보건학박사학위논문

Thyroid, neural, and kidney toxicities of major organic UV filters and related mechanisms in zebrafish of different life stages

주요 유기계 자외선 차단 물질이 여러 생애 단계의 제브라피쉬의 갑상선, 신경 및 신장에 미치는 독성과 관련 기전 연구

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권 바 름

Thyroid, neural, and kidney toxicities of major organic UV filters and related mechanisms in zebrafish of different life stages

A dissertation submitted in partial fulfillment of the requirements for the degree of
Doctor of Philosophy in Public Health

To the faculty of the Graduate School of Public Health at **Seoul National University**
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Abstract

Thyroid, neural, and kidney toxicities of major organic UV filters and related mechanisms in zebrafish of different life stages

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Organic UV filters are widely used for skin protection in personal care products such as sunscreen, cosmetics, and hair products. Although several UV filter agents, such as benzophenone-3 (BP-3) and octyl methoxycinnamate (OMC), have been banned for use in sunscreens in some regions, many organic UV filters are still extensively used for various applications. Most organic UV filters can penetrate the skin following dermal application and reach the bloodstream at levels warranting caution. Growing laboratory and epidemiologic evidence shows that organic UV filter agents can affect the thyroid endocrine system and possibly affect behavior and damage the kidneys. However, toxicological information is mostly limited to the endocrine-disrupting effects of BP-3. Therefore, the present study aims to investigate thyroid endocrine disruption and related toxicities following exposure to different organic UV filters.

In the first study (Chapter 1), to identify the knowledge gaps related to UV filters, we reviewed the current uses, regulations and known endocrine disrupting potencies of major organic UV filters. Considering environmental exposures and ecological significance, AVB, BP-3, OC and OMC were chosen as test UV filters for further toxicological evaluations.

In the second study (Chapter 2), the effects of the 'test UV filters' on thyroid hormone dysregulation were evaluated in embryo-larval (~120 hours post-fertilization), early-life (~30 days post-fertilization), and adult (>6 months, 21-day exposure) male zebrafish (*Danio rerio*). After exposure, thyroid stimulating hormone (TSH) and thyroid hormones (T4 and T3) were measured with enzyme-linked immunosorbent assay (ELISA), and genes related to thyroid hormone regulation (*trh, trhr, tshβ, tshr, nis, tpo, tg, dio1, dio2, dio3, trα, trβ, ugt1ab, sult1 st5, ttr*) were evaluated by using real-time quantitative polymerase chain reaction (RT–qPCR). After exposure to several UV filters, including AVB, BP-3, OC, and OMC, the levels of thyroid hormone and hypothalamus-pituitary thyroid (HPT) axis and hepatic metabolism genes were significantly altered in embryo-larval, juvenile and adult male zebrafish. Thus, our study suggests that commonly used UV filters could cause thyroid system imbalance in a life stage-specific manner. In addition, the results of this study indicate that early life stages are specifically vulnerable to UV filter exposure.

In the third study (Chapter 3), the neurotoxicity of these UV filters was examined, and a possible link between thyroid hormone dysregulation and neurotoxicity was investigated in embryo-larval (~120 hours post-fertilization), early-life (~30 days post-fertilization), and adult (>6 months, 21 days of exposure) male zebrafish. Neurobehavioral changes in zebrafish were analyzed during open-field and novel tank tests using Daniovision Ethovision® (Nodulus). Hypo- or hyperactivity and thigmotaxis, which are anxiety-like behavior phenotypes, were also evaluated. The mRNA expression levels of neurogenesis and neurotoxic markers (*mbp*, *gap43, gfap, c-fos, syn2a, syt1a, stxbp1b*) were quantified to address possible underlying mechanisms. Neurobehavioral changes were observed, along with changes in key genes related to neurodevelopment or neurogenesis. Our results suggest that locomotion and anxiety-like behavior changes in embryo-larval and juvenile zebrafish are likely the result of altered gene expression in the central nervous system and/or direct neurotoxic effects on neuronal cells. Moreover, UV filter displayed stronger effects in terms of neurogenesis, which implies that the developing brain is particularly vulnerable to UV filter exposure.

In the last study (Chapter 4), we demonstrated kidney toxicity caused by the study UV filters in embryo-larval (~120 hours post-fertilization), early-life (~30 days post-fertilization), and adult (>6 months, 21-day exposure) male zebrafish (*Danio rerio*). At the end of the exposure period, embryo-larval and juvenile fish were transferred to clean culture media for proteinuria analysis. The kidneys of the adult male zebrafish were harvested for histological observations. For nephrotoxicity- and nephrogenesis-related mRNA quantification, whole embryo-larval and juvenile zebrafish and the kidney tissues of adult male zebrafish were collected. In embryo-larval and juvenile fish, only genes that were expressed specifically in the kidneys were evaluated (*wt1a, podocin, nephrin, kim-1, cdh17, sim1a*). For adult male fish, genes known to play a role in nephrogenesis and kidney injury were analyzed (*ppargc1a, tbx2a, tbx2b, etv4, etv5, wt1a, podocin, nephrin, kim-1, cdh17, sim1a, pax2a*). Subsequently, it was found that exposure to UV filters induced significant changes in the proteinuria of embryonic larvae and zebrafish. In addition, transcriptional changes in genes related to kidney structure and injury were observed in embryo-larval, juvenile and adult zebrafish. The results from this study clearly demonstrate that exposure to UV filters increases kidney toxicity in a concentration-dependent manner during various life stages (e.g., embryo-larval, juvenile, and adult). This study provides important insights into kidney health consequences associated with UV filters.

The series of studies demonstrated important health effects of UV filters. We found that many organic UV filters disrupt the thyroid hormone balance of the embryo-larval, early-life and adult stages of zebrafish. Moreover, these UV filters impaired neurobehavior and damaged the normal kidney function of the fish. Because UV filters are designed to stay on the skin for a long time and can penetrate the skin to reach the circulation, their effects on human health are of great public health concern. Moreover, many UV filters are used in combination with others, and hence, it is urgent to understand and model their combined toxicity to guarantee the safety of sunscreens. Finally, the consequences of UV filter exposure during susceptible periods, e.g., fetal life, infancy, and puberty, are in question and warrant rigorous experimental and epidemiologic investigations.

Keywords: UV filters; endocrine disruption; thyroid hormone disruption; neurotoxicity; kidney toxicity, zebrafish

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Chapter 1 Introduction

1.1 Background

Ultraviolet (UV) light is a major component of sunlight and is essential for the endogenous synthesis of vitamin D from 7-dehydrocholesterol in human skin (Wacker and Holick. 2013). UV radiation, however, can cause skin injuries that range from premature aging and wrinkles to cancer. It can also damage the integrity of many consumer products, such as plastic packaging and fabrics, and hence reduce their durability (Briasco et al. 2017; Li and Kannan 2018). To protect both skin and other materials from the harmful effects of UV light, various UV filters have been developed and added to numerous products, ranging from cosmetics and personal care products, including sunscreens, to food packaging and home products (Gago-Ferrero et al. 2012; Ramos et al. 2015; Wang et al. 2016).

