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Are BPA-free plastics safe for aquatic life? - Fluorene-9-bisphenol induced thyroid-disrupting effects and histopathological alterations in adult zebrafish (*Danio rerio*)

Selin Özkan-Kotiloğlu [a,*], Pınar Arslan [b], Gülçin Akca [c], Aysel Çağlan Günal [d]

[a] Department of Molecular Biology and Genetics, Faculty of Science and Art, Kırşehir Ahi Evran University, Kırşehir, Turkey
[b] Biology Department, Faculty of Science, Çankırı Karatekin University, Çankırı, Turkey
[c] Department of Medical Microbiology, Faculty of Dentistry, Gazi University, Ankara, Turkey
[d] Department of Biology Education, Faculty of Gazi Education, Gazi University, Ankara, Turkey

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ABSTRACT

Fluorene-9-bisphenol (BPFL) is used as an alternative compound for bisphenol A, an endocrine disruptor compound which is present in various materials including plastic bottles and packaging. Although it is used extensively in products that are labelled BPA-free, its effect on wildlife and humans have not been fully studied. Therefore, this study aimed to investigate the effects of BPFL in adult zebrafish. In the preliminary experiments of the study, the median lethal concentration value (LC_{50}) of BPFL was 0.25 mg/L (95 % confidence interval 0.15–0.41) for 96 h. Following exposure to three different sublethal concentrations of BPFL after 96 h and 15 days, T4 hormone levels, expression levels of genes involved in thyroid metabolism and histopathological alterations were assessed. T4 hormone levels were found to be significantly higher in females at the lowest BPFL concentration following 96 h exposure ($P < 0.05$). Expression levels of *trh*, *tshba* and *trhrb* genes were upregulated following 96 h exposure at 0.025 mg/L concentration and *crh* was upregulated following 15 days exposure at 0.025 mg/L concentration in female zebrafish ($P < 0.05$). The most prominent histopathological findings in zebrafish exposed to 0.025 and 0.125 mg/L of BPFL were observed in the gill, liver, kidney and testis tissues. The gill tissues showed some hyperemia, lamellar fusion, hyperplasia, epithelial lifting, and telangiectasis, while passive hyperemia, hydropic degeneration, and necrosis were observed in the liver tissues. The BPFL is highly toxic to zebrafish even in sublethal concentrations according to the molecular and histopathological responses.

1. Introduction

Accelerating number of natural and synthetic compounds have been described to disrupt the normal function of the endocrine system and to make adverse effects on animal and human health (Preda et al., 2012). These compounds are referred to as Endocrine Disrupting Compounds (EDCs). They are chemical or chemical mixtures altering the synthesis, transport, binding, or destruction of natural hormones of organisms that are responsible for homeostasis, reproduction, development, and behavior (Kavlock et al., 1996; Kabir et al., 2015) and lead to adverse effects of progeny (Preda et al., 2012) of the organisms and subpopulations.

EDCs are classified into two categories according to occurrence: natural like phytoestrogens and synthetic chemicals including plastics, plasticizers, pesticides, and pharmaceutical agents (Diamanti-Kandarakis et al., 2009; Shahjahan et al., 2022; Archunan et al., 2022; Merola et al., 2022; Dar et al., 2022). Among EDCs, Bisphenol A (BPA; 4, 4′-dihydroxy-2, 2-diphenylpropane) is a chemical substance widely used in the production of polycarbonate, epoxy resins, dental materials, and plastic additive materials including polyvinyl chloride used in water pipes, toys, medical tubing, and polyethylene terephthalate used in water bottles (Welshons et al., 2006). There have been many studies reporting that BPA has altered estrogen (Kim et al., 2014; Pelch et al., 2019), androgen (Pelch et al., 2019), and thyroid (Zhang et al., 2018) metabolisms in various species.

Fluorene-9-bisphenol (BPFL, also known as BHPF, 9,9-bis(4-hydroxyphenyl)-fluorene), which is claimed as a harmless alternative to bisphenol A (BPA), has been widely used in the manufacture of "BPA-

* Corresponding author.
E-mail addresses: selin.ozkan@ahievran.edu.tr, selin.ozkan11@alumni.imperial.ac.uk (S. Özkan-Kotiloğlu).

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free" plastics. As the use of BPA is currently restricted due to its endocrine disrupting impacts, the variety and number of products consisting of BPA substitutes such as BPFL are dramatically increased (den Braver-Sewradj et al., 2020). BPFL has good thermal stability and is extensively used to synthesize polyesters, epoxy resins, polycarbonates, and utilized in the manufacturing of a wide range of consumer products including building materials and plastic bottles (Dai et al., 2009), leading to releasing the BPFL concentrations to the environment. For instance, Jin and Zhu (2016) showed that the concentrations of BPFL were found between 0.056 ng/L and 0.069 ng/L in Liaohe River Basin (China). Zhang et al. (2017b) found that the concentration of BPFL could reach 81.47 ng/L in plastic bottles.

Research reporting the toxic and endocrine disrupting effects of BPFL have being accumulated. It has been reported that BPFL is released into water from BPA-free plastic bottles, interferes with blood circulation, and has anti-estrogenic effects on mouse (Zhang et al., 2017b). Moreover, it has been stated that BPFL exposure had disruptive and inhibitory effects on both mouse and porcine oocyte maturation (Jiao et al., 2018; Jiao et al., 2019). Recently, relatively low levels of BPFL exposure have been shown to cause liver injury in CD-1 mice (Yang et al., 2021). The BPFL exposure showed toxic and lipid peroxidation effects of freshwater green algae *Chlorella vulgaris* (Zhang et al., 2021). Moreover, in zebrafish, exposure to BPFL has adverse impacts on lipid metabolism, development, reproduction, nervous system, behaviors, and locomotor activity (Mi et al., 2019; Mi et al., 2020; Sun et al., 2020; Jin et al., 2021; Zhang et al., 2022).

Zebrafish (*Danio rerio*) is a preferred model organism for investigating the effects of EDCs due to providing inherent advantages in terms of size, cost, life cycle, and genetic information (Blahova et al., 2020; Jijie et al., 2021; Zicarelli et al., 2022). Zebrafish also reflects the aquatic environment which is constantly polluted by many chemicals including plasticizers. In addition, it shares many common features in terms of anatomy, genetics and physiology with mammals (Segner, 2009).

The thyroid system is crucial for body homeostasis and the hormones of this system are essential for both physiological and psychological health (Qiao et al., 2021). Hypothalamus-pituitary-thyroid (HPT) axis is the primary control body of the thyroid endocrine system in fish (Carr and Patino, 2011). It controls the synthesis and secretion of thyroid hormones, for instance thyrotropin releasing hormone (TRH) is synthesized by the hypothalamus and stimulates thyroid-stimulating hormone (TSH) secretion from the pituitary gland. It has been also reported that corticotropin-releasing hormone (CRH) is an inducer of TSH secretion in non-mammalian vertebrate classes such as fish (de Groef et al., 2006). There has been an accelerating number of reports stating the disruption of thyroid metabolism by environmental contaminants (Liu et al., 2011; Liu et al., 2016; Guo et al., 2019; Wu et al., 2020; Qiao et al., 2021). To our knowledge there has been only one study investigating the role of BPFL on the thyroid metabolism of zebrafish larvae (Jin et al., 2021).

