Marine Pollution Bulletin 213 (2025) 117628

Contents lists available at ScienceDirect

Marine Pollution Bulletin

journal homepage: www.elsevier.com/locate/marpolbul

Ecotoxicological responses of marine fish to the organophosphate flame-retardant tris (2-chloroisopropyl) phosphate (TCPP) dietary exposure: Juvenile gilthead seabream (*Sparus aurata*) as a case study

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ARTICLE INFO

Keywords:
High production volume chemicals
Organophosphate esters
Emerging contaminants
Chronic exposure
Ecotoxicity

ABSTRACT

High Production Volume Chemicals (HPVCs) are contaminants that pose serious threats to aquatic environments and species that inhabit them, given their massive production and ubiquitous distribution across biological compartments. Among them, organophosphate esters (OPEs) are of particular concern, as they are widely used as plasticizers and flame-retardants, and linked to various forms of toxicity in marine organisms. In this study, we investigated the ecotoxicological response of juvenile gilthead seabream *Sparus aurata* to the OPE tris (2-chloroisopropyl) phosphate (TCPP) following chronic dietary exposure to three different concentrations (low, D1: 0.2 mg kg^{-1}; ecologically relevant, D2: 2 mg kg^{-1}; and high, D3: 10 mg kg^{-1}). Different biomarkers indicative of antioxidant defence mechanisms (catalase, CAT, glutathione S-transferase, GST, activities), metabolism (citrate synthase, CS, lactate dehydrogenase, LDH, activities) and endocrine disruption (vitellogenin content, VTG), as well as cell (lipid peroxidation levels, LPO) and protein damage (ubiquitin content, UBI) were analyzed in liver and muscle to assess TCPP toxicity. High concentrations of TCPP affected *S. aurata* growth, but not overall fitness condition. Furthermore, metabolic disruption and severe oxidative damages were observed, regardless of exposure dose. VTG content significantly decreased after exposure to all TCPP dosages, indicating a possible masculinization effect. These findings provide new insights to the scientific knowledge on TCPP ecotoxicological attributes and impacts on marine ichthyofauna. In addition, our results confirm the relevance of conducting integrated multi-biomarker approaches to disclose the ecotoxicological effects of poorly studied chemical contaminants and, ultimately, implement wastewater treatment strategies and legislation to protect marine ecosystems from pollution.

1. Introduction

High Production Volume Chemicals (HPVCs) are substances manufactured or imported in quantities above 1000 t per year in at least one member country, for the Organization for Economic Cooperation and Development (OECD) and the European Union (EU), or above 454 t per year, for the United States of America (Nikfar et al., 2014). The OECD has established a Cooperative Chemicals Assessment Programme to derive worldwide hazard assessments of these chemicals. Additionally, a few countries have developed or are currently developing their own systems to specifically manage them, though the exact approaches differ (Park et al., 2008; Nikfar et al., 2014; Ha et al., 2016). HPVCs comprise a wide range of compounds, which include organophosphate esters (OPEs), widely used nowadays as plasticizers and flame-retardants,

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https://doi.org/10.1016/j.marpolbul.2025.117628
Received 28 November 2024; Received in revised form 20 January 2025; Accepted 30 January 2025
Available online 5 February 2025

more so since the ban on some brominated flame-retardants. Hence, OPEs are essential to various industries and everyday appliances, being increasingly used in the production of food packages, furniture and textiles, and electronic equipment (van der Veen and de Boer, 2012; ECHA, 2018), among others.

Tris (2-chloroisopropyl) phosphate (TCPP) is an OPE employed primarily as an additive flame-retardant and used in the production of polyurethane foam in construction and furniture (ECCC, 2020). TCPP is found across the globe in sediments (Brandsma et al., 2015), surface waters (Cristale et al., 2021), wastewaters (Pantelaki and Voutsa, 2022), dust (Tran et al., 2020), air (Castro-Jiménez and Sempéré, 2018), tap water (He et al., 2018), agricultural products (He et al., 2018), and human milk (Sundkvist et al., 2010). In the marine environment, these compounds has been detected in seawater samples worldwide, with concentrations ranging from 3 to 1300 ng L^{-1} in the North Sea (Bollmann et al., 2012), Aegean Sea (Pantelaki and Voutsa, 2021), Mediterranean Sea (Schmidt et al., 2021), in the Bohai, Yellow and East China Seas (Hu et al., 2014; Zhong et al., 2017), Arctic Ocean (Na et al., 2020), North Pacific (Na et al., 2020), South Pacific (Li et al., 2023), North Atlantic (Schmidt et al., 2019) and Antarctic Ocean (Li et al., 2023). TCPP is often the dominant OPE in both water and sediments (e.g. Bollmann et al., 2012; Brandsma et al., 2015; Zhong et al., 2017; Schmidt et al., 2019, 2021; Na et al., 2020), and its persistence in the marine environments is related to its stability in water and low biodegradability (van der Veen and de Boer, 2012; ECCC, 2020).

As a result, marine organisms inhabiting contaminated coastal areas, particularly those subjected to strong anthropogenic pressures and industrial wastewater inputs, are inevitably exposed to TCPP, likely uptaking this substance via water, sediments and trophic transfer, as confirmed in several environmental monitoring studies on fish (Brandsma et al., 2015; Hallanger et al., 2015; Wang et al., 2022; Castro et al., 2023; Borrull et al., 2024), mammals (Hallanger et al., 2015; Sala et al., 2019), birds (Hallanger et al., 2015; Fu et al., 2020), turtles (Sala et al., 2021), crustaceans (Castro et al., 2023), bivalves (Castro et al., 2023), gastropods (Fu et al., 2020), cephalopods (Sala et al., 2021; Castro et al., 2023), zooplankton (Brandsma et al., 2015; Schmidt et al., 2021) and algae (Fu et al., 2020). Although these studies suggest that bioaccumulation and biomagnification of TCPP can occur in marine trophic webs, information is extremely limited regarding the way marine organisms cope with the presence of this compounds from an ecotoxicological standpoint (Pantelaki and Voutsa, 2020), most likely due to TCPP's rapid biotransformation and elimination (Sala et al., 2019; ECCC, 2020).

TCPP has been linked to reproductive and developmental toxicity and carcinogenicity, while its potential genotoxicity and neurotoxicity to humans, and biota effects, still require further investigation (ECHA, 2018; ECCC, 2020). Due to health concerns, its use is restricted in children's toys in the EU (Directive 2009/48/EC). TCPP is regarded as moderately toxic to aquatic biota (ECCC, 2020). In both bivalves and fish, it may affect larvae development and survival (Mercurio et al., 2021, 2022; Xia et al., 2021), lead to oxidative stress and altered metabolism pathways (Ji et al., 2020; Zhong et al., 2020; Aluru et al., 2021; Xia et al., 2021; Deng et al., 2022; Yan et al., 2022) and act as an immunosuppressor (Wu et al., 2018; Yan et al., 2022). It has also been shown to cause behavioural abnormalities in zebrafish (Noyes et al., 2015; Dach et al., 2019; Xia et al., 2021). In echinoderms, it has been considered to have an estrogenic effect (Campoy-López et al., 2020). Yet, to the best of our knowledge, so far only one study using marine fish (rockfish) as model has explored the bioaccumulation and ecotoxicological effects of TCPP upon chronic dietary exposure (Ji et al., 2020).

