., 2018). In *Corbicula fluminea*, exposure to BPS increased significantly GR activity, while CAT activity and LPO levels remained unchanged (Seoane et al., 2021). In *R. philippinarum,* it has been demonstrated a significant reduction of gill total antioxidant capacity after 14 days of exposure to 300 ng/L of BPS and MIX (BPS + BPF + BPAF) (Fabrello et al., 2023b). Overall, the results of the present study suggested that BPA analogues can induce important alterations in the antioxidant defence line of crabs. However, further studies are necessary to better understand the relationship between exposure to such contaminants and oxidative stress in crabs.

As for oxidative damage biomarkers, in this study, PCC levels were significantly increased in gills after exposure for 7 days to BPAF, while a significant reduction was observed after 14 days of exposure of crabs to BPS and MIX. In hepatopancreas, PCC values significantly increased following exposure for 14 days of crabs to BPF and MIX. Regarding LPO, we demonstrated that exposure of crabs for 7 days to BPF increased gill LPO levels, whereas exposure to MIX caused a reduction after 14 days. We have recently demonstrated that exposure to BPA analogues caused lipid peroxidation in *R. philippinarum*, with LPO levels increasing in both gills and digestive gland (Fabrello et al., 2023b). Increased oxidative damage was also observed in both the respiratory tree and digestive tubes of *Holothuria poli* after exposure to BPA, BPS, and their mixture (Jenzri et al., 2023). Lastly, LPO levels increased significantly in the crab *Charybdis japonica* exposed to BPA (Peng et al., 2018). Overall, the results of this study and those available in the literature suggested that BPA and its analogues can induce oxidative damage in aquatic invertebrates, although the extent of such effects depends greatly on the experimental conditions adopted (e.g., tested concentrations, duration of exposure, model species).

Based on the results obtained in our study, we can exclude that BPA analogues are neurotoxic to crabs, at least under the experimental conditions tested (AChE and BChE activities). However, further studies are necessary to better understand the neurotoxic profile of such contaminants in aquatic species. In the present study, we observed a significant increase in AChE activity in gills from crabs exposed for 7 and 14 days to BPF. Conversely, AChE and carboxylesterase activities decreased significantly in the hepatopancreas of the crayfish *A. leptodactylus* exposed to BPA (Uçkun, 2022). However, it is important to highlight that the concentrations of BPA tested in that study were much higher than those tested in the present survey. A significant reduction in AChE activity was also observed in the claw muscles of the artic spider crab *Hyas araneus* after 3 weeks of exposure to 50 μg/L of BPA (Minier et al., 2008). Furthermore, exposure to BPA, BPS and their mixture caused a significant inhibition of AChE activity in the respiratory tree of the sea cucumber *Holothuria poli* (Jenzri et al., 2023).

Overall, we can exclude that exposure to BPA analogues causes neurotoxic effects in crabs, at least under the experimental conditions adopted in our survey. At the same time, we highlight that neurotoxic effects have been detected in previous studies in which organisms were exposed to higher BPA and BPA analogue concentrations than those tested in our study.

4.3. Physiological parameters

In this study, we measured two pivotal physiological parameters, namely respiration rate and excretion rate, to evaluate possible negative effects of BPA analogues on the physiological performance of crabs. The respiration rate of crabs significantly increased after exposure for 14 days to MIX, suggesting an increased metabolism. On the contrary, no significant alteration in excretion rate was found. To the best of our knowledge, this is one of the first reports concerning the effects of bisphenols on the respiration rate of crabs. In a previous study, exposure for 14 days of the white-leg shrimp *Litopenaeus vannamei* to 2 μg/L of BPA, 0.69 mg/L of polystyrene microplastics, or their mixture reduced oxygen consumption rate in both males and females, while ammonia excretion rate significantly increased in males (Han et al., 2022). As for other contaminants, previous studies focused on evaluating the effects of heavy metals and polycyclic aromatic hydrocarbons (PAH) on the physiological responses of animals (Bao et al., 2020; Camus et al., 2002; Dissanayake et al., 2008). For instance, in the freshwater crayfish *Cambaroides dauricus,* both acute and sub-chronic exposure to Cu caused a reduction in oxygen consumption rate and ammonium excretion rate. Interestingly, the authors concluded that the shift in O:N ratio indicated a metabolic substrate shift towards lipid and carbohydrate metabolism under Cu exposure (Bao et al., 2020). In a recent study, a long-term exposure of the mussels *Lithophaga lithophaga* to 0.25, 1, 2, and 5 μg/L of BPA caused a reduction of both filtration rate and burrowing behaviour (Elkader and Al-Shami, 2024). Overall, higher oxygen consumption found in the present study in crabs exposed to BPA analogues suggests the animals increased metabolism and expended more energy to counteract the effects of exposure.