Avobenzone (AVB), benzopheone-3 (BP-3), homosalate (HS), octocrylene (OC), octisalate (OS) and octyl methoxycinnamate (OMC) are among the major organic filters that have been used in large quantities worldwide. These filters can directly enter the aquatic environment from recreational activities and indirectly from wastewater treatment plants (WWTPs). Due to their high lipophilicity and environmental persistence (ECHA, 2017) (Table 1), as well as their low removal efficiencies in wastewater treatment, they are detected frequently in aquatic environments (Ramos et al. 2016; Apel et al. 2018). Considering their heavy uses in sunscreens, it is not surprising that sites such as those near recreational beaches and swimming areas or WWTPs are hot spots for UV filter contamination. For example, there has been a huge coastal influx of several organic UV filters associated with tourism along the Mediterranean coast, which is estimated to receive 132 million international tourists (or 2.9 tourists per meter of coast) during the summer season (Tovar-Sánchez et al. 2019). Indeed, approximately 68% of the beachgoers interviewed replied that they applied sunscreen, with an average frequency of 2.6 times per visit, and approximately 92% applied it to the whole body. It was estimated that every

1000 beachgoers would release an average mass of 52.5 kg UV filters per day (1.4 ton/month) into the aquatic environment on the French coast of the Mediterranean Sea (Labille et al. 2020).

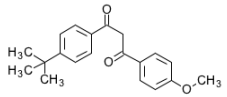
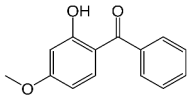
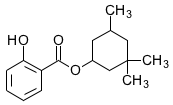
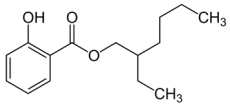
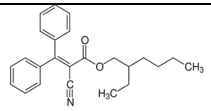
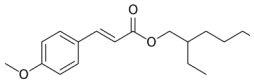
Some organic UV filters have been reported to have serious ecological consequences, especially on the marine coral population; a few UV filters have been reported to have adverse effects on zooxanthellae density, total polyp retraction, planular development, and coral cell mitochondrial function (Danovaro et al. 2008; He et al. 2019; Mitchelmore et al. 2019; Raffa et al. 2018; Sirois et al. 2019; Stien et al. 2019; Tsui et al. 2017). For this reason, the state of Hawaii, USA, has banned the use of sunscreen products containing BP-3 or octinoxate (OMC) (Ouchene et al. 2019; Raffa et al. 2019). Other countries, such as Palau, Thailand, and the Philippines, have also banned the use of some "reef toxic" organic UV filters (Battistin et al. 2020). More recently, Hawaii has additionally approved Senate Bill 132, prohibiting all sunscreens containing avobenzone or octocrylene as of January 2023 because of their potential toxic impacts on aquatic organisms (SB132, SD2, 2021).

Some organic UV filters are also known to cause adverse effects on endocrine systems. Endocrine activities of BP-3 and OMC were reported in both in vitro and in vivo experimental models and potentially in humans (Krause et al. 2012; Wang et al. 2016; Siller et al. 2019; Suh et al. 2020; Carve et al. 2021). Previous studies have shown that BP-3 and OMC induce weak or moderate estrogenicity through activation of estrogen receptors (ERα and ERβ) and regulatory changes in the hypothalamic-pituitary-gonad (HPG) axis (Krause et al. 2012; Wang et al. 2016; Carve et al. 2021). Both UV filters were also demonstrated to have anti-androgenic (Kunz and Fent, 2006, Wang et al. 2016) and anti-progesterone activities (Krause et al. 2012). Moreover, BP-3 and OMC showed anti-thyroid hormone activities through the inhibition of thyroid peroxidase (TPO) and 5'-deiodinase (DIO) activity, respectively (Krause et al. 2012; Wang et al. 2016). Endocrine disruption can result in adverse effects on reproduction and hence may lead to adverse consequences on the sustainability of aquatic ecosystems (Taylor and Harrison 1999; US EPA 1997). While data indicating endocrine disruption are accumulating for BP-3 and

OMC, other commonly used UV filters, such as AVB, HS, OC, and OS, have received less attention, and their endocrine disruption potentials are relatively unknown.

As many UV filters are used in increasing amounts in modern society and eventually reach the aquatic environment, it is important to identify priority chemicals based on potential environmental occurrences and toxicological effects. The present study focuses on major organic UV filters and aims to synthesize the currently available information on the amount in which they are used, their environmental occurrences, and up-to-date toxicological information with a focus on endocrine disruption. For this purpose, six frequently used organic UV filters, namely, AVB, BP-3, HS, OC, OMC, and OS (hereafter referred to as 'study UV filters'), were chosen. The results of this study will help to identify the knowledge gaps that warrant further investigation to develop measures for appropriate ecological risk management for this group of emerging aquatic contaminants.

Table 1. Chemical structure of study UV filters

Compound	CAS. #	Chemical structure	Formula[a]	MW[a] (g/mol)	Boiling point[a] (25℃)	Log Kow[b]	Vapor pressure[b] (mm Hg) at 25℃	BCF[b]	Half-life[b] in water (days) at 25℃	Overall environmental persistence[b] (days)
Avobenzone; butyl methoxydibenzoylmethane	70356-09-1		$C_{20}H_{22}O_3$	310.4	398	4.51	1.41×10^{-6}	1096	60	119
Benzophenone-3; oxybenzone	131-57-7		$C_{14}H_{12}O_3$	228.3	370	3.79	6.62×10^{-6}	502[c]	38	51
Homosalate; Homomenthyl salicylate	118-56-9		$C_{16}H_{22}O_3$	262.3	341	5.56	4.5×10^{-6}	223.8	38	46
Octisalate; Ethyl hexyl salicylate	118-60-5		$C_{15}H_{22}O3$	250.3	327	5.57	2.1×10^{-5}	47.7	15	20
Octocrylene; 2-Ethylhexyl 2-cyano-3,3-diphenylacrylate	6197-30-4		$C_{24}H_{27}NO_2$	361.5	327	7.35	2.1×10^{-5}	547	15	15
Octinoxate; Octyl methoxycinnamate	5466-77-3		$C_{18}H_{26}O_3$	290.4	358	5.43	2.3×10^{-5}	588.1	15	20

[a] Data are compiled from the US EPA Chemistry Dashboard, available at https://comptox.epa.gov/dashboard, as of Mar. 23rd, 2022.

[b] Values were estimated by using EPI Suite™ v4.11 from the US Environmental Protection Agency. Aquatic half-lives were estimated assuming a steady state. For the overall environmental persistence time, the mass amount (%), half-life (hours), and emissions (kg/hour) in air, water, soil, and sediment were considered.

[b] Kim and Choi (2014)

MW: Molecular weight

BCF: Bioconcentration factor

1.2 Current uses and regulations

The study UV filters, i.e., AVB, BP-3, HS, OC, OMC, and OS, are permitted for use not only in sunscreen products and cosmetics (Juliano and Magrini 2017; Manová et al. 2012; Mikkelsen et al. 2015; Stiefel and Schwack 2015) but also in consumer products, plastic packaging, and/or building materials (Table 2). Sunscreens, in which UV filters are most abundantly used, are generally classified as cosmetics and therapeutics in many countries, including Australia, Canada, Korea, and the European Union, or as nonprescription therapeutic drugs (over-the-counter products) in the USA (Table 3). Depending on the type of UV filter or the sun protection factor (SPF) of the products, they can be classified as drugs. For example, in Canada, sunscreens containing AVB, BP-3, HS, OC, OMC, or OS are categorized as nonprescription drugs (Health Canada, 2018), whereas products with SPF >4 are classified as drugs in Australia (Therapeutic Goods Administration, 2019). The different classifications of sunscreens explain the dissimilar regulations on sunscreens by country (Kumar and Gupta 2013). For example, chemicals used in drugs or cosmetics are not regulated under the Toxic Substance Control Act (TSCA) in the USA, but in Europe, the substances in articles including cosmetics are covered by Registration, Evaluation, Authorization and Restriction of Chemicals (REACH) (EC No 1907/2006). Currently, the maximum allowed concentrations of organic UV filters in the sunscreens range between 3% and 15% (Table 3). Recently, the European Commission submitted an updated draft of the Regulation amending Annex VI to the EU cosmetic regulations amended the maximum concentration of BP-3 up to 6% in cosmetics, 2.2% in baby products, and OC up to 9% in propellent spray products and 10% in other products as of the second quarter of 2022 (European Commission, 2021).