Gathering such information is crucial to accurately define xenobiotics' toxicity and determine the environmental risk they pose, especially for compounds that present a rather lipophilic behaviour, such as OPEs (OECD, 2014). Hence, this study investigated the ecotoxicological responses of the commercially important marine fish *Sparus aurata* (gilthead seabream) to chronic exposure to TCPP at low, moderate, and high doses, following a multi-biomarker approach that combined whole organism (fitness indicators) and biochemical (antioxidant activity, lipids and proteins damage and endocrine disruption) endpoints. Given its wide distribution, commercial significance, and extensive research background, *S. aurata* has been frequently used to assess the ecotoxicity of xenobiotics present in the marine environment (e.g. Albendín et al., 2021; Solomando et al., 2021; Dias et al., 2024). In addition, juvenile specimens were used as models, as this species uses coastal areas as nurseries and, thus, early-life stages are particularly exposed to high loads of chemical contaminants before migrating to the open ocean as adults.

2. Materials and methods

2.1. Ethical statement

The trial was performed by researchers certified in animal experimentation (EU functions A and B), and the animal handling and sampling procedures were conducted in compliance with the ARRIVE guidelines (Animal research: reporting of in vivo experiments) and ethics for the care and use of animals, following the recommendations of the Federation of European Laboratory Animal Science Associations (FELASA) and the Portuguese legislation (EU Directive 2010/63; Decree-Law n° 113/2013). The study was approved by IPMA's Animal Welfare and Ethics Body (ORBEA) and overseen by the Portuguese National Authority for the use of live animals (General Directorate of Food and Veterinary, DGAV; authorization reference: 77418/24-S).

2.2. Experimental feeds

Two commercial feeds and three TCPP contaminated feeds (containing three different concentrations of this compound, i.e., Low – D1 = 0.2 mg TCPP kg^{-1}, Reference/Medium – D2 = 2 mg TCPP kg^{-1}, and High – D3 = 10 mg TCPP kg^{-1}) were prepared with the collaboration of the feed producer company SPAROS Lda. (Olhão, Portugal). Feeds composition had in consideration juvenile *S. aurata* nutritional requirements (ingredients and proximate chemical composition can be consulted in Table S1). Solid ingredients were powdered (below 250 μm) in a micropulverizer hammer mill (model SH1, Hosokawa-Alpine, Germany) and mixed according to the target formulation in a double-helix mixer (model 500 L, TGC Extrusion, France). No oils were incorporated at this stage. Pellets (size: 3.0 mm) were produced in a twin-screw extruder (model BC45, Clextral, France; screw diameter = 55.5 mm; temperature = 105–109 °C) and dried in a vibrating fluid bed dryer (model DR100, TGC Extrusion, France). In contaminated feeds, the corresponding amounts of TCPP (97 % purity; Sigma-Aldrich, Germany) required to attain the nominal TCPP concentrations in D1, D2 and D3 feeds were first dissolved in a small volume of ethanol (5 mL, 100 % *v*/v) and then mixed with the oils before pellets extrusion. Oils were subsequently added to the extruded pellets by vacuum coating (model PG-10VCLAB, Dinnissen, The Netherlands). To enable appropriate comparisons between treatments, a similar amount of solvent (i.e. 5 mL of ethanol) was also added to the oils used in the coating of CTR feed pellets (see section 2.3).

TCPP was quantified in all feeds through QuEChERS extraction and GC–MS/MS analysis, as described in Borrull et al. (2024). TCPP real concentrations are presented in Table S2. The ecologically relevant concentration (D2) was chosen based on the range of values found in marine biological matrices (values up to 0.7 mg kg^{-1}; e.g. Sundkvist et al., 2010), and the concentrations reported in seawater and other environmental matrices, such as air, dust and sediments (values up to 2.5 mg kg^{-1} (Pantelaki and Voutsa, 2019)).

2.3. Fish rearing and experimental design

Sparus aurata (n = 105) were reared until juvenile stage (55.23 ±

7.10 g total weight, 14.76 ± 0.72 cm total length; mean ± standard deviation) in the Aquaculture Research Station of the Portuguese Institute for the Sea and Atmosphere (EPPO-IPMA, Olhão, Portugal) under routine hatchery conditions, and then transported to IPMA's Live Marine Organisms Bioterium (LABVIVOS, Algés, Portugal). For the present work, 30 specimens were used, whereas the remaining animals were utilized in a parallel study devoted to TCPP toxicokinetics (to be published separately). Fish were first kept in quarantine for ten days. Afterwards, they were randomly assigned into the experimental systems, which consisted of 15 rectangular glass tanks (200 L each) within independent recirculation aquaculture systems (RAS), at a density of 7 fish per tank (2 of which used for this study). Detailed description of the equipment used in RAS is presented in Marmelo et al. (2024). Fish were acclimated to the experimental tanks for a week. During quarantine and acclimation, fish were hand-fed twice a day with a commercial feed (CF) following the nutritional requirements of juvenile *S. aurata* (2 % average body weight, BW; detailed feed composition can be found in Table S1).

Once animals were acclimated to laboratory conditions, the exposure trial was initiated and fish from each treatment were fed (~2 % BW/day) for 28 days with one feed. The experimental setup included five treatments (each composed by three replicate tanks; Fig. S1): one commercial feed corresponding to the negative control (CF, non-contaminated), three contaminated (D1, D2 and D3), as well as an additional carrier control non-contaminated treatment (CTR) in which fish were fed with the commercial feed with no TCPP, but with the addition of the solvent used for contaminant solubilisation, i.e. ethanol (as explained in section 2.2). TCPP concentration was also determined in CTR feed following the method previously described (see section 2.2), as to rule out any unintentional contamination. The system temperature was maintained at 18.0 ± 0.5 °C, dissolved oxygen >5 mg L^{-1}, salinity at 35 ± 1 psu, and pH at 8.0 ± 0.1 units. These abiotic parameters were monitored with a multi-parameter measuring instrument (HI98194, HANNA instruments, Italy). The photoperiod followed a 12 h light/12 h dark cycle. Ammonia (NH_3/NH_4^+), nitrite (NO_2^-) and nitrate (NO_3^-) were measured weekly using colorimetric tests (Tropic Marin, USA), with ammonia and nitrite being kept below detectable levels, and nitrates below 50 mg L^{-1}. Additionally, welfare assessments were done on a daily basis to ensure the welfare and health of the fish, by monitoring signs of distress or suffering (e.g. disturbed behaviour, including loss of appetite, lethargy or increased activity, loss of balance, signs of anxiety, body lesions and abdomen distension).