4.4. Bioaccumulation

C. aestuarii showed a reduced capability to bioaccumulate the three BPA analogues tested in this study. Indeed, only BPAF was detected in whole soft tissues of crabs, in the order of a few ng/g. These findings are consistent with the larger hydrophobicity of BPAF (log K_{ow} 3,69), with respect to that of BPF and BPS (log K_{ow} 2,91 and 1,29, respectively) (Fabrello and Matozzo, 2022). However, the low bioaccumulation is not surprising, since it has been demonstrated that BPA analogues are relatively quickly metabolized by organisms and transformed into bisphenol conjugates. Indeed, in a recent study conducted on marine organisms, it was demonstrated that after an initial enzymatic hydrolysis to cleave the conjugates, the detectable concentrations of the free-form BPA analogues increased (Zhao et al., 2021). In detail, BPA, BPZ, BPF, BPS, and BPAF were present in conjugated forms with an average percentage of 74, 52, 49, 48, and 45%, respectively (Zhao et al., 2021). In our previous study, we demonstrated that the clam *R. philippinarum* can bioaccumulate BPA analogues at a higher extent (hundreds of ng/g) (Fabrello et al., 2023b) than crabs (the present survey). Probably, the difference in bioaccumulation capability between bivalves and crustaceans is due to their different lifestyle, the former being excellent filter feeders, while in crabs and other larger decapods filter feeding is of minor importance and restricted to capture of nutrient-rich zooplankton (Riisgård, 2015).

5. Conclusions

In conclusion, the results of this study demonstrated that environmentally realistic concentrations of BPA analogues can induce significant alterations in cellular, biochemical, and physiological parameters of the marine crab *C. aestuarii*. However, we are aware that other studies are necessary to evaluate the ecotoxicological risk posed by these emerging contaminants to aquatic species, not just marine ones. We also suggest that future studies should adopt a multi-biomarker approach like that of the present study to evaluate more fully the effects of these compounds on aquatic species. Our study demonstrated also that crabs, at least under the experimental conditions tested, do not bioaccumulate high levels of BPA analogues. It will be interesting to assess whether the same contaminants provided via contaminated food can induce greater bioaccumulation. To this end, new studies are already underway in our laboratories.

Since most of the effects were observed in crabs exposed to MIX, we think that it will be important in the future to evaluate the effects of mixtures of contaminants to better reproduce in the laboratory the environmental conditions to which the organisms are subjected. At the same time, we think that it will also be important to evaluate the combined effects of contaminants and changing environmental parameters (temperature, salinity, oxygen, pH and so on) to better understand the mechanisms of action of emerging environmental contaminants, such as BPA analogues, in a context of climate change.

CRediT authorship contribution statement

Jacopo Fabrello: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Maria Ciscato:** Methodology, Investigation. **Davide Asnicar:** Methodology, Formal analysis. **Jacopo Giorgi:** Methodology, Investigation. **Marco Roverso:** Writing – original draft, Methodology, Investigation, Conceptualization. **Sara Bogialli:** Writing – original draft, Methodology, Investigation, Conceptualization. **Valerio Matozzo:** Writing – original draft, Supervision, Project administration, Conceptualization.

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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.marenvres.2024.106800.

Data availability

The data that has been used is confidential.