Table 2. The study UV filters, and target UV types, and their uses in consumer and industrial applications.

Compound	UV type	Consumer uses	Industrial uses
Avobenzone	A	Beverages, pharmaceuticals, fragrances, cosmetics, sunscreens for children, hair products (shampoo, hairspray, conditioner, hair dyes), body wash, spray, 3D-printed materials[a]	NA
Benzophenone-3	A and B	Food additives/packaging, pharmaceuticals, fragrances, cosmetics, hair products (shampoo, hairspray, conditioner hair dyes), nail polish, baby products (lotions, oils, powders, creams), textiles[b], home products (cleaners, air fresheners), building materials, pesticides	
Homosalate	B	Food additives/packaging, pharmaceuticals, fragrances, cosmetics, sunscreens, hair products (shampoo, hairspray, conditioner, hair dyes), body wash, pesticides	Odor agents
Octisalate	B	Food additives/packaging, beverage, pharmaceuticals, fragrances, cosmetics, sunscreens, hair products (shampoo, hairspray, conditioner, hair dyes), body wash, toothpaste, home products (cleaners, laundry products, air fresheners)	Odor agents
Octocrylene	A and B	Food additives/packaging, pharmaceuticals, fragrances, cosmetics, sunscreen for children or babies, hair products (shampoo, hairspray, conditioner, hair dyes), body wash, toothpaste, home products (cleaners, air fresheners, laundry detergents, soaps, degreasers, spot removers, dry cleaners, furniture, bedding), building materials (flooring, insulation, caulk, tile, wood, glass, cement), machinery (air/spacecraft, electrical), polishes (shoe, car, floor, leather products), pesticides	Odor agents, paint and coating additives, plastics
Octinoxate	B	Cosmetics, sunscreens, hair products (shampoo, hairspray, conditioner, hair dyes), body wash, nail polish	Odor agents

Data are compiled from the US EPA Chemistry dashboard (https://comptox.epa.gov/dashboard) and EPA Chemicals under the Toxic Substance Control Act (TSCA; https://www.epa.gov/chemical-data-reporting).

NA: not available.

a: Warr et al. (2020)

Table 3. Regulations on UV filters permitted for use in cosmetics and therapeutics by country.

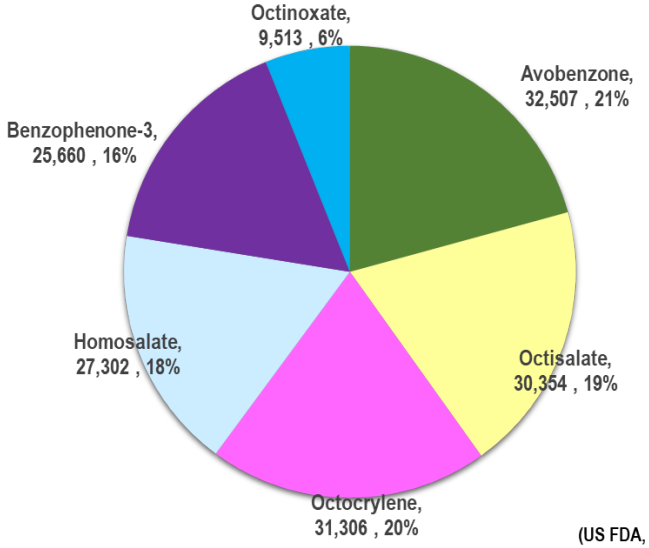
	Australia	**Canada**	**EU**	**Korea**	**USA**
Classification	Therapeutic /cosmetic	Therapeutic	Cosmetic	Cosmetic	OTC
Maximum allowed concentrations (%)					
Avobenzone	5	3	5	5	3
Benzophenone-3	10	6	6	5	6
Homosalate	15	15	10	10	15
Octinoxate	6	7.5	10	7.5	7.5
Octisalate	NA	5	5	5	5
Octocrylene	NA	10	10	10	10

NA: not available.

OTC: over-the-counter (non-prescription) drugs.

The amount of UV filter use varies by country. In the USA, UV filters are used in approximately 1,100~1,900 sun care products, with use in sunscreens ranging between 9,513 and 32,507 tons annually; AVB is used in the greatest amount, followed by OC, OS, HS, BP-3 and OMC (Fig. 1; US FDA, 2019). In the USA, BP-3, HS, OC, OS, and OMC were also registered in the range of 39.5-5,000 tons/year in TSCA, i.e., for use in items other than cosmetics and drugs. In Europe, the amounts of the five study UV filters registered by REACH are all in the range of 1,000-10,000 tons/year each. In Europe, the active ingredients of cosmetic products are classified by the European Commission and are reviewed for safety evaluation by the European Scientific Committee on Consumer Products (SCCS) (SCCS/1625/20; SCCS Opinions 2016-2021).

(A) Estimated consumption of UV filter agents in sunscreen products



(US FDA, 2019)

Active ingredient	Products*	Estimated consumption in 2016 (tons/year)*
Avobenzone	1,461	32,507(26,302-38,712)
Octocrylene	1,432	31,306 (25,230-37,382)
Octisalate	1,791	30,354 (23,642-37,067)
Homosalate	1,167	27,302 (23,181-31,423)
Benzophenone-3	1,342	25,660 (22,772-28,548)
Octinoxate	1,940	9,513 (5,266-13,760)

(B) Production of UV filters (tons/year)

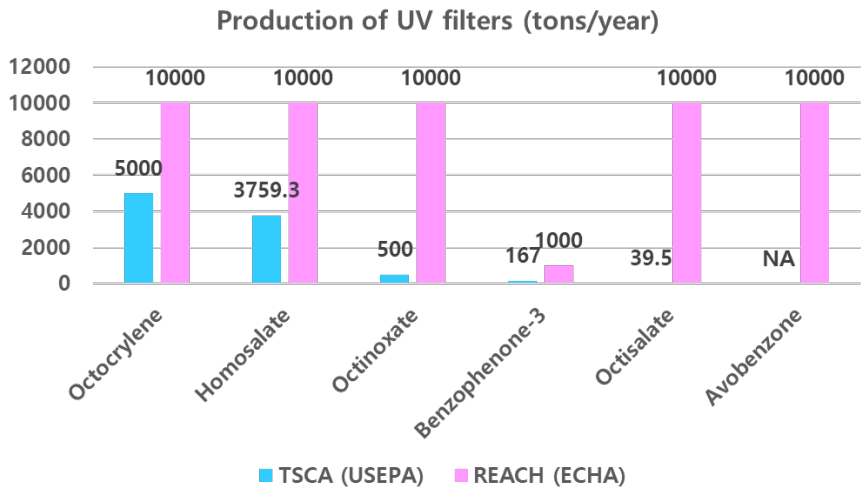


Fig. 1. Annual estimated production level of UV filters. (A) Estimated consumption of UV filter agents (US FDA, 2019). * If a product contains >1 activ e ingredient, the US FDA included it in the number of products and the consumption volume. Parentheses indicate lower and upper confidence intervals of estimated consumption. (B) UV filters registered under the Toxic Substance Control Act (TSCA, US EPA) and Registration, Evaluation, Authorisatio

n and Restriction of Chemicals (REACH, ECHA). NA: not available.

The frequency of use of UV filters in consumer products also varies by country (Fig. 2). In surveys of sunscreen products in Australia (N=100) (O'Malley et al. 2021), Denmark (N=75) (Rastogi 2002), and Germany (N=462) (Uter et al. 2014), OC (90%), OMC (49%), and AVB (74%) were most frequently used, respectively. In cosmetics studied in Germany (N=4,447; Uter et al. 2014), Korea (N=7,908; Choi et al. 2020), and the United Kingdom (N=337; Kerr 2011), the most frequently used UV filters were AVB, OMC, and AVB, respectively. It is noteworthy that many organic UV filters are used in combination to provide greater protection against different types of UV light (Fourtanier et al. 2012; Gilbert et al. 2013; Nash and Tanner, 2014; US FDA, 2019; Uter et al. 2014).

