Master’ s Thesis of Public Health

# Thyroid disrupting effects of mixtures of three major organic UV-filters in laval zebrafish

## 유기계 자외선차단물질 3종의 혼합노출에 따른 제브라피쉬 치어의 갑상선 교란 영향

August 2023

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Environmental Health Science Major

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# Thyroid disrupting effects of mixtures of three major organic UV-filters in laval zebrafish

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이 논문을 보건학석사 학위논문으로 제출함

2023 년 5 월

서울대학교 보건대학원
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# Abstract

# Thyroid disrupting effects of mixtures of three major organic UV-filters in laval zebrafish

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Organic ultraviolet (UV) filters such as diethylamino hydroxybenzoyl hexyl benzoate (DHHB), octocrylene (OC), and avobenzone (AVB) have been widely used in sunscreens. Recently, dermal absorption and their adverse health outcomes of several UV-filters are of increasing concern. While being used in combination in many sunscreen products, most studies have been focused on exposure to single chemicals.

In the present study, thyroid-disruption toxicities of binary mixtures of DHHB, OC and AVB were assessed using embryo-larval zebrafish (*Danio rerio*). Following 120 h exposure, the whole-body contents of total T4, free T3, and thyroid stimulating hormone (TSH) were measured and the transcription levels of thyroid hormone-related genes were analyzed. Individual UV-filters lowered thyroid hormone levels in larval zebrafish. Following exposure to binary mixtures, similar pattern of thyroid hormone decreases was observed. The extent of thyroid hormone changes did not suggest deviation

from additivity of mixture interaction. At transcription level, upregulation of *sult1 st5* gene, was observed in the fish exposed to all three binary mixtures, along with down-regulation of several thyroid specific genes such as *tpo* in some mixtures, indicating that enhanced metabolic excretion and decreased thyroid hormone synthesis may cause decreased thyroid hormones in the larval fish. The present observations revealed that the short-term exposure to UV-filter mixtures could lead to reduced thyroid hormones in an additive way in larval zebrafish. As multiple UV-filters are used together in a sunscreen, safety management for sunscreens should not limit to individual ingredients but to consider toxicity of multiple UV-filters added together in the products.

**Keyword:** UV-filter, thyroid hormone, zebrafish, DHHB, OC, AVB, mixture toxicity

**Student Number:** 2021-27873

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# 1. Introduction

UV-filters are present in a wide range of daily-use products such as personal care products (PCPs) as well as many other industrial goods, to protect the products from harmful effects of ultraviolet (UV) radiation. Diethylamino hydroxybenzoyl hexyl benzoate (DHHB), octocrylene (OC), and avobenzone (AVB) have been widely used as UV-filters in many products (Boyd et al., 2021; Muñiz-González & Martínez-Guitarte, 2018; Park et al., 2017). Human uses of these products caused environmental pollution not only by recreational activities, but also through domestic and industrial sewage (Gago-Ferrero et al., 2012). The presence of UV-filters has been previously reported in coastal waters, river and lake waters, swimming pool waters, wastewaters (Ramos et al., 2015). The widespread use of UV-filters has resulted in significant amounts of their occurrence not only in the environment but also in organisms (Fàbrega et al., 2013; Molins-Delgado et al., 2016). Due to their high lipophilicity and stability in the environment, some of UV-filters have been identified to have high bioaccumulation factors in fish (Brausch & Rand, 2011).

In most sunscreen products, UV-filters are used in combination. Most studies have focused on the effects of individual chemicals despite most UV-filters are used in combination. Thus, there is a lack of information about the joint interaction of UV-filter mixtures.

Similar to many endocrine disrupting compounds (EDCs), several UV-filters have been reported for sex hormone disrupting effects in vertebrates (Díaz-Cruz and Barceló, 2009), antiandrogenicity (Ma et al., 2003, Schreurs et al., 2005), and estrogenicity (Kunz and Fent, 2006, Schreurs et al., 2002, Schreurs et al., 2005).

Several UV-filters have already reported as HPT function disruptor (Axelstad et al., 2011, Schmutzler et al., 2007), especially when exposed to during the early stages of development (Krause et al., 2012). These actions can directly affect the gland, the hypothalamus, and the pituitary, affecting the levels of thyrotropin releasing hormone (TRH) and thyroid-stimulating hormone (TSH), which are directly related to the synthesis of thyroid hormones.

Thyroid hormones play major roles in ontogenesis, acting on embryonic and fetal tissues, via active transport of maternal thyroid hormone across the placenta to ensure normal development, until the fetal thyroid gland reaches maturity (Obregon et al., 2007). Thyroid hormones are important coordinators of embryonic and early postnatal development, metabolism, thermogenesis, the stimulation of growth and the development of various tissues; thus, abnormalities of thyroid hormone levels in infancy and childhood may result in dysfunctional effects in adults.

Zebrafish is widely used in evaluating thyroid disrupting effects of chemicals due to its rapid organogenesis and similar thyroid system to mammalian species (Guo & Zhou, 2013; Persani & Marelli, 2017). In zebrafish, mRNA expression of most genes of HPT axis could be detected before 96 hpf, and thyroid follicle tissue became visible at 60 hpf and kept growing until 5 dpf (Alt et al., 2006, Opitz et al., 2011, Raldúa et al., 2012), suggesting that thyroid endocrine system might become relatively stable after 5 dpf.

Studies defining the joint interaction of UV-filter mixtures are limited and lack toxicological information, especially thyroid hormone disruption. In order to fill the knowledge gap on the mixture toxicity of UV-filters, this study aims to determine the thyroid-disruption toxicities of the selected UV-filters through TH

levels and gene analysis on embryo–larval zebrafish (*Danio rerio*) and explored the mixture interaction of binary mixtures of DHHB, OC, and AVB.

# 2. Materials and Methods

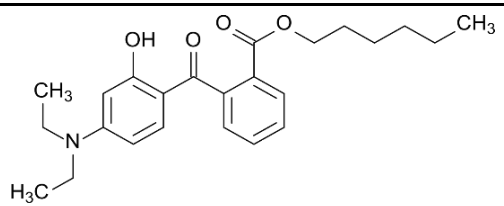
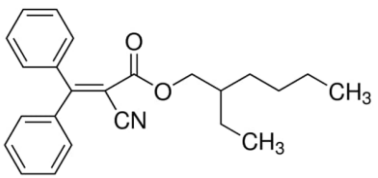
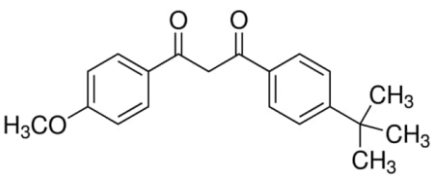
## 2.1. Chemicals

Diethylamino hydroxybenzoyl hexyl benzoate (DHHB, CAS No. 302776-68-7, purity: 99%), octocrylene (OC, CAS No. 6197-30-4, purity: 99%), and avobenzone (AVB, CAS No. 70356-09-1, purity: 99%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Physicochemical properties of DHHB, OC, and AVB were described in Table 1. The solvent dimethyl sulfoxide (DMSO, CAS No. 67-68-5, purity: 99%) was purchased from Junsei Chemical Co. (Tokyo, Japan). All the test chemicals used DMSO as a solvent and the concentration of DMSO was 0.01% volume/volume (v/v) or weight/volume (w/v).