References

Abd Elkader, H.-T.A.E., Al-Shami, A.S., 2023. Chronic exposure to bisphenol A induces behavioural, neurochemical, histological, and ultrastructural alterations in the ganglia tissue of the date mussels *Lithophaga lithophaga*. Environ. Sci. Pollut. Res. Int. 30, 109041–109062. https://doi.org/10.1007/s11356-023-29853-3.

Aebi, H., 1984. [13] Catalase *in vitro*. In: Methods in Enzymology, Oxygen Radicals in Biological Systems. Academic Press, pp. 121–126. https://doi.org/10.1016/S0076-6879(84)05016-3.

Andújar, N., Gálvez-Ontiveros, Y., Zafra-Gómez, A., Rodrigo, L., Álvarez-Cubero, M.J., Aguilera, M., Monteagudo, C., Rivas, A., 2019. Bisphenol A analogues in food and their hormonal and obesogenic effects: a review. Nutrients 11, 2136. https://doi.org/10.3390/nu11092136.

Apak, R., Güçlü, K., Özyürek, M., Karademir, S.E., 2004. Novel total antioxidant capacity index for dietary polyphenols and vitamins C and E, using their cupric ion reducing capability in the presence of neocuproine: CUPRAC method. J. Agric. Food Chem. 52, 7970–7981. https://doi.org/10.1021/jf048741x.

Bao, J., Xing, Y., Feng, C., Kou, S., Jiang, H., Li, X., 2020. Acute and sub-chronic effects of copper on survival, respiratory metabolism, and metal accumulation in *Cambaroides dauricus*. Sci. Rep. 10, 16700. https://doi.org/10.1038/s41598-020-73940-1.

Baum, H., Dodgson, K.S., Spencer, B., 1959. The assay of arylsulphatases A and B in human urine. Clin. Chim. Acta 4, 453–455. https://doi.org/10.1016/0009-8981(59)90119-6.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254. https://doi.org/10.1016/0003-2697(76)90527-3.

Buege, J.A., Aust, S.D., 1978. Microsomal lipid peroxidation. Methods Enzymol. 52, 302–310. https://doi.org/10.1016/S0076-6879(78)52032-6.

Camus, L., Jones, M.B., Børseth, J.F., Regoli, F., Depledge, M.H., 2002. Heart rate, respiration and total oxyradical scavenging capacity of the Arctic spider crab, *Hyas araneus*, following exposure to polycyclic aromatic compounds via sediment and injection. Aquat. Toxicol. Amst. Neth. 61, 1–13. https://doi.org/10.1016/s0166-445x(02)00013-9.

Chen, D., Kannan, K., Tan, H., Zheng, Z., Feng, Y.-L., Wu, Y., Widelka, M., 2016. Bisphenol analogues other than BPA: environmental occurrence, human exposure, and toxicity—a review. Environ. Sci. Technol. 50, 5438–5453. https://doi.org/10.1021/acs.est.5b05387.

Crapo, J.D., McCord, J.M., Fridovich, I., 1978. [41] Preparation and assay of superioxide dismutases. In: Fleischer, S., Packer, L. (Eds.), Methods in Enzymology, Biomembranes - Part D: Biological Oxidations. Academic Press, pp. 382–393. https://doi.org/10.1016/S0076-6879(78)53044-9.

Damiens, G., Gnassia-Barelli, M., Loquès, F., Roméo, M., Salbert, V., 2007. Integrated biomarker response index as a useful tool for environmental assessment evaluated using transplanted mussels. Chemosphere 66, 574–583. https://doi.org/10.1016/j.chemosphere.2006.05.032.

Diler, Ö., Özil, Ö., Nane, I.D., Naziroglu, M., Minaz, M., Aslankoc, R., Ozmen, O., Atsatan, K., 2022. The effects of bisphenol A on oxidative stress, antioxidant defence, histopathological alterations and lysozyme activity in narrow-clawed crayfish (*Pontastacus leptodactylus*). Turk. J. Fish. Aquat. Sci. 22. https://doi.org/10.4194/TRJFAS19877.