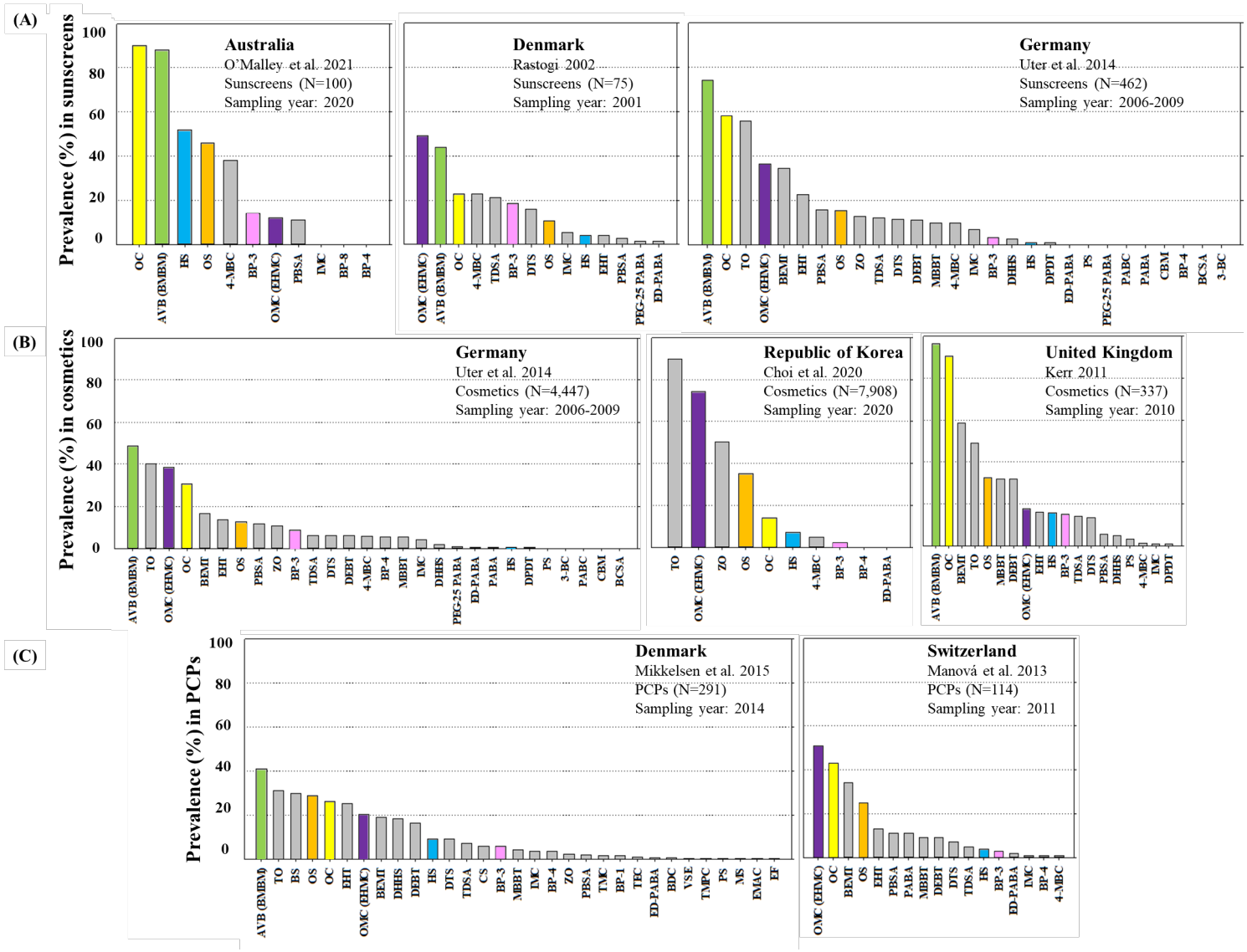


Fig. 2. Prevalence of UV filters in (A) sunscreens, (B) cosmetics, and (C) personal care products (PCPs) in the markets of different

countries. Countries are presented in alphabetical order. In Australia, mineral (inorganic) UV filters were not included (O'Malley et al. 2021). Cosmetics in Germany, the Republic of Korea and the United Kingdom include sunscreen products (B). In Germany, cosmetic products include any products with or without UV filters (Uter et al. 2014). Personal care products (PCPs) in Denmark include sunscreens, make-ups, facial wash, face cream, lip care, soap, body care products, mouth wash, and fragrances. (Mikkelsen et al. 2015). In Switzerland, PCPs include sunscreens, lip care products, lipsticks, face creams, liquid foundation makeup, aftershaves, and hand creams. Due to the presence of multiple UV filters in the given products, the percentages may total > 100%. Abbreviations are used to represent each chemical substance as follows: 3-BC: 3-benzylidene camphor, 4-MBC: 4-methylbenzylidene camphor, AVB (BMBM): avobenzone; butyl methoxydibenzoylmethane, BCSA: benzylidene camphor sulfonic acid, BDC: benzotriazolyl dodecyl p-cresol, BEMT: bis-ethylhexyloxyphenol methoxyphenyl triazine, BP-1: benzophenone-1, BP-3: benzophenone-3, BP-4: benzophenone-4, BP-8: benzophenone-8, BS: benzyl salicylate, CBM: camphor benzalkonium methosulfate, CS: camellia sinensis leaf extract, DEBT: diethylhexyl butamido triazone, DHHS: diethylamino hydroxybenzoyl hexyl benzoate, DPDT: disodium phenyl dibenzimidazole tetrasulfonate, DTS: drometrizole trisiloxane, ED-PABA: ethylhexyl dimethyl PABA; octyl dimethyl PABA, EF: ethyl ferulate, EHT: ethylhexyl triazone; octyl triazone, EMAC: ethylene/methacrylate copolymer, HS: homosalate, IMC: isoamyl p-methoxycinnamate, MBBT: methylene bis-benzotriazolyl tetramethylbutylphenol (incl. the nano form), MS: menthyl salicylate, OC: octocrylene, OMC (EHMC): octinoxate; ethylhexyl methoxycinnamate; octyl methoxycinnamate, OS: octisalate; ethylhexyl salicylate; octyl salicylate, PABA: PABA (aminobenzoic acid), PABC: polyacrylamidomethyl benzylidene camphor, PBSA: phenylbenzimidazole sulfonic acid and its salts (including potassium, sodium and triethanolamine), PEG-25 PABA: polyethylene glycol-25 PABA, PS: polysilicone-15, TDSA: terephthalylidene dicamphor sulfonic acid and its salts, TEC: triethoxy caprylylsilane, TMC: trimethoxy caprylylsilane, TMPC: tris(tetramethylhydroxypiperidinol) citrate, TO: titanium dioxide (incl. the nano form), VSE: *Vitis vinifera* seed extract, ZO: zinc oxide.

Safety information for most organic filters is insufficient, and others are classified as not safe (Table 4). According to the US FDA, most organic UV filters, including all of the study UV filters, are currently classified as "Insufficient safety data to determine GRASE status" (US FDA, 2019), suggesting that their potential toxicities should receive further appropriate attention (Table 4). Similarly, ECHA included AVB, OS, and OC in the Community Rolling Action Plan (CoRAP), i.e., as chemicals that warrant investigation for potential environmental and human health risks. The ECHA decision was primarily based on the high aggregated tonnage, wide dispersive use, consumer use, exposure to the environment, and suspected 'persistent, bioaccumulative, and toxic' (PBT)/'very persistent and very bioaccumulative' (vPvB) characteristics of these organic UV filters. Moreover, OS is proposed to be listed as a potential endocrine disruptor by ECHA and subject to evaluation in 2022 (ECHA, 2020c). In addition, OMC has been listed in the surface water "Watch list" under the Water Framework Directive of the European Union due to its PBT characteristics and potential endocrine activity (EU Commission, 2016).