## 2.2. Fish Maintenance

The test was initiated with fertilized eggs of zebrafish (*Danio rerio*) obtained from non-exposed adults reared in the Shinsung fish farm, Korea. All the eggs and larvae were maintained in a temperature-controlled culture room (27 ± 1 ℃, 14:10 h light/dark photoperiod) with dechlorinated filtered-tap water at the Environmental Toxicology Laboratory in Seoul National University (Seoul, South Korea). Water quality parameters (dissolved oxygen, temperature, pH, conductivity) were measured every day.

Table 1. Physicochemical properties of DHHB, OC, and AVB

| Compound | DHHB | OC | AVB |
|---|---|---|---|
| Molecular formula | $C_{24}H_{31}NO_4$ | $C_{24}H_{27}NO_2$ | $C_{20}H_{22}O_3$ |
| CAS number | 302776-68-7 | 6197-30-4 | 70356-09-1 |
| Molecular weight | 397.5[a] | 361.5[a] | 310.4[a] |
| Log $K_{ow}$ | 6.2 | 6.1 | 4.51 |
| Molecular structure |  |  |  |

[a] PubChem

## 2.3. Chemical Exposure

### 2.3.1. Single Chemical Exposure

Within 4 hours post fertilization (hpf), embryos were exposed to 0.05, 0.15, 0.5, 1.5 μM of DHHB, 0, 1, 3, 10, 30 μM of OC, and 0, 1, 3, 10, 30 μM of AVB with each solvent control (DMSO 0.01%). Those concentrations were determined as non-lethal by a preliminary range-finding test (results not shown). For dilution, dechlorinated tap water was used. DMSO was used as a solvent with final concentration of 0.01% (v/v). During the exposure period, all media were renewed every day, to keep the concentration consistent. Following 120 h exposure, 5 dpf embryos were collected and stored at −80 °C for subsequent thyroid hormone quantification and gene transcription analysis.

### 2.3.2. Single and Mixture Exposure

Within 4 hours post fertilization (hpf), embryos were exposed to DHHB 1.5 μM, OC 30 μM, and AVB 30 μM, either alone or in combination with each solvent control (DMSO 0.01%). The maximum concentrations that were determined as non-lethal by a preliminary test were used for this mixture exposure. The study was conducted in 6 groups of 3 single chemicals and their combination (Table 2). For dilution, dechlorinated tap water was used, and DMSO was used as a solvent with a final concentration of 0.01% (v/v). During the exposure period, exposure media were renewed every day, to keep the concentration consistent. 5 dpf embryos were collected and stored at −80 °C for subsequent thyroid hormone quantification and gene transcription analysis.

Table 2. Exposure combination and concentration

| | Compound | Concentration |
|---|---|---|
| Single exposure | DHHB | 0.05, 0.15, 0.5, 1.5 μM of DHHB |
| | OC | 0, 1, 3, 10, 30 μM of OC |
| | AVB | 0, 1, 3, 10, 30 μM of AVB |
| Single and mixture chemical exposure | DHHB | 1.5 μM of DHHB |
| | OC | 30 μM of OC |
| | AVB | 30 μM of AVB |
| | DHHB+OC | 1.5 μM of DHHB + 30 μM of OC |
| | DHHB+AVB | 1.5 μM of DHHB + 30 μM of AVB |
| | OC+AVB | 30 μM of OC + 30 μM of AVB |

## 2.4. TH Quantification by ELISA Method

A total of 300 embryos were randomly allocated to 1 L beakers with a 400 mL exposure medium. Each treatment included four replicates (n = 4). During the 120 h exposure period, the exposure solution was renewed daily. The mortality of embryo and mortality, hatchability, and morphological changes (e.g., yolk sac edema, bent spine, tail malformation) of larvae were observed every 24 hours. At 5 dpf, total of 30 larvae per replicates were randomly sampled for gene analysis, and 220 larvae per replicates were selected randomly for hormone analysis in each test.

For thyroid hormone analysis, collected larvae were washed by 1 × PBS, and weighed the wet body weight after removing as much remaining water as possible. Those were stored at −80 ℃ until homogenization for each analysis. For gene analysis, collected larvae were washed by 1× PBS, and stored at −80 ℃ until homogenization for each analysis.

TH levels from whole body larvae were measured by using enzyme−linked immunosorbent assay (ELISA) following the protocol of Yu et al. (2010) with slight modifications. Frozen larvae (n = 220) were thawed from −80 ℃ and then homogenized in 250 μL of 1× PBS. After sonication in water for 20 min at 4 ℃, the homogenates were centrifuged for 20 min at 10,000× g at 4 ℃, and the supernatant was collected and stored at −80 ℃ until further TH measurement. Hormone levels of total triiodothyronine (tT3), total thyroxine (tT4), free triiodothyronine (fT3), free thyroxine (fT4), and thyroid stimulating hormone (TSH) were quantified using commercial ELISA kits according to the protocol by manufacturer.

For normalization of TH levels, body weight (bw) of larval zebrafish were used. Wet body weight from 220 larvae and the volume of supernatant from larval homogenates were measured for body weight correction.

tT3 ELISA kit (Cat No. TF E-2300), tT4 ELISA kit (Cat No. TF E-2400), fT3 ELISA kit (Cat No. TF E-2100), and fT4 ELISA kit (Cat No. TF E-2200) were purchased from LDN (Nordhorn, Germany). Fish TSH ELISA kit (Cat No. CSB-EQ027261FI) were purchased from Cusabio Technology LLC (Houston, TX, USA). The optical density for all THs and proteins of the BCA assay was determined with a Tecan Spark® (Tecan Group Ltd, Männedorf, Switzerland).

## 2.5. RNA Extraction and Quantitative Real-time PCR

After 120 h exposure, each replicate was sampled for 30 larval zebrafish. The fish were homogenized with a tissue grinder and the total RNA was extracted using RNeasy mini kit (Qiagen). Extracted RNA was stored at −80 ℃ until complementary DNA (cDNA) synthesis. Quality (260/280 ratio) and concentration of RNA were quantified with a Nanodrop ND−1000 spectrometer (Nanodrop Technologies, Wilmington, DE, USA). 1−2 μg of total RNA were reverse−transcribed into cDNA using an iScriptTM cDNA synthesis kit (BioRad, Hercules, CA, USA). For quantitative real− time polymerase chain reaction (qRT−PCR), 20 μL reaction mix consisted of 10 μL of the PCR master mix (Power SYBR Green Master Mix, Applied Biosystems by Lift Technologies, Carlsbad, CA, USA), 0.1 μL of each 10 μM PCR primer, 7.8 μL of nuclease−free water (Qiagen), and 2 μL of 25ng cDNA. The thermal cycle profile included preincubation at 95 ℃ for 10 min, 40 cycles of amplification at 95 ℃ for 15 s and 60 ℃ for 1 min with melting curve analysis. qRT−PCR was performed with QuantStudioTM 3 Real−Time PCR System (Applied Biosystems).

Nineteen genes related to thyroid hormone regulation were analyzed: central regulation and thyroid related receptors (*crhβ, trh, trhr, tshβ, tshr, trα, trβ*), TH synthesis (*nis, tpo, tg*), thyroid development (*nkx2.4b*), TH transport (*ttr, mct8*), TH metabolism (*dio1, dio2, dio3a, dio3b, ugt1ab, sult1 st5*). Primer sequences for the reference gene and target genes are listed in Table 3. The relative mRNA expression level of each target gene was normalized to *rpl8* (housekeeping gene) and quantified using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001).

