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Ecotoxicological effects and bioaccumulation of BPA analogues and their mixture in the clam *Ruditapes philippinarum*

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ABSTRACT

Bisphenol A is recognized as an endocrine disruptor that can affect several biological processes in marine species. Consequently, its use has been restricted and it has been replaced with other similar compounds named bisphenol A analogues (BPA analogues). BPA analogues are speculatively considered safer compounds than BPA and their usage is increasing with a consequent higher environmental release. In this study, specimens of the clam *Ruditapes philippinarum* were exposed to three main BPA analogues, namely BPAF, BPF, BPS and their mixture at an environmentally relevant concentration of 300 ng/L for 7 and 14 days. Effects on biomarkers indicative of cytotoxicity, oxidative stress and damage and neurotoxicity were evaluated. In addition, bioaccumulation of the compound tested was analysed in clam soft tissues. Results showed that BPA analogues at an environment concentration affected cellular parameters and antioxidant system causing also oxidative damage, suggesting that BPA analogues can be harmful compounds for clams.

1. Introduction

Bisphenols are synthetic and widely used compounds and the most important of them is bisphenol A (BPA). It is manly used as plasticizer in the manufacture of polycarbonate plastic and its production increased over the years and is expected to reach 10.6 million metric tons in 2022 (Fabrello and Matozzo, 2022). BPA is also used as a colour developer in thermal paper and its use in thermal paper manufactured in the EU and placed on the EU market was 2776 tons in 2017 (Fabrello and Matozzo, 2022). BPA is considered as a chemical compound of very high concern due to its toxicity on reproductive system and its endocrine disrupting effects both in humans and other animals. This has led to BPA restrictions with its ban in the manufacture of baby bottles in USA, Canada, and EU. Furthermore, the EU has limited the BPA usage to less than 0.02% by weight in thermal paper since January 2020 (EU, 2016).

In consequence of this ban, BPA has been replaced by other similar compounds named bisphenol A analogues (BPA analogues), a group that includes 17 bisphenols and many other bisphenol derivatives. The main BPA analogues recently used in the production of polycarbonate plastics and epoxy resins are bisphenol F (BPF), bisphenol S (BPS), and bisphenol AF (BPAF). BPF is also used in food packaging, liners, water pipes, dental sealants, industrial floors, grouts, electrical varnishes, coatings, lacquers, plastics, adhesives, and tissue substitutes (Chen et al., 2016). In addition, BPS has also many uses, such as epoxy glues, thermal receipt papers, sulfonated poly (ether ketone ether sulfone), and as additive in dyes agents (Chen et al., 2016). Lasty, BPAF is used in common polymer applications, such as a cross-linker in fluoroelastomers, electronics, and optical fibres, as a high-performance monomer for polyimides, polyamides, polyesters, polycarbonate copolymers and in specialty polymer applications such as plastic optical fibres and waveguides (Chen et al., 2016).

Information regarding the BPA analogues production is scarce, even if it is considered increasing. Indeed, BPS usage has increased twice from 200 tons to 397 tons between 2016 and 2017, and the European Food Safety Authority (EFSA) reports that the annual production of BPS is 1000–10,000 tons (ECHA, 2018; EFSA, 2020). Regarding BPAF, its annual production in the USA was in the range of approximately 4.5–220 tons from 1986 to 2002, while its manufacture/import in the EU is 100–1000 tons per year (Hu et al., 2019; ECHA 2023).

Despite their recent adoption, BPA analogues are considered hormonally active due to their similar chemical structure to BPA, showing endocrine-disrupting effects acting as estrogenic, progesteronic and

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anti-androgenic compounds (Rochester and Bolden, 2015; Usman et al., 2019). Bisphenols reach aquatic environments from wastewater treatment plants (WWTPs), discharge from urban and industrial areas, and they are also continuously released and adsorbed by microplastics in the environment. After their release, bisphenols are mainly biodegraded. However, their persistence in water is highly variable. Indeed, some bisphenols can be rapidly degraded in river water as demonstrated for BPF, which can be completely biodegraded in 37 days (Frankowski et al., 2020). On the contrary, the biodegradation of BPAF, BPE, and BPB was minimal, while the biodegradation of BPS appeared to be higher than that of BPA, but lower than that of BPF in rivers. In addition, BPAF has a half-life that ranges from 15 to 180 days in water (Chen et al., 2016). In aquatic environments, the BPA analogues are often found at lower concentrations than BPA, in the range of a few ng/L. However, it has been reported that their concentrations can reach hundreds and thousands of ng/L (Fabrello and Matozzo, 2022), exceeding the predicted no-effect concentration (PNEC) for BPA settled by EU at 1500 ng/L for freshwater and 150 ng/L in marine water (EU, 2008). BPAF was recorded at a mean concentration of 140 ng/L in surface water in China, while BPF reached 2850 ng/L and BPS reached 65,600 ng/L in surface water of Japan and China, respectively (Wang et al., 2017; Yamazaki et al., 2015; Huang et al., 2018). In marine ecosystems, bisphenol analogues can reach concentrations from very few ng/L up to tens of ng/L, even if their concentrations can increase, as in the case of BPF with a maximum concentration of 282 ng/L and 1470 ng/L in seawater in South China and in the Tokyo Bay, respectively (Zhao et al., 2019; Yamazaki et al., 2015).