Table 4. Commonly used UV filters and GRASE determination by the U.S. Food and Drug Administration (2019)

Category		**UV filters**
I	GRASE (generally recognized as safe and effective)	Zinc oxide, titanium oxide
II	Not GRASE	Para-aminobenzoic acid, trolamine salicylate
III	Insufficient safety data to determine GRASE status	Cinoxate, dioxybenzone, ensulizole, **homosalate**, eradimate, **ocinoxate**, **octisalate**, **octocrylene**, padimate O, sulisobenzone, **benzophenone-3**, **avobenzone**

Chemicals in boldface are the UV filters included in the present study.

1.3 Physicochemical characteristics and environmental occurrences

The study UV filters are lipophilic, as indicated by their octanol/water partition coefficient (log Kow) values ranging between 3.8 and 7.4, and exhibit reasonably high bioconcentration potential with bioconcentration factors (BCFs) up to 1,096 (Table 1). In addition, their half-lives in water ranged between 15 and 60 days, indicating their low degree of dissipation from the aquatic environment. Hence, exposure throughout the aquatic food web can be significant. Relevant information on the biomagnification of some UV filters can be found in Gago-Ferrero et al. (2012), Ramos et al. (2015) and Pawlowski et al. (2019). For these reasons, organic UV filters have been frequently detected in both marine and freshwater environments, including water, sediment, WWTPs, and biota, worldwide.

Based on a PubMed search for environmental occurrences, with keywords of chemical substance names, 'UV filters', 'UV blockers', 'UV absorbers', 'sunscreen', 'sunblock', 'aquatic, environment', 'occurrence', 'distribution', and/or 'analytical method'), a total of 77 articles published between 1996 and 2020 were identified. For BP-3, for which environmental occurrence and toxicity are relatively well documented (Kim and Choi, 2014), only recently published studies (from 2015-2022) were collected. The reported levels of the study UV filters in water along with the location of the survey are summarized in Fig. 3. When the median (or average) levels were compared, BP-3, OC, AVB, and HS were generally detected to be higher in the recreational beach waters. In contrast, OMC and OS were detected at the highest median concentrations in the estuaries of Melbourne, Australia, which are influenced by discharges from wastewater treatment plants. These observations support that recreational input and municipal sewage are major sources of organic UV filters in the aquatic environment.

The UV filters that were detected at high concentrations in recreational marine waters of the USA and its territories, e.g., OC and OMC, are those with the highest amounts of use in cosmetics (US FDA 2016). These were detected at up to 3,730 and 756 ng/L, respectively, in two recreational locations, Folly Beach (South Carolina, USA) and Puerto Rico (Bratkovis et al. 2015; Sánchez Rodríguez et al. 2015) (Fig. 3). In recreational waters popular for watersports

near Folly Beach, OC, AVB, and OMC were detected at average concentrations of 711 ng/L [range: below limit of detection (<LOD)~3,730 ng/L], 234 ng/L (range: <LOD~1,298 ng/L), and 96.9 ng/L (range: <LOD~341.1 ng/L), respectively (Bratkovics et al. 2015). Notably, BP-3 showed the highest level among UV filters in recreational water compared to other studies described above. The most recent study, conducted by Downs and colleagues (Downs et al. 2022), reported that the concentration of BP-3 ranged from 136 to 27,880 ng/L and suggested beach showers as a possible source of sunscreen environmental contamination (Downs et al. 2022).

The influence of municipal or industrial discharges was also clearly observed. In Australia, higher concentrations of OMC and OC were detected in the estuaries of Port Philip Bay, Victoria (Allinson et al. 2018). For example, in the Maribyrnong River, which is the second largest river running through the metropolitan Melbourne area of Australia, with two wastewater treatment plants upstream, OMC and OC were detected at 640 ng/L and 109 ng/L, respectively. In the Pearl River estuary of the South China Sea, relatively high concentrations of OC, OMC, HS, and OS were found, and the levels decreased as the location of measurement moved farther away from highly industrialized and urbanized areas and toward the South China Sea coastal region (Tsui et al. 2015; Tsui et al. 2017; Tsui et al. 2019). Illegal discharge of untreated wastewater was also suggested as the source of contamination in the South China Sea water system (Tsui et al. 2019). Moreover, BP-3 has been detected at a mean concentration of 1,180 (68.5~5,010 ng/L) in the Huangpu River and 176 ng/L (121~297 ng/L) in the Suzhou River, which are influenced by cosmetic factories (Wu et al. 2018). In addition, BP-3 was detected at levels as high as 45.1 ng/L in the Yangtze River, probably due to the incomplete coverage of the wastewater treatment system (Ma et al. 2018).

In freshwater, BP-3, OMC, OC, and HS were predominant, but these observations are exclusively made within Asia. OMC, OC, and HS were detected in the ranges of 1.1-3.1 ng/L, 0.4-0.9 ng/L, and 4.5-7.9 ng/L, respectively, in the Qinhuai River, which receives effluents from industrial and/or sewage treatment plants in the highly urbanized Nanjing area (Tsui et al. 2019; Yang et al. 2020). BP-3 was found at 24.1 ng/L (2.5~331 ng/L) in the Mariana watershed,

Singapore. The Mariana watershed covers one-sixth of the total land area of Singapore, passing through metropolitan cities (Mao et al. 2018). BP-3 also occurs in other highly urbanized regions. The measured levels were reported to be up to 2,100 ng/L, 13.6 ng/L, and 52.7 ng/L in Brazil, Italy, and Spain, respectively (Serra-Roig et al. 2016; Palmiotto et al. 2018; Pompei et al. 2019).

Among the studied UV filters, OS is the least reported on organic UV filter in terms of aquatic occurrence. Recreational activities and municipal and industrial wastewater discharges were suggested as potential sources of contamination for this UV filter as well (Tang et al. 2018; Tashiro and Kameda, 2013; Tsui et al. 2019). In recreational waters, OS was detected at relatively high levels, up to 1,030 ng/L in Victoria Harbor of Hong Kong, followed by 128 ng/L in the South China Sea near Chaozhou and 121 ng/L in Hawaii, USA (Michelmore et al. 2019; Tsui et al. 2015). In ambient water with negligible recreational activities, OS was detected at lower levels, ranging from 18.0-31.6 ng/L, 13.4-29.4 ng/L, 0.85-27.8 ng/L, and <LOD-1.8 ng/L in estuaries of Port Philip Bay in Australia, Chaohu Lake in China, the coastal region of the South China Sea and Okinawa, Japan, respectively (Allison et al. 2020; Tashiro and Kameda; Tang et al. 2018; Tsui et al. 2019). In Australia, the highest concentration of OS was reported in Kororoit Creek near Port Philip Bay. Because there are no WWTP sewer overflows in this area, storm water runoffs are suspected to be potential sources of OS (Allison et al. 2018).

