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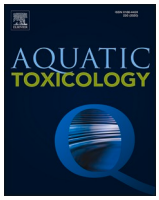

A multibiomarker approach to assess the effects of a BPA analogue-contaminated diet in the crab *Carcinus aestuarii*

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ABSTRACT

Bisphenol A analogues are largely used plasticisers that are going to replace bisphenol A in many sectors. Due to this replacement, their discharge and presence in the marine coastal areas are increasing, with unknown consequences for organisms and the trophic chain. This study assessed the effects of three different bisphenols (BPAF, BPF and BPS) - alone or as a mixture - provided via food (exposed clams) to the crab *Carcinus aestuarii*. First, clams were exposed for two weeks to 300 ng/L of each of the three bisphenols and their mixture (100 ng/L of each) to allow the bioaccumulation of the contaminants in bivalves. Then, crabs were fed for two weeks with BPA analogue-exposed clams, while unexposed clams were used to feed control crabs. After 7 and 14 days, haemolymph, gills and hepatopancreas were collected from crabs to measure a battery of biomarkers indicative of cytotoxicity, oxidative stress and damage, neurotoxicity, physiological performance (respiration and excretion rate) and electron transport system activity. Lastly, bioaccumulation of BPA analogues was assessed by UHPLC-HRMS in crabs. Our findings revealed that BPA analogue-exposed clams were able to alter total haemocyte count, haemocyte size and their proliferation. The activity of immune enzymes, such as phosphatases and phenoloxidase was altered. Moreover, we observed an impairment of antioxidant and detoxifying enzymes like SOD, CAT, GST and GPX activities. Alterations of metabolism-involved enzymes and physiological parameters and increased oxidative damage to macromolecules like proteins, lipids, and DNA were also observed in crabs. Among BPA analogues, only bioaccumulation of BPAF, which has the highest Log_{kow} value among the tested bisphenols, was evidenced in crabs. Overall, the obtained results indicated that crabs, under the tested experimental conditions at least, underwent alterations in cellular, biochemical and physiological responses following a diet of bisphenol-exposed clams, suggesting a potential ecotoxicological risk in the marine food chain.

1. Introduction

Bisphenols are widely used compounds, with the best-known representative being bisphenol A (BPA) (Almeida et al., 2018). BPA is a commercially important chemical that was synthesised for the first time in 1891 by condensation of phenol and acetone while in 1952 the first plastic product was made with BPA (vom Saal and Welshons, 2006; Vandenberg et al., 2007). BPA has been used in the production of plastic and plastic-derived products including food and beverage containers, water and baby bottles, toys, sports equipment and compact discs (Eladak et al., 2015) as well as in the manufacture of polyvinyl chloride as an antioxidant and stabilizer (Staples et al., 1998). Other uses of BPA were in dental sealants and thermal papers (Vandenberg et al., 2007). However, as a consequence of many studies that linked BPA to several health issues, some countries have restricted the use of BPA in consumer products, like in baby bottles and thermal paper (EU, 2011; EU, 2016; Michałowicz, 2014). However, BPA was replaced with other similar compounds, named BPA analogues. At least 17 different BPA analogues have been synthesized, from which derive more than 130 chemical compounds that share the same BPA core structure (two phenolic groups). Three of the most known BPA analogues are BPAF, BPF and BPS, being largely used in the products that were BPA-based (Chen et al., 2016). This replacement is causing a higher release into ecosystems, with BPA analogues' environmental concentrations commonly in the order of few ng/L in both freshwater and seawater (Fabrello and Matozzo, 2022). However, their concentration can be higher, as

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reported for BPAF that was detected up to 245.69 ng/L in river water samples from Hangzhou Bay in China (Yang et al., 2014), and up to 15300 ng/L in the Xitang River in an area around a BPAF manufacturing plant in China (Song et al., 2012). Similarly, BPF concentrations can exceed 1 μg/L, as observed in river samples from Japan, Korea and China, reaching a concentration of 2850 ng/L in the Tamagawa River in Tokyo (Yamazaki et al., 2015; Yan et al., 2017). Furthermore, high levels of BPS were recorded in rivers and the Taihu Lake (up to 65.6 μg/L and 1.6 μg/L, respectively) in China (Huang et al., 2018; Yan et al., 2017).

In the marine environment, the concentrations of BPA analogues are generally lower than those recorded in freshwater (Fabrello and Matozzo, 2022), with some exceptions. Indeed, high concentrations of BPF (282 ng/L and 1470 ng/L) were detected in seawater in South China and Tokyo Bay (Yamazaki et al., 2015; Zhao et al., 2019). Moreover, a recent study conducted in estuarine and coastal areas in Malaysia revealed that BPF had a concentration range of 160.47–1890.51 ng/L, while BPS had a concentration range of 2.95–30.52 ng/L (Zainuddin et al., 2023). Interestingly, these values are higher than the BPA concentration (1.32–133.91 ng/L) observed in the same sites revealing how the bisphenol production is shifting from BPA to its analogues (Zainuddin et al., 2023).

To our knowledge, only few studies have been conducted to evaluate the effects of BPA analogues on both freshwater and marine animals. We recently demonstrated that waterborne exposure to BPA analogues can induce cell alterations and oxidative stress in the crab *C. aestuarii* (Fabrello et al., submitted). In detail, alterations of total haemocyte count and cell proliferation were found in BPS- and MIX-exposed crabs. In addition, there was an imbalance of the antioxidant capacity in both gills and hepatopancreas, mainly following exposure to BPF and MIX. Interestingly, BPF significantly increased the acetylcholinesterase (AChE) activity, whereas BPAF and BPF caused the highest oxidative damage to macromolecules. Regarding crab physiological parameters, exposure to the mixture of the three bisphenols increased the respiration rate of crabs. Lastly, no bioaccumulation was detected for BPF and BPS and only slightly for BPAF (Fabrello et al., submitted). Despite this evidence, there are still questions regarding the capacity of these compounds to be transferred through the marine food chain and if they can exert toxic effects even with indirect contamination via food. In this regard, the present study tries to fill this knowledge gap by assessing for the first time the effects of BPA analogue-exposed food in the same crab species to evaluate whether the ingestion of BPA analogue-exposed food can also be an ecotoxicological risk for crabs. In detail, the hypotheses that we tested were: *i)* if BPA analogue-exposed food can alter the haemocyte parameters and the immune-related enzyme activities, *ii)* if these diets can exert oxidative stress causing an alteration of the antioxidant system and increase the oxidative damage on macromolecules with also genotoxic effects, *iii)* if BPA analogues provided via food can be neurotoxic, *iv)* if the adopted diets can alter the activity of metabolic-involved enzymes and the physiological parameters in crabs *v)* if BPA analogues can be transferred through a marine food chain passing from primary consumers, namely clams, to secondary consumers represented by crabs. For these purposes, firstly, clams (*Ruditapes philippinarum*) were exposed for 14 days to 300 ng/L of BPAF, BPF, BPS and their mixture (100+100+100 ng/L) that are environmentally relevant concentrations. We have previously evaluated the BPA analogues bioaccumulation in clams, demonstrating that BPAF and BPS were significantly bioaccumulated (Fabrello et al., 2023). Then, BPA analogue-exposed clams were fed to crabs for two weeks. After 7 and 14 days of diet based on BPA analogue-exposed clams, many biomarkers indicative of cell integrity, oxidative stress, neurotoxicity and energy metabolism, as well as physiological performance, were measured. We chose to adopt this time lag for tissue sampling to evaluate the medium- and long-term responses of crabs and to easily compare the findings of this study with the results obtained in our study previously performed exposing crabs for 7 and 14 days to BPA analogue-contaminated water (Fabrello et al., submitted). In detail, we measured total haemocyte count (THC), the size of haemocytes, and the activity of some important enzymes being haemocytes involved in immune responses in *C. aestuarii* (Matozzo and Marin, 2010). To evaluate the onset of oxidative stress, the level of total antioxidants and the activity of several antioxidant enzymes were measured. Moreover, we evaluated the oxidative damage on macromolecules measuring the DNA strand breaks, and the physiological performance evaluating the oxygen consumption rate and the excretion rate. Lastly, bioaccumulation of BPA analogues was also measured in crabs.

2. Materials and methods

2.1. Clam acclimation and exposure

Specimens of *R. philippinarum*, with similar size, were collected from a clam fishing area in the Lagoon of Venice (Italy) in January 2024. The animals were acclimated in large aquaria with a sandy bottom and aerated seawater (salinity 35 ± 1, temperature of 19 ± 0.5 °C). The stock solutions of BPAF and BPF (100 mg/L) were prepared in methanol, while BPS (100 mg/L) was dissolved in distilled water. A total of five experimental conditions consisting of control (unexposed), BPAF, BPF, BPS and their mixture (MIX) were prepared using ten glass aquaria (two aquaria per condition) of 30 L capacity each posed in a single automatically controlled-temperature chamber with a light-dark photoperiod of 10:14 hours. Clams (100 per experimental condition) were exposed for 14 days to 300 ng/L of each bisphenol or 100 ng/L of each of them in the mixture treatment. The clams under the control condition were maintained in clean seawater without the addition of any bisphenol. Bisphenol concentrations tested in this study have an order of magnitude close to those already detected in coastal areas (see Introduction section). 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 molluscs and crustaceans (Hutchinson et al., 2006; Kaviraj et al., 2004).

During the exposure, seawater from tanks of all the experimental conditions was renewed every two days, pumping from the same stock seawater tank (about 5000 litres capacity). Where necessary, contaminants were re-added. After seawater renewal, clams were fed with 250 mL of a suspension of the uncontaminated microalgae *Phaeodactylum tricornutum*. After 14 days, both treated and untreated (=control) clams were dissected and whole soft tissues were frozen at -20 °C and then provided as food to crabs.