2.4. Sample preparation

After 28 days of trial, 6 fish per treatment (2 per replicate tank), were randomly caught and anaesthetized by immersion in an overdosed tricaine methanesulfonate solution (MS-222, 2 g L^{-1}; Acros Organics, Belgium) buffered with sodium bicarbonate in a 1:2 ratio of MS-222: $NaHCO_3$. For enzymatic biomarkers, a sample of 4 to 8 fish has been shown to suffice in obtaining statistical significance with a power of 95 % (Gagnon and Hodson, 2012). Fish were sacrificed, weighted (g, total weight, W), measured (cm, total length, TL) and dissected. Liver and muscle were removed and weighted, and a portion of each tissue sample (~50 mg) was homogenized (Ultra-Turrax device, Ika, Germany) in ice-cold conditions in different buffer solutions according to specificities of each procedure, i.e.: i) for antioxidant activity, lipid and protein damage, and vitellogenin content: 0.14 M NaCl, 0.003 M KCl, 0.01 M Na_2HPO_4, 0.002 M KH_2PO_4 (Sigma-Aldrich, Germany, pH = 7.4); ii) citrate synthase: 20 mM HEPES (Gibco, USA), 1 mM EDTA (Triplex, Merk, Switzerland), Triton X-100 1 % (Sigma Aldrich); and iii) lactate dehydrogenase: 150 mM Imidazole (Carl Roth, Germany), 1 mM EDTA (Triplex), Triton X-100 1 %. Tissue homogenates were centrifuged at 10,000*g* and 4 °C for 10 min., and the supernatants frozen at −80 °C until further analysis.

2.5. Fish fitness

Fulton's condition factor (K) was calculated to determine fish condition, as proposed by Ricker (1975):

$$K = \frac{W}{TL^3} \times 100$$

where W represents the total weight (g) and TL the total length (cm). The relation between fish weight and its respective liver weight (hepatosomatic index, HSI), as well as viscera weight (viscerosomatic index, VSI), provide information on liver and visceral condition, respectively. They were calculated as:

$$HSI = \frac{\text{Liver weight}}{W} \times 100$$

$$VSI = \frac{\text{Visceral weight}}{W} \times 100$$

2.6. Ecotoxicological biomarkers

The homogenate samples were used to determine different biomarkers indicative of fish physiological fitness: i) antioxidant response (catalase (CAT) and glutathione S-transferase (GST) activities); ii) protein (ubiquitin, UBI) and lipid (malondialdehyde, MDA, content) damage; iii) metabolism (citrate synthase (CS) and lactate dehydrogenase (LDH) activities); and iv) endocrine disruption (vitellogenin content). CAT and GST activities, lipid peroxidation (LPO, measured as MDA content) and UBI content were assessed in both liver and muscle, while VTG content only was quantified in liver. These methods are described in detail in Maulvault et al. (2019). CS and LDH activities were assessed in liver and muscle, based on kinetic spectrophotometric assays, as described in Rosa et al. (2016). Further details regarding these methodologies are available in the Supplementary Materials (Methodologies). All samples were analyzed, at least, in duplicate, using 96-well microplates (Greiner Bio-one, Austria) and a Multiskan GO 1510 microplate reader (ThermoFisher Scientific, USA). The reagents and standards were of pro analysis grade. To normalize the results, total protein levels were quantified for each sample via the Bradford method (Bradford, 1976), and results are expressed per mg of protein. The biomarkers chosen are universally employed in ecotoxicological studies due to their reliability and aptness in determining the effects of xenobiotics, including HPVC's (e.g. He et al., 2019; Ramesh et al., 2020; Kim et al., 2022), and in particular, TCPP (e.g. Wu et al., 2018; Yan et al., 2022).

2.7. Statistical analysis

Data was tested for normality and homoscedasticity through the Shapiro-Wilk and Levene tests, respectively. A Student's t-test (or Welch's t-test if variances were not equal and a Wilcoxon test when no parametric assumptions were met) was done to test differences on body condition indexes and biomarker levels between the two non-contaminated treatments (i.e. CF and CTR). Additionally, the presence of significant differences in the same variables between the contaminated (D1, D2 and D3) and control (CTR) treatments were investigated via a one-way ANOVA. *Post-hoc* Tukey HSD tests were subsequently carried out to perform multiple comparisons. When data did not meet the parametric assumptions, a Kruskal-Wallis test and a subsequent Dunn Test were carried out instead. Significant correlations (*r*) between relevant endpoints were determined through Pearson's correlation analysis (or Spearman correlation if its assumptions were not met). All statistical analyses were performed at a significance level of 0.05, using RStudio interface software (4.1.1, 2021).

3. Results

The negative control (CF) included to assess the toxicological effects induced by ethanol used for TCPP solubilisation revealed that, with the exception of LPO (which was significantly lower in fish muscle from the carrier control CTR treatment; $p = 0.01$), no significant differences were observed between CTR and CF in the remaining ecotoxicological biomarkers ($p > 0.05$). Overall, these results confirm the absence of ethanol-induced toxicity in CTR, D1, D2 and D3 treatments and, thus, the variations observed in contaminated treatments (in relation to CTR) are only due to the exposure to TCPP. Hence, to facilitate data interpretation, the subsequent sections undertake comparisons between CTR, D1, D2 and D3 treatments.

3.1. Fish fitness

No mortality occurred throughout the exposure trial. Body condition indexes and morphometric measurements are presented in Table 1. TL and W ranged from 13.9 cm and 44.5 g (D3 treatment) to 16.8 cm and 85.98 g (CTR), respectively. K values varied between 1.47 (D1) and 1.94 (D2), HSI from 0.71 (D2) to 1.84 (D1), and VSI from 4.52 (D3) to 6.93 (D1). TL (−9.8 %, $p = 0.004$) and W (−25.6 %, $p = 0.039$) were significantly lower in the highest concentration of TCPP (D3) when compared to CTR (Table 1–2). However, no significant differences were found for the Fulton's condition factor (K), hepatosomatic index (HSI) and viscerosomatic index (VSI) ($p > 0.05$). A strong positive correlation was found between body weight and length ($r = 0.92$, $p < 0.001$), but not between the remaining fitness indexes (Table S4).

3.2. Ecotoxicological biomarkers

Tissue biomarker levels in *S. aurata* are presented in Fig. 1–4 (and Table S3). All ecotoxicological biomarkers were significantly affected by the presence of TCPP, though dose-dependent responses were observed in most cases (Fig. 1–4; Table S3). Starting with tissue antioxidant responses, CAT activity levels varied between 0.62 (CTR) and 1.57 nmol min^{-1} mg^{-1} protein (D3) in the liver, whereas values in the muscle varied between 0.21 (D1) and 0.60 nmol min^{-1} mg^{-1} protein (D3). A significant increase was found between CTR and D3 treatments, in both tissues (+60.7 %, $p = 0.006$ and + 88.5 %, $p = 0.004$, respectively; Fig. 1A and B, Table 2). GST activity levels varied between 13.93 (D2) and 36.30 (D3) nmol min^{-1} mg^{-1} protein in liver. Comparing to CTR, GST activity showed a significant increase of 69.8 % in D3 treatment ($p = 0.021$; Fig. 1C, Table 2). In muscle, it varied between 3.82 (D1) and 6.44 (CTR) nmol min^{-1} mg^{-1}, and there was a decrease in D1 and D2 (−33.0 %, $p < 0.001$ and − 32.2 %, $p = 0.041$, respectively; Fig. 1D, Table 2). MDA content ranged between 0.04 (CTR) – 0.24 nmol mg^{-1} protein (D3) in the liver and 0.10 (CTR) – 0.90 (D3) nmol mg^{-1} protein in the muscle. Values were significantly higher in D2 and D3 in both tissues compared to the CTR, with the latter treatment showing the most significant increase (equivalent to 3.8-fold in liver ($p < 0.001$; Fig. 2A) and 6.25-fold in muscle ($p = 0.001$; Fig. 2B). In liver, LPO levels were correlated with VSI ($r = −0.65$; $p = 0.026$) and in muscle, LPO was significantly and negatively correlated with TL ($r = −0.80$, $p = 0.002$) and W ($r = −0.75$, $p = 0.005$) (Table S4). Additionally, CAT activity showed a strong positive correlation with LPO levels in both tissues ($r = 0.78$, $p = 0.008$ and $r = 0.88$, $p < 0.001$, in liver and muscle, respectively; Table S4). Regarding UBI, concentrations ranged between 0.99 (D2) and 1.43 (D1) ng mg^{-1} protein in the liver, with concentrations being higher in treatment D1 in comparison to D2 ($p = 0.039$), though no significant differences were found between contaminated treatments and the control ($p > 0.05$, Fig. 2C). In muscle, concentrations ranged between 8.07 (D1) and 11.89 (D3) ng mg^{-1} protein. In this tissue, there was a significant reduction in D1 and D2 (equivalent to −19.6 % and − 14.8 %, respectively) in relation to CTR ($p = 0.007$ and $p = 0.002$, respectively; Fig. 2D, Table 2).