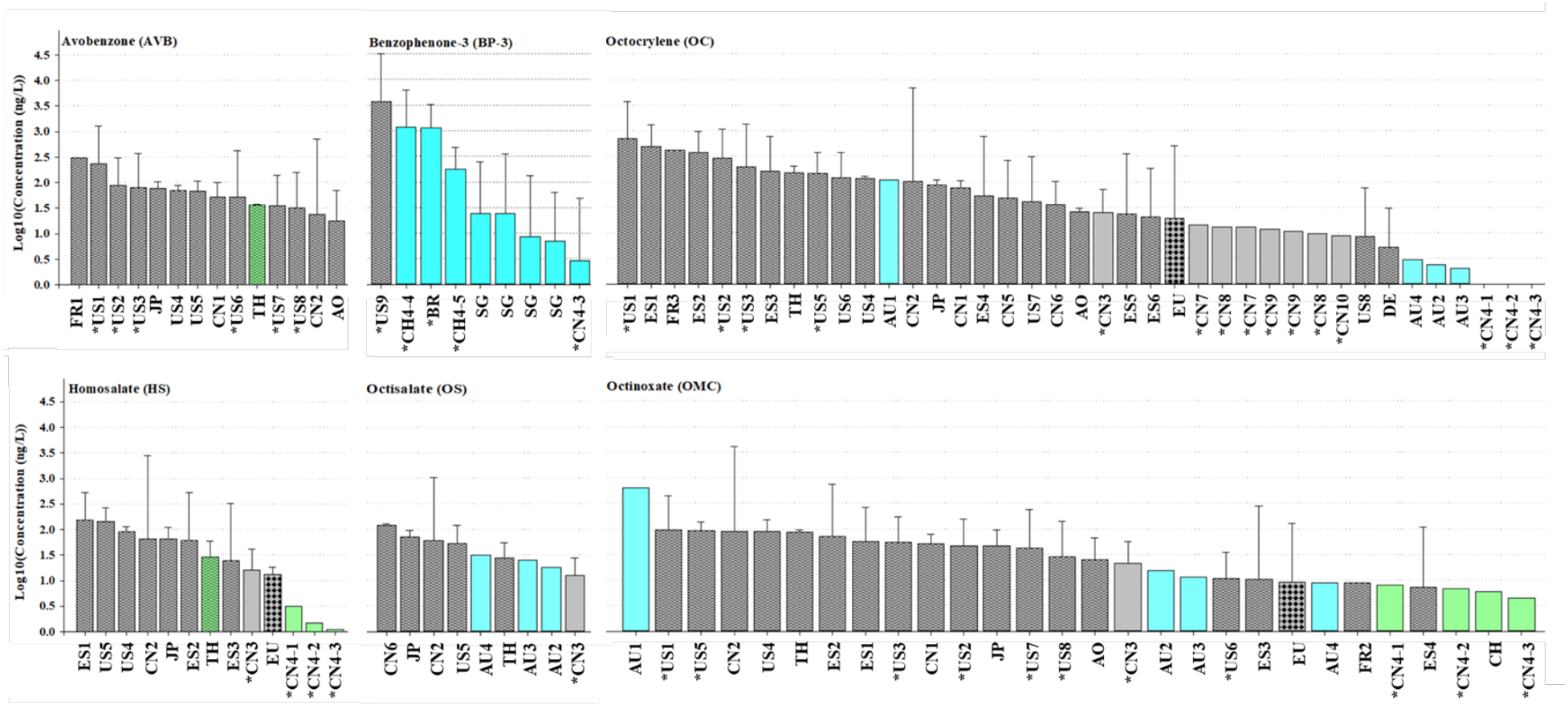


Fig. 3. Concentrations (median) of the study UV filters reported in water environments worldwide. Data compiled from Kwon and Choi (2021). Gray, green, and blue colors indicate UV filter concentrations reported in marine, freshwater, and estuarine environments, respectively. Error bars indicate the maximum level detected. Zigzag and diamond patterns indicate locations with recreational activity and aquaculture areas, respectively. Single-grab sampling was conducted for the measurement of UV filters in Australia (blue bars) (Allinson et al. 2018). *Arithmetic mean is shown because the median was not available. Two-letter codes are used to represent countries/regions as follows: AO, Atlantic Ocean; AU, Australia; BR: Brazil; CN, China; CH, Switzerland; DE, Germany; ES, Spain; EU, Europe (Iberian); FR, France; JP, Japan; SG: Singapore; TH, Thailand; US, USA. Numbers next

to the country code represent the sampling locations in a given country, as found in Kwon and Choi (2021). For example, CN4-1, CN4-2, and CN4-3 indicate sampling locations in the New Qinhuai River, Qinhuai River, and Yangtze River, respectively.

1.4 Endocrine disrupting potencies

Studies on cell lines (Table 5) suggest that the study UV filters possess endocrine activity. Laboratory animal studies also showed sex and thyroid hormone disruption, but this evidence is limited to BP-3, HS, OC, and OMC (Tables 6-7). Human observations for outcomes related to endocrine disruption have also been made, but only for BP-3, HS, OC, and OMC (Table 8).

1.4.1 *In vitro* observations

AVB, BP-3, HS, and OMC have been reported to possess anti-androgenic or estrogenic activities in different cell lines (Table 5). AVB exhibited strong anti-androgenic activity in human breast cancer (MDA-kb2) cells (median inhibitory concentration, IC_{50}: 2.0×10^{-11} M) (Klopci et al. 2017). However, in a different human breast cancer (MCF-7) cell line, estrogenic activity was not observed (Schlumpf et al. 2001). OMC demonstrated weak estrogenic activity in both human embryonic kidney (HEK-293) cells transfected with human androgen and progesterone receptors (Schreurs et al. 2005) and MCF-7 cells (Alamer and Darbre 2017; Schlumpf et al. 2001); however, it demonstrated an antiestrogenic effect in hER recombinant yeast (IC_{50}: 4.3×10^{-3}) (Kunz and Fent, 2006). For BP-3, few investigations have been conducted between 2015 and 2022. One research group reported an anti-androgenic effect in hER recombinant yeast (EC_{50}: 1.5×10^{-5}) (Balázs et al. 2016). In addition, Altamirano et al. (2020) and Wnuk et al. (2018) demonstrated significant

upregulation of *Er* expression in isolated mammary glands of C57BL female mice (EC_{50}: 1.5×10^{-5}) and primary neocortical and hippocampal neural cells (EC_{50}: 1.5×10^{-5}), respectively. The most recent study and identified significant estrogen receptor (*ERα*) modulation in BP-3-treated MCF-7 cells in a microarray experiment (Rooney et al. 2021).

In addition, OMC was able to induce alterations in hypothalamic gonadotropin-releasing hormone (Gn-RH) and luteinizing hormone-releasing hormone (LH-RH) levels in isolated rat brain tissue (Carbone et al. 2010; Szwarcfarb et al. 2007). HS showed anti-androgenic activity in both MDA-kb2 cells (Ma et al. 2003) and PALM cells transfected with human androgen receptors (Jiménez-Díaz et al. 2013) and induced estrogenic activity in MCF-7 cells (Alamer and Darbre, 2017; Jiménez-Díaz et al. 2013; Schlumpf et al. 2001). For OS, only one study has been performed to examine its endocrine activity (Jiménez-Díaz et al. 2013). However, no androgenic or estrogenic activities were observed, even at the maximum experimental concentration of 10 μM (i.e., lowest observable effect concentration (LOEC) >10 μM).

For thyroid-related disruption, AVB exhibited anti-thyroid activity in a rat pituitary (GH3-TRE-Luc) cell line when coadministered with T3. The maximum luciferase inhibition of AVB was reached at 2.5×10^{-5} M, which was a quarter of the dose of the positive control, bisphenol A (Klopci et al. 2017). For BP-3, Lee and colleagues (2018) suggested T3-like activities in GH3 cells by showing downregulation of *Trhr*, *Tshb*, and *Trb* (LOEC $\geq3.2\times10^{-2}$ M) and decreased thyroid hormones in rat thyroid follicular cells (FRTL-5) attributed

to significant changes in genes related to thyroid hormone synthesis (*Nis, Tg, Tpo*). For OS, two studies were performed to examine its endocrine activity (Kunz and Fent, 2006; Jiménez-Díaz et al. 2013). OS showed antiestrogenic activity in hAR recombinant yeast (IC_{50}: 6.8×10^{-3}) (Kunz and Fent, 2006); however, no active responses were observed (androgenic and estrogenic LOEC>1.0×10^{-5} M).