Limited information on the effects of BPA analogues to aquatic species pose several questions on the BPA analogues ecotoxicological profile. Consequently, in this study we evaluated for the first time the effects of BPAF, BPF BPS and their mixture on cellular and biochemical parameters of the clam *Ruditapes philippinarum*. We chose *R. philippinarum* as a model organism because it is a sensitive species, easy to collect (it is widely distributed in estuarine areas, such as the Lagoon of Venice) and maintain under laboratory conditions. In addition, this clam species is largely used in ecotoxicological studies to assess the effects of environmental contaminants in bivalves. The hypotheses we tested were **i)** if BPA analogues can pose a potential ecotoxicological risk to marine bivalves at environmentally realistic concentrations, **ii)** if a mixture of BPA analogues can be more harmful than singular BPA analogues, and **iii)** if clams can bioaccumulate BPA analogues.

2. Materials and methods

2.1. Clam acclimation and exposure

R. philippinarum specimens (mean length = 36.0 mm) were sampled in a licensed area for bivalve culture in the southern basin of the Lagoon of Venice (Italy) and acclimated for 7 days in large aquaria filled with sand and aerated seawater (salinity of 35 ± 1, temperature of 12 ± 0.5 °C). A mixture of the microalgae *Tetraselmis chuii* and *Phaeodactylum tricornutum* was provided *ad libitum* as food.

The stock solutions (100 mg/L) of BPAF and BPF were prepared in methanol, while BPS stock solution was prepared in distilled water. Clams (80 in total per each concentration tested) were exposed for 7 and 14 days to 300 ng/L of BPAF, BPF, BPS and MIX (100 ng/L of each) in 35 L glass tanks (two tanks per each concentration, with 40 clams per tank) without sandy bottom. The nominal concentration was chosen based on data on BPA analogue levels in aquatic ecosystems (see the Introduction section). A solvent control was omitted because the concentration of methanol used in BPAF and BPF exposure tanks was very low (0.0003%), while the solvent can exert acute and chronic effects at higher concentrations (tens and hundreds of mg/L) in aquatic species (Kaviraj et al., 2004; Hutchinson et al., 2006). Seawater, chemical compounds, and food supply (microalgae mixture at an initial concentration of about 1.6×10^{10} cells/L) in exposure tanks were renewed every 24 h.

2.2. Tissue collection

Haemolymph was collected from the anterior adductor muscle by a 1-mL plastic syringe and stored in Eppendorf tubes at 4 °C. At each sampling time, 5 pools of haemolymph (from six clams each) from each experimental condition were prepared. A volume of pooled haemolymph was immediately used to measure total haemocyte count (THC), haemocyte diameter and volume, lactate dehydrogenase (LDH) activity and haemocyte proliferation (XTT assay). The remaining part 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) samples, frozen in liquid nitrogen and stored at −80 °C until analyses.

Gills and digestive gland from clams were then excised, pooled to obtain five different pools of six clams each, divided in aliquots, frozen in liquid nitrogen, and stored at −80 °C until analyses.

2.3. Haemocyte parameters

THC, as well as haemocyte diameter and volume, were determined using a Scepter™ 2.0 Automated Cell Counter (Millipore, FL, USA). Briefly, 20 μL of haemolymph were added to 2 mL of Coulter Isoton II diluent. The THC was expressed as the number of haemocytes (10^7)/mL of haemolymph, while haemocyte diameter and volume were expressed in μm and picolitres (pL), respectively.

A commercial kit (*Cytotoxicity Detection* Kit, Roche) was used to measure lactate dehydrogenase activity (LDH) in cell-free haemolymph (CFH). Pooled haemolymph (500 μL) from each experimental condition was centrifuged at 780×*g* for 10 min, and the 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, as described in Fabrello et al. (2021). In detail, a volume of 200 μL of the mixture provided by the kit was added to 400 μL of pooled haemolymph and incubated for 4 h in a dark humidified chamber. The absorbance at 450 nm was then recorded using a Beckman 730 spectrophotometer. The results were normalized to THC values of each experimental groups and expressed as optical density (OD) at 450 nm.

The lysozyme activity was measured in haemocyte lysate (HL) from pooled haemolymph (500 μL). 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 acid phosphatase activity was measured both in HL and CFH. In detail, the substrate 4-nitrophenyl phosphate was hydrolysed by the enzyme 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.

The arylsulfatase activity was measured in HL samples according to Zucker-Franklin et al. (1983), measuring the conversion of the substrate p-nitrocatechol sulfate to p-nitrocatechol and sulfate. 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.

Total protein concentrations in HL and CFH samples were quantified according to Bradford (1976).

2.4. Gill and digestive gland enzyme activity assays

Gills and digestive gland samples were homogenised for 5 min and 50 oscillation 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), and centrifuged at 12,000 g for 30 min at 4 °C. Supernatants (SN) were collected for analyses. The protein concentration in SN samples was quantified according to Bradford (1976).

The total antioxidant capacity was evaluated using the CUPRAC method according to Apak et al. (2004). The cupric reduction product reacts with neocuproine and the complex was measured at 450 nm using a microplate reader. Results are expressed as mM of Trolox equivalents.