Our present work is directed toward determining the acute and the chronic effects of BPFL as limited information is available on the effects of BPFL on zebrafish thyroid hormone metabolism. For this purpose, firstly, it was aimed to determine the median lethal concentration values (LC_{50}) of BPFL for zebrafish, and then to analyze the thyroid hormone metabolism and histopathological alterations in zebrafish exposed to sublethal concentrations of BPFL. In order to assess the effect of BPFL and reveal the possible mode of action, transcriptional effects of BPFL were also investigated by determining the expression levels of genes involved in thyroid metabolism. Moreover, impacts of BPFL on plasma T4 levels and also on tissues were searched to provide further insight into the thyroid disrupting and histopathological effects of BPFL.

2. Materials and methods

2.1. Chemicals

Fluorene-9-bisphenol (BPFL; CAS No. 3236-71-3, purity: 97 %) was purchased from Sigma-Aldrich (St. Louis, USA). Stock solution of BPFL (1 mg/L) was dissolved in dimethyl sulfoxide (DMSO, 0.1 % (*v*/v)) and used immediately. Both BPFL and DMSO were analytical grade. TRIzol reagent (USA) and T4 hormone ELISA kit were obtained from Invitrogen and Cloud-Clone Corp (USA), respectively.

2.2. Zebrafish maintenance

The zebrafish experiments were done at Biology Education Laboratory 4, Faculty of Gazi Education, Gazi University (Ankara, Turkey). Healthy adult zebrafish, *Danio rerio*, of both sexes, were obtained from a commercial supplier in Ankara, Turkey. The average length and weight of adult zebrafish were 4.02 ± 0.26 cm and 0.57 ± 0.12 g, respectively. They were acclimated to the laboratory conditions for 2 weeks prior to the experiments. During the acclimation and experimental periods, the aquarium water was provided using filtered tap water. The physiochemical parameters of water were measured every day (pH 7.5–7.8; temperature 27.6 ± 0.7 °C; 14 h light:10 h dark photo period). The tops of the aquariums were covered with glass covers that were half transparent and half black. The water of the aquariums was continuously aerated. Zebrafish were kept in 20 L water (1 animal/L) in 30 L aquariums. The fish were fed with commercial aquarium fish food containing *Artemisa* sp. at the rate of ad libitum of their body weight per day with. The movements of fish were monitored for the normal behavior of the species. To remove metabolic waste and food waste, 2 h after feeding, the whole aquaria water was cleaned by siphoning and renewed by filtered tap water. All experiments were performed following the guidelines and regulations set forth by the Local Ethics Committee for Animal Experiments of Gazi University, Ankara, Turkey (G.Ü.ET-18.049).

2.3. Determination of LC_{50}

The experiments which were carried out within the scope of the study were done according to the methods of ZSF determination method APHA, OECD, TSE, ISO and FAO in two stages as pre-test and the main experiment. Before sublethal BPFL exposure, preliminary experiments were conducted in order to determine the median lethal concentration (LC_{50}) of BPFL. 10 female and 10 male zebrafish were distributed randomly to aquariums. Preliminary experiments were conducted for 96 h to determine the median lethal concentration (LC_{50}) in three replicates using the static bioassay method. LC_{50} values are based on Finney's probit analysis method in the U.S. Calculated according to the EPA computer program.

2.4. Experimental design and BPFL exposure

10 male and 10 female adult zebrafish (same size and weight) were randomly placed to aquariums each 30 L glass-aquaria (1 animal/L). The fish were divided into four treatment groups, in duplicate (20 fish per aquaria, in a total of 40 fish per group): a control group maintained in 0.1 % (*v*/v) DMSO; $^1/_{100}$ as 0.0025 mg/L BPFL; $^1/_{10}$ as 0.025 mg/L BPFL; and $^1/_2$ as 0.125 mg/L BPFL; and exposed for 96 h as acute exposure and 15 days as chronic exposure. During the experimental period, the aquariums were continuously aerated. Around 50 % of each aquarium solution water were changed every 48 h. On each renovation, the bottom and side of the aquariums were cleaned to keep water quality. The water physicochemical parameters, the feeding process and photoperiod during the experiments were maintained as described in the previous section. After the length and weight measurements of the fish were taken at the end of the BPFL exposure times, the fish were anesthetized in ice

water (Chen et al., 2014). Then, brain and bodies of 3 male and 3 female individuals from each group were taken for biochemical and bodies of 7 male and 7 female individuals were taken for histopathological analysis.

2.5. RNA isolation and qRT-PCR

Following exposure to BPFL for 96 h and 15 days, fish was anesthetized on ice and brains were removed by dissection from male and female animals. Seven brains of same sex in each group were pooled and soaked in liquid N_2 and then stored at -80 °C until RNA isolation. Total RNA was isolated using Trizol (Invitrogen) according to the manufacturer's protocol after grinding frozen brain tissue using glass sticks. To eliminate DNA contamination, total RNAs were treated with Turbo DNase free kit (Thermo). cDNA synthesis was performed by using RevertAid cDNA synthesis kit (Thermo) with random hexamers as kit's manual described. Quantitative RT-PCR was performed using Rotor-Gene SYBR Green PCR Kit (Qiagen) and analyzed on a Rotor Gene-Q Series Software version 1.7 (Qiagen). The PCR conditions were as follows 95 °C for 10 min, 40 cycles of 95 °C for 5 s, 60 °C for 10 s. The expression levels of the genes related to the HPT axis (*trh:* thyrotropin releasing hormone, *trhrB:* thyrotropin releasing hormone receptor b, *tshba:* thyroid stimulating hormone subunit beta a and *crh:* corticotropin releasing hormone) were measured. *GAPDH* gene was used as a reference gene. The primer sequences of each gene were demonstrated in Table 1.

2.6. ELISA assay for T4 hormone

The concentration of the T4 hormone was determined by using an enzyme-linked immunosorbent assay (ELISA). For each concentration and duration of BPFL exposure, whole bodies of three adult male and three adult female zebrafish as duplicate were homogenized in 1× phosphate buffer saline (PBS; pH:7) in 1:9 ratio. Following centrifugation at 5000 ×*g* for 10 min at 4 °C, supernatant was collected and T4 levels were measured duplicate using T4 ELISA kit (Cloud-Clone Corp, Wuhan, China) according to the manufacturer's instruction at 470 nm using BioTek ELx800 absorbance microplate reader.