Studies on cell lines illustrated that UV filters may impair the normal function of the central nervous system (CNS). One research group evaluated the effects of BP-3 and OMC on cell viability and caspase-3 activity in human neuroblastoma cells (SH-SY5Y) and found that exposure to these UV filters significantly decreased cell viability and induced apoptosis by activating caspase-3 activity at very low concentrations (from 10^{-8} to 10^{-7} M) (Broniowska et al. 2016). Additionally, Wnuk and colleagues (2018) demonstrated elevated ROS production and caspase-3 activity and neurite outgrowth deficit along with alterations of estrogen receptors (*Erα* and *Erβ*) upon BP-3 exposure in isolated primary neocortical and hippocampal neural cells from Swiss mouse embryos. This study also suggested the possibility of sex hormone-mediated apoptosis by attenuation of *Erα* and stimulation of *Erβ* in mouse neuronal cells (Wnuk et al. 2018). Moreover, *in vitro* experiments in hypothalamic cells isolated from male and female Wistar rats demonstrated that an equal dose of OMC (1×10^{-7} M) significantly inhibited neurotransmitter systems, i.e., aspartate (Asp), glutamate (Glu), and gamma-aminobutyric acid (GABA) (Table 5) (Szwarcfarb et al. 2007; Carbone et al. 2010).

Table 5. Endocrine disrupting activities of UV filters reported *in vitro*

Compound	Hormonal activity	Cell line	Observation (assay)	Endpoint	Effect concentration (M)	Reference
AVB	Anti-androgenic	MDA-kb2	Luciferase activity	IC_{50}	2.0×10^{-11} (↑)	Klopci et al. 2017
	Estrogenic	HEK923	Luciferase activity	EC_{50}	Weak estrogenic	Schreurs et al. 2005
		MCF-7	Cell proliferation	EC_{50}	Inactive	Schlumpf et al. 2001
				LOEC	$>5.0\times10^{-5}$ (-)	
	Glucorticoid-like	MDA-kb2	Luciferase activity	EC_{50}	2.7×10^{-6} (↑)	Klopci et al. 2017
	Thyroid hormone-like	GH3-TRE-Luc	Luciferase activity	EC_{50}	1.0×10^{-9} (-)	
	Anti-thyroid hormone-like			IC_{50}	7.2×10^{-5} (↑)	
BP-3	Anti-androgenic	hARα-Yeast (*Saccharomyces cerevisiae*)	Luciferase activity	EC50	1.0×10^{-5} (↓)	Balázsetal. 2016
	Estrogenic	Mammary gland (C57BL/6 female mice)	mRNA expression	LOEC (*Esr1*)	$<1.0\times10^{-12}$ (↑)	Altamirano et al. 2020
		Primary neocortical and hippocampal neural cells (Swiss mouse embryos)		LOEC (*Erβ*)	$<2.5\times10^{-5}$ (↑)	Wnuk et al. 2018
		hERα-Yeast (*Saccharomyces cerevisiae*)	Luciferase activity	EC_{50}	6.4×10^{-6} (↑)	Balázsetal. 2016
	Thyroid hormone-Like	GH3	mRNA expression	NOEC (*Trhr, Tshβ, Trβ*)	1.0×10^{-2}	Lee et al. 2018

			LOEC (*Trhr, Tshβ, Trβ*)	3.2×10^{-2} (↓)	
Anti-thyroid hormone-like	FRTL-5		NOEC (*Nis, Tg*)	1.0×10^{-2}	
			LOEC (*Nis, Tg*)	3.2×10^{-2} (↑)	
			NOEC (*Tpo*)	1.0×10^{-2}	
			LOEC (*Tpo*)	3.2×10^{-2} (↓)	
Neurotoxicity	SH-SY5Y (neuroblastoma)	Apoptotic cells (%)	LOEC	$<1.0\times10^{-8}$	Broniowska et al. 2016
	Primary neocortical and hippocampal neural cells (Swiss mouse embryos)		LOEC	$<2.5\times10^{-5}$ (↓)	Wnuk et al. 2018
		Caspase-3 activity	NOEC	1.0×10^{-8}	
			LOEC	1.0×10^{-7} (↑)	
	Primary neocortical and hippocampal neural cells (Swiss mouse embryos)		NOEC	$<1.0\times10^{-5}$ (↑)	
		Cell cytotoxicity	NOEC	1.0×10^{-4}	Broniowska et al. 2016
			LOEC	1.0×10-5 (↑)	
		Neurite growth	LOEC	$<2.5\times10^{-5}$ (↓)	Wnuk et al. 2018
		ROS production	LOEC	$<2.5\times10^{-5}$ (↑)	

HS	Androgenic	hAR-Yeast (*Saccharomyces cerevisiae*)	Luciferase activity	EC_{50}	1.7×10^{-4} (104% of DHT)	Kunz and Fent 2006
		PALM-hAR	Luciferase activity	EC_{50}	Inactive	Jiménez-Díaz et al. 2013
	Anti-androgenic	hAR-Yeast (*Saccharomyces cerevisiae*)		IC_{50}	1.1×10^{-4} (104% of FT)	Kunz and Fent 2006
		MDA-kb2		IC_{50}	1.56 (↑)	Ma et al. 2003
		PALM-hAR		IC_{50}	2.7×10^{-6} (↑)	Jiménez-Díaz et al. 2013
		U2-OS (human osteosarcoma)		IC_{50}	1.7×10^{-6}	Schreurs et al. 2005
	Anti-estrogenic	hERα-Yeast (*Saccharomyces cerevisiae*)		IC_{50}	2.1×10^{-3} (98% of 4HT)	Kunz and Fent 2006
	Estrogenic	HEK923		EC_{50}	1.6×10^{-6}	Schreurs et al. 2005
		hERα-Yeast (*Saccharomyces cerevisiae*)		EC_{50}	Inactive	Kunz and Fent 2006
		MCF-7	Cell proliferation	EC_{50}	5.3×10^{-6} (↑)	Jiménez-Díaz et al. 2013
		MCF-7		EC_{50}	1.6×10^{-6}	Schlumpf et al. 2001
		MCF-7		LOEC	$<1.0\times10^{-5}$ (↑)	Alamer and Darbre 2017
		MCF-7		LOEC	1.0×10^{-6} (↑)	Schlumpf et al. 2001
		MCF-7		NOEC	1.0×10^{-7}	
		MCF-7	Luciferase activity	NOEC	$>1.0\times10^{-5}$	Alamer and Darbre 2017
	Anti-progetagenic	U2-OS (human	Luciferase activity	IC_{50}	3.0×10^{-6}	Schreurs et al.