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

CAT activity was measured in gills and digestive gland SN in triplicate following the method proposed by Aebi (1984). The enzyme activity in a volume of 30 μL of tissue SN were measured at 240 nm and expressed as U/mg protein. One unit of CAT was defined as the amount of enzyme that catalysed the dismutation of 1 μmol of H_2O_2/min.

The method of Ellman et al. (1961) was used to measure acetylcholinesterase (AChE) activity in gill SN, following the colorimetric reaction between acetylthiocholine and the reagent dithiobisnitrobenzoate. Changes in absorbance were then recorded at 405 nm for 5 min on a microplate reader at room temperature. The results were expressed as nmol/min/mg of protein. The same parameters were adopted to measure the butyrylcholinesterase (BChE) activity according to Escartín and Porte (1997) using butyrylthiocholine as substrate. Results are expressed as nmol/min/mg protein.

Glutathione S-transferase (GST) activity was measured in digestive gland 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 protein.

Glutathione reductase (GR) activity was evaluated 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 protein.

Oxidative damage in gills and digestive gland was measured through both protein carbonyl content (PCC) and the lipid peroxidation assays. 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 protein. 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 protein. 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).

2.5. Chemical analysis

Methanol, acetonitrile, ammonium acetate, BPF, BPAF, 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).

Seawater samples were collected after three timepoints, namely 0, 7 and 14 days to determine actual concentrations of bisphenol analogues. As the seawater in tanks was changed daily for the entire duration of the experiment, seawater samples were collected after 0 and 24 h from the daily addition BPA analogues. Seawater samples were treated by a 0.2 μm RC Syringe Filter (Phenomenex, Bologna, Italy) before being 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 phase A and B were water and methanol, 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. Injection volume was 400 μL. 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 1 spectrum/s. The QTOF calibration was performed daily with the manufacturer's solution in this mass range. The MS were analysed by the Mass Hunter Qualitative Analysis software (Agilent Technologies, Palo Alto, Santa Clara,CA, USA).

For quantitative purpose, a matrix-matched seven points external calibration curve was made in the range 20–600 ng/L. Artificial seawater, prepared according to ASTM Standards D 1141–98, was used as matrix. Peak areas for each analyte, obtained by the $[M\text{-}H]^-$ extracted ion chromatogram (mass accuracy 10 ppm), were normalized for the area of the internal standard, added at the final concentration of 600 ng/L. Linearity was evaluated by the least squares regression and $R^2 > 0.995$ was obtained for all the analytes. LODs were 1 ng/L, 5 ng/L and 30 ng/L for BPAF, BPS and BPF, respectively. Samples and calibration solutions were analysed in duplicate.

Bioaccumulation of BPA analogues was evaluated in whole animals after tissue homogenisation (about 1 g for each clam) and protein precipitation with 7 mL of cold acetonitrile added with internal standard at the concentration of 20 μg/L (corresponding approximately to 140 ng/g in clams). After centrifugation (13000 rpm, 10 min, 4 °C), 20 μL of the supernatant were analysed by UHPLC-HRMS as previously described. Ten no-treated clams spiked with the internal standard were extracted and pooled to obtain a matrix-matched seven points external calibration curve, in the range 14–1400 μg/L (approximately 98–9800 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 were 10 ng/g, 70 ng/g and 400 ng/g for BPAF, BPS and BPF, respectively. Each treated animal was analysed separately, and results are reported as mean and standard deviation.

2.6. Statistical analysis

Data from each biomarker were analysed using the Permutational multivariate Analyses of Variance (PERMANOVA, with 9999 permutations) to detect significant effects of treatment, exposure time and their interaction (treatment*time) using the software PRIMER 6 PERMANOVA Plus (PRIMER-E Ltd, Plymouth, UK). Then, the post hoc test (Fisher test) was used for pairwise comparisons of results using the software OriginPro 2022. All results were expressed as the mean ± standard deviation (SD).

3. Results

3.1. Biomarker responses

Results of PERMANOVA analysis are reported in supplementary materials (Table SM1, SM2 and SM3). The THC value was altered by the factor "time*treatment interaction" ($p < 0.05$) (Table SM1, Fig. 1A). In addition, both diameter and volume of haemocytes were affected by treatment ($p < 0.01$) and the pairwise comparison revealed a significant reduction of both after exposure for 7 days to BPF, BPS and MIX (Table SM1, Fig. 1B-C). We did not observe any cytotoxic effects (LDH assay) (Table SM1, Fig. SM1A) while treatment affected significantly ($p < 0.001$) cell proliferation, and the pairwise comparison revealed increased values after 7 and 14 days of exposure to the MIX (Fig. 1D).