2.7. Histological analysis

For each concentration and duration of BPFL including the control groups, the whole bodies of seven male and seven female zebrafish were taken and kept in 10 % buffered formalin solution for two weeks in histology tissue processing cassettes. Then, the routine histological techniques were carried out. For dehydration procedures, the tissues were passed through 50 %, 70 %, 80 %, 90 %, 96 %, and absolute ethanol series. In order to remove alcohol, the tissues were kept in xylol for 2 h and embedded in paraffin and paraffin with xylol at 56-58 °C. For histopathological examination, whole body sagittal sections of 5–6 μ thickness were cut from the paraffin blocks with Rotary Thermo Shandon microtome and placed on the slide in the hot water bath. After the sections were kept in an oven at 50-60 °C overnight, they were stained with hematoxylin and eosin (H&E) according to the Presnell and Schreibman (1997). The histology slides were examined under the light microscope (Zeiss Primo Star). The criterion of the histopathological alteration of the tissues was scored according to the intensity of the lesions as*(−) none (no histological alterations), which represents normal histological structure; (+) histopathology in >20 % of fields (mild); (++) histopathology in 20–60 % of fields (moderate) and (+++) histopathology in <60 % of fields (severe) (Benli et al., 2008).

2.8. Molecular docking

The molecular docking of the chemical compounds-proteins binding site was performed using Autodock 2.2.6 software (Morris et al., 1998). The docked complexes were visualized using PYMOL (DeLano, 2002) and Chimera (Goddard et al., 2007). The 3D dimensional structure of proteins was obtained from the UniProt database (https://www.uniprot.org/). All proteins were simulated using the open-source server PROCHECK, and the Ramachandra plot for the proteins were examined. The Protox-II webserver (Banerjee et al., 2018) was used to estimate the organ toxicities and toxicological end points of the title complex and their LD_{50}.

2.9. Statistical analysis

Data were tested for normality and homogeneity of variances using Kolmogorov-Smirnov test prior to statistical analysis. One-way ANOVA test was used to analyze the results by using GraphPad Prism Software 9.0 (USA). $P < 0.05$ was considered as significant and error bars were done by using SEM. Significance was set as $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***).

3. Results

3.1. LC_{50} values

The median lethal concentrations (LC_{50}) of BPFL were found to be 1.28 (0.69–2.44) mg/L, 0.59 (0.33–1.06) mg/L, 0.32 (0.18–0.51) mg/L, and 0.25 (0.15–0.41) mg/L at 95 % confidence interval for 24, 48, 72 and 96 h, respectively (Fig. S1). Therefore, three concentrations below LC_{50} (1/100, 1/10 and 1/2 of LC_{50}) were selected to determine the sublethal effects of BPFL on adult zebrafish.

3.2. Effects of BPFL on T4 hormone levels

In female zebrafish, the concentration of T4 hormone was significantly higher at 0.0025 mg/L concentration group when compared to the control group ($P < 0.05$). Additionally, there was a statistically significant decrease between 0.0025 mg/L 96 h group and 0.025 mg/L 15d group ($P < 0.05$; Fig. 1a).

In male zebrafish, while T4 hormone levels decreased upon 96 h exposure for all concentration groups, following 15d exposure an increase was observed for all concentration groups when compared to the control group but these differences were not statistically significant. However, for 15d exposure T4 hormone level was higher at 0.025 mg/L concentration group than 0.0025 mg/L concentration group ($P < 0.05$; Fig. 1b). Moreover, following 96 h exposure T4 hormone levels were significantly higher in females than males at the lowest concentration ($P < 0.05$; Fig. 1c).

Table 1
Primer sequences and accession numbers of the genes of interest.

Gene name	F primer	R primer	Accession number	Reference
crh	TTCGGGAAGTAACCACAAGC	CTGCACTCTATTCGCCTTCC	NM 001007379	Chen et al., 2012
tshb	GCAGATCCTCACTTCACCTACC	GCACAGGTTTGGAGCATCTCA	AY135147	Chen et al., 2012
trh	CAGAACAGCGAGAACGATCA	AGCAGCATCAGGTAGCGTTT	NM_001012365.2	This study
trhrb	GCAGCATCCAGAAGACAGGTT	GTCCTGGAGGAAGGAGTTGAC	NM_001114688.1	This study
gapdh	CTGGTGACCCGTGCTGCTT	TTTGCCGCCTTCTGCCTTA	NM001115114	Liu et al., 2016

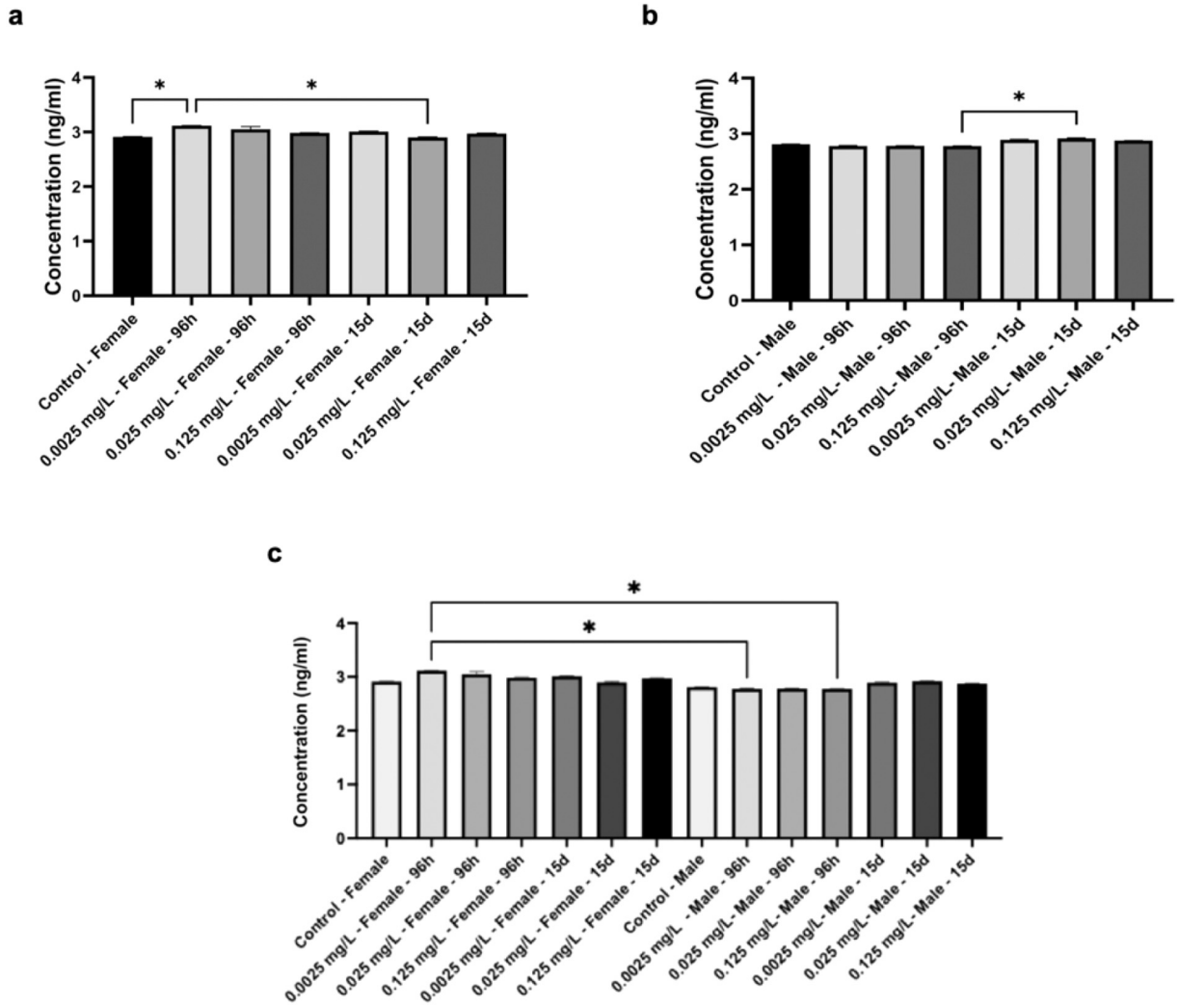

Fig. 1. Levels of T4 hormone concentration upon BPFL exposure and control groups. The values were expressed as mean ± standard deviation ($n = 3$). Asterix indicated significant difference ($P < 0.05$).