Dissanayake, A., Galloway, T.S., Jones, M.B., 2008. Physiological responses of juvenile and adult shore crabs *Carcinus maenas* (Crustacea: Decapoda) to pyrene exposure. Mar. Environ. Res. 66, 445–450. https://doi.org/10.1016/j.marenvres.2008.07.006.

Elkader, H.-T.A.E.A., Al-Shami, A.S., 2024. Acetylcholinesterase and dopamine inhibition suppress the filtration rate, burrowing behaviours, and immunological responses induced by bisphenol A in the hemocytes and gills of date mussels, *Lithophaga lithophaga*. Aquat. Toxicol. 272, 106971. https://doi.org/10.1016/j.aquatox.2024.106971.

Ellman, G.L., Courtney, K.D., Andres, V., Featherstone, R.M., 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem. Pharmacol. 7, 88–95. https://doi.org/10.1016/0006-2952(61)90145-9.

Escartín, E., Porte, C., 1997. The use of cholinesterase and carboxylesterase activities from *Mytilus galloprovincialis* in pollution monitoring. Environ. Toxicol. Chem. 16, 2090–2095. https://doi.org/10.1897/1551-5028(1997)016<2090:TUOCAC>2.3.CO;2.

EU, 2008. European Union Updated Risk Assessment Report. Environment Addendum of April 2008 (To Be Read in Conjunction with Published EU RAR of BPA, 2003) 4,4′-ISOPROPYLIDENEDIPHENOL (Bisphenol-A) Part 1 Environment, European Commission, Joint Research Centre. Institute for Health and Consumer Protection, Luxembourg.

EU, 2016. Commission regulation 2016/2235 of 12 December 2016 amending annex XVII to regulation (EC) No 1907/2006 of the European parliament and of the council concerning the registration, evaluation, authorisation and restriction of chemicals (REACH) as regards bisphenol A. Off. J. Eur. Union L337, 3–5.

EU, 2020. European Parliament and Council of the European Union (2020). DIRECTIVE (EU) 2020/2184 of the european parliament and of the council of 16 December 2020 on the quality of water intended for human consumption. Off. J. Eur. Union. L 435/1. Availanle online: https://eur-lex.europa.eu/eli/dir/2020/2184/oj.

Fabrello, J., Ciscato, M., Moschin, E., Dalla Vecchia, F., Moro, I., Matozzo, V., 2023a. Can BPA analogs affect cellular and biochemical responses in the microalga *Phaeodactylum tricornutum* Bohlin? Journal of Xenobiotics 13 (3), 479–491. https://doi.org/10.3390/jox13030030.

Fabrello, J., Ciscato, M., Munari, M., Vecchiatti, A., Roverso, M., Bogialli, S., Matozzo, V., 2023b. Ecotoxicological effects and bioaccumulation of BPA analogues and their mixture in the clam *Ruditapes philippinarum*. Mar. Environ. Res. 192, 106228. https://doi.org/10.1016/j.marenvres.2023.106228.

Fabrello, J., Matozzo, V., 2022. Bisphenol analogs in aquatic environments and their effects on marine species—a review. J. Mar. Sci. Eng. 10, 1271. https://doi.org/10.3390/jmse10091271.

Gürkan, M., 2018. Effects of three different nanoparticles on bioaccumulation, oxidative stress, osmoregulatory, and immune responses of *Carcinus aestuarii*. Toxicol. Environ. Chem. 100, 693–716. https://doi.org/10.1080/02772248.2019.1579818.

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, 7130–7139.

Han, Y., Shi, W., Tang, Y., Zhou, W., Sun, H., Zhang, J., Yan, M., Hu, L., Liu, G., 2022. Microplastics and bisphenol A hamper gonadal development of whiteleg shrimp (*Litopenaeus vannamei*) by interfering with metabolism and disrupting hormone regulation. Sci. Total Environ. 810, 152354. https://doi.org/10.1016/j.scitotenv.2021.152354.

Huang, C., Wu, L.-H., Liu, G.-Q., Shi, L., Guo, Y., 2018. Occurrence and ecological risk assessment of eight endocrine-disrupting chemicals in urban river water and sediments of south China. Arch. Environ. Contam. Toxicol. 75, 224–235. https://doi.org/10.1007/s00244-018-0527-9.