2.2. Crab acclimation and treatment

C. aestuarii specimens were sampled in the Lagoon of Venice (Italy) and acclimated for 7 days in large aquaria filled with aerated seawater (salinity of 35 ± 1, temperature of 19 ± 0.5 °C). To avoid the effects of gender, only male crabs were used for experiments. During acclimation, crabs were fed *ad libitum* every two days with uncontaminated clams (*R. philippinarum*). After the acclimation phase, crabs with similar size and age were randomly divided into 10 tanks (2 per experimental condition, 30 L capacity and 30 crabs per tank) and filled with clean aerated seawater pumped from the same stock seawater tank and under constant temperature. During 14 days of the experiment, control crabs were fed with untreated clams (maintained in clean seawater without the addition of bisphenols), while treated crabs received a diet of BPAF-, BPF-, BPS-, or MIX-exposed clams. A photoperiod of 10:14 hours light: dark was applied every day. In detail, each crab was fed on one-half clam of the corresponding experimental condition every 48 hours. Crabs were not fed daily to avoid a buildup of not ingested food in the tanks. Accordingly, seawater was renewed 1 hour after feeding, removing not ingested material at the bottom of exposure tanks to avoid the release of bisphenols from clam tissues to the seawater. Crab mortality was

checked daily.

2.3. Haemolymph collection

A syringe was used to collect haemolymph from the abdominal segment of crabs and stored in tubes on ice. At each tissue sampling time (7 and 14 days), 5 pools of haemolymph (from four crabs each) for each experimental condition were prepared. Immediately after sampling, total haemocyte count (THC), haemocyte diameter and volume, lactate dehydrogenase (LDH) activity and haemocyte proliferation (XTT assay) were measured. The remaining pooled haemolymph samples were then centrifuged (780 × *g* for 10 min) to obtain haemocytes (=pellets) and cell-free haemolymph (CFH). Haemocyte pellets were then resuspended in distilled water to obtain haemocyte lysate (HL) samples. Both HL and CFH samples were frozen in liquid nitrogen and stored at −80 °C until analyses. Lastly, gills and hepatopancreas were excised from each crab and pooled to obtain 5 different pools, (replicates) of four crabs each for each experimental condition, frozen in liquid nitrogen, and stored at −80 °C until analyses.

2.4. Haemolymph and haemocyte biomarkers

As reported in the previous section, THC, haemocyte diameter and volume, were instantly determined. In detail, we used a Scepter™ 2.0 Automated Cell Counter (Millipore, FL, USA) and the haemolymph samples were diluted 1:100 using 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.

The lactate dehydrogenase (LDH) activity in CFH samples was measured using the *Cytotoxicity Detection* Kit (Roche) 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 the reagent mixture provided by the kit was added to two volumes of pooled haemolymph and incubated for 4 h. After the incubation period we recorded the absorbance at 450 nm using a spectrophotometer. Results were normalized to THC values of each experimental group and expressed as optical density (OD) at 450 nm.

The acid phosphatase, alkaline phosphatase and phenoloxidase (PO) activity enzymes were measured both in HL and CFH. In detail, the acid phosphatase activity was recorded measuring the hydrolyzation of the substrate 4-nitrophenyl phosphate at 405 nm using a microplate reader. Results were expressed as U/mg of proteins. 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-phenylalanine, Sigma) as substrate in which one hundred μL of HL or CFH were added to 900 μL of 1 mg L-DOPA/mL of phosphate buffered saline and incubated for 30 min at 37 °C. Then, the absorbance at 490 nm was recorded and results were expressed as U/mg of proteins (Matozzo et al., 2011).

The arylsulfatase activity was measured only in HL samples according to Zucker-Franklin et al. (1983). The amount of produced p-nitrocatechol was quantified at 515 nm after 1 h of incubation 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 proteins.

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

The hemocyanin concentration in haemolymph was quantified at 335 nm using an extinction coefficient of 17,5 mmol L^{-1} cm^{-1} as proposed by Weber et al. (2008) based on the $A_{1\%,1\ cm}$ value of 2.33 reported for *C. maenas* hemocyanin (Nickerson and Van Holde, 1971) and a functional-unit mass of 75 kDa. Results were expressed as mM of hemocyanin.

The total antioxidant capacity in haemolymph was measured following the CUPRAC method (Apak et al., 2004). The assay is based on the production of cupric ions that directly react with the neocuproine colorimetric indicator originating a chemical complex with an absorbance peak at 450 nm. The results are expressed as mM of Trolox equivalents/mg of proteins.

2.5. Antioxidant and oxidative damage biomarkers

Gills and hepatopancreas samples were homogenised using TissueLyser LT (Qiagen) for 5 min and 50 oscillations per second in four volumes of 10 mM Tris-HCl buffer, pH 7.6, containing 0.15 M KCl, 0.5 M sucrose, 1 mM EDTA and protease inhibitor cocktail. After the centrifugation at 12000 g for 30 min at 4 °C, supernatants (SN) were collected for analyses.

The total antioxidant capacity in both gills and hepatopancreas was measured as described for the haemolymph (Apak et al., 2004). The results are expressed as mM of Trolox equivalents/mg of proteins.

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

CAT activity was measured following the method proposed by Aebi (1984) in both gills and hepatopancreas. In detail, the enzyme activity was measured at 240 nm and expressed as U/mg of proteins in which 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). Briefly, we measured the 5-thio-2-nitrobenzoic acid (TNB) production at 412 nm and the enzyme activity was expressed as U/mg of proteins.

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

Selenium-dependent glutathione peroxidase (GPX) activity was measured according to Livingstone et al. (1992). In detail, H_2O_2 was used as a substrate, while reduced glutathione (GSH) as an electron donator converting itself into oxidized glutathione (GSSG). The GSH was reconstituted by glutathione reductase using NADPH as an energy source. The consequent oxidation of NADPH was recorded at 340 nm using the spectrophotometer. One unit of activity is defined as the amount of enzyme that leads to the oxidation of 1 μmol of NADPH per minute. GPX results are expressed as nmol/min/mg of proteins.

Acetylcholinesterase (AChE) activity was measured in gills following the colourimetric reaction between acetylthiocholine and the reagent 5,5′ dithiobis(2-nitrobenzoic acid) (Ellman et al., 1961). Absorbance was recorded at 405 nm for 5 min on a microplate reader and the results were expressed as nmol/min/mg of proteins. A similar procedure was adopted to measure butyrylcholinesterase (BChE) activity, using butyrylthiocholine as a substrate (Escartín and Porte, 1997). Results are expressed as nmol/min/mg of proteins.

Amylolytic enzyme activity was measured in hepatopancreas SN following the method proposed by Oliveira et al. (2019). In detail, the starch was hydrolysed by α-amylase and amyloglucosidase enzymes 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 of proteins.

The electron transport system activity (ETS) was measured following the procedure proposed by King and Packard (1975) and modifications

reported by De Coen and Janssen (1997) in both gills and hepatopancreas SN. In detail, the p-Iodonitrotetrazolium (8 mM) was used as an artificial electron acceptor, while NADH and NADPH were used to saturate both the mitochondrial and microsomal ETS, respectively. The reaction caused the formation of formazan salt that was quantified at 490 nm. Results are reported as nmol /min/mg of proteins.

Oxidative damage in the gills and hepatopancreas was measured by assessing the protein carbonyl content (PCC), lipid peroxidation (LPO) and DNA strand breaks. Briefly, PCC was measured in duplicate using the method of Mecocci et al. (1999) following the reaction with 2,4-dinitrophenylhydrazine (DNPH). Results were expressed as nmol carbonyl group/mg of proteins. The LPO was quantified using the malondialdehyde (MDA) assay, according to the method of Buege and Aust (1978) in which the absorbance was read at 532 nm and results were expressed as nmoles of thiobarbituric reactive substances (TBARS)/mg of 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).

The DNA strand breaks were measured using a fluorescence method adapted from an alkaline precipitation assay (Olive, 1988). Fluorescence was measured using a fluorimeter at 360 nm (excitation) and 450 nm (emission) against blanks. Salmon sperm genomic DNA standards were used for DNA calibration, and the results were expressed as μg/mg of proteins. The protein concentration in all samples was quantified according to Bradford (1976).

2.6. Physiological biomarkers

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

The ammonia excretion rate was measured in 10 mL samples of seawater collected from each respirometry chamber after 30 min. We measured the ammonia concentration following the procedure proposed by Solórzano (1969). Ammonia excretion values were expressed as μmol NH_4/g/h.

2.7. Bioaccumulation

Methanol, acetonitrile, ammonium acetate, bisphenol AF (BPAF), bisphenol F (BPF), bisphenol S (BPS) and bisphenol A d-16, used as internal standard, were purchased from Merck (Milan, Italy). Ultrapure-grade water was produced with a Pure-Lab Option Q apparatus (Elga Lab Water, High Wycombe, UK). Bioaccumulation of bisphenols was evaluated in five crabs collected after 7 and 14 days of exposure and stored at -20 °C. Each 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 minutes, and centrifuged for 5 minutes at 5000 rpm. After a further centrifugation step (13000 rpm, 10 minutes, 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): t0–4 0% B; t4–22 0–100% B, t22–25 100% B; t25–32 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 spectrum/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). Extracted ion chromatograms (EIC) were obtained by selecting the *m/z* value corresponding to the $[M\text{-}H]^-$ species for each considered analyte with a mass tolerance of 5 ppm (*m/z* 335.0412 for BPAF, *m/z* 199.0765 for BPF, *m/z* 249.0227 for BPS, and *m/z* 241.1956 for internal standard). Chromatographic peaks were integrated by selecting the Agile 2 algorithm.