3.3. Expression levels of the genes involved in thyroid metabolism

To better understand the impact of BPFL on thyroid metabolism, the expression of the genes involved in the HPT axis was measured and changes in transcriptional profiles were determined by sex.

Corticotropin-releasing hormone gene (*crh*) was significantly increased following 15 days of exposure to 0.025 mg/L in female zebrafish when compared to the control group ($P < 0.05$). There were also statistically significant differences between 0.025 mg/L concentration group and 0.125 mg/L concentration group after 96 h exposure. Moreover, expression of *crh* gene was significantly higher in 0.025 mg/L 15 days concentration group than the same concentration group for 96 h in female zebrafish ($P < 0.001$) (Fig. 2). In male zebrafish, there was no statistical significance in the expression of *crh* gene.

The expression level of thyroid stimulating hormone subunit beta a gene (*tshba*) was significantly upregulated in 0.025 mg/L concentration group after 96 h exposure in females ($P < 0.05$). The expression level of *tshba* gene was significantly higher in 0.025 mg/L 96 h exposure group than both 0.0025 mg/L 15d and 0.125 mg/L 15d exposure groups ($P < 0.001$ and $P < 0.05$, respectively). There are also statistically significant differences between 0.125 mg/L 96 h group and 0.0025 mg/L 15d group and between 0.0025 mg/L 15d group and 0.025 mg/L 15d exposure group in female zebrafish (Fig. 2). In males, none of the exposure groups was significantly different from the control group. However, expression of *tshba* gene was significantly lower in 0.0025 mg/L 15d exposure group than in both 0.0025 mg/L 96 h and 0.025 mg/L 96 h exposure groups ($P < 0.01$). Furthermore, expression of *tshba* gene was significantly lower in 0.125 mg/L 15d exposure group than both 0.0025 mg/L 96 h and 0.025 mg/L 96 h exposure groups ($P < 0.05$).

The expression level of thyrotropin-releasing hormone (*trh*) was significantly upregulated in females following 96 h exposure to 0.025 mg/L BPFL ($P < 0.05$). There was a significant difference observed between 0.025 mg/L 96 h exposure group and 0.125 mg/L 15d exposure group where the expression of *trh* decreased significantly in the latter (Fig. 2). In males, there was no statistically significant difference between the control group and concentration groups. However, at the highest concentration and longest exposure the expression level of *trh* gene significantly downregulated when compared to both 0.0025 mg/L 96 h and 0.025 mg/L 96 h exposure groups ($P < 0.05$; Fig. 2).

In females, when compared to the control group, the transcription of the thyrotropin-releasing hormone receptor beta (*trhrb*) gene was significantly risen after 96 h of exposure to 0.025 mg/L ($P < 0.05$) and 0.125 mg/L BPFL ($P < 0.001$) concentration groups and also after 15 days of exposure to 0.025 mg/L ($P < 0.05$) concentration group (Fig. 2). Furthermore, in female zebrafish there was a significant difference detected at 0.025 mg/L concentration between 96 h and 15d exposure groups ($P < 0.05$). In males, the level of *trhrb* showed different trends in different exposure groups both at 96 h and 15d, however, none of them