Table 3. Primer sequences of measured genes

| Organism | Endpoint | Gene | Primer Sequence (5' - 3') | |
|---|---|---|---|---|
| | | | Forward | Reverse |
| Zebrafish | Thyroid | *rpl8* | ttgttggtgttgttgctggt | ggatgctcaacagggttcat |
| | | *crhβ* | caattacgcacagattctcctcg | gaagtactcctcccccaagc |
| | | *trh* | gctctctccgtcggtctgtt | gcgagatccgtgctgatga |
| | | *trhr* | cagtgccatcaaccctctga | ggcagcgcggaacttct |
| | | *tshβ* | gcagatcctcacttcacctacc | gcacaggtttggagcatctca |
| | | *tshr* | gcgagaagggagaggaggtt | tcctcgcaagggttgaactc |
| | | *trα* | gccgcttcctgcacatg | agcggcgggaacagttc |
| | | *trβ* | tggcatggctacagacttggt | tcagcttccgcttggctaa |
| | | *nis* | ggtggcatgaaggctgtaat | gatacgggatccattgttgg |
| | | *tpo* | gttcggtctgccaggacact | tccaagcgcttcagcagagt |
| | | *tg* | gtctcttgagtgttcgaatgacaag | aaaggcgggccattaagg |
| | | *nkx2.4b* | aggacggtaaaccgtgtcag | caccatgctgctcgtgtact |
| | | *ttr* | cgggtggagtttgacacttt | gctcagaaggagagccagtg |

| | | | |
|---|---|---|---|
| | *mct8* | cttcggatgtcggaaaacgg | cccagagtcgtggcgaag |
| | *dio1* | aacttggaggagaggcttgct | agggcatggagggtcttctt |
| | *dio2* | cgcgaaatgggcttgct | ccaggcaaaatctgcaaagtta |
| | *dio3a* | tcgcacctgtattctccgtg | cgagcgtcccgtattcagac |
| | *dio3b* | tcctgaccgcccttcatgac | tgcgcctcctcgatgtacac |
| | *ugt1ab* | gccagctttgatgaacttgcc | aactcctccagttccttggtt |
| | *sult1 st5* | cccatccaacttttgcctcg | Ggatccccataccaattgtcct |

2

## 2.6. Statistical Analysis

Normality of distribution and homogeneity of variance were evaluated by Shapiro-Wilk's test and Levene's test, respectively. For multiple comparisons among the solvent control and each treatment, one-way analysis of variance (ANOVA) was used with Dunnett's or Dunnett's T3 test as a post- hoc analysis. When data was not normally distributed, a non- parametric Kruskal-Wallis test was conducted. To assess the linear trend between concentration and response, Spearman's rank correlation test was used. To compare statistical differences between individual chemical and mixture chemical treatment, t-test was applied. A p-value less than 0.05 was considered statistically significant. All data are shown as mean values with the standard deviation of the mean (SD). All statistical analysis was performed using IBM SPSS Statistics 26 (SPSS Inc., Chicago, IL, USA).

# 3. Results

## 3.1. Thyroid Hormone Changes by Individual Chemicals

In the larval fish, TSH, tT4, fT4, and fT3 significantly decreased dose-dependent after exposure to DHHB (Figure 1a). After exposure to OC, also TSH, tT4, fT3, fT4, and fT3 showed significant decreases (Figure 1b). After exposure to AVB, statistically significant results were not obtained, but a tendency to decrease was observed (Figure 1c).

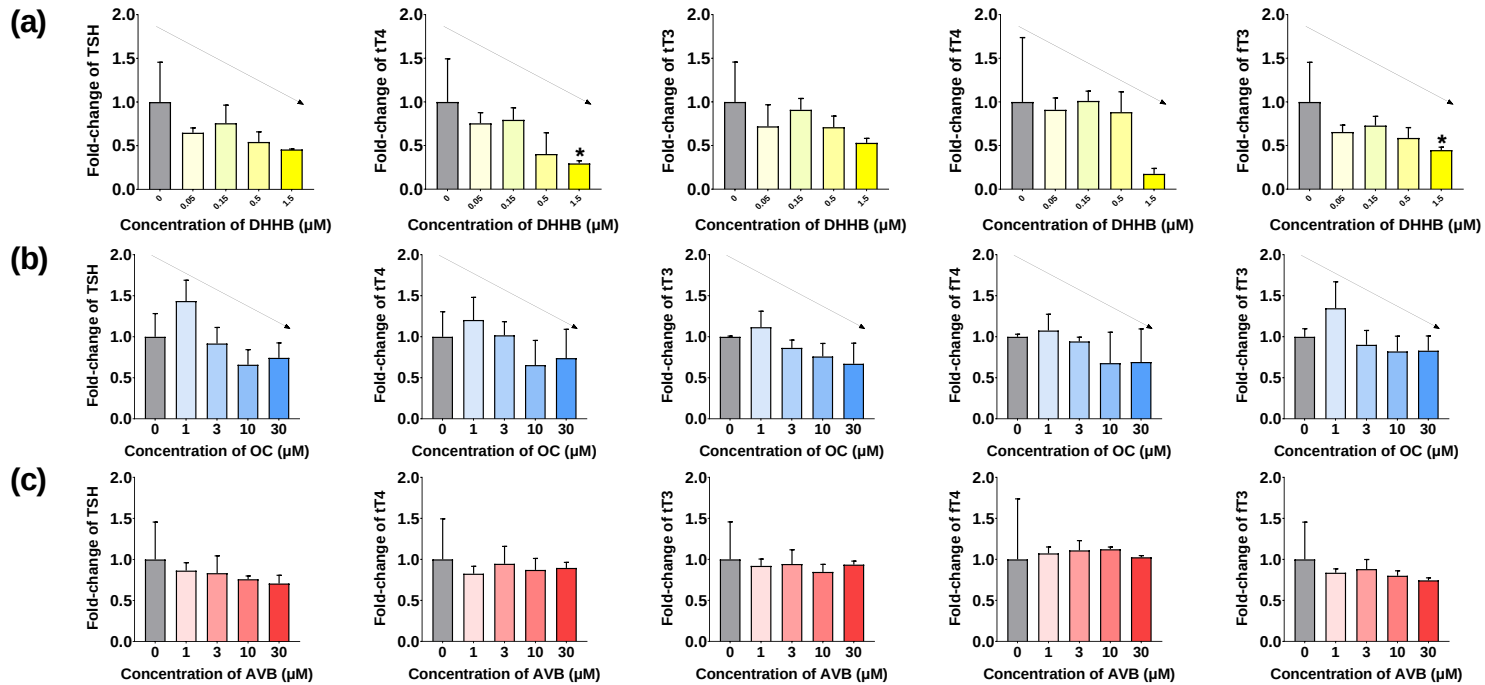



**Figure 1.** Thyroid hormone and TSH changes in embryo-larval zebrafish following 120 h exposure to (a)DHHB, (b)OC, and (c)AVB. Asterisk (*) and arrow mean the statistically significant changes ($p < 0.05$). Values are expressed as mean±SEM of three replicate samples. The error bar represents the standard deviation (SD) (n = 3 replicates consisting of a group of 220 fish).

### 3.2. Thyroid Hormone Changes by Binary Mixtures

A binary combination of DHHB+OC, as well as either DHHB or OC significantly decreased TSH compared to the control group (Figure 2a). DHHB and OC decreased TSH by 30%, 27.0%, respectively, compared to the control group. In TSH, the decrease in DHHB+OC is 36.1%, 21.0% less than the sum of the decreases in DHHB and OC of 57.0%. DHHB, OC, and DHHB+OC significantly decreased tT4 compared to the control group (Figure 2b). DHHB and OC decreased tT4 by 39.5%, 30.9%, respectively, compared to the control group. In tT4, the decrease in DHHB+OC is 36.6%, 33.9% less than the sum of the decreases in DHHB and OC of 70.4%. In fT3, DHHB, OC, DHHB+OC did not show any statistically significant differences compared to the control group (Figure 2c). DHHB and OC decreased by 15.2%, 11.4%, respectively, compared to the control group. In fT3, the decrease in DHHB+OC is 20.5%, 6.1% less than the sum of the decreases in DHHB and OC of 26.6%.