Lysozyme activity and acid phosphatase in HL were affected only by the factor "time of exposure" ($p < 0.001$) (Table SM1, Fig. SM1B–1C), whereas CFH acid phosphatase activity was affected significantly ($p < 0.001$) by time of exposure, and by treatment ($p < 0.001$) in HL

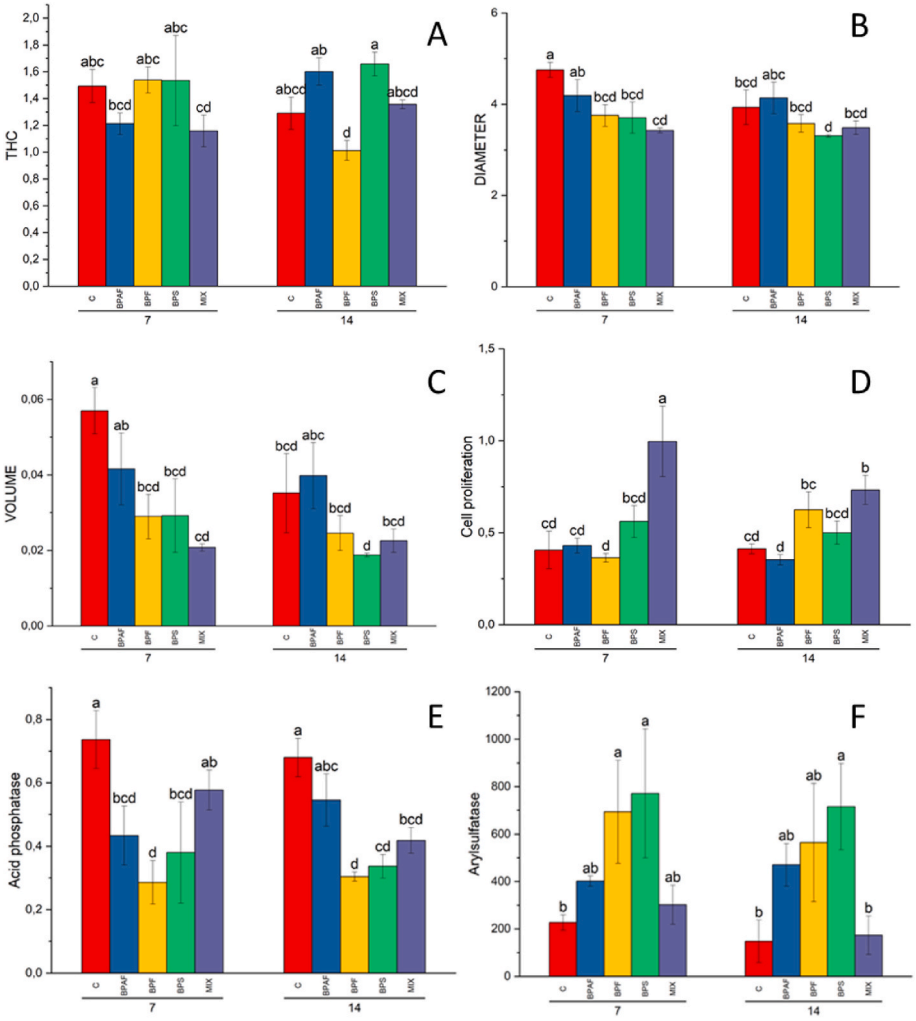

Fig. 1. Total haemocyte count (THC), expressed as n° haemocytes (10^7)/mL haemolymph (A), haemocyte diameter, expressed in μm (B), haemocyte volume, expressed in pL (C), haemocyte proliferation, expressed as OD_{450} (D), haemocyte lysate acid phosphatase activity, expressed as U acid phosphatase/mg protein (E), and haemocyte lysate arylsulfatase activity, expressed as μg/h of p-nitrocatechol produced/mg protein (F). The values are mean ± SD (n = 5). The letters indicate significant differences in comparison with the related control.

(Table SM1). In addition, the post-hoc test revealed that enzyme activity was dropped by BPAF, BPF and BPS after 7 days of exposure and by BPF, BPS and MIX after 14 days (Fig. 1E). Similarly, the arylsulfatase activity measured in HL was affected by treatment ($p < 0.01$), with an increased activity after exposure for 7 days to BPF and BPS and the effect was also revealed in the BPS-treated animals after 14 days (Table SM1, Fig. 1F).

The total antioxidant capacity measured with the CUPRAC assay was altered significantly ($p < 0.05$) by treatment in gills and by the factor "treatment*time interaction" in digestive gland ($p < 0.05$) (Table SM2, SM3). Moreover, there was a significant reduction in the total antioxidant capacity in gills of animals exposed for 14 days to BPS and MIX (Fig. 2A-B).

The total SOD activity was influenced significantly ($p < 0.05$) by exposure time in clam digestive gland (Fig. SM2A), while both two independent parameters alone (treatment and exposure time) and their interaction affected SOD activity in gills ($p < 0,001$ and $p < 0.05$, respectively) (Table SM2, SM3). In addition, there was an increased enzyme activity in animals exposed to BPS (14 days) and MIX (7 and 14 days) (Fig. 2C).

As for CAT, gill enzyme activity resulted influenced significantly ($p < 0.001$) by exposure time and treatment factors, while in digestive gland only the time of exposure had an effect ($p < 0.05$) (Table SM2, SM3; Fig. SM2B). Again, an increased activity was observed in gills of clams exposed for 7 and 14 days to MIX (Fig. 2D).

Like SOD and CAT, the main effects on GR activity were recorded in gills (treatment*time interaction; $p < 0.05$) with a significant decrease in GR activity after 14 days of exposure of clams to BPS (Table SM2; Fig. 2E). In digestive gland, only the effect of the factor "exposure time" were observed ($p < 0.001$) (Table SM3; Fig. SM2C).