CS activity values ranged between 2.77 (CTR) and 4.05 (D3) nmol min^{-1} mg^{-1} protein in liver, and a significant increase was found in all the contaminated treatments regardless of dose (+21.8 %, $p = 0.024$, +21.6 %, $p = 0.026$, +23.3, $p = 0.017$ in D1, D2 and D3 respectively; Fig. 3A, Table 2). In this tissue, this enzyme's activity was significantly and negatively correlated with VSI ($r = −0.73$, $p = 0.007$; Table S4). In the muscle, the lowest CS activity was found in D2 treatment (2.15 nmol min^{-1} mg^{-1} protein), whereas the highest value was found in D3 treatment (3.65 nmol min^{-1} mg^{-1} protein). There was a significant increase of 20.8 % in D1 ($p = 0.021$) and 31.6 % in D3 ($p = 0.002$) in comparison to CTR, as well as a significant reduction of 18.1 % in D2 treatment ($p = 0.041$) (Fig. 3B, Table 2). As for the anaerobic pathway, LDH activity ranged between 38.99 (D2) – 79.01 (D3) nmol min^{-1} mg^{-1} protein in the liver. The activity of this enzyme was significantly reduced in the liver of *S. aurata* exposed to treatment D2 in comparison to CTR (−32.4 %, $p = 0.041$; Fig. 3C, Table 2). Additionally, statistically higher LDH activities were found in livers from D3 treatment compared to D1 ($p = 0.009$) and D2 ($p = 0.005$), but not compared to CTR ($p = 0.394$; Fig. 3C, Table 2). It is noteworthy that, in this tissue, D2 led to LDH activity inhibition but CS activity induction. LDH activity varied between 36.51 (D1) – 63.44 (D3) nmol min^{-1} mg^{-1} protein in the muscle. As in liver, it was also significantly increased in D3 in comparison to D1 ($p = 0.035$) in muscle, but no differences to CTR were found ($p > 0.05$; Fig. 3D, Table 2).

Finally, hepatic VTG content ranged from 19.65 (D3) to 98.62 (CTR) ng mg^{-1} protein. The average VTG content was higher in CTR, being diminished in fish exposed to TCPP, regardless of the exposure dose (−54.0 %, $p = 0.004$, −58.1 %, $p = 0.002$, −65.1 %, $p < 0.001$ for D1, D2 and D3, respectively; Fig. 4). A significant correlation was found between VTG content and HSI ($r = 0.63$, $p = 0.029$; Table S4).

4. Discussion

OPEs are a global environmental risk that potentially pose a threat to aquatic life. The present findings add to the comprehension of the toxicological repercussions of TCPP and highlight its potential hazards to marine juvenile fish, such as gilthead seabream, *S. aurata*. The two examined tissues (liver and muscle) presented different ecotoxicological responses, due to their differing basal protein levels, individual functions and unique sensibilities. TCPP revealed a detrimental effect in fish growth at the highest concentration tested. Growth is a reflection of food availability and efficiency in its utilization. Although food ingestion was

Table 1
Total length (TL), total weight (W), Fulton's condition index (K), hepatosomatic index (HSI) and viscerosomatic index (VSI) in *S. aurata* for each treatment (mean ± SD) at the end of the exposure to TCPP. Different letters indicate significant differences ($p < 0.05$) between treatments. The absence of letters indicates no significant differences ($p > 0.05$). CTR: control treatment; D1: low concentration (0.2 mg TCPP kg^{-1}); D2: medium concentration (2 mg TCPP kg^{-1}); D3: high concentration (10 mg TCPP kg^{-1}).

	TL (cm)	W (g)	K	HSI	VSI
CTR	16.12 ± 0.63^{a}	71.74 ± 12.27^{a}	1.70 ± 0.15	1.52 ± 0.21	6.11 ± 0.25
D1	15.24 ± 0.51^{ab}	60.53 ± 7.10^{ab}	1.71 ± 0.16	1.54 ± 0.28	6.01 ± 0.54
D2	15.44 ± 0.61^{ab}	65.81 ± 9.33^{ab}	1.78 ± 0.10	1.23 ± 0.37	5.89 ± 0.33
D3	14.54 ± 0.68^{b}	53.35 ± 7.67^{b}	1.73 ± 0.14	1.11 ± 0.27	5.74 ± 0.90

Table 2
Percentage of change (%) in fitness indexes and ecotoxicological biomarkers induced by each TCPP contaminated treatment in comparison to the CTR treatment, and statistical comparisons (Tukey HSD or Dunn test) between CTR and each treatment (*p-value*). "+" indicates a significant increase and "-" indicates a significant decrease compared to CTR values; NS: no significant alteration ($p < 0.05$) compared to CTR values. CTR: control treatment; D1: low concentration (0.2 mg TCPP kg^{-1}); D2: medium concentration (2 mg TCPP kg^{-1}); D3: high concentration (10 mg TCPP kg^{-1}). TL: total length; W: total weight; K: Fulton's condition index; HSI: hepatosomatic index; VSI: viscerosomatic index; CAT: catalase activity; GST: glutathione S-transferase activity; LPO: lipid peroxidation levels; UBI: ubiquitin content; CS: citrate synthase activity; LDH: lactate dehydrogenase activity; VTG: vitellogenin content.