Homogenates from no-treated organisms were used to build a matrix-matched seven-point external calibration curve, in the range 0.5-100 μg/L. Linearity was evaluated by the least squares regression and R^2>0.998 was obtained for all the analytes. LODs were 0.08 ng/g for BPAF, 40 ng/g for BPF and 2 ng/g for BPS. Each treated organism was analysed separately, and results are reported as mean and standard deviation.

2.8. Statistical analysis

Data normal distribution (Shapiro-Wilk's test) and variance homogeneity (Bartlett's test) were assessed. The results obtained were compared by a two-way ANOVA analysis, using "exposure time", "treatment" (=diet) and "exposure time-treatment interaction" as independent factors. Pairwise comparisons among experimental conditions were performed using Fisher's LSD post-hoc test with a significant threshold 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). Lastly, PCA analyses were performed to evaluate the effect of treatment time and diet, both on the whole data set and individual tissues (haemolymph, gills and hepatopancreas). The software package Origin 2023 (Origin lab) was used for the statistical analyses.

3. Results

A table summarising the results of two-way ANOVA analysis for each biomarker is reported in supplementary materials (SM1).

3.1. Haemolymph and haemocyte biomarkers

THC was significantly altered by the factor "treatment" (two-way ANOVA analysis: $p<0.05$). The post-hoc test showed that all diets caused a significant decrease in THC values after 7 days, with BPS- and MIX-exposed food that reduced by 50% THC value with respect to control, while no significant differences were found after two weeks of diet (Fig. 1A).

Haemocyte diameter and volume were affected by "time*treatment interaction" (ANOVA: $p<0.01$) and the Fisher's test showed that a 7-day diet based on BPF-exposed clams caused an increase of both haemocyte diameter and volume in crabs (Fig. 1B-C).

LDH was altered only by the factor "time of exposure" (ANOVA: $p<0.05$) (data not shown), while haemocyte proliferation was affected by the factor "treatment" (ANOVA: $p<0.001$). In the latter case, the post-hoc test revealed that crabs fed for 14 days with BPS- and MIX-exposed clams had higher cell proliferation, with double median values in comparison to that of the related control (Fig. 1D).

HL acid phosphatase activity was affected by the factor "time*-treatment interaction" (ANOVA: $p<0.01$), and the post-hoc test showed a significant increase in enzyme activity in crabs fed for 14 days with

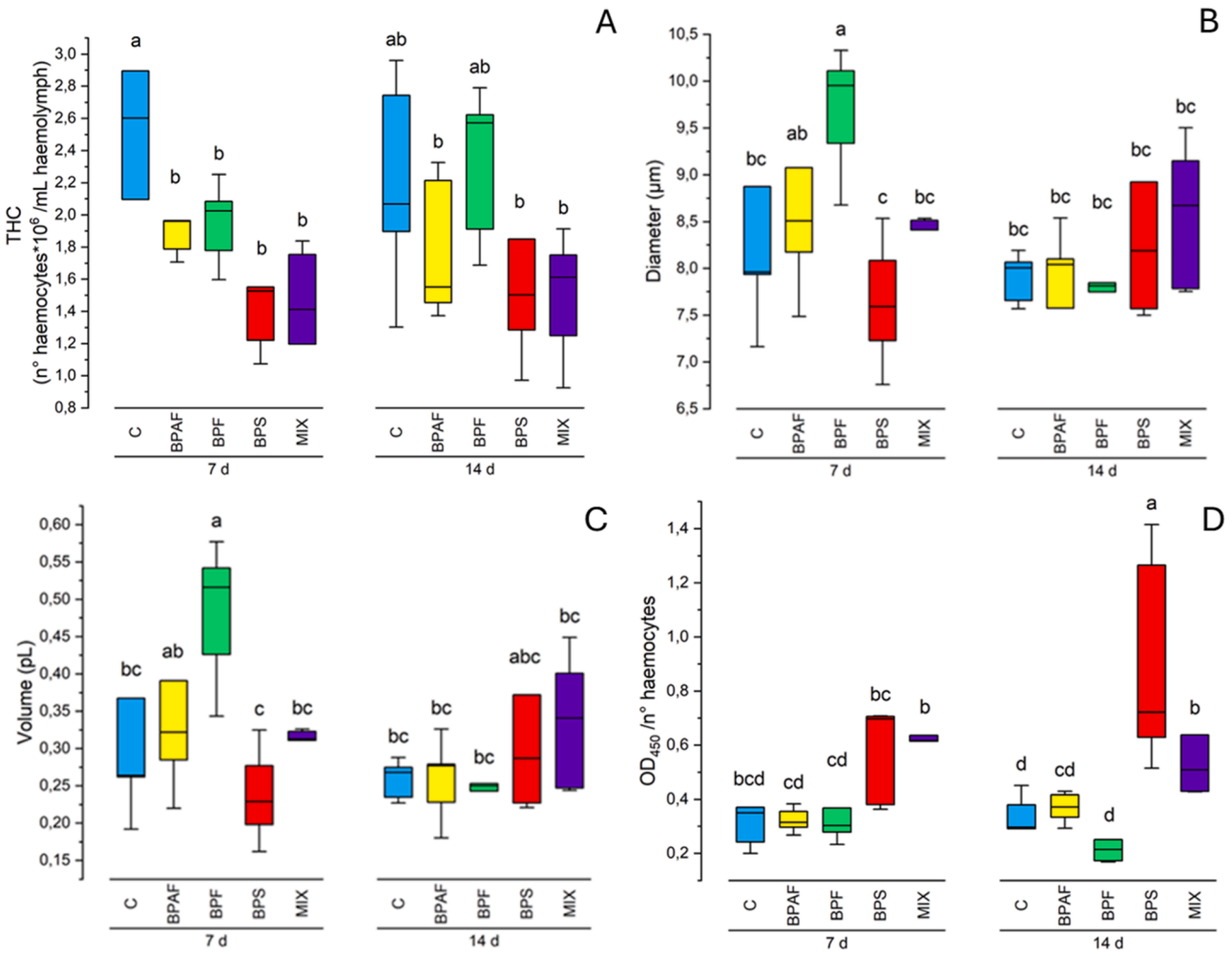

Fig. 1. Total haemocyte count (THC), expressed as n° haemocytes (10^6)/mL haemolymph (A), haemocyte diameter, expressed as µm (B), haemocyte volume, expressed as pL (C), and haemocyte proliferation, expressed as OD450/ n° haemocytes (D) in crabs fed with exposed clams. Different letters indicate significant differences among all treatments. N=5.

BPF and BPS-exposed clams (Fig. 2A). A similar result was observed for acid phosphatase in CFH (Fig. 2B). Indeed, the factors "exposure time" (ANOVA: $p<0.05$) and its interaction with "treatment" significantly (ANOVA: $p<0.05$) altered enzyme activity. Moreover, the Fisher's test indicated a significant reduction in enzyme activity in the haemolymph of crabs fed for 7 days with BPF- and BPS-exposed clams. Alkaline phosphatase was significantly altered in HL by the factors "treatment" and its interaction with "exposure time" (ANOVA: $p<0.001$ and $p<0.01$, respectively). The post-hoc test revealed a significant inhibition of enzyme activity in the crabs fed for 7 days with MIX-exposed clams in which we measured an enzyme activity value close to zero. Similarly, BPAF-, BPF- and MIX-exposed clams caused a significant decrease in enzyme activity in crabs after 14 days of diet, passing from a control value of 0.1 to values close to 0.05 U/mg of proteins in treated crabs (Fig. 2C). Regarding alkaline phosphatase activity in CFH, the two-way ANOVA revealed that all the independent factors caused a significant effect. In detail, we observed that "exposure time" (ANOVA: $p<0.01$), "treatment" (ANOVA: $p<0.05$) and their "interaction" (ANOVA: $p<0.05$) altered such enzyme activity. Interestingly, all the diets caused an enzyme inhibition after 7 days, while all treatments were not significantly different in comparison to the related control after 14 days (Fig. 2D).

Phenoloxidase activity was altered only by the factor "exposure time" (ANOVA: $p<0.001$) in HL (data not shown). On the contrary, in CFH it was altered by the factors "exposure time*treatment interaction" (ANOVA: $p<0.01$) and the post-hoc test showed a significant increase in enzyme activity (about 250%) in crabs fed for 14 days with BPS-exposed clams (Fig. 2E).

As for arylsulfatase activity, it was altered only by "exposure time" (ANOVA: $p<0.05$), whereas no significant effects caused by the different diets were recorded on hemocyanin amount in haemolymph (data not shown). Moreover, glucose levels and total antioxidant capacity in haemolymph were altered only by the "exposure time" factor (ANOVA: $p<0.05$ and $p<0.01$, respectively) (data not shown).