Similarly, a statistically significant ($p < 0.001$) effect of the factor "treatment*time interaction" was recorded in digestive gland GST activity, and the pairwise comparisons revealed significant differences between treated animals and the related controls at 7 and 14 days of exposure (Table SM3; Fig. 2F). Indeed, the GST activity increased significanlty after 7 days of exposure to BPS and MIX, while it decreased significantly after 14 days of exposure at the same experimental conditions.

Exposure did not cause neurotoxic effects in clams, at least in the experimental conditions tested in our study. Indeed, no significant alterations on AChE and BChE have been observed, and only the factor "exposure time" affected significantly ($p < 0.001$) BChE activity (Table SM2; Fig. SM3A-3B).

As for oxidative damage, a significant effect of treatment ($p < 0.05$) of digestive gland PCC was observed, with a significant reduction in BPAF, BPF, MIX after 7 days of exposure (Table SM3). Conversely, no alteration of PCC content was observed in gills (Table SM2; Fig. 3A-B).

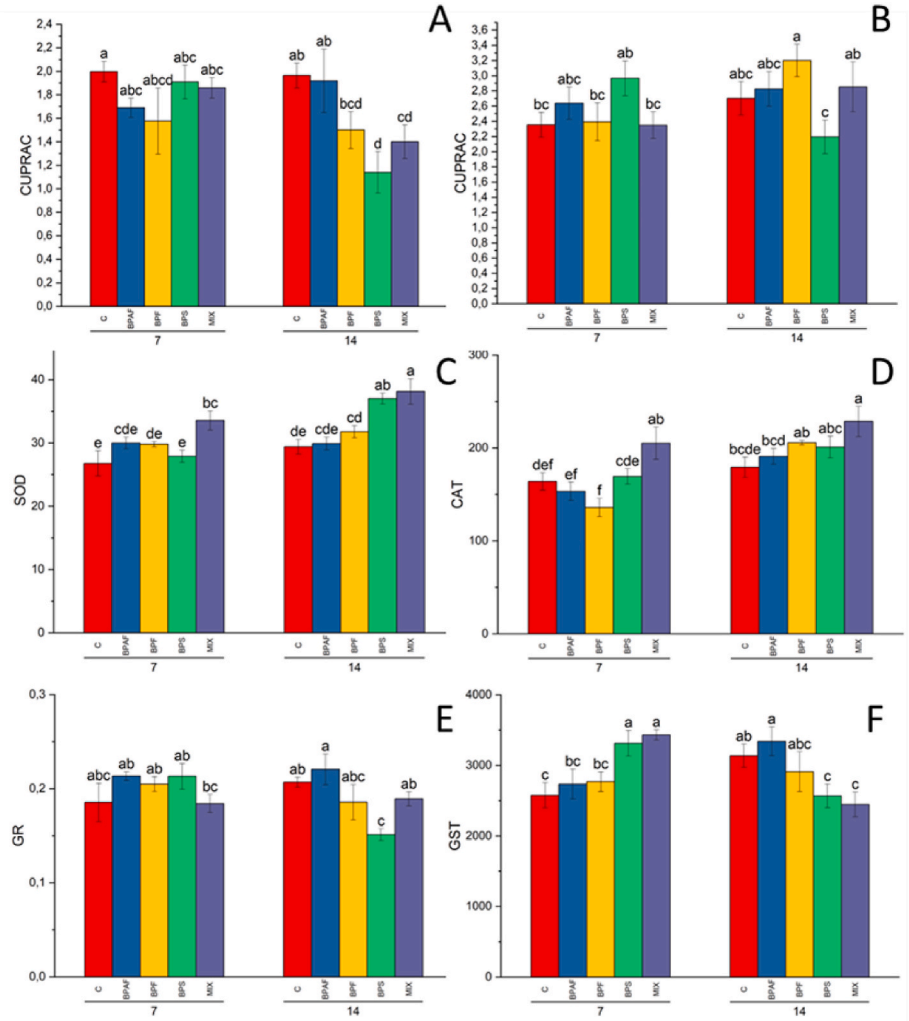

Fig. 2. CUPRAC levels expressed as mM of Trolox equivalents/mg protein in gills (A) and digestive gland (B), SOD activity in gills, expressed as U SOD/mg protein (C), CAT activity in gills, expressed as U CAT/mg protein (D), GR activity in gills expressed as U GR/mg protein (E), GST activity in digestive gland expressed as nmol/mg protein (F). The values are mean ± SD (n = 5). The letters indicate significant differences in comparison with the related control.

Furthermore, LPO was altered significantly ($p < 0.01$) by treatment in gills (Table SM2), with significant increased levels after the exposure for 7 days of clams to both BPS and MIX (Fig. 3C). In digestive gland, LPO was affected by the factors time, treatment, and their interaction ($p < 0.001$, $p < 0.05$, $p < 0.05$, respectively) (Table SM3). After 14 days of exposure, significant increased LPO levels were observed in clams exposed to BPF, BPS and MIX (Fig. 3D).

3.2. Chemical analyses

Actual concentrations of the three bisphenols in seawater tanks, measured by UHPLC-HRMS, at different timepoints are reported in Table SM4. At Time 0, it was noted that the actual concentrations were very similar to the nominal ones. At time 24 (before seawater renewal), results showed a decrease in the concentrations of bisphenols, more pronounced for BPAF.