Huang, Z., Zhao, J.-L., Yang, Y.-Y., Jia, Y.-W., Zhang, Q.-Q., Chen, C.-E., Liu, Y.-S., Yang, B., Xie, L., Ying, G.-G., 2020. Occurrence, mass loads and risks of bisphenol analogues in the Pearl River Delta region, South China: urban rainfall runoff as a potential source for receiving rivers. Environ. Pollut. 263, 114361. https://doi.org/10.1016/j.envpol.2020.114361.

Hutchinson, T.H., Shillabeer, N., Winter, M.J., Pickford, D.B., 2006. Acute and chronic effects of carrier solvents in aquatic organisms: a critical review. Aquat. Toxicol. 76, 69–92. https://doi.org/10.1016/j.aquatox.2005.09.008.

Jenzri, M., Gharred, C., Bouraoui, Z., Guerbej, H., Jebali, J., Gharred, T., 2023. Assessment of single and combined effects of bisphenol-A and its analogue bisphenol-S on biochemical and histopathological responses of sea cucumber *Holothuria poli*. Mar. Environ. Res. 188, 106032. https://doi.org/10.1016/j.marenvres.2023.106032.

Juhel, G., Bayen, S., Goh, C., Lee, W.K., Kelly, B.C., 2017. Use of a suite of biomarkers to assess the effects of carbamazepine, bisphenol A, atrazine, and their mixtures on green mussels, *Perna viridis*. Environ. Toxicol. Chem. 36, 429–441. https://doi.org/10.1002/etc.3556.

Kaviraj, A., Bhunia, F., Saha, N.C., 2004. Toxicity of methanol to fish, Crustacean, oligochaete worm, and aquatic ecosystem. Int. J. Toxicol. 23, 55–63. https://doi.org/10.1080/10915810490265469.

Konkel, L., 2013. Thermal reaction: the spread of bisphenol S via paper products. Environ. Health Perspect. 121, A76. https://doi.org/10.1289/ehp.121-a76.

Livingstone, D.R., Lips, F., Martinez, P.G., Pipe, R.K., 1992. Antioxidant enzymes in the digestive gland of the common mussel *Mytilus edulis*. Mar. Biol. 112, 265–276. https://doi.org/10.1007/BF00702471.

Matozzo, V., Gallo, C., Marin, M.G., 2011. Can starvation influence cellular and biochemical parameters in the crab *Carcinus aestuarii*? Mar. Environ. Res. 71, 207–212. https://doi.org/10.1016/j.marenvres.2011.01.004.

Mecocci, P., Fanó, G., Fulle, S., MacGarvey, U., Shinobu, L., Polidori, M.C., Cherubini, A., Vecchiet, J., Senin, U., Beal, M.F., 1999. Age-dependent increases in oxidative damage to DNA, lipids, and proteins in human skeletal muscle. Free Radic. Biol. Med. 26, 303–308. https://doi.org/10.1016/S0891-5849(98)00208-1.

Michałowicz, J., 2014. Bisphenol A – sources, toxicity and biotransformation. Environ. Toxicol. Pharmacol. 37, 738–758. https://doi.org/10.1016/j.etap.2014.02.003.

Minier, C., Forget-Leray, J., Bjørnstad, A., Camus, L., 2008. Multixenobiotic resistance, acetyl-choline esterase activity and total oxyradical scavenging capacity of the Arctic spider crab, *Hyas araneus*, following exposure to bisphenol A, tetra bromo diphenyl ether and diallyl phthalate. Mar. Pollut. Bull. 56, 1410–1415. https://doi.org/10.1016/j.marpolbul.2008.05.005.

NORMAN Ecotoxicology Database. Available online: https://www.norman-network.com/nds/ecotox/lowestPnecsIndex.php–. (Accessed 6 June 2024).

Odebunmi, E., Owalude, S., 2007. Kinetic and thermodynamic studies of glucose oxidase catalysed oxidation reaction of glucose. J. Appl. Sci. Environ. Manag. 11, 95–100.