3.2. *Gills and hepatopancreas biomarkers*

According to the two-way ANOVA analysis, CUPRAC levels were altered only by the factor "exposure time" (ANOVA: $p<0.05$) in both gills and hepatopancreas (data not shown). On the contrary, SOD activity in gills was altered by "exposure time" (ANOVA: $p<0.05$), "treatment" (ANOVA: $p<0.01$) and their "interaction" (ANOVA: $p<0.001$). Interestingly, the pairwise comparison showed that crabs fed with BPF-, BPS- and MIX-exposed clams showed a significantly reduced SOD activity after 7 days. A similar inhibition in SOD activity was observed after 14 days in crabs under the BPAF-contaminated diet in which SOD activity was decreased by about 30%, with respect to the value observed in the control condition (Fig. 3A). In hepatopancreas, we did not observe any significant alteration due to diet and only "exposure time" had a significant effect (ANOVA: $p<0.01$) (data not shown). A

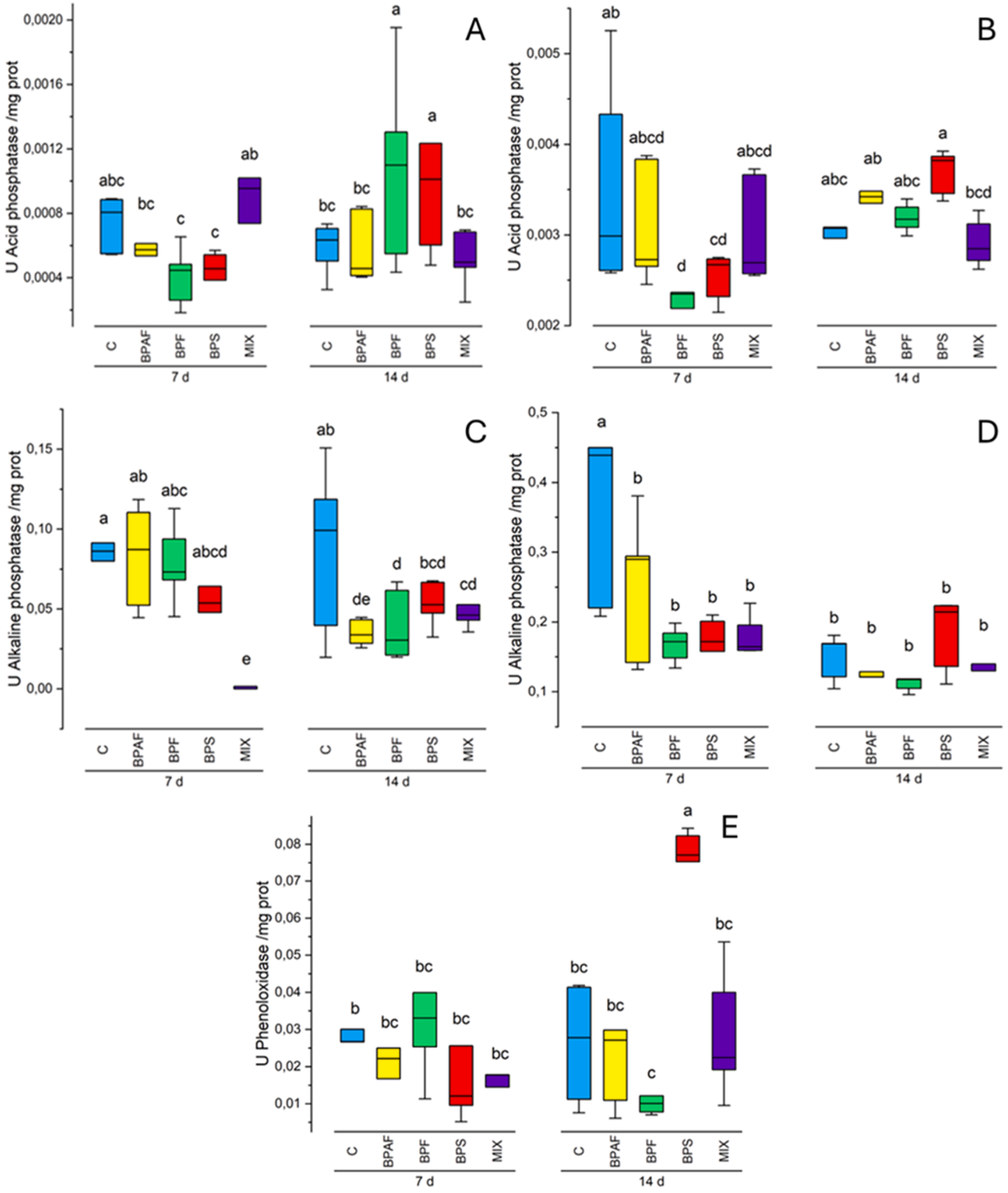

Fig. 2. Acid phosphatase activity in HL, expressed as U/mg of proteins (A), acid phosphatase activity in CFH, expressed as U/mg of proteins (B), alkaline phosphatase activity in HL, expressed as U/mg of proteins (C), alkaline phosphatase activity in CFH, expressed as U/mg of proteins (D), phenoloxidase activity in CFH, expressed as U/mg of proteins (E) in crabs fed with exposed clams. Different letters indicate significant differences among all treatments. N=5.

similar pattern was observed for CAT activity. Indeed, CAT was altered in gills by "exposure time" (ANOVA: $p<0.05$), "treatment" and their "interaction (ANOVA: $p<0.01$), while in hepatopancreas only "exposure time" altered CAT activity (ANOVA: $p<0.05$). The post-hoc test showed that CAT activity in gills was significantly increased in crabs fed with BPAF- and BPF-exposed clams for 7 days, when compared with the related control, as well as in crabs fed for 14 days with BPS- and MIX-exposed clams (Fig. 3B).

We observed that GR activity was altered by the factor "exposure time" in gills (ANOVA: $p<0.001$) and by "treatment" in hepatopancreas (ANOVA: $p<0.05$). However, there were no statistically significant differences between experimental conditions and the related control

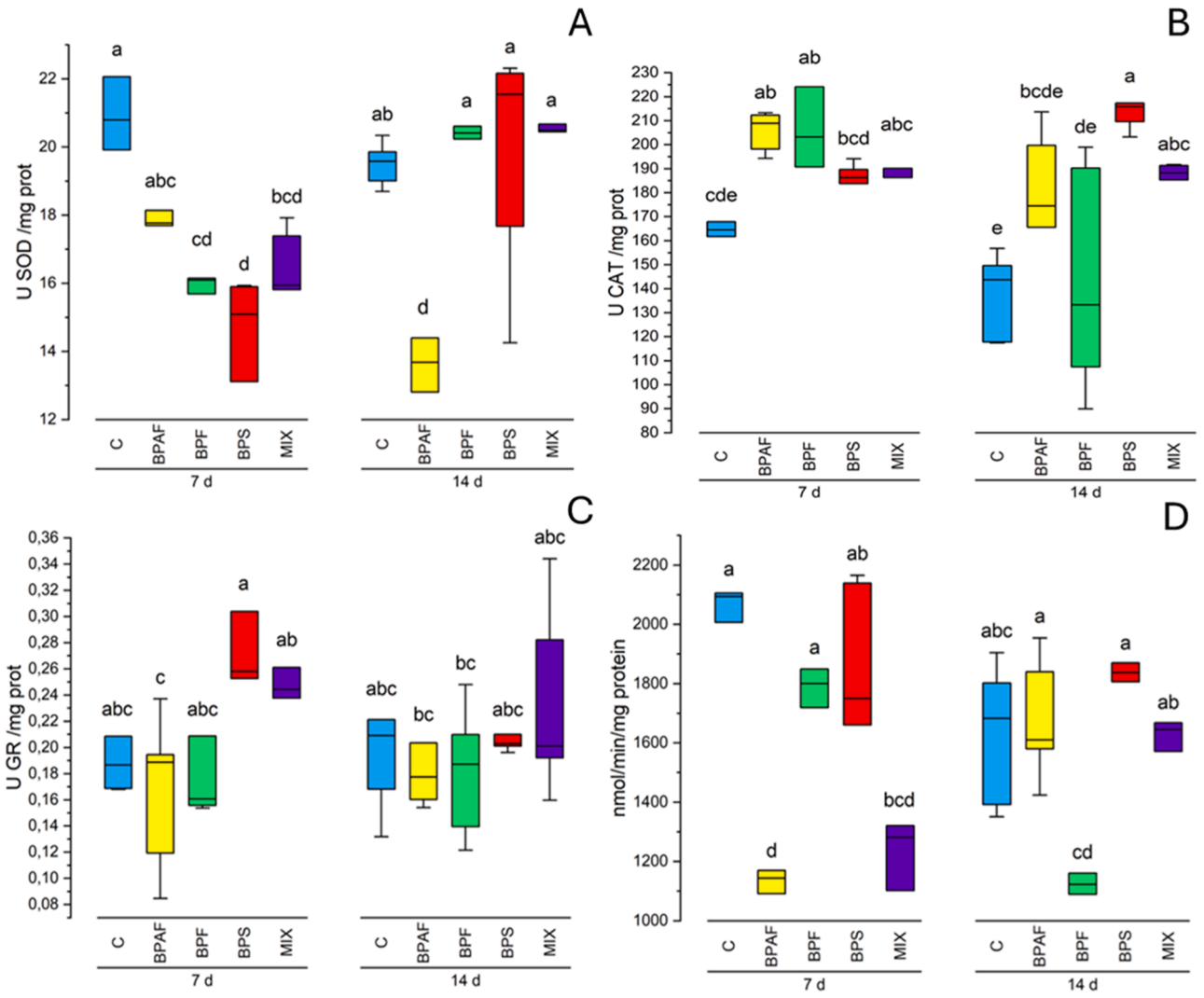

Fig. 3. SOD activity in gills (A), expressed as U/mg of proteins, CAT activity in gills (B), expressed as U/mg of proteins, GR activity in hepatopancreas (C), expressed as U/mg of proteins, GST activity in hepatopancreas (D), expressed as nmol/min/mg of proteins in crabs fed with exposed clams. Different letters indicate significant differences among all treatments. N=5.

(Fig. 3C).

Conversely, GST activity was altered by the factors "treatment" (ANOVA: $p<0.05$) and "exposure time*treatment interaction" (ANOVA: $p<0.01$). In detail, we observed a significant reduction of GST activity in hepatopancreas, with almost halved values in comparison to the control, from crabs fed for 7 days with both BPAF- and MIX-exposed clams. Similarly, there was a significant reduction of the GST activity in crabs fed for 14 days with BPF-exposed clams (Fig. 3D).