A binary mixture of DHHB+AVB, along with either DHHB or AVB significantly decreased TSH compared to the control group (Figure 2a). DHHB and AVB decreased TSH by 30%, 28.9%, respectively, compared to the control group. In TSH, the decrease in DHHB+AVB is 35.7%, 23.2% less than the sum of the decreases in DHHB and AVB of 58.9%. DHHB and DHHB+AVB significantly decreased tT4 compared to the control group. AVB did not show any statistically significant differences compared to the control group (Figure 2b). DHHB+AVB significantly decreased tT4 compared to AVB. DHHB and AVB decreased by 39.5%, 13.5%, respectively, compared to the control group. In tT4, the decrease in DHHB+AVB is 56.3%, 3.3% more than the sum of the decreases in DHHB and AVB of 53.0%. In fT3, DHHB, AVB, DHHB+AVB did not

show any statistically significant differences compared to the control group (Figure 2c). DHHB and AVB decreased fT3 by 15.2%, 6.8%, respectively, compared to the control group. In fT3, the decrease in DHHB+AVB is 20.7%, 1.4% less than the sum of the decreases in DHHB and AVB of 22.1%.

A binary mixture of AVB+OC, along with either AVB or OC significantly decreased TSH compared to the control group (Figure 2a). AVB and OC decreased by 28.9%, 27.0%, respectively, compared to the control group. In TSH, the decrease in AVB+OC is 32.9%, 23.0% less than the sum of the decreases in AVB and OC of 55.9%. DHHB and DHHB+AVB significantly decreased tT4 compared to the control group. Also, AVB did not show any statistically significant differences compared to the control group. AVB+OC significantly decreases compared to AVB (Figure 2b). AVB and OC decreased by 13.5%, 30.9%, respectively, compared to the control group. In tT4, the decrease in AVB+OC is 46.4%, 2.0% more than the sum of the decreases in AVB and OC of 44.5%. fT3 was significantly decreased only in AVB+OC compared to the control group (Figure 2c). AVB, OC did not show any statistically significant differences compared to the control group (Figure 2c). AVB and OC decreased by 6.8%, 11.4% respectively, compared to the control group. In fT3, the decrease in AVB+OC is 21.8%, 3.6% more than the sum of the decreases in DHHB and AVB of 18.2%.

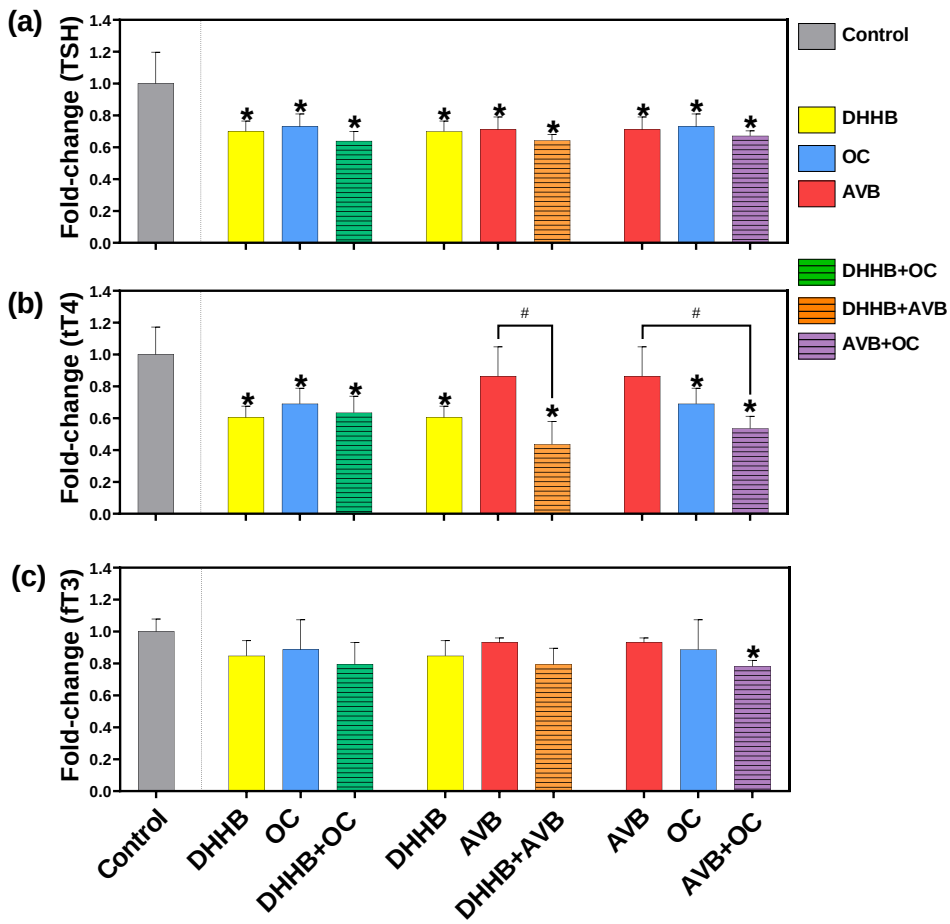



Figure 2. Comparison of (a)TSH, (b)tT4, and (c)fT3 levels following 120 h exposure in DHHB, OC, AVB, and their mixture. * $p < 0.05$ substantial alteration by comparison with the control; # $p < 0.05$ significant difference relative to the corresponding single chemical exposure. The error bar represents the standard deviation (SD) (n = 5 replicates consisting of a group of 220).

### 3.3. Transcription Changes of Genes Related to HPT Axis

A binary mixture of DHHB+OC down-regulated *trh*, while DHHB and OC individually showed no significant differences (Figure 3b). DHHB+OC down-regulated *tpo* compared to the control group, while DHHB and OC did not show any statistically significant differences (Figure 3g). DHHB+OC up-regulated *trβ* significantly compared to the control group while DHHB and OC did not show any statistically significant differences. Also, there are statistically significant differences between single chemicals and their binary mixture (Figure 3k). *ttr* showed statistically significant differences in DHHB+OC compared to the individual chemicals (Figure 3l). DHHB+OC up-regulated *mct8* compared to the control group, while DHHB and OC did not show any statistically significant differences (Figure 3m). DHHB+OC down-regulated *dio3a* compared to the control group, while DHHB, OC did not show any statistically significant differences (Figure 3p). OC and DHHB+OC down-regulated *dio3b* compared to the control group. DHHB did not show any statistically significant differences (Figure 3q). DHHB+OC up-regulated *sult1 st5* compared to the control group, while DHHB and OC did not show any statistically significant differences (Figure 3s).

DHHB+AVB up-regulated *trβ* significantly compared to the control group, while DHHB and AVB did not show any statistically significant differences. Also, there are statistically significant differences between single chemicals and their binary mixture (Figure 3k). DHHB+AVB up-regulated *mct8* compared to the control group, while DHHB and AVB did not show any statistically significant differences. Also, there are statistically significant differences between DHHB, AVB, and their binary mixture (Figure 3m). AVB up-regulated *ugt1ab* significantly compared to the control group. Conversely,

DHHB+AVB did not show any statistically significant differences compared to the control group (Figure 3r).