Oliveira, H.M., Pinheiro, A.Q., Fonseca, A.J.M., Cabrita, A.R.J., Maia, M.R.G., 2019. Flexible and expeditious assay for quantitative monitoring of alpha-amylase and amyloglucosidase activities. MethodsX 6, 246–258. https://doi.org/10.1016/j.mex.2019.01.007.

OriginPro, 2023. OriginPro, Version 2023. OriginLab Corporation, Northampton, MA, USA.

Park, J.C., Lee, M.-C., Yoon, D.-S., Han, J., Kim, M., Hwang, U.-K., Jung, J.-H., Lee, J.-S., 2018. Effects of bisphenol A and its analogs bisphenol F and S on life parameters, antioxidant system, and response of defensome in the marine rotifer *Brachionus koreanus*. Aquat. Toxicol. 199, 21–29. https://doi.org/10.1016/j.aquatox.2018.03.024.

Park, K., Kim, W.-S., Kwak, I.-S., 2019. Endocrine-disrupting chemicals impair the innate immune prophenoloxidase system in the intertidal mud crab, *Macrophthalmus japonicus*. Fish Shellfish Immunol. 87, 322–332. https://doi.org/10.1016/j.fsi.2019.01.025.

Peng, Y.Q., Wang, M.J., Chen, H.G., Chen, J.H., Gao, H., Huang, H.H., 2018. Immunological responses in haemolymph and histologic changes in the hepatopancreas of *Charybdis japonica* (A. Milne-Edwards, 1861) (Decapoda: Brachyura: portunidae) exposed to bisphenol A. J. Crustac Biol. 38, 489–496. https://doi.org/10.1093/jcbiol/ruy046.

Pu, C., Liu, Y., Ma, J., Li, J., Sun, R., Zhou, Y., Wang, B., Wang, A., Zhang, C., 2024. The effects of bisphenol S exposure on the growth, physiological and biochemical indices, and ecdysteroid receptor gene expression in red swamp crayfish, *Procambarus clarkii*. Comp. Biochem. Physiol. Toxicol. Pharmacol. CBP 276, 109811. https://doi.org/10.1016/j.cbpc.2023.109811.

Qyli, M., Aliko, V., Faggio, C., 2020. Physiological and biochemical responses of Mediterranean green crab, *Carcinus aestuarii*, to different environmental stressors: evaluation of hemocyte toxicity and its possible effects on immune response. Comp. Biochem. Physiol., Part C: Toxicol. Pharmacol. 231, 108739. https://doi.org/10.1016/j.cbpc.2020.108739.

R Core Team, 2022. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria. Version 4.2.2, 2022-10-31). https://www.r-project.org/.

Resende, A., Pereira, D.M.C., 2023. _IBRtools: Integrating Biomarker-Based Assessments and Radarchart Creation_. R package version 0.1.3. https://CRAN.R-project.org/package=IBRtools.

Riisgård, H.U., 2015. Filter-feeding mechanisms in crustaceans. In: Thiel, M., Watling, L. (Eds.), Life Styles and Feeding Biology, Volume II in the Natural History of Crustaceans, 1 ed., Vol. 2. Oxford University Press, pp. 418–463.

Rodrigues, E.T., Pardal, M.Â., 2014. The crab *Carcinus maenas* as a suitable experimental model in ecotoxicology. Environ. Int. 70, 158–182. https://doi.org/10.1016/j.envint.2014.05.018.

Ross, N.W., Firth, K.J., Wang, A., Burka, J.F., Johnson, S.C., 2000. Changes in hydrolytic enzyme activities of naïve Atlantic salmon *Salmo salar* skin mucus due to infection with the salmon louse *Lepeophtheirus salmonis* and cortisol implantation. Dis. Aquat. Org. 41, 43–51. https://doi.org/10.3354/dao041043.

RStudio Team, 2023. RStudio: Integrated Development for R. RStudio. PBC, Boston, MA (Build 369, 33206f75, 2023-20212-17).