GPX activity was significantly altered in both gills and hepatopancreas. Indeed, in the first tissue, the two-way ANOVA showed a significant effect of "exposure time" (ANOVA: $p<0.01$) and its "interaction" with treatment (ANOVA: $p<0.05$) and the pairwise comparison highlighted a significant decrease of more than 50% in GPX activity in crabs fed for 14 days with clams previously exposed to BPF (Fig. 4A). In hepatopancreas, we observed a significant effect of both "treatment" and "exposure time*treatment interaction" (ANOVA: $p<0.001$). In detail, there was a significant increase in enzyme activity caused by a 7-day diet of BPAF- and BPF-treated clams. On the contrary, there was a significant decrease caused by BPF-, BPS- and MIX-treated clams after 14 days (Fig. 4B).

In hepatopancreas, the activity of amylolytic enzymes was altered by "exposure time" and "treatment" (ANOVA: $p<0.01$ and 0.001, respectively). In general, the activity was reduced by all the diets, and in particular, BPS- and MIX-based diets were able to inhibit the amylolytic activity after 7 days, while all the bisphenol-exposed food inhibited enzyme activity after 14 days (Fig. 4C).

Regarding the neurotoxicity biomarkers, the factors "exposure time" (ANOVA: $p<0.001$) and "treatment" (ANOVA: $p<0.05$) altered gill AChE activity, with an increased enzyme activity caused by a 7-day BPF- and BPS-treated clams with an increased enzyme activity of about 30% and 10%, respectively (Fig. 4D). Conversely, BChE activity was altered only by the factor "exposure time" (ANOVA: $p<0.001$) (data not shown).

The ETS was not altered in hepatopancreas, while in gills only "exposure time" significantly affected this biomarker (ANOVA: $p<0.001$) (data not shown).

Regarding oxidative damage, PCC levels were affected only by "exposure time" (ANOVA: $p<0.01$) in gills, while in hepatopancreas it was altered by "treatment" (ANOVA: $p<0.01$) and its "interaction" with exposure time (ANOVA: $p<0.01$). In the latter tissue, the post-hoc test showed that crabs fed for 14 days with BPS- and MIX-exposed clams had higher PCC values with respect to the control (Fig. 5A). Interestingly, crabs fed with MIX-treated clams showed a PCC level twice that of control crabs. Conversely, LPO levels in hepatopancreas were affected only by "exposure time" (ANOVA: $p<0.001$) (data not shown), while in gills the factors "exposure time" (ANOVA: $p<0.001$) and its "interaction" with treatment (ANOVA: $p<0.01$) significantly affected LPO levels.

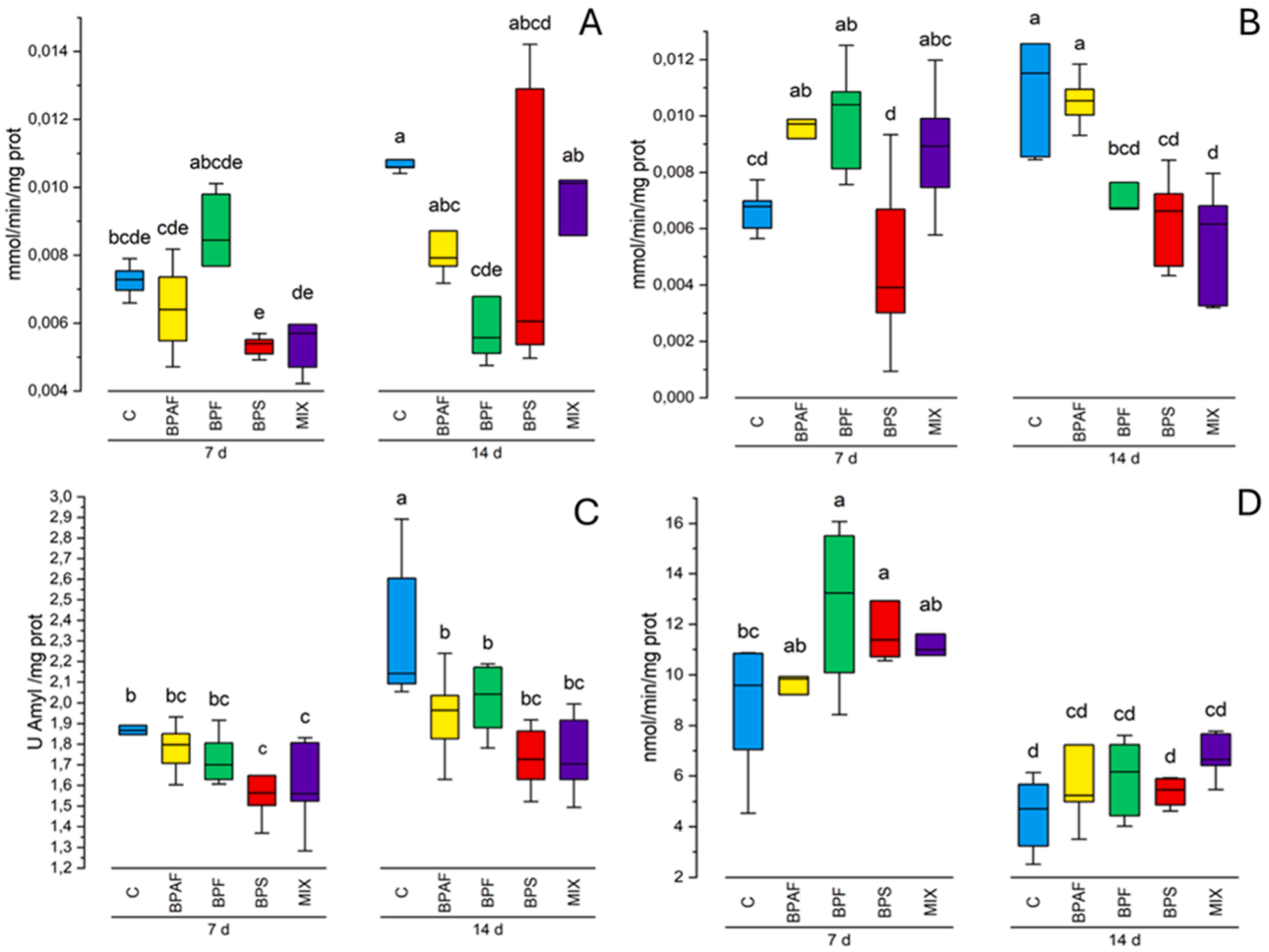

Fig. 4. GPX activity in gill (A), expressed as mmol/min/mg of proteins, GPX activity in hepatopancreas (B), expressed as mmol/min/mg of proteins, amylolytic enzymes in hepatopancreas (C), expressed as U/mg of proteins, AChE activity in gills (D), expressed as nmol/min/mg of proteins in crabs fed with exposed clams. Different letters indicate significant differences among all treatments. N=5.

In particular, there was a significant reduction of LPO levels in crabs fed for 7 days with BPF-exposed clams (Fig. 5B).

Lastly, DNA strand breaks were affected by the factor "treatment" (ANOVA: $p<0.05$) in gills and the post-hoc test showed that crabs fed for 14 days with MIX-exposed clams had a doubled DNA damage level, in comparison to the related control (Fig. 5C). In crab hepatopancreas, statistical analysis showed that "treatment" significantly altered the DNA damage level (ANOVA: $p<0.05$). However, no significant differences were observed after the post-hoc test (data not shown).

3.3. Physiological parameters

The oxygen consumption rate was significantly affected by the factors "treatment" (ANOVA: $p<0.05$) and "exposure time" (ANOVA: $p<0.001$). Moreover, the post-hoc test indicated that crabs fed for 14 days with BPAF- and MIX-exposed clams had an increased respiration rate, in comparison to the control animals (Fig. 6A). Ammonia excretion rate was significantly altered by the factors "exposure time", "treatment" and their "interaction" (ANOVA: $p<0.05$). The pairwise comparison revealed that all the diets based on BPA analogue-exposed clams caused a significant reduction in the crab ammonia excretion rate after 7 days (Fig. 6B).

3.4. PCA results

The PCA analysis showed that the two components explained 15.10% and 10.74% of the variance, respectively. Moreover, the PCA conducted to assess the effect of "time of exposure" on the whole dataset (SM2) showed a clear distinction of the biomarker response between 7 and 14 days of diet. Regarding the PCA analysis based on the diet typology (SM3), there is not a clear clusterization between the different diets. The only cluster seems to be that composed of the biomarkers obtained under the BPS-diet condition. In addition, further PCA analyses were performed on the whole dataset of each tissue. In detail, no clear separation was recorded, and the two components explained 25.8 % and 17.46 % of the variance for haemolymph, 26.76 % and 17.26% for the gills, and 19.63% and 16.21% for the hepatopancreas, respectively (SM4-6).

3.5. Bioaccumulation

No bioaccumulation of both BPF and BPS, which had a concentration below the LOD, was recorded in crabs following the diet. On the contrary, detectable concentrations of BPAF were measured in crabs, with 1.007 ng/g f.w. and 0.666 ng/g f.w. after 7 and 14 days of a diet of BPAF-contaminated clams, respectively (Table 1). In the case of MIX treatment, a concentration of 0.55 ng/g f.w. of BPAF was detected in 14-day-fed crabs.