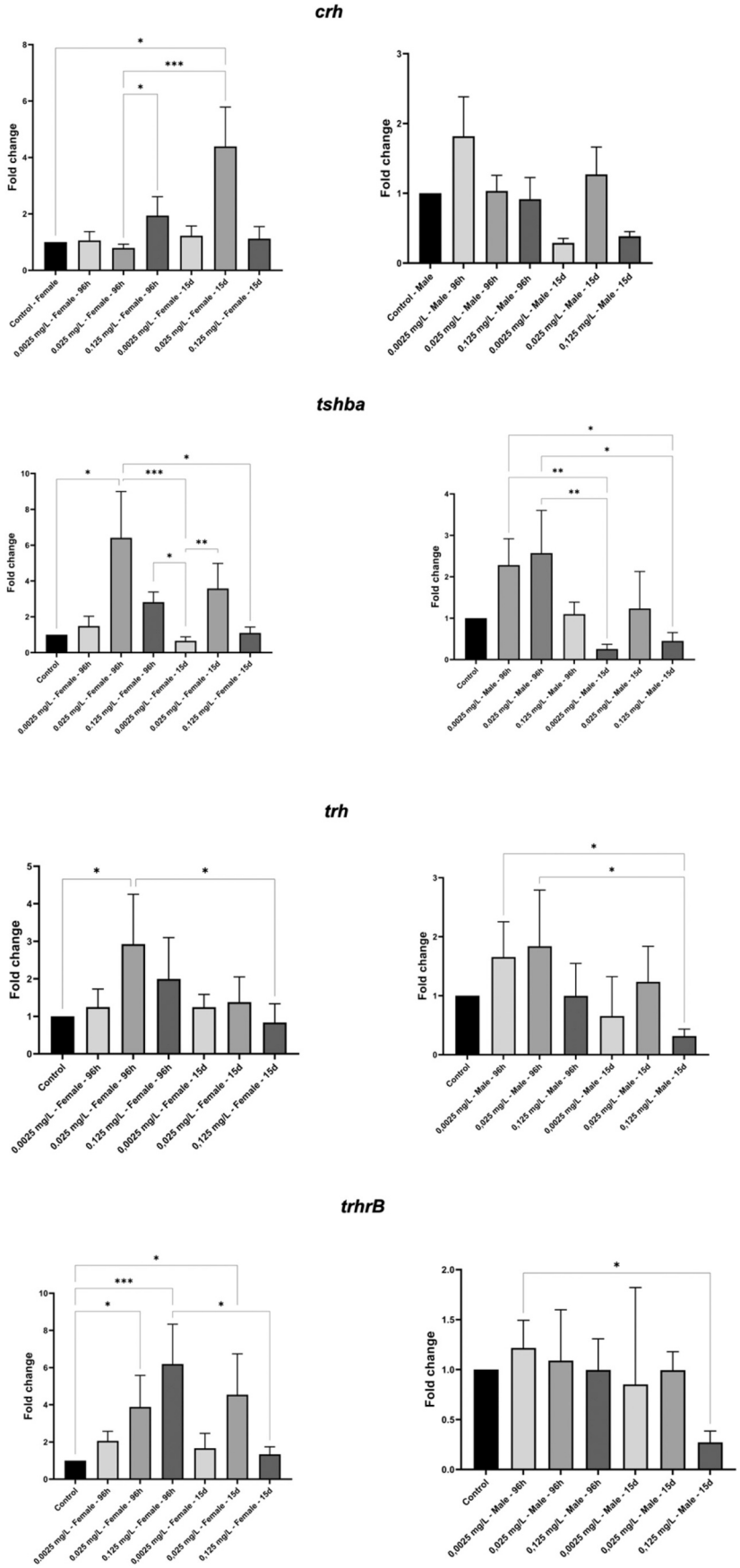

Fig. 2. The expression levels of *crh, tshba, trh* and *trhrB* genes relative to *GAPDH* in female and male zebrafish following 96 h and 15d of exposure to 0.0025 mg/L, 0.025 mg/L and 0.125 mg/L BPFL and control groups.

was different from the control group. The only statistically significant difference was observed between 0.0025 mg/L 96 h group and 0.125 mg/L 15d group ($P < 0.05$; Fig. 2).

Interestingly, in both female and male groups, at the 0.025 mg/L concentration for 96 h, expression of all four genes were higher however these were not statistically significant.

3.4. Histological examination

The histopathological findings of zebrafish exposed to BPFL and the control groups for two exposure times are summarized in Table 2. As seen in the Table 2, the control groups in both exposure times and zebrafish exposed to 0.0025 mg/L BPFL for 96 h did not exhibit any histopathological alterations in the whole-body samples.

The most severe lesions were detected as necrosis in the liver and kidney tissues of 15d for 0.125 mg/L BPFL exposed group. The most prominent lesions were observed in the gill, liver, kidney and testis after exposure to 0.025 and 0.125 mg/L of BFPL. The gill tissues of BPFL exposed zebrafish showed some hyperaemia, lamellar fusion, hyperplasia, epithelial lifting, and telangiectasis depending on the concentration of BPFL and exposure times (Fig. 3).

Passive hyperemia, hydropic degenerations, and necrosis were observed in the liver tissues (Fig. 4), while tubule degeneration and necrosis were noticed in the kidney tissues depending on the duration and the concentration of BPFL (Fig. 5).

The degeneration of the Leydig cells in the testis tissues was obvious compared to the control groups (Fig. 6).

3.5. Molecular docking

As shown in Table S1, the docking parameters calculated inhibition constant, binding energy, and hydrogen bond distance all play a role in determining the type of the molecule. The binding interactions of the compound with three protein targets are depicted in Fig. 7.

From the docking results in Table S2 and Fig. 7, the Fluorene-9-Bisphenol and TRHRB (receptor) with a binding energy and inhibition constant values are −8.00 Kcal/mol and 1.37 μM. TSHB protein formed a hydrogen bond with complex with binding energy of −8.19 Kcal/mol. For all proteins, the hydrogen-bonded amino acids bond length is with the <3 Å which indicate they are all strong hydrogen bonding interaction. The biological activity of the title compound is confirmed by the docking of all three proteins with the molecule. Furthermore, the protein targets under consideration, the titled compound, showed strong binding ability to TRHRB with lower binding energies and Ki value. In addition, the electrostatic energy has a negative value, and this value shows that the molecule is linked to a protein (Tüzün and Kaya, 2018).

The Ramachandran plot provides essential information for determining, predicting, and validating protein structure. This indicates that most of the residues lie in the allowed region of the structure and thus may be suitable for molecular docking studies (Fig. S2).

Acute toxicity prediction findings, such as toxicity class classification 1 (toxic) to 6 (non-toxic), indicated that the named chemical was classified as acute toxicity class 5 (harmful if swallowed). According to the ProTox-II server, the predicted LD_{50} value of the compound was 5000 mg/kg and the results suggested that the compound is non-Hepatotoxicity in molecule and has no effect on immunotoxicity, mutagenicity, and cytotoxicity but has some carcinogenicity (Table S2).

4. Discussion

Endocrine disruptive chemicals are the exogenous chemicals leading to health issues including cancer, infertility, and obesity by interfering with hormonal activity. These exogenous pollutants have (Zlatnik, 2016) potential to accumulate in wildlife as well as human, interact with the endocrine system of the organisms and cause severe harm in hormonal secretion. Several studies have shown the adverse effects of EDCs on the secretion of estrogen, androgen and thyroid hormones (Huang et al., 2020; Burgos-Aceves et al., 2021).

EDCs usually show their impacts by interacting with hormone receptors so that they can mimic and alter the production, storage, or action of the hormones (Zlatnik, 2016). Among EDCs, bisphenols, especially bisphenol A has been shown to mimic estrogen hormone, be able to bind to its receptors and have adverse effects on the hypothalamic-pituitary-gonadal axis resulting in disruption in reproductivity (Burgos-Aceves et al., 2021). In recent years, thyroid metabolism has caught attention as a target for EDCs especially for bisphenol substitutes as thyroid hormones have crucial roles in development, differentiation, homeostasis, and well-being in all organisms including zebrafish (Spaan et al., 2019). As zebrafish shares many physiological, anatomical and genetical features with mammals, it has been considered as a model organism for research on human endocrine system (Howe et al., 2013).

As aquatic organisms may be exposed to a wide range of waterborne or anthropogenic contaminants affecting the HPT axis, it is crucial to reveal the effects of chemicals on this system (Carr and Patino, 2011).

In aquatic toxicology studies, fish acute toxicity evaluation is highly important for hazard and risk assessment of chemicals including EDCs. This data is provided by bioassays conducted in the laboratory conditions on the acute exposure tested chemicals of fish for 96 h according to OECD guideline (OECD, 1992; Birke and Scholz, 2019). In this study, first we evaluated the acute toxic effect of BPFL on the adult zebrafish under laboratory conditions. For adult zebrafish specimens, the median lethal concentration (LC_{50}) values were evaluated 1.28 mg/L at 24 h, 0.59 mg/L at 48 h, 0.32 mg/L at 72 h, and 0.25 mg/L at 96 h. Mi et al.

Table 2
Histopathological findings* of the zebrafish (*Danio rerio*) after exposure to Fluorene-9-bisphenol concentrations.

Histopathology		Control		Fluorene-9-bisphenol					
				0.0025 mg/L		0.025 mg/L		0.125 mg/L	
		96 h	15 d	96 h	15 d	96 h	15 d	96 h	15 d
Gill	Hyperemia	–	–	–	+	+	++	+	++
	Lamellar hyperplasia	–	–	–	–	+	++	+	++
	Epithelial lifting	–	–	–	–	+	++	+	++
	Telangiectasis	–	–	–	–	+	++	+	++
	Lamellar fusion	–	–	–	–	+	++	+	++
Liver	Passive hyperemia	–	–	–	+	+	++	+	++
	Hydropic degeneration	–	–	–	–	–	++	++	+
	Necrosis	–	–	–	–	–	+	–	+++
Kidney	Tubule degeneration	–	–	–	–	–	++	++	++
	Necrosis	–	–	–	–	–	–	–	+++
Testis	Degeneration of the Leydig cells	–	–	–	–	+	++	+	+++

* (−) none (no histological alterations), which representing normal histological structure; (+) mild; (++) moderate and (+++) severe histopathological alterations in the tissues.