Table SM5 reports the concentrations of bisphenols in clam soft tissues, depicting the bioaccumulation capability of clams under the experimental conditions tested. Results are reported as the mean concentration measured in five different animals per experimental conditions.

4. Discussion

In this study, three of the main BPA analogues have been tested at an environmentally realistic concentration (alone and as a mixture) for the first time on the clam *Ruditapes philippinarum*, an ecological and commercial important species. To our knowledge similar concentrations have not been tested yet in marine species, but similar results have been reported in other studies testing higher concentrations.

Haemolymph results showed that BPA analogues can alter haemocyte parameters in clams. Indeed, we observed a general reduction of both diameter and volume of haemocytes with a contemporary slight reduction of THC after 7 days of exposure. Furthermore, there was an increased cellular proliferation. Increased cell proliferation can be suggested as a response of animals to compensate - partially at least - the reduction of circulating haemocyte number. Because of increased cell proliferation, new and smaller haemocytes than the older ones were produced, as suggested by reduction in cell diameter and volume. As for hydrolytic enzyme with immunological role, we observed that acid phosphatase activity in haemocyte lysate was generally reduced by treatment. On the contrary, the arylsulfatase activity was enhanced during the two weeks of exposure. Interestingly, this enzyme, similarly to β-glucuronidase, is involved in the de-conjugation of the bisphenol-sulfate conjugates that can be produced by other biochemical pathways (Ginsberg and Rice, 2009). Our results are like those concerning

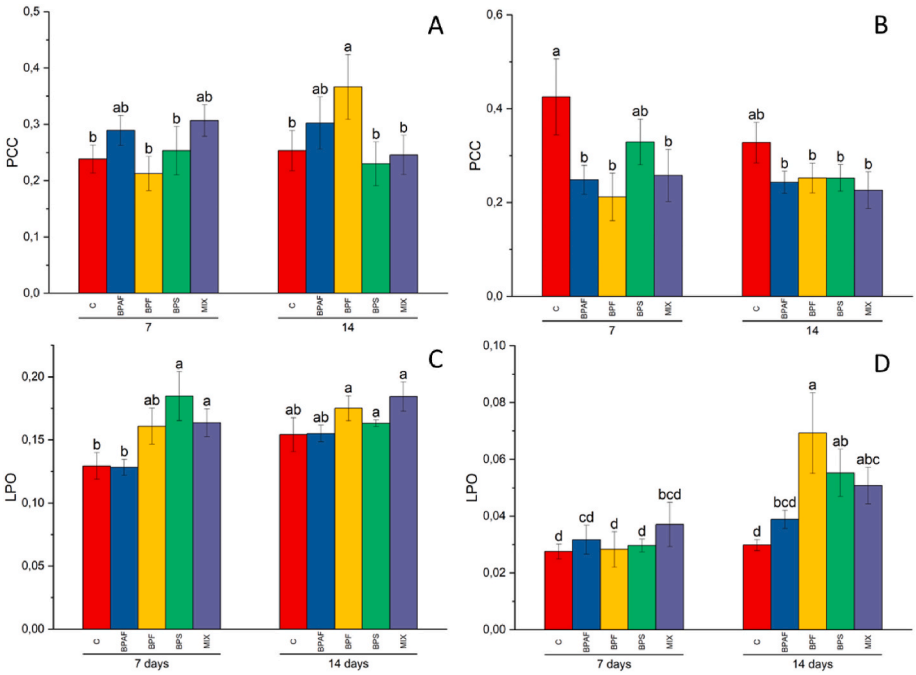

Fig. 3. Protein carbonyl content, expressed as nmol/mg protein, in gills (A) and digestive gland (B), and lipid peroxidation, expressed as nmol TBARS/mg protein, in gills (C) and digestive gland (D). The values are mean ± SD (n = 5). The letters indicate significant differences in comparison with control.

the evaluation of the effects of BPA on immune systems in other invertebrate species. Indeed, in the bivalve *Tegillarca granosa*, THC was reduced after 2 weeks of exposure to 10 ng/L and 100 ng/L of BPA. Moreover, the authors observed a decreased percentage of red granulocytes and an increased percentage of both basophil granulocytes and hyalinocytes (Tang et al., 2020). Furthermore, there was a significantly reduction of both phagocytic activity of haemocytes and the expression of four immune-related genes (Tang et al., 2020). In the study of Peng et al. (2018), reduced THC values were recorded in the crab *Charybdis japonica* exposed for 1, 3, and 6 days to 1 mg/L. Like our results, the activity of the immune-related enzymes lysozyme and phenoloxidase reached the highest values after one week of exposure and then decreased during the second week of exposure in both haemolymph and hepatopancreas. Regarding the cytotoxicity assay (LDH), we did not observe cytotoxic effects caused by the tested compounds. Instead, Kaptaner et al. (2021) observed that BPS caused cytotoxic effects in a concentration-dependent manner in hepatocytes of the rainbow trout *Oncorhyncus mykiss* treated for 24 h with 0, 15.63, 31.25, 62.50, 125, 250, and 500 μM. Similarly, BPF induced cytotoxic effects in a dose-dependent manner in the hepatocytes of rainbow trout (Aykut and Kaptaner, 2021).