		D1		D2		D3	
		%	*p-value*	%	*p-value*	%	*p-value*
Fitness indexes							
TL		NS	0.145	NS	0.327	**−9.8**	0.004
W		NS	0.266	NS	0.748	**−25.6**	0.039
K		NS	0.999	NS	0.773	NS	0.979
HSI		NS	0.999	NS	0.364	NS	0.112
VSI		NS	0.991	NS	0.921	NS	0.726
Ecotoxicological Biomarkers							
CAT	Liver	NS	0.994	NS	0.964	**+60.7**	0.006
	Muscle	NS	0.999	NS	0.069	**+88.5**	0.004
GST	Liver	NS	0.992	NS	0.861	**+69.8**	0.021
	Muscle	**−33.0**	<0.001	**−32.2**	0.041	NS	0.999
LPO	Liver	NS	0.403	**+220.2**	0.002	**+282.7**	<0.001
	Muscle	NS	0.766	**+513.0**	0.002	**+539.8**	0.001
UBI	Liver	NS	0.299	NS	0.492	NS	0.670
	Muscle	**−19.6**	0.007	**−14.8**	0.002	NS	0.193
CS	Liver	**+21.8**	0.024	**+21.6**	0.026	**+23.3**	0.017
	Muscle	**+20.8**	0.021	**−18.1**	0.041	**+31.6**	0.002
LDH	Liver	NS	0.082	**−32.4**	0.041	NS	0.394
	Muscle	NS	0.216	NS	0.690	NS	0.579
VTG	Liver	**−54.0**	0.004	**−58.1**	0.002	**−65.1**	0.001

not targeted in this study, the daily observations do not suggest differences in feeding behaviour among treatments. However, altered metabolism (CS and LDH activities) and evidence of tissue damage (i.e. increased LPO) under the highest TCPP level likely explains the observed reduced growth. Wang et al. (2015) revealed significantly decreased growth of zebrafish (*Danio rerio*) exposed to another chlorinated OPE [TDCPP: tris (1,3-dichloro-2-propyl) phosphate], and Hu et al. (2022) observed the same in freshwater yellow catfish (*Pelteobagrus fulvidraco*) exposed to TCEP [tris(2-chloroethyl)]. TCPP has been found to also affects other organism's metabolism, with Antonopoulou et al. (2022) mentioning reduced microalgae growth. Nonetheless, despite the myriad of changes revealed at the cell level herein presented, there were no effects on overall fish fitness condition and wellbeing, which could imply that TCPP requires longer exposure times or greater concentrations to severely impact fish at more complex levels of organization (i.e. whole organism). Still, the lack of a clear effect on fish condition could otherwise be related to the rearing environment, i.e. animals are well-fed and able to meet all their energetical and nutritional demands, as well as free from any additional stress they would suffer in their natural habitat (e.g. fleeing from predators). Fish might have been, therefore, able to circumvent some of the effects caused by TCPP exposure, as a result of the extremely controlled laboratory conditions in which they were kept. In nature, parallel to the many environmental challenges faced by *S. aurata*, TCPP contamination may have had a greater impact on this species' fitness.

S. aurata antioxidant mechanisms were likewise affected by TCPP. The antioxidant defence system consists of enzymes, such as CAT, superoxide dismutase (SOD) and GST. CAT plays a defensive role against reactive oxygen species (ROS) produced due to exposure to environmental stressors, by converting hydrogen peroxide – previously formed by SOD during the scavenging of ROS – into oxygen and water (Halliwell and Gutteridge, 2015). The main task of GST lies on the detoxification of xenobiotics through the conjugation of reduced glutathione, and its activity is generally increased due to oxidative stress (Yan et al., 2022). Moreover, this enzyme protects the cell from toxic endogenous compounds, like the byproducts of lipid peroxidation, which can cause membrane damage if not detoxified (Sainas et al., 2018). CAT activity was upregulated in both studied tissues thanks to high TCPP concentrations, which implies a need of antioxidant protection against the effects of this chemical. GST activity was downregulated in the muscle of fish subjected to low and medium doses of TCPP, and upregulated in the liver of those subjected to the highest dose. Inhibition of GST could be linked to concentration-dependent mechanisms that interfere with specific pathways, and its suppression can occur when the tissue's detoxification mechanisms are exhausted, for instance through a decrease in the formation of reduced glutathione (Maulvault et al., 2019). In biologically functional organisms and/or cells not disrupted by stress, GST is able to ligate unwanted substances and detoxify cells, and antioxidant enzymes such as CAT break down the ROS that arise, restoring cellular equilibrium (Maulvault et al., 2017; Yan et al., 2022). Nonetheless, the activity of antioxidant enzymes can be either induced or inhibited depending on exposure time and the severity of the stressors (Feng et al., 2013), and when the quantity of ROS surpasses a certain threshold, cell damage or apoptosis can occur (Maulvault et al., 2017; Yan et al., 2022). Additionally, adaptation to oxidative stress does not always involve elevated antioxidant defences (Halliwell and Gutteridge, 2015).

Cell damage was assessed through MDA content, as a proxy for lipid peroxidation levels, which we found to substantially increase under mid and high TCPP concentrations. Additionally, we found LPO levels to be positively correlated with CAT activity in both tissues examined, therefore confirming that, despite the activation of cells' antioxidant scavenging system, the stress induced by the exposure to TCPP was so intense that LPO could not be prevented. Matching these results, a recent study following a metabolite approach showed that TCPP can affect the antioxidant system and lipid homeostasis of zebrafish gut in concentrations of 40 μg L^{-1} (Wang et al., 2024). Yan et al. (2022) also found CAT activity and MDA levels to be enhanced by TCPP in zebrafish at concentrations of 25 mg L^{-1}, and GST related genes were overexpressed in the specimens exposed to 5–15 mg L^{-1}. This work, in particular, demonstrates that changes at the biochemical level (e.g. enzyme activity) are observed at greater concentrations, whereas changes at the molecular level (e.g. gene expression) become evident at lower doses. This ties back to the above-mentioned notion that higher TCPP exposure