4. Discussion

The evaluation of the toxic effects of contaminated food in a

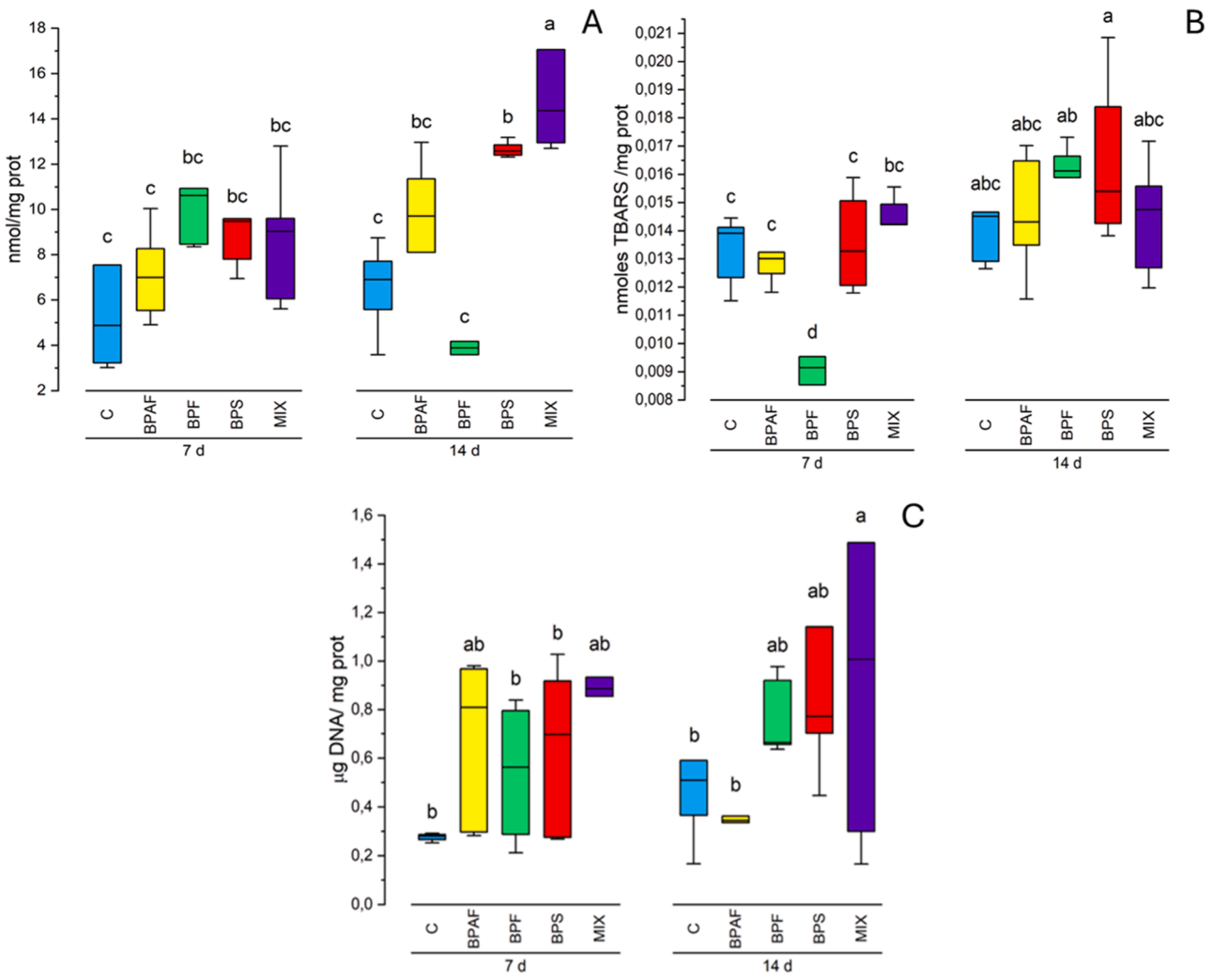

Fig. 5. PCC in hepatopancreas (A), expressed as nmol/mg of proteins, LPO in gills (B) expressed as nmol TBARS/mg of proteins, DNA oxidative damage in gill (C), expressed as µg DNA/mg of proteins in crabs fed with exposed clams. Different letters indicate significant differences among all treatments. N=5.

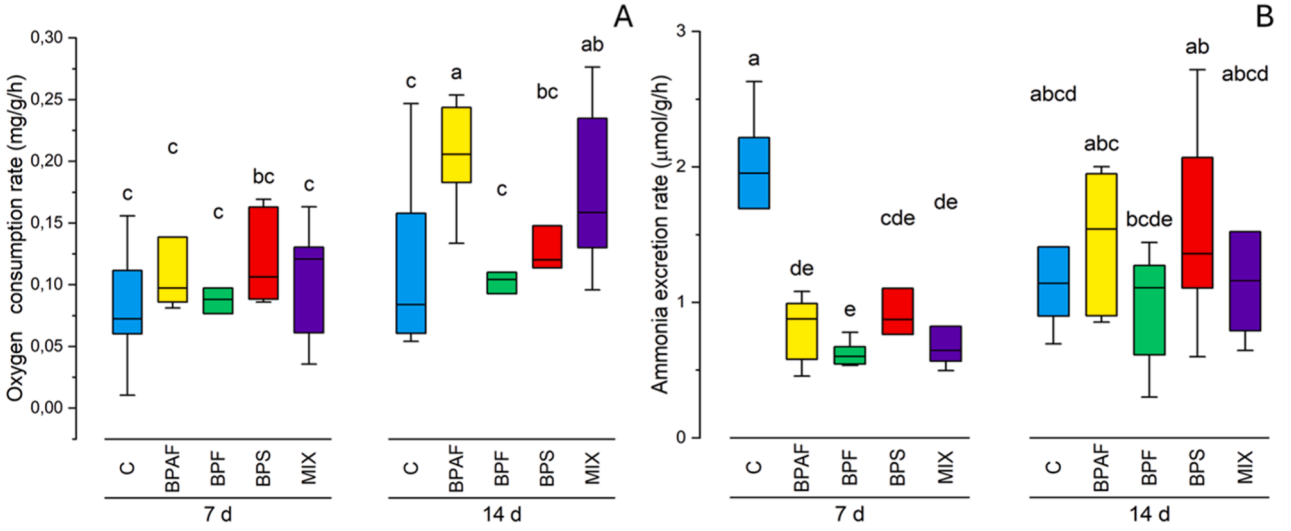

Fig. 6. Oxygen consumption rate (A), expressed as mg/g/h, and ammonia excretion rate (B), expressed as µmol/g/h in crabs fed with exposed clams. Different letters indicate significant differences among all treatments. N=6.

Table 1
Concentrations of BPA analogues in the whole soft tissues of crabs. Results are reported as mean ± standard deviation in brackets (n=5) and expressed as ng/g of fresh weight (ng/g f.w.). LODs were 0.08, 40 and 2 ng/g for BPAF, BPF and BPS, respectively.

		T = 7 days	T = 14 days
BPAF [ng/g f.w.]		1.007 (0.377)	0.666 (0.699)
BPF [ng/g f.w.]		<40	<40
BPS [ng/g f.w.]		<2	<2
MIX	BPAF [ng/g f.w.]	<0.08	0.550 (0.441)
	BPF [ng/g f.w.]	<40	<40
	BPS [ng/g f.w.]	<2	<2

simplified trophic chain requires a long time for both the preparation of contaminated food and the consequent exposure of target species. However, this experimental procedure can provide important and remarkable information on the risk posed by contaminants present in food. It is important to highlight that the information available in the literature regarding the effects of contaminated food on target species is very scarce, mainly regarding molluscs and crustaceans. Therefore, the discussion of our results is mainly based on available data referring to water exposure of target species. In previous surveys, an experimental set-up similar to that adopted in the present study was used to assess the toxicity and trophic transfer of heavy metals, pesticides, toxins and microplastics in crustaceans (Oikawa et al., 2005; Farrell and Nelson, 2013; Petrocelli et al., 1975; Simon et al., 2000). In this study, we evaluated for the first time the potential ecotoxicological risk posed by bisphenol-exposed food (clams) to the crab *C. aestuarii*.

4.1. Haemocytes and immune parameters

In our study, BPA analogues provided with the diet were able to cause marked alterations in cell parameters in crabs, including immune-related responses. THC is considered a valuable immunomarker that can provide a general view of the immune system activity in marine invertebrates (Matozzo and Gagné, 2016). In this study, THC was significantly dropped by all the diets after 7 days, while THC levels were not different after two weeks between treated and untreated crabs. We also observed that crabs fed with BPF-exposed clams had higher haemocyte diameter and volume after 7 days of diet. An increased haemocyte diameter and volume can be explained by an increased phagocytic and or endocytotic activity. However, in our opinion, more in-depth research is required on this specific aspect. Recently, some studies showed an impairment of the immune system caused by bisphenols in marine invertebrates. Indeed, the clam *Tegillarca granosa* exposed for two weeks to BPA-contaminated seawater (10 ng/L and 100 ng/L) showed a significant decrease in THC value (Tang et al., 2020). A reduction of THC values was also found in the shrimp *Charybdis japonica* after 1, 3 and 6 days of exposure to 1 mg/L of BPA (Peng et al., 2018). Regarding the haemocyte diameter and volume, in a previous study conducted on the clam *Ruditapes philippinarum* in which the animals were exposed to bisphenol-contaminated seawater (300 ng/L) for 14 days, both haemocyte diameter and volume were reduced by BPF, BPS and MIX after 7 days (Fabrello et al., 2023).

No cytotoxic effects caused by the BPA analogue-exposed food were observed in this study, whereas crabs fed for 14 days with BPS- and MIX-exposed clams had higher cell proliferation in comparison to the related control. Similarly, cell proliferation increased significantly in *R. philippinarum* specimens exposed to 300 ng/L of a mixture composed of BPAF, BPF and BPS after both one and two weeks of exposure (Fabrello et al., 2023). In this study, increased cell proliferation may be an attempt by crabs to balance the reduced THC levels, mostly in crabs fed BPS- and MIX-exposed clams.