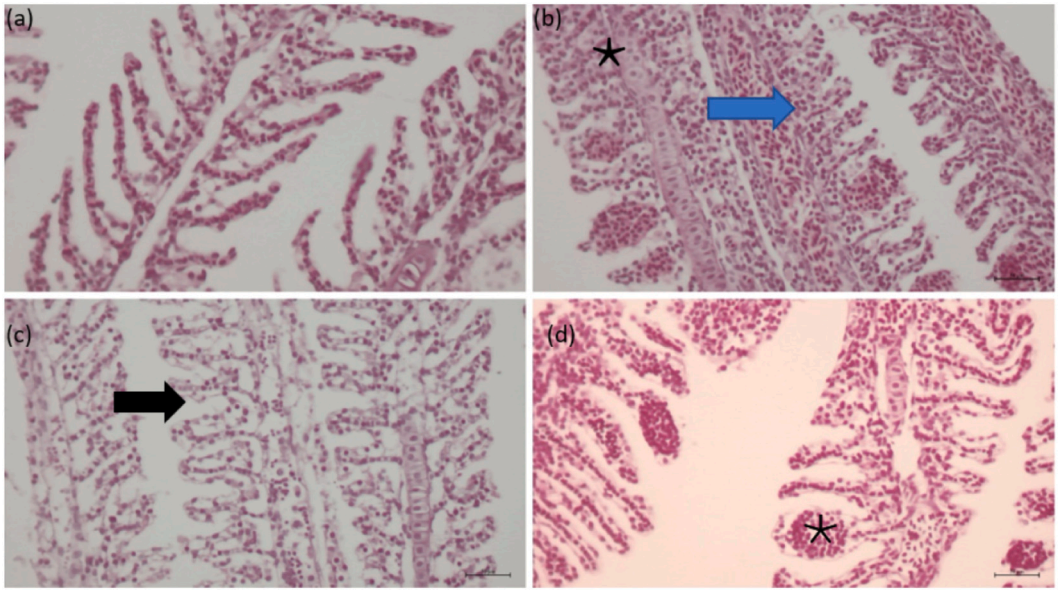

Fig. 3. The images of the gill tissues (a) control (b) hyperplasia (blue arrow) and fusion (black asterix) at 0.025 mg/L for 15 d (c) epithelial lifting (black arrow) at 0.125 mg/L for 15 d (d) talengiectasis (black asterix) at 0.125 mg/L for 15 d. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

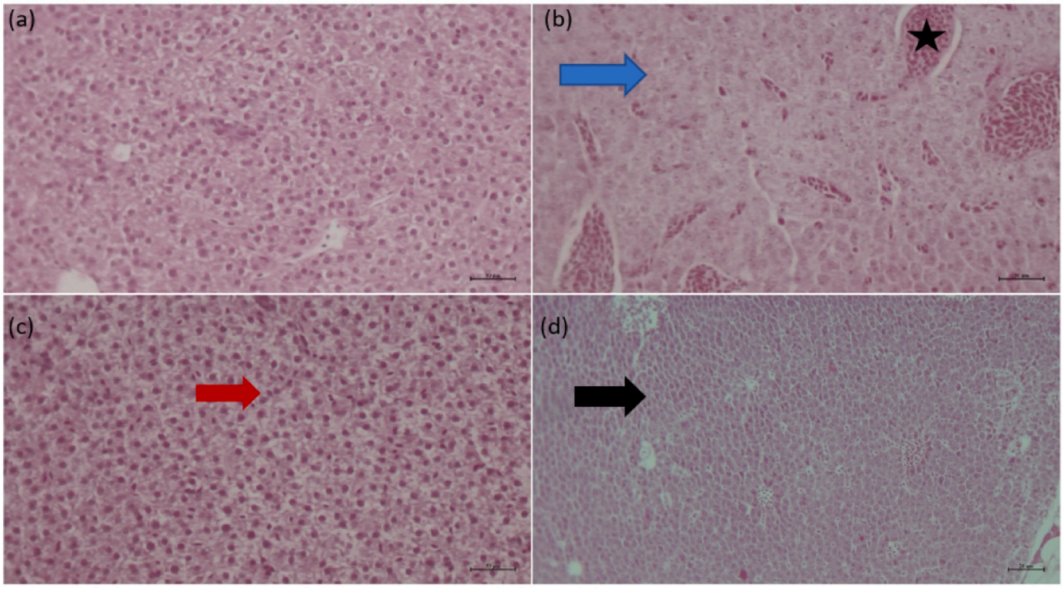

Fig. 4. The images of the liver tissues (a) control (b) passive hyperemia (black asterix) and necrosis (blue arrow) at 0.025 mg/L for 15 d (c) hydropic degeneration (red arrow) at 0.125 mg/L for 96 h (d) necrosis (black arrow) at 0.125 mg/L for 15 d. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(2020) found that the LC_{50} values for zebrafish embryos were determined 4.395 μM (1.54 mg/L) at 24 h post fertilization (hpf), 4.170 μM (1.46 mg/L) at 48hpf, 3.834 μM (1.34 mg/L) at 72hpf, and 2.88 μM (1.01 mg/L) at 96 hpf. The studies show that similar sensitivity has been found in acute embryo toxicity (ZFET) with acute fish toxicity (AFT) (Birke and Scholz, 2019). Therefore, decreases in LC_{50} from 24 h to 96 h observed in adult individuals in our study were also observed for embryos in Mi et al. (2020).

Thyroid hormone homeostasis has been accepted as a biomarker to determine any disruption of the thyroid metabolism. Therefore, the secretion of the T4 hormone, the main hormone of the system in fish, is crucial to assess in order to detect potential thyroid disruption (Spaan et al., 2019). Many studies have shown alterations on plasma T4 hormone level upon exposure to various EDCs on zebrafish (Liu et al., 2011; Chen et al., 2012; Kwon et al., 2016; Guo et al., 2019; Gao et al., 2020). In the current study, sex-dependent alterations in T4 hormone levels were determined in the adult zebrafish following 96 h exposure to 0.0025 mg/L BPFL. The level of the T4 hormone was higher in female zebrafish when compared to male zebrafish and also the level of the T4 hormone increased in females when compared to controls. These results suggested that the exposure of adult to BPFL induced sex-dependent disruption of thyroid status in the adult zebrafish. Consistent with this, the level of whole-body T4 hormone was found to be increased in female zebrafish after exposure to triphenyltin chloride (Wu et al., 2020). Similarly, it is demonstrated that exposure to some organophosphate flame retardants led to a decrease in T4 hormone level in