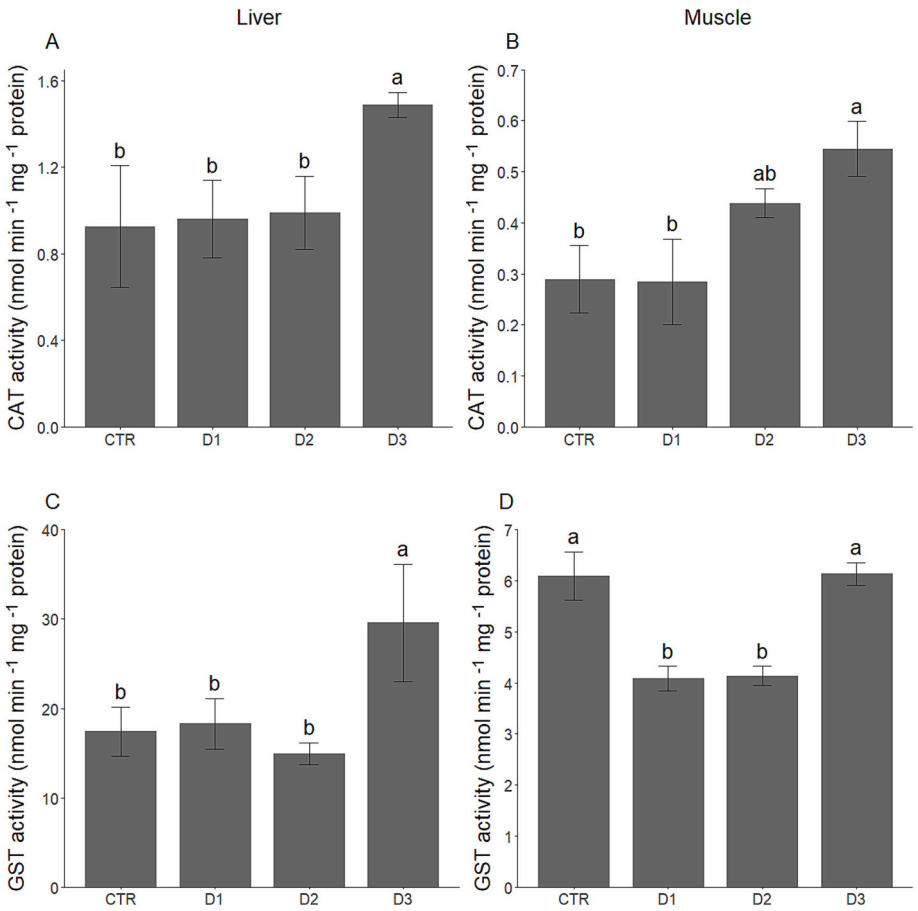

Fig. 1. Antioxidant enzymes activity (CAT, nmol min^{-1} mg^{-1} protein; GST, nmol min^{-1} mg^{-1} protein) in liver (A, C) and muscle (B, D) of juvenile *S. aurata* (mean ± standard deviation) after 28 days of feed-exposure to different TCPP treatments ($n = 6$). Different letters indicate significant differences ($p < 0.05$) between treatments. CTR: control treatment; D1: low concentration (0.2 mg TCPP kg^{-1}); D2: medium concentration (2 mg TCPP kg^{-1}), D3: high concentration (10 mg TCPP kg^{-1}).

doses may be necessary to trigger visible effects in fish from the whole organism and/or biochemical standpoints. Overall, the trends observed in the present study and reported in recent literature evidence that organism's response to TCPP is dose-dependent.

In *Mytilus galloprovincialis*, TCPP exposure induced SOD activity and LPO at concentrations of 100 nmol L^{-1} (Wu et al., 2018). SOD and CAT activity increased in *Escherichia coli* exposed to TCPP, yet the same study found altered membrane potential and ATPase activities, which the authors attributed to significantly elevated MDA levels (Jin et al., 2024). Our findings and those of various studies here mentioned indicate that chronic exposure to this OPE (regardless of route, i.e. either via water or diet) causes failure of the antioxidant system in multiple tissues and species. Even a possible activation of antioxidant defences, e.g. the enhancement in CAT or GST activity herein reported, as well as in the studies of Yan et al. (2022) or Jin et al. (2024), it seems insufficient to tackle the overgeneration of ROS and the subsequent oxidative damage that arises due to this compound, as indicated by elevated LPO levels. Interestingly, in the present study, LPO in muscle showed a very strong and negative correlation with fish length and weight, suggesting that smaller and/or early-life stage organisms might be more sensitive to TCPP exposure. Possibly, this could be related to differing bioaccumulation kinetics according to size (Merciai et al., 2014). This response, however, was not found for liver at a significant level, nor was it found for antioxidant defence enzymes (CAT and GST). Still, these results merit further research regarding the influence of fish size and maturity on the effects of this OPE. Exposure to stressors and subsequent oxidative damage can also incur in protein damage. The ubiquitin proteasomal pathway is the main system for the elimination of irreversibly anomalous and misfolded proteins in the cells, and its dysregulation can enhance cellular stress (Jannuzzi et al., 2022). More specifically, it might cause mislocalization or accumulation of damaged proteins, inaccurate signal transductions or aberrant enzymatic activities (Sheng et al., 2024). Antioxidant enzymes can actually be a target of ubiquitination, hence modulating cellular responses to oxidative stress (Sheng et al., 2024). Our results show an inhibition of ubiquitin synthesis in the muscle of fish exposed to low and medium dosages of TCPP, a result similar to the trend observed in GST activity. Therefore, this decrease in UBI content can also be linked to TCPP's concentration-dependent mechanisms that should be further examined. In the liver, despite the lack of significance in differences between CTR and the contaminated, UBI synthesis also tended to be supressed by medium and high concentration, whereas an enhancement occurred at low concentrations of TCPP. Indeed, although ubiquitination can be induced upon exposure to pollutants (Zhong et al., 2020; Sheng et al., 2024), an inhibition can also be observed under more extreme or long-term conditions, due to energetic imbalances, i.e. the need to prioritize energy allocation towards physiological homeostasis and survival under stress, rather than for protein synthesis processes (Maulvault et al., 2018). Modifications of the metabolic apparatus and performance can alter the energy production mode. The activity of enzymes that participate in aerobic and anaerobic pathways, such as CS and LDH, serves as biochemical markers that reflect these shifts (Rosa et al., 2016). Both these enzyme activities are modulated by other contaminants, suggesting that the responses observed in CS and LDH are a universal mechanism through which fish manage dietary exposure of such compounds (Cohen et al., 2005). TCPP can alter lipid metabolism, which in turn potentially affects energy

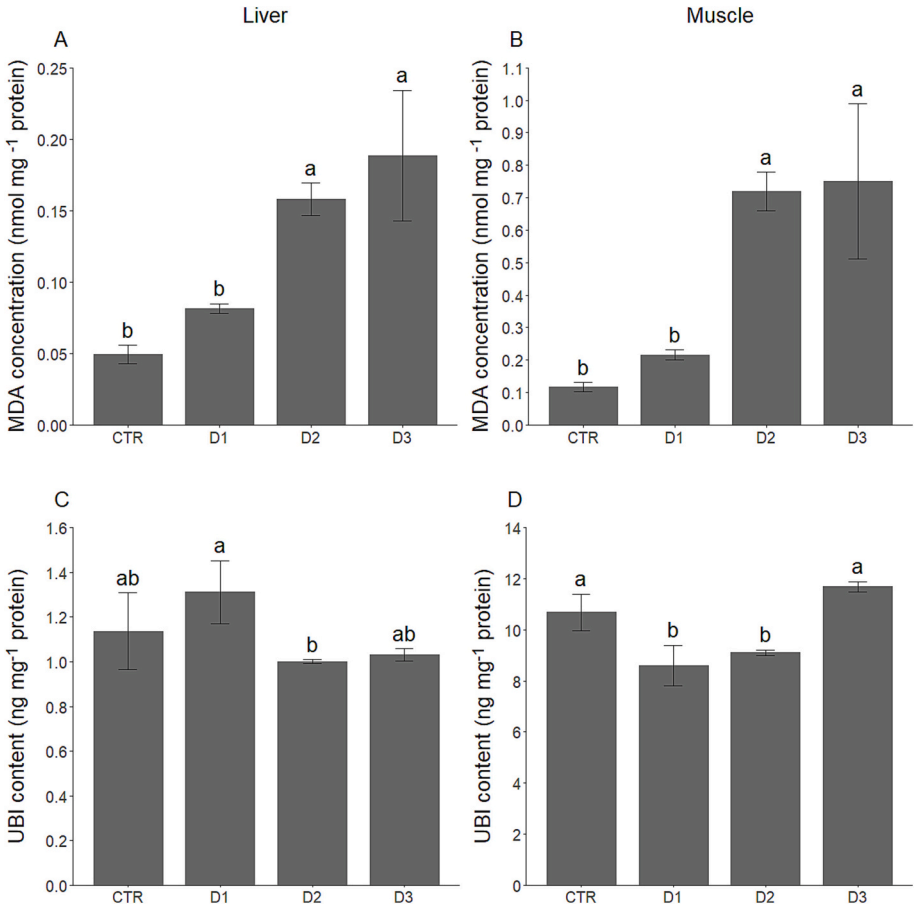

Fig. 2. Lipid peroxidation (MDA concentration, nmol MDA mg^{-1} protein) and protein degradation (UBI content, ng mg^{-1} protein) in liver (A, C) and muscle (B, D) of juvenile *S. aurata* (mean ± standard deviation) after 28 days of feed-exposure to different TCPP treatments ($n = 6$). Different letters indicate significant differences ($p < 0.05$) between treatments. CTR: control treatment; D1: low concentration (0.2 mg TCPP kg^{-1}); D2: medium concentration (2 mg TCPP kg^{-1}), D3: high concentration (10 mg TCPP kg^{-1}).