As for immunomarkers, acid phosphatase activity in HL increased in crabs fed for 14 days with BPF and BPS-exposed clams, while in CFH acid phosphatase activity was reduced in crabs fed for 7 days with BPF- and BPS-exposed clams. Alkaline phosphatase in HL was markedly inhibited in crabs fed for one week with MIX-exposed clams, while BPAF-, BPF- and MIX-exposed clams caused a significant reduction of the enzyme activity after 14 days. In CFH, alkaline phosphatase activity was also inhibited by all the tested diets after 7 days. Measurement of hydrolytic enzyme activity is largely used as an immunomarker because of the immune role of enzymes against pathogens. In addition, these enzymes can be altered also in response to xenobiotic presence (Ellis et al., 2011). These results are in accordance with other previous results. Indeed, it has been reported that BPA analogues can inhibit acid phosphatase activity in clams (Fabrello et al., 2023). In addition, in the hepatopancreas of the crayfish *Procambarus clarkii* exposed to BPS (1, 10 and 100 μg/L) both acid and alkaline phosphatase activities were significantly decreased after 14 days (Pu et al., 2024). Our results suggest that ingestion of exposed food can significantly reduce the activity of some important immune-related enzymes.

As for phenoloxidase activity, results demonstrated that it was altered only in CFH, where enzyme activity increased in crabs fed for 14 days with BPS-exposed clams. Alterations (both increased and reduced gene expression) of the prophenoloxidase and impairment in the phenoloxidase activity were observed in gills and hepatopancreas of the intertidal mud crab *Macrophthalmus japonicus* exposed to 1, 10 and 30 μg/L of BPA (Park et al., 2019). The activity of phenoloxidase was also measured by Peng et al. (2018) in *Charibdys japonica* exposed to BPA-contaminated water. The authors observed an increased PO activity in the haemolymph of shrimps exposed for 1, 3 and 6 days to 0.25 and 0.5 mg/L of BPA. In addition, in that study lysozyme activity increased significantly after 3 and 6 days of exposure to 0.25 mg/L of BPA, while it reduced after 9 and 15 days of exposure to the two highest tested concentrations (0.5 and 1 mg/L).

No significant alterations in hemocyanin levels and arylsulfatase activity were observed in the present study. Oppositely, BPF- and BPS-contaminated seawater increased the arylsulfatase activity in the clam *R. philippinarum* during the two weeks of exposure (Fabrello et al., 2023). Regarding the glucose level, we did not observe altered levels, however, it is known that contaminants can alter the glucose level in the crab haemolymph. Indeed, Saravanan et al. (2018), demonstrated that heavy metals (Cadmium and Mercury) induced hyperglycaemia and a contemporary decline of the hepatopancreas glycogen level in the blue swimmer crab *Portunus pelagicus* exposed for 48 hours. Moreover, glucose levels in the haemolymph were increased in the crayfish *Astacus leptodactylus* oral-exposed to 25, 50, and 100 mg of polyethylene microplastic per 1 kg feed after eight weeks (Gholamhosseini et al., 2023).

4.2. Gill and hepatopancreas biomarkers

Total antioxidant capacity provides a general oxidant status of a tissue, revealing if its non-enzymatic antioxidant components are altered. However, in the present study, BPA analogue-exposed food did not alter the total antioxidant capacity in both tissues. Both SOD and CAT enzymes represent the first antioxidant barriers against ROS and for this reason, they play an important role in the maintenance of healthy tissue. In this case, oppositely to the CUPRAC results, SOD activity in gills was reduced in crabs fed with BPF-, BPS- and MIX-exposed clams after 7 days, while after 14 days the BPAF-contaminated diet caused an inhibition of SOD activity. As for CAT activity, it was significantly increased in the gills of crabs fed for 7 days with BPAF- and BPF-exposed clams. Interestingly, CAT activity was increased also in crabs fed for two weeks with BPS- and MIX-exposed clams. In a recent study, the crab *Cyrtograpsus angulatus* fed with the polychaete *Laeonereis acuta* previously exposed to sediments contaminated with BDE-47, a polybrominated diphenyl ether, for 2 weeks showed a reduction of total antioxidant capacity in hepatopancreas after 7 days, while in gills the total antioxidant capacity was increased after 14 days of exposure (Díaz-Jaramillo et al., 2016). The dietary effects of BPA were tested in

the juveniles of the fish *Sparus aurata* exposed for 28 days to pentabromodiphenyl ether (BDE-99), BPA and their mixture (average oral exposure doses of 0.9 μg /day and 4.8 μg /day of BDE-99 and BPA, respectively) (Dias et al., 2024). The authors reported that SOD activity was significantly reduced in the BDE99 + BPA treatment, and both CAT and GST activity were reduced by all the contaminated diets (Dias et al., 2024). An impairment of the antioxidant system was also reported in animals directly exposed to bisphenol-contaminated water. Indeed, in the hepatopancreas of the crab *Charybdis japonica*, exposure to 0.25, 0.5 and 1 mg/L of BPA significantly increased SOD activity after 3 and 6 days (Peng et al., 2018). In addition, in the same study, the authors reported an increased CAT activity at the same BPA concentrations after 3 days of exposure. In the crayfish *Procambarus clarkii*, Pu et al. (2024) evaluated the toxicity of BPS after 14 days of exposure and demonstrated that SOD activity significantly decreased at 1, 10 and 100 μg/L, while CAT activity significantly decreased after 14 days of exposure to 10 μg/L and 100 μg/L (Pu et al., 2024). Lastly, a significant reduction of gill total antioxidant capacity has been demonstrated in *R. philippinarum* after 14 days of exposure to 300 ng/L of BPS and MIX (BPAF+BPF+BPS) (Fabrello et al., 2023). In that study, SOD activity increased in animals exposed for 14 days to BPS, and to MIX after 7 and 14 days. The MIX treatment was also able to increase CAT activity in gills after both 7 and 14 days (Fabrello et al., 2023). Overall, the results regarding the antioxidant system indicate a clear impairment of the antioxidant enzymes in crabs fed with BPA analogue-exposed clams. On the contrary, the level of total non-enzymatic antioxidant components was not affected.

Regarding the glutathione-related enzymes, the GSH represents an abundant antioxidant peptide, and it is also used by antioxidant enzymes like GPX and GST to counteract oxidative stress and help to excrete xenobiotics. After the biochemical reactions, the GSH level has to be reconstituted by GR enzyme. In the present study, the GR activity was not altered by any of the tested diets. Contrarily, GPX activity was significantly altered in both the gills and hepatopancreas of crabs. An alteration of GPX activity was also recorded in a recent study in which the authors tested the dietary exposure on an estuarine food chain model composed of three levels: *Artemia salina* as a primary consumer, *Litopenaeus vanamei* as a secondary consumer, and *Oreochromis niloticus* as a tertiary consumer (Saikumar et al., 2024). In detail, they exposed *A. salina* to polystyrene microplastics at a concentration of 10^6 particles/mL. *A. salina* was then supplied as food to *L. vannamei*, which in turn was supplied as food to *O. niloticus*. The authors evaluated the gene expression of two antioxidant genes, such as *GPX* and mitochondrial SOD (*cMnSOD*) after both 24 and 48 hours post-feeding. Interestingly, the authors observed an upregulation of both genes. In the tertiary consumer, the authors reported that the antioxidant *GPX* gene was upregulated while *SOD3* gene was downregulated after 24 h. However, both *SOD3* and *GPX* were upregulated 48 hours post-feeding, in comparison to the control (Saikumar et al., 2024).

The third glutathione-related enzyme measured in the present study was GST, which has a pivotal role in the conjugation of xenobiotics with GSH to allow their excretion. We observed a significant reduction of GST activity in crabs fed with both BPAF- and MIX-exposed clams after 7 days, and BPF-exposed clams after 14 days. Conversely, an increased GST activity was reported in the hepatopancreas after 7 days and in gills after 7 and 14 days in crabs *Cyrtograpsus angulatus* fed with BDE-47 contaminated polychaete (Díaz-Jaramillo et al., 2016).

In another study that investigated the effects of BPA, the GPX activity increased after both 3 and 6 days of exposure of *Charybdis japonica* to 0.5 and 1 mg/L of BPA (Peng et al., 2018). Furthermore, a significant increment of GPX activity was observed in the crayfish *Astacus leptodactylus* exposed for 96 hours to 24.11, 12.06 and 96.45 mg/L of BPA (Uçkun, 2022). The same concentrations caused also an increased GST activity, while the GR activity was significantly reduced (Uçkun, 2022). Moreover, GST and GR activities were significantly decreased in the muscle of the narrow-clawed crayfish *Pontastacus leptodactylus* after 5 days of exposure to 10, 50 and 100 μg/L of BPA-contaminated water (Diler et al., 2022). The toxicity of BPF, BPS, and BPA was assessed in the marine rotifer *Brachionus koreanus* (Park et al., 2018). The three bisphenols caused ROS production, with BPS and BPF increasing both ROS and GST levels at almost all the tested concentrations for 24 h (1, 5, and 10 mg/L) (Park et al., 2018). In addition, BPF and BPA significantly influenced the expression levels of both cytochrome P450 (CYP) and GST genes (Park et al., 2018). Interestingly, BPS (300 ng/L) caused a significant decrease of GR activity in *R. philippinarum* after 14 days , while GST activity increased significantly after 7 days of exposure to BPS and MIX and decreased after 14 days of exposure to the same compounds (Fabrello et al., 2023).