Bisphenols tested in our study, alone or as a mixture, also caused oxidative stress, as alterations of several antioxidant enzyme activity demonstrated. However, more marked alterations have been observed in the gills rather than in the digestive gland. Indeed, we observed a significant reduction in the total antioxidant capacity in gills of animals exposed for 14 days to BPS and MIX. In an opposite way, SOD activity in gills increased after exposure to BPS and MIX, while gill CAT activity increased after both 7 and 14 days of exposure to MIX. Regarding the glutathione related enzymes, we observed that GR activity was significantly reduced in gills after 14 days of exposure to BPS, while the detoxifying enzyme GST was affected by the factor "treatment*time interaction", with an increased enyme activity caused by BPS and MIX after 7 days of exposure followed by a significant decrease after 14 days of exposure to the same experimental conditions. The alterations of antioxidant enzymes caused by the three BPA analogues tested in this study are not different to those caused by the BPA. Indeed, Peng et al. (2018) observed that the activities of SOD, CAT and glutathione peroxidase reached the highest values during the first week and then decreased during the second week of exposure in both haemolymph and hepatopancreas from *Charybdis japonica* exposed to high concentrations of BPA. In another study, specimens of the mussel *Mytilus galloprovincialis* injected with nominal concentrations of BPA of 3, 15, and 60 ng/g dry weight showed a downregulation of antioxidant genes, CAT activity and metallothionein levels one day post injection (Canesi et al., 2007). Furthermore, BPA altered the activity of several antioxidant enzymes such as CAT, GST, glutathione reductase, and the level of total glutathione (Canesi et al., 2007). Likewise, also BPA analogues can cause alterations of the antioxidant system, as reported by recent surveys. For instance, oxidative stress was observed in both the digestive tract and respiratory tree of sea cucumber specimens exposed to 200 μg/L of BPS. In addition, the authors observed an increased oxidative stress in the animals exposed to the mixture of BPA + BPS with both CAT and GST activity significantly increased. Furthermore, the authors evidenced the presence of several types of histopathological lesions in the respiratory system (Jenzri et al., 2023). Ecotoxicological consequences of BPF, BPS, and BPA were also evaluated on *Brachionus koreanus*, a marine rotifer, during a 24h exposure (Park et al., 2018). The results highlighted that the three compounds increased ROS levels, with both BPS and BPF that increased both ROS and GST levels at almost all the tested concentrations (1, 5, and 10 mg/L). Moreover, BPF - as well as BPA - significantly altered the expression levels of cytochrome P450 and GST genes (Park et al., 2018). Similar results were obtained in the tropical freshwater cladoceran *Moina micrura* exposed to high concentrations of BPA, BPF and BPS: a significantly upregulation of the expression of the GST and hemoglobin genes was observed, whereas the sex determination genes, such as doublesex and juvenile hormone analogues, were not significantly different (Razak et al., 2022). Exposure of *Danio rerio* specimens to 1000 nmol/L of BPA, BPS and TBBPA caused the alteration of several antioxidant genes (Shan et al., 2023). Moreover, the activities of SOD, CAT, and GSH-Px/GPX in zebrafish brain tissue of the 1000 nmol/L BPA/BPS/TBBPA-treated group were significantly reduced after 30 days of exposure (Shan et al., 2023).

Recently, the effects of BPS were compared to those of BPA in the clam *Corbicula fluminea* by Seoane et al. (2021) during a short-term exposure. After 96 h of exposure to BPA (3.75, 7.5, 15, 30 mg/L) and

BPS (2.5, 5, 10, 20 mg/L), such compounds provoked valve closure and decreased filtration capability of clams in a concentration-dependent manner. Furthermore, *C. fluminea* exposed to the highest concentrations of BPA showed a significant increase in lipid peroxidation, CAT, and glutathione reductase activities, with respect to controls. On the contrary, BPS caused less effects than BPA, inducing alterations in filtration and in GR activity only at the two highest concentrations tested, indicating that BPS was less toxic than BPA to clams (Seoane et al., 2021). An impairment of the antioxidant system was also recorded in cultured hepatocytes from the rainbow trout *Oncorhyncus mykiss* and treated for 24 h with various concentrations of BPS (0, 15.63, 31.25, 62.50, 125, 250, and 500 μM) (Kaptaner et al., 2021). In that study, a reduction in SOD activity was observed, while CAT and GPX activities were increased at higher concentrations. The GST activity was significantly increased at 31.25 μM or higher, whereas the GST Theta 1-1 activity and the reduced glutathione content were decreased at the same concentrations (Kaptaner et al., 2021). In a similar study performed with BPF, an increased SOD activity was recorded, while CAT activity decreased at all the concentrations tested in *O. mykiss*. In that study, concentrations of BPF between 15.63 and 250 μM caused an increase of reduced glutathione, but it decreased significantly at a concentration of 500 μM. Lastly, the two glutathione-related enzyme, GPX and GST, were significantly increased at concentrations of 31.25–500 μM and at 250 μM respectively (Aykut and Kaptaner, 2021). Regarding the BPAF, a long-term exposure (120 days) of female marine medaka (*Oryzias melastigma*) to 6.7 μg/L, 73.4 μg/L, and 367.0 μg/L caused a significant down-regulation of the gene expression of SOD, CAT and GPX in the liver (Chen et al., 2022). The authors also observed detrimental effects on the immune system, with complement system-related genes that were significantly up-regulated at 73.4 and 367.0 μg/L (Chen et al., 2022). Toxicity of BPAF - alone or in combination with microplastics - was also tested on adults of zebrafish. Fish were exposed to 1 mg/L of microplastics and 200 μg/L BPAF for 45 days. Both BPAF and BPAF + microplastics caused an increased gene expression in neurodevelopmental, inflammation, apoptosis, and oxidative stress-related genes in offsprings (Wang et al., 2023). Moreover, the same experimental conditions caused oxidative stress with an increased gene expression of CAT, Cu/Zn SOD and Keap1 (Wang et al., 2023).