AVB and AVB+OC down-regulated *trh* compared to the control group. OC did not show any statistically significant differences (Figure 3b). AVB+OC down-regulated *trhr*, *tshr*, and *nis* compared to the control group, while AVB and OC did not show any statistically significant differences (Figure 3c, Figure 3e, and Figure 3f). AVB and AVB+OC down-regulated *tpo* compared to the control group. OC did not show any statistically significant differences (Figure 3g). AVB+OC down-regulated *dio2* compared to the control group, while AVB and OC did not show any statistically significant differences (Figure 3o). AVB and AVB+OC down-regulated *dio3a* compared to the control group. OC did not show any statistically significant differences (Figure 3p). AVB, OC, and AVB+OC down-regulated *dio3b* compared to the control group (Figure 3q). AVB up-regulated *ugt1ab* significantly compared to the control group. Conversely, AVB+OC did not show any statistically significant differences compared to the control group (Figure 3r).

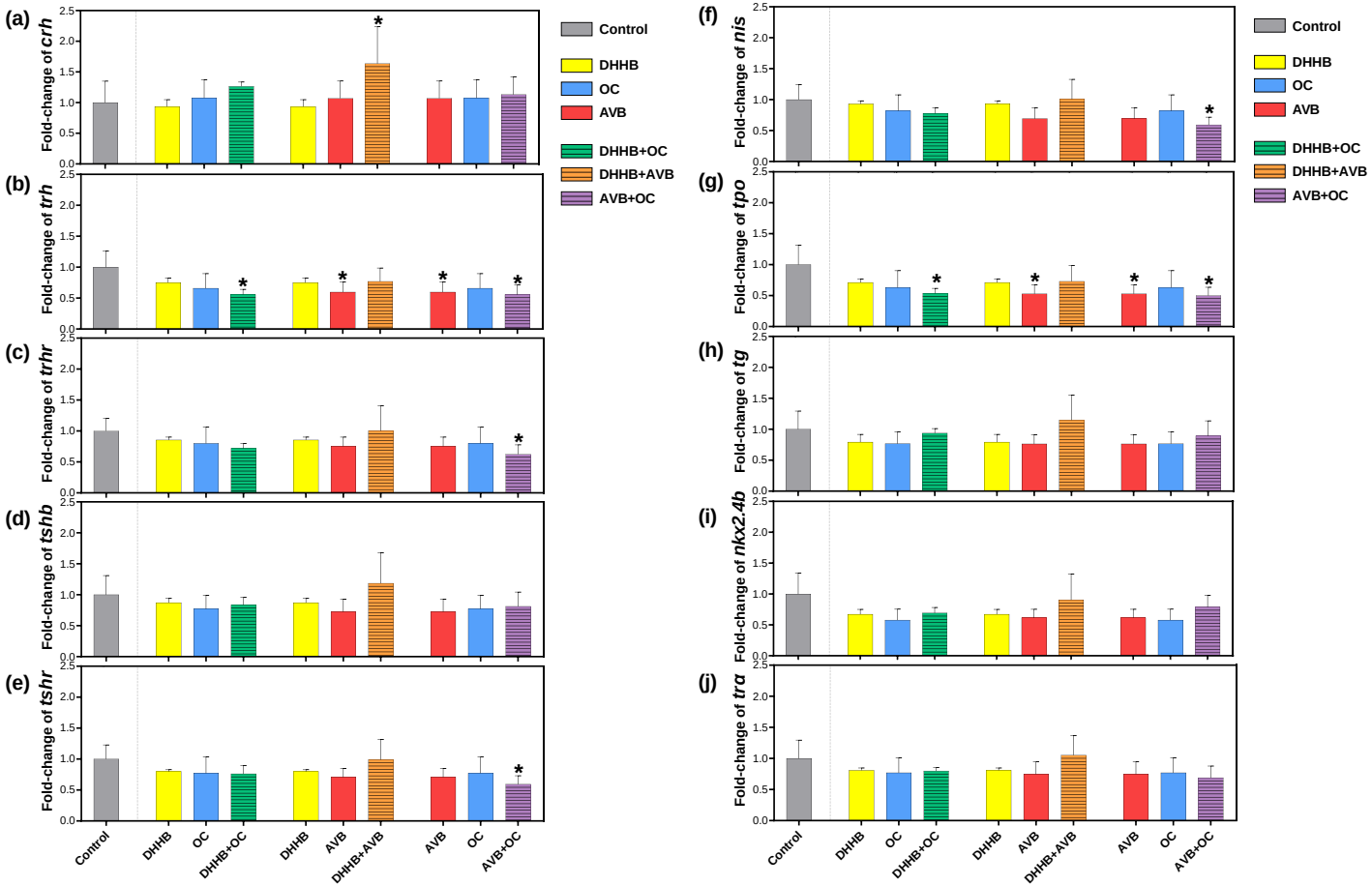



**Figure 3.** Influences on gene transcriptions associated with HPT axis in embryo–larval zebrafish following 120 h exposure to DHHB, OC, AVB, and their combinations. (a) *crh*, (b) *trh*, (c) *trhr*, (d) *tshβ*, (e) *tshr*, (f) *nis*, (g) *tpo*, (h) *tg*, (i) *nkx2.4b*, (j) *trα*, (k) *trβ*, (l) *ttr*, (m) *mct8*, (n) *dio1*, (o) *dio2*, (p) *dio3a*, (q) *dio3b*, (r) *ugt1ab*, and (s) *sult1 st5*. Each bar represents the mean ± standard deviation (n = 4 replicates consisting of a group of 30 fish). * p <0.05 substantial alteration by comparison with the control; # p <0.05 significant difference relative to the corresponding single chemical exposure.