metabolism (Aluru et al., 2021; Yan et al., 2022). Disrupted energy production can consequently provoke further dysregulation of other biological processes (Ji et al., 2020). Being a vital enzyme on the Krebs cycle (Giraudo et al., 2017), enhanced CS activity usually translates in higher aerobic potential (Rosa et al., 2016). Regardless of dose, TCPP exposure heightened aerobic capacity in the livers of juvenile *S. aurata*. In muscle, both high and low concentrations of TCPP enhanced CS activity, while the medium (and most ecologically relevant) inhibited it. When animals are exposed to stressful conditions, such as the exposure to pollutants, an activation of the aerobic pathway can take place as a way to counteract increased oxidative stress induced by chemical substances (Giraudo et al., 2017). In contrast, the inhibition of this pathway (as observed in fish from D2 treatment) might be related with hampered mitochondria functionality and ATP synthesis, which later translates to decreased antioxidant scavenger capacity (Feidantsis et al., 2018). Chlorinated OPEs have been shown to cause mitochondrial dysfunction and morphological abnormality (Le et al., 2021), and the accompanying disruption of energy metabolism damages the fish's organ structures (Wang et al., 2024). In mussels (*M. galloprovincialis*) exposed to TCPP via water (100 nmol L^{-1}), it was observed an alteration in the expression of important enzymes pertaining to this Krebs cycle (Zhong et al., 2020). In rockfish (*Sebastes schlegeli*), β-oxidation was enhanced due to TCPP exposure (10–100 nmol L^{-1}), which could indirectly affect the Krebs cycle and thus CS activity (Ji et al., 2020). Conversely, a helpful indicator for evaluating anaerobic potential (Rosa et al., 2016) is LDH, an important enzyme involved in anaerobic glycolytic metabolism, i.e., the conversion of lactate to pyruvate and vice-versa (Ramesh et al., 2020). An inhibition of this enzyme occurred in the liver of fish exposed to the medium dose. In the study carried out by Deng et al. (2022), TCPP, at a concentration of 100 μg L^{-1}, also reduced LDH activity in mussels (*M. coruscus*), suggesting that this substance affects the anaerobic metabolism across multiple marine taxa. This reduction may also be due to changes in mitochondrial function or impaired carbohydrate metabolism (Ambili et al., 2013). We also hypothesize that, at lower or mild doses of TCPP, both this OPE and its metabolites could be leading to direct suppressing processes concerning the aforementioned enzymes (such as forming an enzyme-inhibitor complex, increase formation of substrate, or acting on the regulatory site of the enzyme and thus modifying its active site), as explained by Gupta (2021). However, these mechanisms may become saturated when the organism is exposed to elevated quantities of TCPP, obliging them to reach internal homeostasis and resulting in baseline levels of enzyme activity (e.g. LDH in the fish subjected to the highest dose). Interestingly, some metabolites of TCPP might be more detrimental to biota than the parental form itself (Noyes et al., 2015), meaning that compound metabolization and the toxicological effects of predominant metabolites is a topic that warrants more attention in upcoming research.

VTG acts as a biomarker for estrogenic endocrine disruption (Wang et al., 2015), and is a protein associated with female oocyte maturation (García Hernández et al., 2020). Hence, the drastic inhibition of VTG synthesis in fish exposed to TCPP pointed out to an endocrine-disrupting effect elicited by this compound. As a protandrous hermaphrodite species, early-life stage *S. aurata* specimens present a male phenotype which, in some cases, gradually shift to a functional female upon reaching sexual maturity (approximately, at the age of three; García Hernández et al., 2020). As such, a decrease in VTG content can indicate

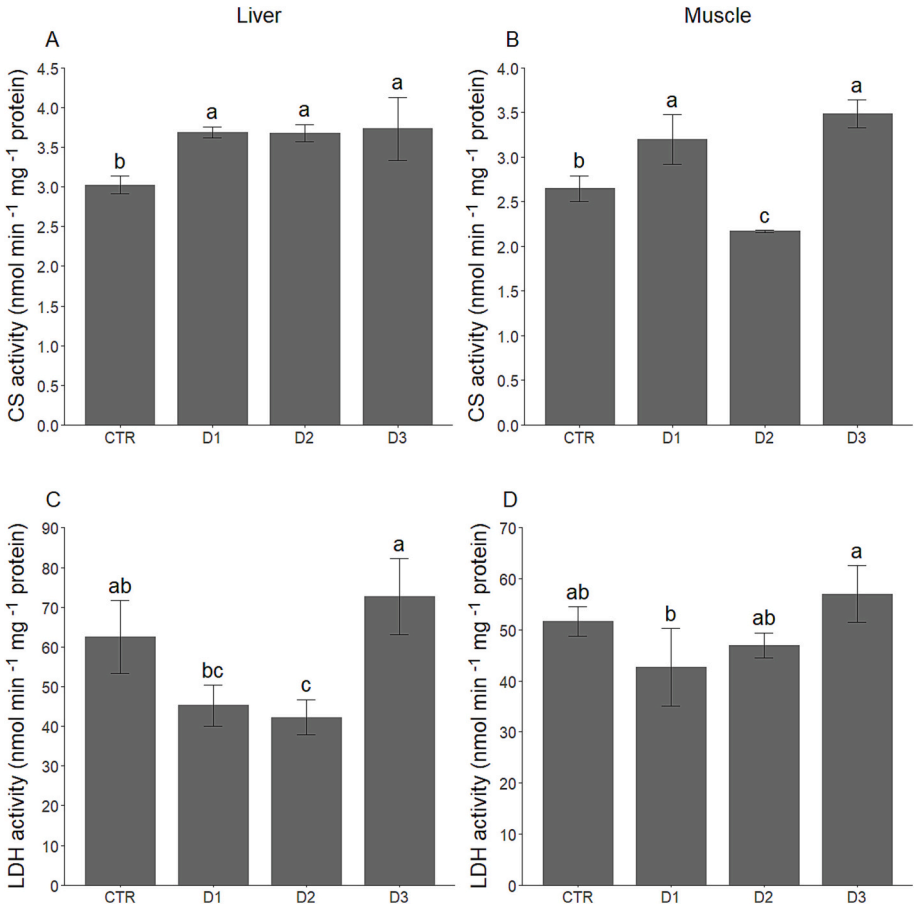

Fig. 3. Metabolism enzymes activity (CS, nmol min^{-1} mg^{-1} protein; LDH, nmol min^{-1} mg^{-1} protein) in liver (A, C) and muscle (B, D) of juvenile *S. aurata* (mean ± standard deviation) after 28 days of feed-exposure to different TCPP treatments (n = 6). Different letters indicate significant differences ($p < 0.05$) between treatments. CTR: control treatment; D1: low concentration (0.2 mg TCPP kg^{-1}); D2: medium concentration (2 mg TCPP kg^{-1}), D3: high concentration (10 mg TCPP kg^{-1}).