Regarding neurotoxic effects, we did not observe negative effects of bisphenols on both AChE and BChE activity. In this context, it is important to highlight that BPS has been indicated as a probable AChE inhibitor in a study with both an *in vitro* and *in silico* approach on the electric eel *Electrophorus electricus* (Yilmaz et al., 2022). Moreover, Jenzri et al. (2023) observed neurotoxic effects of BPS with a significant reduction of AChE activity in the sea cucumber *Holothuria poli*. An impairment of the neuro system was also observed in the claw muscles of the artic spider crab *Hyas araneus* with a significant reduction in AChE activity after 3 weeks of exposure to 50 μg/L of BPA (Minier et al., 2008). Recently, specimens of medaka (*Oryzias melastigma*) were exposed to 200 μg/L of BPA, BPF, BPAF, and their mixture for 70 days (Li et al., 2022). The histological analysis showed that exposure to bisphenol led to vacuolization and local lesions in the liver, especially in the BPAF group. Furthermore, medaka showed altered antioxidant enzyme activities, with a reduction of both SOD and CAT activity in the liver of males exposed to BPAF or MIX (Li et al., 2022). The mixture tested by Li et al. (2022) did not show additive effects of the selected bisphenols, in accordance with our results that indicated no interaction between the three bisphenols in the MIX treatment.

Regarding the oxidative damage, we observed an altered level of protein carbonyl content in digestive gland, with a significant reduction in BPAF, BPF, MIX after 7 days. Interestingly, LPO was increased in gills of clams exposed for 7 days to both BPS and MIX, while in digestive gland LPO levels were increased after 14 days of exposure to BPF, BPS and MIX. Similarly, malondialdehyde content gradually increased in both the haemolymph and hepatopancreas of the crab *Charybdis japonica* exposed to BPA (Peng et al., 2018). Lastly, both BPS and BPF tested in the marine amphipod *Gammarus aequicauda* at 0.25, 0.5, and 1 mg/L for 24 h increased DNA damage in both haemocytes and spermatozoa, BPF significantly increasing DNA damage level in haemocytes at all the concentrations, and in spermatozoa at 1 mg/L. However, both BPF and BPS caused less DNA damage than BPA (Cosentino et al., 2022).

Chemical analysis evidenced a different uptake of the bisphenols tested in clams from exposure tanks, depending either on the specific compounds or the exposure to single compounds or MIX. The lowest concentrations measured in seawater were recorded in BPAF exposure tank, both as a single (nominal concentration 300 ng/L) and as MIX (nominal concentration 100 ng/L each one), followed by BPF and BPS. These results suggested a major uptake of BPAF by clams, with respect to the other two compounds. The concentrations measured in clams highlighted BPAF and BPS as the congeners more prone to be bioaccumulated, as BPF was always < LOD. Even if these are preliminary results, it is interesting to observe that BPS seems to maintain a rate of bioaccumulation quite comparable over 7 and 14 days, both as single compound and in MIX. BPAF, which showed the higher uptake from seawater and the larger bioaccumulation in clams, evidenced a more limited concentration in clams when they were exposed to the MIX.

These data are consistent with the finding of the biomarker assays. Indeed, this study indicates that BPAF, BPF, BPS and their mixture affected cellular and biochemical parameters of *R. philippinarum*. In particular, we observed oxidative damage mainly in the digestive gland, a well-known tissue responsible of contaminant bioaccumulation. BPS and MIX caused the main alterations, even if in absence of additive effects, we consider that the effects caused by the MIX treatment can be mainly attributed to BPS. These results can be explain considering chemical nature of the compounds tested. Indeed, BPS is hydrophilic, while both BPAF and BPF are hydrophobic. This probably allow BPAF and BPF to be bound to the lipid reserves of the animals without entering its metabolism. On the contrary, BPS highly impacts on many biological pathways. However, further studies are necessary to elucidate better the toxicity of hydrophilic and hydrophobic bisphenols in aquatic organisms. In conclusion, our preliminary study suggests that the recently used BPA analogues are not safer compound than BPA, at least in the experimental conditions tested and in the bivalve species used.

Author statement

Jacopo Fabrello: Conceptualization, Methodology. Jacopo Fabrello, Maria Ciscato, Andrea Vecchiatti, Marco Roverso: Methodology. Jacopo Fabrello, Maria Ciscato, Marco Munari, Andrea Vecchiatti, Marco Roverso, Sara Bogialli, Valerio Matozzo: Data curation, Investigation, Writing- Original draft preparation. Valerio Matozzo: Supervision.

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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.marenvres.2023.106228.

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