Sanchez, W., Burgeot, T., Porcher, J.M., 2013. A novel "Integrated Biomarker Response" calculation based on reference deviation concept. Environ. Sci. Pollut. Res. 20, 2721–2725. https://doi.org/10.1007/s11356-012-1359-1.

Seoane, M., Cid, Á., Herrero, C., Esperanza, M., 2021. Comparative acute toxicity of benzophenone derivatives and bisphenol analogues in the Asian clam *Corbicula fluminea*. Ecotoxicology 30, 142–153. https://doi.org/10.1007/s10646-020-02299-w.

Smith, I.K., Vierheller, T.L., Thorne, C.A., 1988. Assay of glutathione reductase in crude tissue homogenates using 5,5′-dithiobis(2-nitrobenzoic acid). Anal. Biochem. 175, 408–413. https://doi.org/10.1016/0003-2697(88)90564-7.

Solórzano, L., 1969. DETERMINATION of AMMONIA IN NATURAL WATERS by the PHENOLHYPOCHLORITE METHOD 1 1 This Research Was Fully Supported, vol. 14. U.S. Atomic Energy Commission Contract No. ATS (11-1) GEN 10, P.A. 20. Limnol. Oceanogr., pp. 799–801. https://doi.org/10.4319/lo.1969.14.5.0799

Tang, Y., Zhou, W., Sun, S., Du, X., Han, Y., Shi, W., Liu, G., 2020. Immunotoxicity and neurotoxicity of bisphenol A and microplastics alone or in combination to a bivalve species, *Tegillarca granosa*. Environ. Pollut. 265, 115115. https://doi.org/10.1016/j.envpol.2020.115115.

Uçkun, M., 2022. Assessing the toxic effects of bisphenol A in consumed crayfish *Astacus leptodactylus* using multi biochemical markers. Environ. Sci. Pollut. Res. Int. 29, 25194–25208. https://doi.org/10.1007/s11356-021-17701-1.

Wang, H., Tang, S., Zhou, X., Gao, R., Liu, Z., Song, X., Zeng, F., 2022. Urinary concentrations of bisphenol analogues in the south of China population and their contribution to the per capital mass loads in wastewater. Environ. Res. 204, 112398. https://doi.org/10.1016/j.envres.2021.112398.

Yamazaki, E., Yamashita, N., Taniyasu, S., Lam, J., Lam, P.K.S., Moon, H.-B., Jeong, Y., Kannan, P., Achyuthan, H., Munuswamy, N., Kannan, K., 2015. Bisphenol A and other bisphenol analogues including BPS and BPF in surface water samples from Japan, China, Korea and India. Ecotoxicol. Environ. Saf. 122, 565–572. https://doi.org/10.1016/j.ecoenv.2015.09.029.

Yan, Z., Liu, Y., Yan, K., Wu, S., Han, Z., Guo, R., Chen, M., Yang, Q., Zhang, S., Chen, J., 2017. Bisphenol analogues in surface water and sediment from the shallow Chinese freshwater lakes: occurrence, distribution, source apportionment, and ecological and human health risk. Chemosphere 184, 318–328. https://doi.org/10.1016/j.chemosphere.2017.06.010.

Zhang, Y., Mi, K., Xue, W., Wei, W., Yang, H., 2020. Acute BPA exposure-induced oxidative stress, depressed immune genes expression and damage of hepatopancreas in red swamp crayfish *Procambarus clarkii*. Fish Shellfish Immunol. 103, 95–102. https://doi.org/10.1016/j.fsi.2020.04.032.

Zhao, N., Hu, H., Zhao, M., Liu, W., Jin, H., 2021. Occurrence of free-form and conjugated bisphenol analogues in marine organisms. Environ. Sci. Technol. 55, 4914–4922. https://doi.org/10.1021/acs.est.0c08458.

Zucker-Franklin, D., Grusky, G., Yang, J.S., 1983. Arylsulfatase in natural killer cells: its possible role in cytotoxicity. Proc. Natl. Acad. Sci. USA 80, 6977–6981. https://doi.org/10.1073/pnas.80.22.6977.