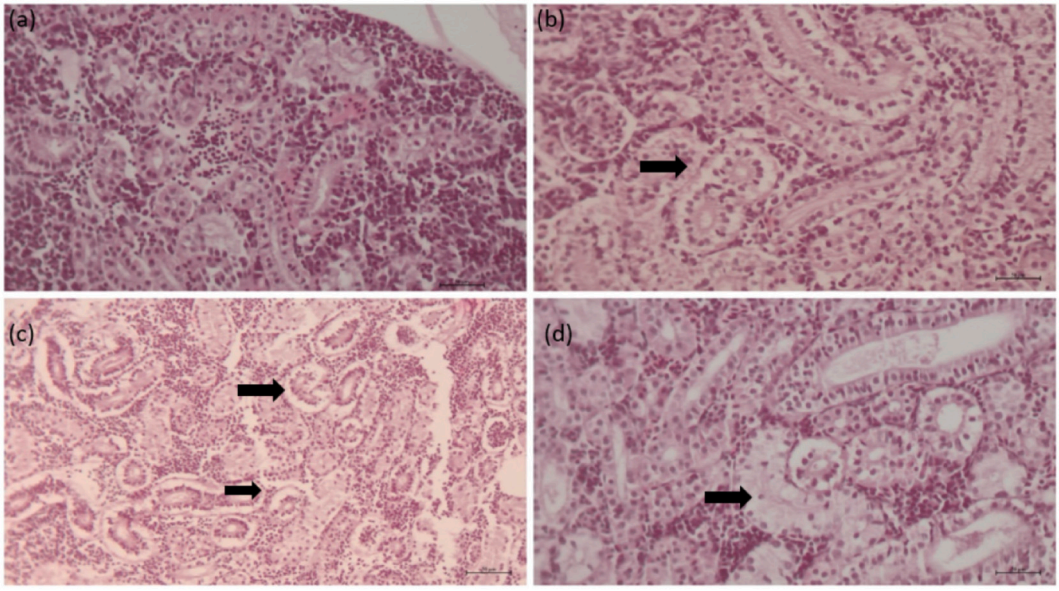

Fig. 5. The images of the kidney tissues (a) control (b) tubule degeneration (black arrow) at 0.025 mg/L for 15 d (c) tubule degeneration (black arrow) at 0.125 mg/L for 15 d (d) necrosis (black arrow) at 0.125 mg/L for 15 d.

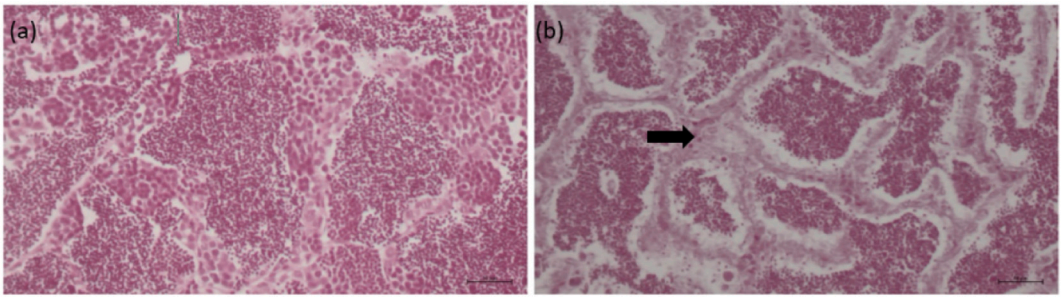

Fig. 6. The images of the testis tissues (a) control (b) degeneration of Leydig cells (black arrow) at 0.125 mg/L for 15 d.

male zebrafish whereas a significant increase was observed in female zebrafish after 14 days (Liu et al., 2019). Moreover, in female rodents, exposure to BPA has led to a significant increase in the level of T4 hormone (Zhang et al., 2017a). There is currently only one research investigating the effect of BPFL on T4 hormone levels in zebrafish reporting an increase in larvae (Jin et al., 2021). Here it can be said that our results on T4 hormone levels are comparable to those following exposure of zebrafish and other organisms to various EDCs.

Even though zebrafish does not possess properly organized thyroid glands, the genes involved in the HPT axis are conserved between mammals and zebrafish. Corticotropin-releasing hormone (CRH) is secreted from the hypothalamus and stimulates TSH secretion along with TRH. TSH then encourages thyroid follicles to secrete thyroid hormones (Spaan et al., 2019). It is also known that TRH has critical role in the synthesis and release of TSH in fish. The expression levels of *trh*, *tshb* and *trhrb* are known to be correlated with the action of TRH and TSH hormones, the expressions of these genes are being used as biomarkers to evaluate the thyroid disruption potential of a chemical (Kim and Ji, 2019). Hence, the genes encoding these hormones in the HPT axis such as *trh*, *crh*, *tshb* and *trhrb* were selected to determine the expression levels. Here we observed a sex and dose-specific effect of BPFL on the expression of genes related to thyroid hormone synthesis and release. Our findings indicated that upon 96 h exposure to 0.025 mg/L BPFL upregulated the expressions of *trh*, *tshb* and *trhrb* genes in female zebrafish. This kind of sex-dependent response has also been reported in adult zebrafish following triphenyltin chloride exposure (Wu et al., 2020). In line with our result, Kim and Ji have reported that the expression levels of genes related HPT-axis, namely *trh*, *tshb* and *trhrb,* increased after exposure to humidifier disinfectants (Kim and Ji, 2019). A recent study done by Jin et al. (2021) has shown that BPFL leads to developmental toxicity in a dose-dependent manner in zebrafish larvae. Researchers assessed the transcriptional and biochemical parameters following exposure to six different BPFL concentrations and reported an apparent increase in the expression of corticotropin-releasing hormone b (*crhb*), thyroid-stimulating hormone subunit b *(tshb)* and *trhb* upon exposure to low doses of BPFL but a remarkable decrease with the increased BPFL dose (Jin et al., 2021).

Our findings clearly indicated a sex-dependent disruption via both T4 hormone levels and expression levels of the genes related to HPT-axis. According to our results, females were more sensitive to BPFL exposure as more thyroid disruption has been observed. This could be explained by the inherent difference between females and males in terms of metabolic capacity and also the hypothalamic-pituitary-gonadal (HPG) axis due to the estrogenic effects of BPFL (Chung et al., 2019; Gao et al., 2020). Another important point to emphasize is how crucial to reveal differences between sexes when evaluating the safety of a chemical (Godfrey et al., 2019).