that this male-to-female sex reversal process might be prevented by TCPP exposure, even at low concentrations. However, as the fish used in this study were immature, future research with protandrous adult fish specimens should be undertaken, as to understand whether these outcomes translate into delayed sexual maturation and altered male-to-female reversal mechanisms. To the best of our knowledge, this is the first study reporting TCPP endocrine disrupting effect in fish species. However, a few reports (on other taxa) have suggested that OPEs (including TCPP) can influence steroidogenic processes, inevitably eliciting an effect on the endocrine system (Liu et al., 2012; Aluru et al., 2021). The estrogenic effects of TCPP in sea urchins (*Paracentrotus lividus*) were found by Campoy-López et al. (2020), and Zhong et al. (2020) raise the possibility of reproductive endocrine disrupting effects in mussels. Besides, other organophosphate flame-retardants (including but not limited to TPP and TDCPP) also led to the disruption of sex hormone balances (Liu et al., 2012) and changes in hepatic VTG expression and other genes in the hypothalamic-pituitary-gonadal axis in zebrafish (Wang et al., 2015). These last authors further argued that OPEs may act directly on estrogenic receptors to alter hepatic VTG synthesis (Wang et al., 2015).

5. Conclusions

The current study suggests that chronic trophic exposure to TCPP (at the low, medium and high concentrations tested) can disrupt the endocrine system and energy metabolism in fish, as well as induce hepatic and muscular oxidative damage, with further deleterious effects on growth. Overall, results highlighted that fish responses to TCPP are: i) dose dependent, e.g. high exposure concentrations (i.e. from 10 mg kg^{-1}) were responsible for a greater enhancement of the antioxidant activity and anaerobic pathway, while increased cell damage (LPO) started to take place at medium exposure concentrations (i.e. from 2 mg kg^{-1}); and ii) tissue-dependent, most likely, as a result of TCPP's mode of action (e.g. CS activity decreased at the medium concentration in fish muscle, whereas an increase was observed in the liver of fish exposed to this concentration; GST decreased at the low and medium concentrations in fish muscle, whereas no significant differences were found in the liver at these concentrations). Hence, our results showcase the necessity of analysing multiple tissues and biomarkers in order to obtain a more insightful and comprehensive assessment of emerging contaminants impacts in marine ichthyofauna. Additionally, as different exposure routes (feed- versus water-based) can take place in the wild, each of them eliciting distinct ecotoxicological responses, further investigations ought to address this subject as to enable appropriate and environmentally relevant data interpretations. As a final remark, since TCPP does not occur alone and is not the only stressor present in the environment, we emphasize the need to research the potential synergies between this chemical and other HPVC's and/or environmental stressors (including abiotic variables), as such approaches would surely enable a more thorough understanding of the consequences of this substance in nature, as well as adequate regulation of potentially hazardous substances, such as OPEs.

CRediT authorship contribution statement

Rita V.C. Gomes: Writing – review & editing, Writing – original

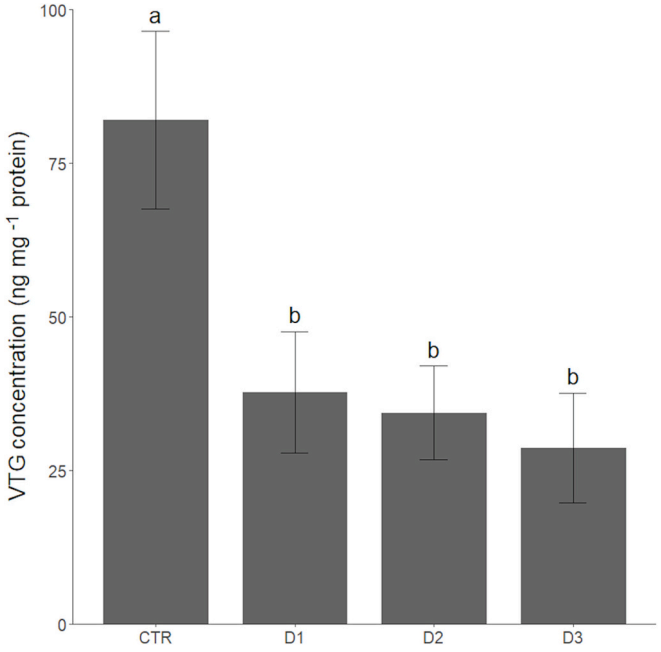

Fig. 4. Vitellogenin content (VTG, ng mg^{-1} protein) in liver of juvenile *S. aurata* (mean ± standard deviation) after 28 days of exposure of feed-exposure to different TCPP treatments (n = 6). Different letters indicate significant differences ($p < 0.05$) between treatments. CTR: control treatment; D1: low concentration (0.2 mg TCPP kg^{-1}); D2: medium concentration (2 mg TCPP kg^{-1}), D3: high concentration (10 mg TCPP kg^{-1}).

draft, Investigation, Formal analysis, Data curation. **Sílvia Borrull:** Writing – review & editing, Investigation. **Alícia Pereira:** Writing – review & editing, Investigation. **Marta Dias:** Writing – review & editing, Investigation. **Rui Cereja:** Writing – review & editing, Investigation. **Marisa Barata:** Investigation. **Pedro Pousão-Ferreira:** Investigation. **Ana M. Faria:** Writing – review & editing, Investigation. **Eva Pocurull:** Writing – review & editing, Investigation. **Rosa Maria Marcé:** Writing – review & editing, Investigation. **António Marques:** Writing – review & editing. **Ana Luísa Maulvault:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Funding acquisition.

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.

Acknowledgments

The research contracts of RG, AP, RC, MD, and AF were supported by the funded projects PRR ABI-2024.NEWPACK.06, PRR PBA-Vertical Algas (C644915664-00000026) (AP and RC), Aqua-CLIMADAPT (PTDC/CTA-AMB/0592/2021), and INNOECOFOOD (101136739, Horizon Europe), respectively. We also acknowledge the financial support from project PID2020-114587GB-I00, funded by MCIN/AEI/10.13039/501100011033 (Ministerio de Ciencia, innovación y Universidades). SB acknowledges Agència de Gestió d'Ajudes Universitàries i Recerca (AGAUR) for her PhD grant (2021 FISDU 00070). The authors also thank SPAROS Lda., for providing the experimental feeds, and the team of EPPO-IPMA in Olhão, for the support provided during animal rearing and transplantation.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.marpolbul.2025.117628.

Data availability

Data will be made available on request.

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