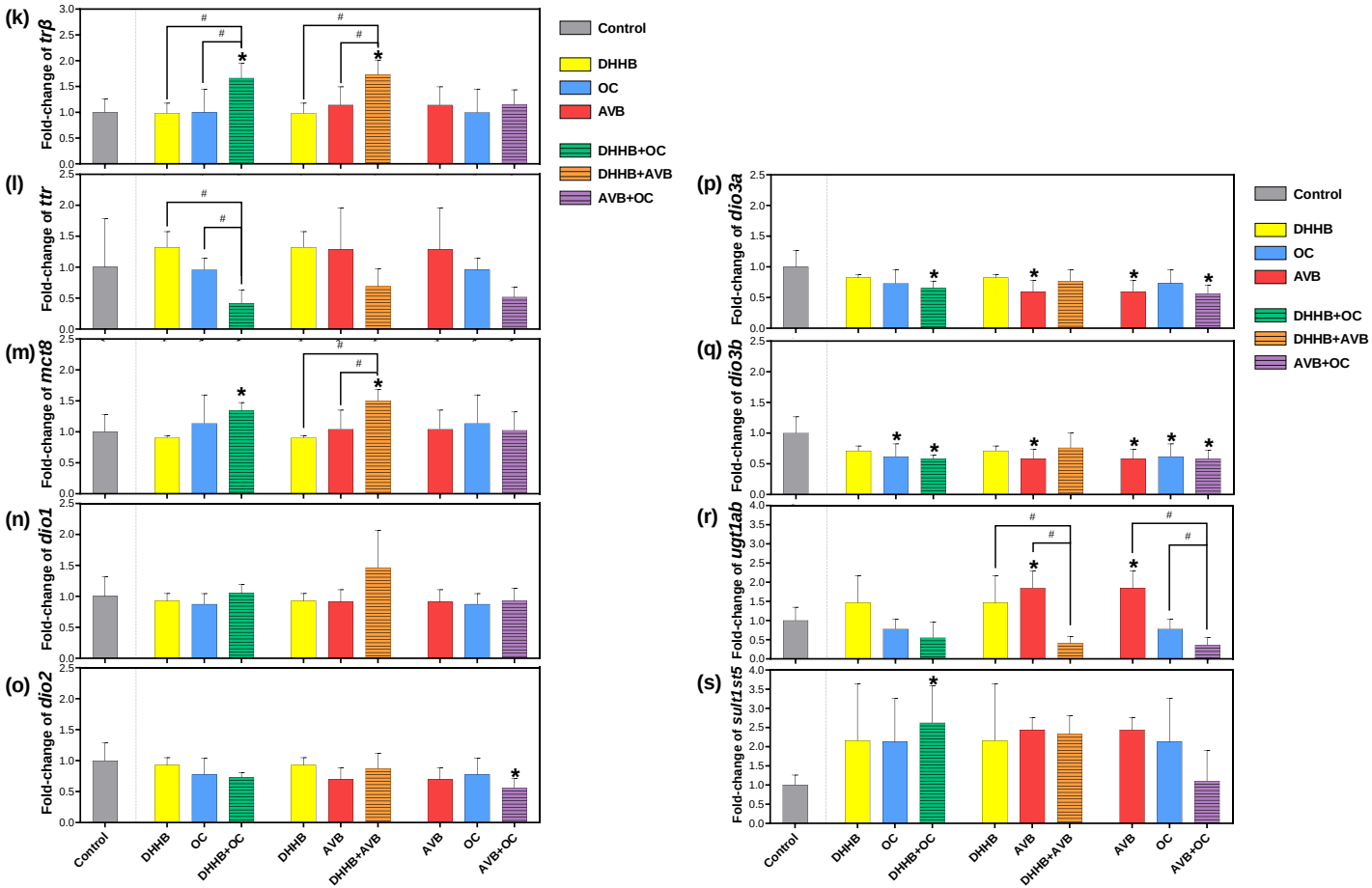



Figure 3. Continued.

# 4. Discussion

## 4.1. Thyroid Hormone Changes

General decreases of TSH, tT4, fT4, and fT3 following exposure to three organic UV-filters (Figure 1) are supported by previous studies (ref). Following 120 h exposure, DHHB, OC, AVB, and their binary mixtures lowered thyroid hormone and altered transcription levels of the genes involved in thyroid regulation in larval zebrafish, which suggests that these UV-filters can disrupt the thyroid hormone regulating system in embryo-larval zebrafish (*Danio rerio*) either individually or in combination.

Some mixture combination caused significantly greater extent of decrease in thyroid hormone levels than single substances. AVB+OC showed a significant decrease in tT4 compared to AVB (Figure 2b). fT3 was significantly decreased compared to control group only in AVB+OC (Figure 2c). These differences may indicate additivity mode of interaction between the test organic UV-filters. Also, there is a difference between the decrease of the mixture and the sum of the decrease of individual substances. For example, in TSH, the decrease in DHHB+OC is 36.1%, 21.0% less than the sum of the decreases in DHHB and OC of 57.0%. In tT4, the decrease in DHHB+AVB is 56.3%, 3.3% more than the sum of the decreases in DHHB and AVB of 53.0%. In fT3, the decrease in AVB+OC is 21.8%, 3.6% more than the sum of the decreases in AVB and OC of 18.2%. Mixture interactions, such as synergy and antagonism, can be determined when the combined effect of constituents is greater or lesser than the expected additive effect (Chen et al., 2016; Hamid et al., 2021; Rodea-Palomares et al., 2015). However, even if the difference in the mixture is lesser than or greater than the sum of the

differences in single substances, it should not be directly interpreted as an antagonistic or synergistic interaction, because of lack of clear understanding on the shape of dose-response relationship. In present study, further decreasing due to the mixing is within the additive effect category.

## 4.2. Transcriptional Changes

After mixture exposure, several genes which are important for thyroid hormones changed in an additive fashion compared to individual exposure. DHHB+OC down-regulated *trh* compared to the control group. DHHB, OC did not show any statistically significant differences, so a decreasing due to the mixing suggests an additivity. Also, AVB and AVB+OC down-regulated *trh* compared to the control group. OC did not show any statistically significant differences, so an increasing due to the presence of AVB suggests an additivity (Figure 3b). AVB+OC down-regulated *trhr*, and *tshr* compared to the control group. AVB and OC did not show any statistically significant differences, so a decreasing due to the mixing suggests an additivity in relation to these genes (Figure 3c and 3e).

The proteins encoded by *tg*, *nis*, and *tpo* play important roles in TH synthesis. As a plasma membrane glycoprotein, Nis mediates the transport of active iodide into the thyroid follicular cells, which is a crucial first step in the synthesis of thyroid hormones (Dohán & Carrasco, 2003). The accumulated iodine is activated by thyroid peroxidase, which is the expression product of the *tpo* gene, which then catalyzes the expression product of the *tg* gene, the iodination of tyrosine on thyroglobulin (Targovnik et al., 2017). These three genes work together to realize the synthesis of thyroid hormones and impacts on them affect thyroid hormone levels (Targovnik et al., 2017). AVB+OC down-regulated *nis* compared to the control group. AVB and OC did not show any statistically significant differences, so a decreasing due to the mixing suggests an additivity (Figure 3f). DHHB+OC down-regulated *tpo* compared to

the control group. DHHB and OC did not show any statistically significant differences, so a decreasing due to the mixing suggests an additivity. Also, AVB and AVB+OC down-regulated *tpo* compared to the control group. OC did not show any statistically significant differences, so a decreasing due to the presence of AVB suggests an additivity (Figure 3g). The *nis* and *tpo* genes are essential for the formation of thyroid hormones, so reductions in these genes can cause a decrease in thyroid hormone levels.

Thyroid organogenesis starts around the 20 hpf with the expression of the early thyroid transcription factors, *nkx2.4b* (Alt et al., 2006). Although not statistically significant, the transcription of *nkx2.4b* tended to down-regulated after UV-filter exposure (Figure 3i). This suggested that the early events responsible for thyroid primordium specification were disturbed by UV-filter exposure.

Thyroid hormone specifically binds to the thyroid hormone nuclear receptor. Thyroid hormone receptors, acting as transcription factors for thyroid hormones, are important in normal functions of THs (Wu and Koenig, 2000). Like vertebrates, zebrafish possess two types of thyroid receptors (TRs), namely, TR$\alpha$ and TR$\beta$ (Refetoff et al., 2014). DHHB+OC and DHHB+AVB up-regulated *tr*$\beta$ significantly compared to the control group. However, DHHB, OC did not show any statistically significant differences. Also, there are statistically significant differences between individual chemicals and their binary mixtures, so this suggests an additivity in relation to this gene. (Figure 3k). Up-regulation of *tr*$\beta$ gene can be considered as the secondary response for compensation. This indicate that mixture exposure altered the expression of genes involved in the TH action.

Thyroid hormone after releasing into the circulation binds to the proteins that exist in the plasma and cross the membrane transporters to enter the intracellular compartment of the cell. Thyroxine-binding globulin (TBG), transthyretin (TTR or thyroxine-binding prealbumin), human serum albumin (HAS), and lipoproteins were among the thyroid hormone-binding proteins (Mimoto & Refetoff, 2020). Compared to individual chemicals, DHHB+OC statistically significantly down-regulated *ttr* gene (Figure 3l). Many environmental contaminants influence levels of circulating THs by competing for binding sites on transport proteins, such as TTR (Morgado et al., 2009). Down-regulation of *ttr* gene therefore would subsequently leave more T4 unbound, making it more susceptible to hepatic catabolism, resulting in a decrease of circulating TH concentration.