Histopathological changes in the fish tissues are very important parameters for deriving maximum admissible concentrations of the xenobiotics. There is a lack of data on the histological alterations of BPFL exposed fish excluding ovarium tissues (Mi et al., 2019). Gills, respiratory and osmoregulatory tissues mirror the water quality with non-

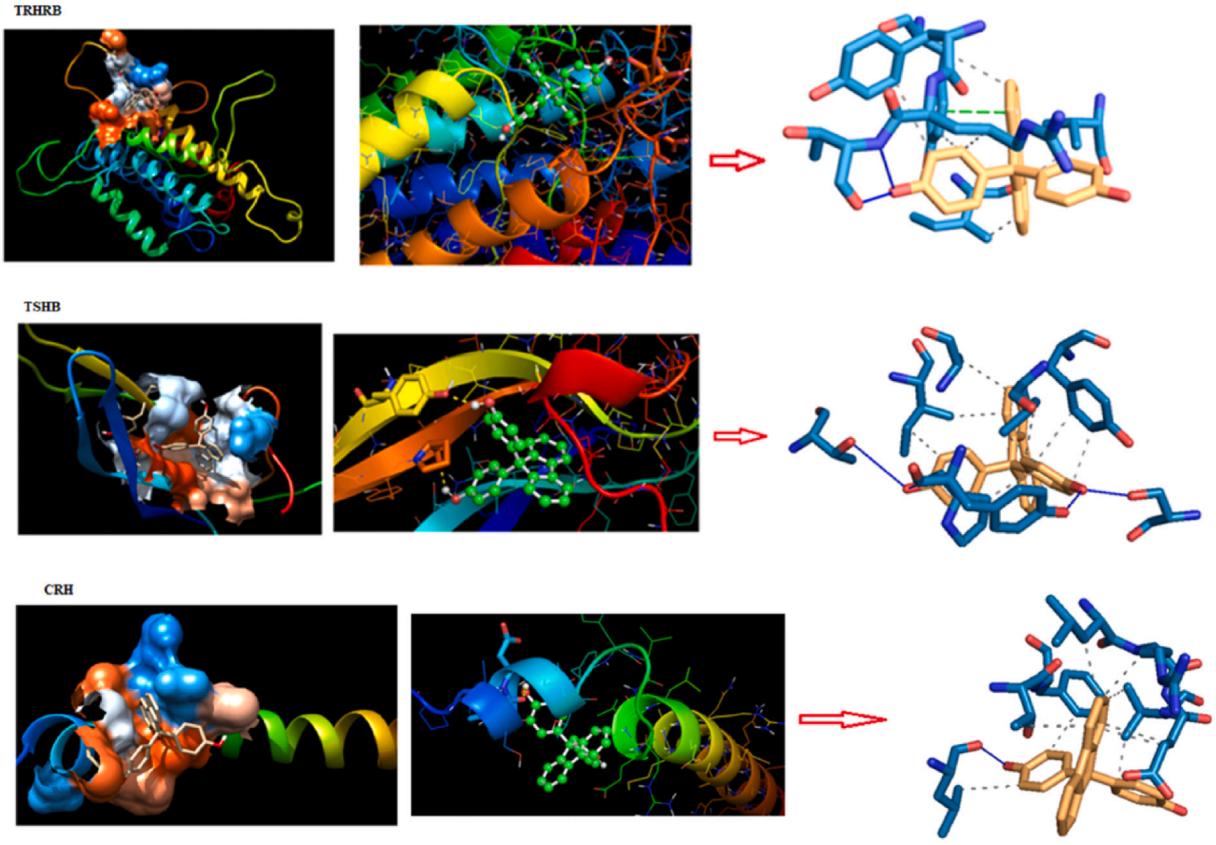

Fig. 7. Ligand BPFL embedded in the docking active sites of protein targets.

specific defense response and gill damage reflect the toxic effects of chemicals (Mallatt, 1985; Marinović et al., 2021). Histopathological alterations in the gill tissues noticed in the present study were well-known and nonspecific findings of exposure to various toxic chemicals from different studies (Benli et al., 2008; Sepici-Dinçel et al., 2009; Erkmen et al., 2017; Günal et al., 2020; Vajargah et al., 2018; Sula et al., 2020; Vajargah et al., 2021; Vali et al., 2022). Similar to the results of the present study, epithelial lifting, hyperplasia and aneurysm of the gill tissues were noticed in zebrafish after 120 h exposure to 34, 85, and 170 µg/L triclosan (Arman, 2021). Elshaer et al. (2013) determined necrosis lifting up of epithelium, fusion of adjacent secondary lamellae and hyperplasia of epithelial cells of *Gambusia affinis* and a *Poecilia reticulata* exposed to 50 µg/l of BPA for 15 and 30 d. Hyperplasia and degeneration were determined in the gills after exposure to 15 d with 1 and 4 mg/L BPA (Faheem et al., 2016).

Fish liver is the main detoxification organ for xenobiotics that is sensitive to toxic responses and the histology of the liver tissue reflects the pollution of the aquatic habitats (Benli et al., 2008; Sepici-Dinçel et al., 2009; Boran et al., 2012; Munoz et al., 2015). Agamy (2013) reported that hepatic cell degenerations were common responses of fish after exposure to xenobiotics. Narrowed hepatic sinuses, dilated central veins, leucocyte infiltration, and cytoplasmic vacuolation were reported in the livers of mice treated with BPFL at dosages of 2 mg/kg bw/3-day for 36 d (Yang et al., 2021). In the present study, necrosis, hydropic degeneration and passive hyperemia were the most prominent histopathological findings depending on the duration and the concentration of BPFL. Similar findings in the liver tissues were also determined by different authors exposure to various xenobiotics (Wolf and Wheeler, 2018). BPA caused central vein congestion, inflammation, edema, degeneration, and hepatocytes necrosis of major carp, *Catla catla* after exposure to 15 d with 1 and 4 mg/L BPA (Faheem et al., 2016). Yang et al. (2016) determined hepatocellular vacuolization of the zebrafish exposed to 1 mg/L bisphenol AF (BPAF) for 28 d. In the present study, kidney tissues displayed tubule degeneration and necrosis, after being exposed to different concentrations of sublethal BPFL. Kidney, a hematopoietic and osmoregulatory organ, is one of the useful organs to assess the toxic effects (Benli et al., 2008). Similar to the findings of the present study, necrosis and degeneration of glomerulus with complete obliteration of bowman's space, shrinkage of tubules and tubule lumen were determined in *C. catla* after exposure to 1 and 4 mg/L BPA for 15 d (Faheem et al., 2016). The degeneration of the Leydig cells in the testis tissues after exposure BPFL was also noticed in the present study. Similarly, the acellular areas were observed in males exposed to 1 mg/L BPAF (Yang et al., 2016).

5. Conclusion

In conclusion, the results of our study provide the first evidence of thyroid-disrupting effects of short- and long-term BPFL exposure in adult zebrafish. It was shown that BPFL has potential to cause modifications of thyroid hormone levels, change the transcription of genes involved in thyroid metabolism and induce histopathological changes. To our knowledge this is the first study investigating the effect of BPFL on adult zebrafish as both in a transcriptional and histopathological manner. The findings of our research represent insights on the histopathological impacts of BPFL exposure during the adulthood period and give an overview of the thyroid disrupting effect of BPFL. Histopathology is a useful tool to understand and assess the effects and the risks of the contaminants to aquatic systems with a key role in the determination of LOEC and NOEC's. More research is required in order to understand the safety of "BPA-free" chemicals for the determination of the environmental risk assessments.

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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

Data will be made available on request.

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