In this study, we tested the hypothesis that BPA analogue-exposed food can cause neurotoxic effects in crabs. Based on the obtained results we can exclude neurotoxic effects of BPA analogue-exposed clams. Interestingly, we observed an increased AChE activity in the crabs fed for 7 days with BPF- and BPS-exposed clams. Similarly, *C. aestuarii* exposed to 300 ng/L of BPF for 7 and 14 days showed an increased AChE activity in gills (Fabrello et al., submitted). Moreover, an increased AChE activity was also reported in *Mytilus galloprovincialis* exposed to 100 μg/L of TBBPA, while at 1 and 10 μg/L enzyme activity was inhibited (Copeto et al., 2024). Neurotoxic effects of BPA were recorded in the hepatopancreas of the crayfish *A. leptodactylus,* in which both AChE and carboxylesterase activities decreased significantly at almost all concentrations tested (96.45 mg/L, 48.23 mg/L, 24.11 mg/L, 12.06 mg/L) (Uçkun, 2022). Furthermore, a significant reduction in AChE activity was 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).

In the case of amylolytic enzymes, we observed that their activity was reduced by all the diets, and in particular, BPS- and MIX-based diets inhibited the amylolytic activity after 7 days, as well as after a 14-day diet. Digestive enzyme activities, such as esterase, β-glucosidase, β-galactosidase, amylase and endoglucanase have been used as biomarkers to evaluate the effects of xenobiotics, such as insecticides and heavy metals. For instance, the insecticide fenvalerate significantly reduced the amylase activity in the gut of zebrafish after 28 days (Mahmoud et al., 2020). Interestingly, Yang et al. (2017) reported that BPS can directly interact with the catalytic site of amylase inhibiting its activity. This can explain why we observed a clear decrease in amylolytic enzyme activity in crabs fed with BPS-contaminated diets (BPS- and MIX-treated clams) after 7 days. Probably, a similar catalytic inhibition activity can be exerted on the amylolytic enzymes also by the other bisphenols (that share a common bisphenol core structure) as demonstrated after 14 days. However, the observed enzymatic inhibition did not correspond to altered glucose levels in haemolymph as previously reported in the results chapter.

Regarding the oxidative damage biomarkers, crabs fed for 14 days with BPS- and MIX-exposed clams had higher PCC values in comparison to those of control crabs. As for LPO, a significant reduction of LPO levels was recorded in the gills of crabs fed for 7 days with BPF-exposed clams. On the contrary, in juveniles of the fish *Sparus aurata* dietary exposure to BPA increased LPO levels after 28 days (Dias et al., 2024). Similarly, a significant increase in LPO levels was observed in the hepatopancreas of *C. granulatus* fed with hexachlorobenzene-contaminated food for one week (Chaufan et al., 2006). Furthermore, LPO levels increased significantly in the crab *Charybdis japonica* after 9 days of exposure to 1 mg/L of BPA and after 15 days of exposure to 0.5 mg/L and 1 mg/L of BPA (Peng et al., 2018).

It is well known that BPA can cause DNA oxidative damage in both animals and humans inducing single and double-strand breaks (Ďurovcová et al., 2022). The obtained results indicate that ingestion of MIX-treated clams significantly increases the DNA damage in the gills of crabs after 14 days of diet. In accordance, BPA increased the percentage of micronucleus in haemolymph, gills and hepatopancreas of *M. galloprovincialis* exposed to all the tested concentrations (0.01, 0.1, 1 10 and 100 ng/L) after 24, 48 and 72 hours (Nalbantlar and Arslan, 2024). Bisphenol E, bisphenol Z and BADGE also caused an increased

micronucleus percentage (Nalbantlar and Arslan, 2024). Similarly, concentrations of 0.25, 0.5 and 1 mg/L of BPA caused genotoxic effects in the haemocytes of the gammarid species *Echinogammarus veneris*, while only the two highest tested concentrations caused DNA damage in *Gammarus aequicauda* (Cosentino et al., 2022). BPF increased DNA damage in all the tested concentrations in the two species, while 1 mg/L of BPS increased the DNA damage in the haemocytes of *E. veneris* (Cosentino et al., 2022). Overall, BPA analogues provided via food were able to increase the oxidative damage on proteins and DNA in crabs, while oppositely, the lipid peroxidation levels remained unaltered or were decreased.

4.3. Physiological parameters

BPA analogue-exposed clams were able to alter physiological parameters in crabs. Indeed, BPAF- and MIX-treated clams increased oxygen consumption rate in crabs fed for two weeks. Moreover, after the first week, we observed a significant reduction in the excretion rate of crabs fed with all the differently exposed clams. These results are in accordance with previous studies conducted on bisphenols, even if in animals exposed to contaminated water. Indeed, *C. aestuarii* exposed to 300 ng/L of bisphenol mixture showed an increased oxygen consumption rate after two weeks of exposure (Fabrello et al., submitted). In addition, it has been reported that BPA can alter physiological parameters in marine invertebrates, such as molluscs and crustaceans (Elkader and Al-Shami, 2024; Han et al., 2022). In detail, Han et al. (2022) reported a significant reduction of oxygen consumption rate in the white-leg shrimp *Litopenaeus vannamei* exposed for 14 days to 2 μg/L of BPA, 0.69 mg/L of polystyrene microplastics, or their mixture. In addition, the ammonia excretion rate significantly increased in treated males (Han et al., 2022). Regarding xenobiotics provided via food, Scarlett et al. (2009), evaluated the effects on behaviour and heart rate in the crabs *Carcinus maenas* fed for 7 days with the mussel *Mytilus edulis* previously exposed to branched alkylbenzenes (BABs). Interestingly, crabs exposed to the BAB-contaminated mussels were slow to respond to stimuli and appeared less aggressive compared to the control. In addition, BAB-exposed crabs took a longer time to detect and open mussels and by day 6 of the exposure they failed to open shells, indicating that contaminated food can alter the animal behaviour (Scarlett et al., 2009). Overall, both crab respiration and excretion rates were altered during this experiment, demonstrating for the first time that BPA analogue-exposed foods can alter the physiology of a secondary consumer.

4.4. Bioaccumulation

It has been demonstrated that xenobiotics can be transferred from molluscs to crabs. For instance, crabs fed with mussels contaminated with branched alkylbenzenes showed bioaccumulation, even if at low levels (Scarlett et al., 2009). A similar result was recorded in the crab *Chasmagnathus granulatus* fed with rabbit food supplemented with algae previously treated with hexachlorobenzene (Chaufan et al., 2006). Moreover, the crab *Cyrtograpsus angulatus* fed with pre-exposed *Laeonereis acuta* polychaetes showed BDE-47, a polybrominated diphenyl ether, bioaccumulation in organs (Díaz-Jaramillo et al., 2016). In our study, we recorded detectable bioaccumulation only for BPAF in crabs fed with BPAF- and MIX-exposed clams, whereas BPF and BPS were not detected in crab tissues. However, the reduced bioaccumulation capability of crabs is partially surprising. First, a potential influence of different hydrophobic characteristics of the compounds tested on crab bioaccumulation cannot be excluded (Log_{kow}values: BPAF 4.47; BPF 2.91; BPS 1.65). Second, it is important to highlight that BPA analogues are relatively quickly metabolized by organisms and transformed into bisphenol conjugates. Indeed, it has been demonstrated that after an initial enzymatic hydrolysis to cleave the conjugates, the detectable concentrations of the free-form BPA analogues increased in aquatic organisms (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). Considering the results obtained in this study and the literature information, it will be important in the future to evaluate whether crabs, as well as other aquatic species, can also metabolize BPA analogues accumulated through food or seawater quickly enough.

5. Conclusions

Results reported in this study demonstrated that BPA analogues can cause detrimental effects even if provided via food, like what was observed after waterborne exposure of crabs (Fabrello et al., submitted). Indeed, crabs fed with clams previously exposed to environmentally realistic concentrations of BPA analogues showed alterations in haemocyte parameters and on several immunomarkers, like hydrolytic enzymes. Moreover, the antioxidant system with its enzymatic effectors like SOD and CAT, as well as the detoxifying enzymes, such as the glutathione-related enzymes, were altered by exposed food. Interestingly, an inhibitory effect on the amylolytic enzyme activity, which can cause a reduction in the energy available, was observed in crabs. In addition, we observed that BPS and MIX induced damage to both proteins and DNA, indicating that BPS and MIX can be genotoxic even if provided through food. Regarding neurotoxicity and physiological parameters, we observed that BPF and BPS were able to increase the AChE activity while all the provided diets induced significant alterations of physiological biomarkers (oxygen consumption rate and ammonia excretion rate). Overall, the MIX-treated clams did not cause higher alterations than clams treated with single bisphenols and there was not a clear difference in toxicity caused by diets based on clams previously exposed to hydrophobic or hydrophilic bisphenols. Lastly, our bioaccumulation results demonstrated that BPAF can be transferred through the first step in a simplified marine food chain, probably due to its higher Log_{kow} value with respect to the other two tested bisphenols. Summarising, the results of this study demonstrated that diets based on BPA analogue-exposed food can induce significant alterations of important biological responses in *C. aestuarii*, suggesting a potential ecotoxicological risk for the marine food chain, at least under the experimental condition of the present survey.

CRediT authorship contribution statement

Jacopo Fabrello: Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Elisabetta Tarussio:** Validation, Methodology, Investigation, Formal analysis. **Alessia Romanello:** Validation, Methodology, Investigation, Formal analysis. **Anna Schiavon:** Validation, Methodology, Investigation, Formal analysis. **Vittorio Rubens Damoli:** Validation, Methodology, Investigation, Formal analysis. **Francesco Luisi:** Validation, Methodology, Investigation, Formal analysis. **Marco Roverso:** Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation. **Sara Bogialli:** Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Conceptualization. **Valerio Matozzo:** Writing – original draft, Validation, Supervision, Methodology, Investigation, Formal analysis, 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.

Data availability

The data that has been used is confidential.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.aquatox.2024.107084.

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