THs use transporter proteins located in cell membranes to reach the inside the cells (Hennemann et al., 2001). Some of the major transporters of THs include the monocarboxylate transporter 8 (MCT8), MCT10 and the solute carrier organic anion transporter 1C1 (OATP1C1), but only MCT8 has been identified as being specific to transport THs (Groeneweg et al., 2017). DHHB, OC, and AVB did not statistically significantly alter the *mct8* gene. However, DHHB+OC, DHHB+AVB up-regulated *mct8* compared to the control group. Also, there are statistically significant differences between DHHB+AVB and single chemicals, so this suggests an additivity in relation to this gene. (Figure 3m). The absence of MCT8 can impair the effective transport of THs. Up-regulation of *mct8* gene can be considered as the secondary response for compensation.

Zebrafish have 3 types of deiodinases encoded by *dio1*, *dio2*, and

*dio3* (Orozco & Valverde-R, 2005). *dio2* involve in the activation whereas *dio3* are associated with the inactivation of the hormone. The transcription of *dio2* gene, which are involved in TH transport and T4 to T3 conversion, was down-regulated compared to the control group after AVB+OC treatment. AVB and OC did not show any statistically significant differences, so a decreasing due to the mixing suggests an additivity in relation to this gene (Figure 3o). Deiodinase 2 is a selenoprotein that catalyzes the transformation of T4 to T3, thyroid hormones regulating neurodevelopment among other processes. It is the most important deiodinase in zebrafish embryonic development, as it controls the quantity of T3 hormone in the tissues (Walpita et al., 2009). Deficiency in *dio2* activity can lead to diminished T3 levels in all tissues causing altered locomotor activity, developmental perturbation, defects in swim bladder inflation (Houbrechts et al., 2016).

DHHB+OC down-regulated *dio3a* compared to the control group. DHHB, OC did not show any statistically significant differences, so this suggests an additivity. AVB and AVB+OC down-regulated *dio3a* compared to the control group. OC did not show any statistically significant differences, so a decreasing due to the presence of AVB suggests an additivity (Figure 3p). OC and DHHB+OC down-regulated *dio3b* compared to the control group. DHHB did not show any statistically significant differences, so a decreasing due to the presence of OC suggests an additivity. AVB, OC, and AVB+OC down-regulated *dio3b* compared to the control group (Figure 3q). Inhibition of *dio3a* and *dio3b* transcription may be a compensatory response to T3 decrease, and is also meaningful in itself. The knockdown of *dio3a* or *dio3b* resulting in substantial decrease in inner-ring deiodinase in the embryo resulted in

impaired embryonic and early larval development (Heijlen et al., 2014). The knockdown of *dio3* resulted in an increase in metabolic rate and stimulation of gluconeogenesis, as well as a delay in hatching and an increase in heart rate and carbohydrate content (Bagci et al., 2015).

AVB up-regulated *ugt1ab* significantly compared to the control group. Conversely, mixtures did not show any statistically significant differences compared to the control group (Figure 3r). UGT is an enzyme responsible for the glucuronidation of T4, which will lead to its biliary excretion. Therefore, increased *ugt1ab* transcription caused by exposure to BPs could lead to enhanced excretion of thyroid hormones and result in decreased thyroid hormone levels in zebrafish larvae. Previous studies also suggested that UGT could reduce thyroid hormone levels through T4 glucuronidation in both animal and in vitro models. DHHB+OC up-regulated *sult1 st5* compared to the control group. DHHB and OC did not show any statistically significant differences, so an increasing due to the mixing suggests an additivity (Figure 3s). *sult1 st5* is very important for the homeostasis of T4 concentrations and can promote the elimination of T4 (Visser, 1994). The increase of *sult1 st5* mRNA expression could lead to T4 decrease.

## 4.3. Thyroid Disrupting Pathways

Toxicity on the HPT axis may be caused by several mechanisms and thyroid disruption may be caused by a variety of mechanisms. Mechanisms of action may involve the sodium−iodide symporter, thyroid peroxidase enzyme, receptors for THs or TSH, transport proteins or cellular uptake mechanisms. The peripheral metabolism of the THs can be affected through effects on iodothyronine deiodinases or hepatic enzymes.

DHHB+OC lowered thyroid hormone and altered transcription levels of the genes involved in thyroid regulation in larval zebrafish. Possible hypotheses are as follows (Figure 4a).

1. The down−regulation of *trh* leads to a decrease in Trh, which may reduce the production of TSH.
2. The *tpo* genes are essential for the formation of thyroid hormones, so down−regulation of *tpo* can cause a decrease in thyroid hormone levels.
3. Up−regulation of *sult1 st5* leads to a decrease in TH by elimination.
4. Down−regulation of *ttr* gene would subsequently leave more T4 unbound, making it more susceptible to hepatic catabolism, resulting in a decrease of circulating TH concentration.
5. Up−regulation of *mct8, trβ* and down−regulation of *dio3a*, *dio3b* may be a secondary response to compensate the decreased THs and TSH.

DHHB+AVB lowered thyroid hormone and altered transcription levels of the genes involved in thyroid regulation in larval zebrafish. Possible hypotheses are as follows (Figure 4b).

1. Up−regulation of *sult1 st5* leads to a decrease in TH by

elimination.
2. Up-regulation of *mct8*, *tr β* may be a secondary response to compensate the decreased THs and TSH.

AVB+OC lowered thyroid hormone and altered transcription levels of the genes involved in thyroid regulation in larval zebrafish. Possible hypotheses are as follows (Figure 4c).
1. The decrease in TRH due to the down-regulation of *trh* and the decrease in TRH response due to the down-regulation of *trhr* may lead to a decrease in TSH production.
2. The down-regulation of *tshr* may reduce response to TSH, resulting in reduced hormone production.
3. The *nis* and *tpo* genes are essential for the formation of thyroid hormones, so down-regulation of these genes can cause a decrease in thyroid hormone levels.
4. Down-regulation of *ttr* gene would subsequently leave more T4 unbound, making it more susceptible to hepatic catabolism, resulting in a decrease of circulating TH concentration.
5. Down-regulation of *dio2* can lead to diminished T3 levels in all tissues
6. Up-regulation of *sult1 st5* leads to a decrease in TH by elimination.
7. Down-regulation of *dio3a*, *dio3b* may be a secondary response to compensate the decreased THs and TSH.

Hormone changes showed similar trends, but transcription changes showed different trends from previous studies. DHHB and AVB are one of benzophenone-like compounds owing to their benzophenone ring and benzophenones (BPs) are regarded as well-known potential thyroid disruptors. Lee et al. (2018) reported that BP-1, BP-3, and BP-8 significantly decrease the whole-body tT3

and tT4 in zebrafish (*Danio rerio*) after 6 d exposure from embryo to larva. The changes in the levels of thyroid hormones after exposure of DHHB, AVB and their mixtures, such as the decrease in tT4 level, are consistent with the effects of other BPs, including BP-1, BP-3, and BP-8 (Lee et al. 2018). In that study, TH metabolism genes, *dio1*, *dio2*, and *ugt1ab*, were up-regulated, which were considered as the primary response to cause the decrease of THs. Also, up-regulation of *tshβ*, *nis*, *tg*, and *tpo* were considered as compensatory efforts against decreased thyroid hormone levels. However, present study showed a different result after exposure of combinations including DHHB. Also, the down-regulation of central regulation genes (*trh*, *trhr*, *tshr*) by exposure to AVB+OC observed in this study is different from that of previous studies that reported elevation of TSH-related genes by BPs (Ka et al., 2022; Chu et al. 2021; Lee et al. 2018).

Although there are some genes that are statistically significant deviating from additivity, they didn't get the biological significance to change the hormone levels. They might not exceed the threshold to make protein level changes. In present study, mixture interaction is within the category of additive effect. The underlying mechanism, however, is not clear in this study. Further studies are needed to know the mechanism of thyroid hormone dysregulation effects by DHHB, OC, AVB, and their binary mixtures.

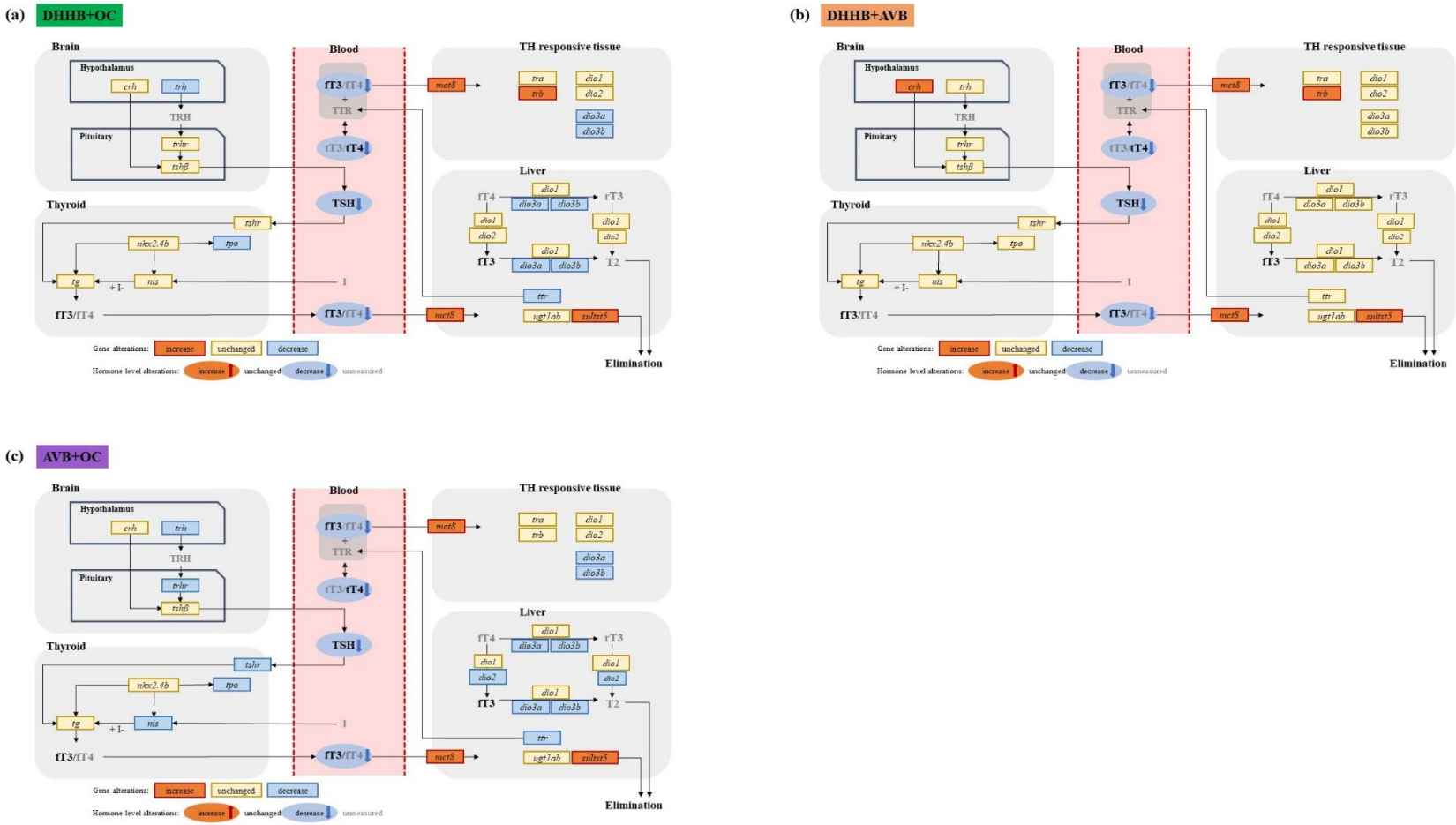



**Figure 4.** Possible toxicity pathways in the HPT axis in embryo–larval zebrafish following 120 h exposure to (a)DHHB+OC, (b)DHHB+AVB, and (c)AVB+OC.

# 5. Conclusion

The present observations revealed that the short-term exposure to UV-filter mixture may have additive effects on thyroid function in zebrafish. Some mixture combination caused greater extent of decrease in thyroid hormone levels than single substances, but within the range that could be interpreted as additive effects. Also, after mixture exposure, several genes which are important for thyroid hormones changed in an additive fashion compared to individual exposure.

Major limitation of this study is that the mixture exposure concentration included only one concentration, so caution in interpretation is needed. Elucidating the mechanism is challenging because a given compound may affect more than one target in the HPT axis or even more than one endocrine axis.

In conclusion, these results indicate that mixtures of UV-filters can produce thyroid hormone disruption in an additive way in zebrafish of early life stage. Safety management of multiple UV-filters in sunscreen products should be improved to include the additivity of the toxicity of individual components into consideration.

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# Abstract
## 국문 초록

# 유기계 자외선차단물질 3종의 혼합노출에 따른 제브라피쉬 치어의 갑상선 교란 영향

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DHHB, OC 및 AVB은 자외선 차단물질로 널리 사용되어 왔으며 잠재적인 내분비 교란이 우려되고 있다. 많은 자외선 차단물질 제품에서 조합으로 사용되는 동안, 이전의 연구들은 단일 화학 물질에 대한 노출에 초점을 맞추어 왔다.

본 연구에서 DHHB, OC 및 AVB의 혼합물의 갑상선 교란 독성을 제브라피쉬(*Danio rerio*)를 사용하여 평가하였다. 120시간 노출 후 Total T4, free T3, 갑상선 자극 호르몬(TSH)의 전신 함량을 측정하고 갑상선 호르몬 관련 유전자의 전사 수준을 분석했다. 개별 자외선 차단물질의 노출은 제브라피쉬 치어에서 갑상선 호르몬 수치를 감소시켰다. 자외선 차단물질의 이진 혼합물에 노출된 후 갑상선 호르몬의 유사한 패턴이 관찰되어 혼합물 상호작용의 additivity를 시사했다. 일부 혼합물에서 *sult1 st5* 유전자의 상향 조절과 *tpo*와 같은 여러 갑상선 특이 유전자의 하향 조절이 관찰되었으며, 이는 대사와 배설의 증가 및 합성의 감소를 통해 제브라피쉬 치어에서 갑상선 호르몬의 감소를 일으킬 수 있음을 시사한다. 이러한 결과는 자외선 차단물질의 혼합물에 대한 단기 노출이 제브라피쉬 치어에서 additive한 방식으로 갑상선 호르몬 감소로 이어질 수 있다는 것을 보여주었다. 이 연구의 결과는 자외선 차단제에 대한 안전 관리가 개별 성분에 국한되지 않고 제품에 첨가된 여러 UV

필터의 독성을 고려해야 한다는 것을 보여준다.

**주요어**: 자외선차단물질, 갑상선 호르몬, 제브라피쉬, DHHB, OC, AVB, 혼합 독성

**학번**: 2021-27873