		osteosarcoma)				2005
	Others	MCF-7	Cell invasion (23 weeks of exposure)	Change in cell index (effect conc.)	1.0×10^{-5} (↑)	Alamer and Darbre 2017
			Cell migration (23 weeks of exposure)	Change in cell index (effect conc.)	1.0×10^{-5} (↑)	
OC	Androgenic	hAR-Yeast (*Saccharomyces cerevisiae*)	Luciferase activity	EC_{50}	6.3×10^{-4} (21% of DHT)	Kunz and Fent 2006
	Anti-androgenic			IC_{50}	2.5×10^{-5} (86% of FT)	
	Estrogenic			EC_{50}	Inactive	
	Anti-estrogenic			IC_{50}	2.6×10^{-3} (118% of 4HT)	
	Others	CHO (Chinese Hamster Ovary cell)	Structural aberrations	No. of cells with aberrations	No change	Odio et al. 1994
OMC	Androgenic	hAR-Yeast (*Saccharomyces cerevisiae*)	Luciferase activity	EC_{50}	1.1×10^{-2} (61% of DHT)	Kunz and Fent 2006
OMC	Anti-androgenic			IC_{50}	3.1×10^{-4} (123% of FT)	
OMC	Anti-estrogenic			IC_{50}	4.3×10^{-3} (182% of 4HT)	
OMC	Anti-progetagenic	U2-OS (human osteosarcoma)		IC_{50}	5.0×10^{-7}	Schreurs et al. 2005
	Estrogenic	HEK923		EC_{50}	Weak estrogenic	
		hERα-Yeast (*Saccharomyces cerevisiae*)		EC_{50}	Inactive	Kunz and Fent 2006
		MCF-7	Cell proliferation	EC_{50}	2.3×10^{-6}	Schlumpf et al. 2001

	MCF-7		LOEC	5.0×10^{-6} (↑)	
	MCF-7		NOEC	$>1.0\times10^{-5}$	Alamer and Darbre 2017
	MCF-7		NOEC	1.0×10^{-6}	Schlumpf et al. 2001
	MCF-7	Luciferase activity	LOEC	1.0×10^{-5} (↑)	Alamer and Darbre 2017
	MCF-7		NOEC	1.0×10^{-7}	
Neuroendocrine disruption	Wistar rats (hypothalamus-pituitary) (F)	Luteinizing hormone-releasing hormone	LH-RH (pg/mL)	1×10^{-7} (↓)	Szwarcfarb et al. 2007
		Neurotransmitter	Aspartate (ASP) (pg/mL)	No change	
			Gamma-aminobutyric acid (GABA) (pg/mL)	No change	
			Glutamate (GLU) (pg/mL)	1×10^{-7} (↓)	
	Wistar rats (hypothalamus-pituitary) (M)	Luteinizing hormone-releasing hormone	LH-RH (pg/mL)	1×10^{-7} (↓)	
		Neurotransmitter	Aspartate (ASP) (pg/mL)	No change	
			Gamma-aminobutyric acid (GABA) (pg/mL)	1×10^{-7} (↑)	
			Glutamate (GLU) (pg/mL)	No change	
		Gonadotropin-releasing hormone (Gn-RH)	Gn-RH (pg/mL)	No change	Carbone et al. 2010
	Wistar rats (hypothalamus-pituitary)[c] (M)	Neurotransmitter	Aspartate (ASP) (pg/mL)	No change	
			GABA (pg/mL)	No change	

		Glutamate (pg/mL)	No change
Wistar rats (hypothalamus-pituitary)$^{c+T}$ (M)	Gonadotropin-releasing hormone (Gn-RH)	Gn-RH (pg/mL)	1×10^{-7} (↓)
	Neurotransmitter	Aspartate (ASP) (pg/mL)	No change
		GABA (pg/mL)	1×10^{-7} (↑)
		Glutamate (pg/mL)	1×10^{-7} (↓)
Wistar rats (hypothalamus-pituitary) (F)	Gonadotropin-releasing hormone (Gn-RH)	Gn-RH (pg/mL)	1×10^{-7} (↓)
	Neurotransmitter	Aspartate (ASP) (pg/mL)	1×10^{-7} (↓)
		Gamma-aminobutyric acid (GABA) (pg/mL)	No change
		Glutamate (GLU) (pg/mL)	1×10^{-7} (↓)
Wistar rats (hypothalamus-pituitary) (M)	Gonadotropin-releasing hormone (Gn-RH)	Gn-RH (pg/mL)	1×10^{-7} (↓)
	Neurotransmitter	Aspartate (ASP) (pg/mL)	No change
		GABA (pg/mL)	1×10^{-7} (↑)
		Glutamate (pg/mL)	1×10^{-7} (↓)
Wistar rats (hypothalamus-pituitary)^{O}	Gonadotropin-releasing hormone (Gn-RH)	Gn-RH (pg/mL)	No change
	Neurotransmitter	Aspartate (ASP) (pg/mL)	No change
		Gamma-aminobutyric acid (GABA) (pg/mL)	No change

				Glutamate (GLU) (pg/mL)	No change	
		Wistar rats (hypothalamus-pituitary)$^{O+E}$ (F)	Gonadotropin-releasing hormone (Gn-RH)	Gn-RH (pg/mL)	1×10^{-7} (↓)	
			Neurotransmitter	Aspartate (ASP) (pg/mL)	1×10^{-7} (↓)	
				Gamma-aminobutyric acid (GABA) (pg/mL)	No change	
				Glutamate (GLU) (pg/mL)	1×10^{-7} (↓)	
	Neurotoxic	SH-SY5Y (neuroblastoma)	Apoptotic cells (%)	LOEC	$<1.0\times10^{-8}$ (↑)	Broniowska et al. 2016
			Caspase-3 activity	LOEC	1.0×10^{-7} (↑)	
				NOEC	1.0×10^{-8}	
			Cell cytotoxicity	LOEC	1.0×10^{-5} (↑)	
				NOEC	1.0×10^{-6}	
	Others	MCF-7	Cell invasion (23 weeks of exposure)	Change in cell index (effect conc.)	1.0×10^{-5} (↑)	Alamer and Darbre 2017
				Change in cell index (effect conc.)	1.0×10^{-5} (↑)	
OS	Androgenic	hAR-Yeast (*Saccharomyces cerevisiae*)	Luciferase activity	EC_{50}	1.1×10^{-4} (44% of DHT)	Kunz and Fent 2006
		PALM-hAR		EC_{50}	Inactive (LOEC >10 uM)	Jiménez-Díaz et al. 2013
	Anti-androgenic	hAR-Yeast (*Saccharomyces cerevisiae*)		IC_{50}	7.3×10^{-6} (77% of FT)	Kunz and Fent 2006

	PALM-hAR		IC_{50}	Inactive	Jiménez-Díaz et al. 2013
Anti-estrogenic	hERα-Yeast (*Saccharomyces cerevisiae*)		IC_{50}	6.8×10^{-3} (164% of 4HT)	Kunz and Fent 2006
Estrogenic			EC_{50}	Inactive	
	MCF-7	Cell proliferation	EC_{50}	Inactive (LOEC >10 uM)	Jiménez-Díaz et al. 2013

MDA-kb2, human breast cancer cell (triple (ER, PR, Her-2) negative); GH3, rat pituitary cell; MCF-7, human breast cancer cell (ER positive); HEK293, human embryonic kidney cell; U2-OS, human osteosarcoma; SH-SY5Y, human neuroblastoma; CHO, Chinese Hamster Ovary cell, IC_{50}: median inhibitory concentration; EC_{50}: median effective concentration; NOEC: no observed effect concentration; LOEC: lowest observed effect concentration; DHT: 4,5-dihydrotestosterone; 4HT: 4-hydrotamoxifen; FT: flutamide; arrows indicate the direction of positive (↑), negative (↓) effects.

1.4.2 *In vivo* observations

Most of the reported *in vivo* observations were made on rodents (Table 6). In Sprague–Dawley (SD) rats, 23 days of BP-3 dermal exposure significantly reduced hypothalamic *Erα*, *Erβ* and *Ar* gene expression (Krzyżanowska et al. 2018). After 7 days of BP-3 dermal exposure in pregnant C57BL/6J mice, placental weight and fetal weight were notably decreased. This study also found a significant increase in the offspring female ratio (Santamaria et al. 2020). In male Wistar rats, oral exposure to OMC significantly decreased prostate weight, sperm count, and serum testosterone levels (Axelstad et al. 2011). In ovariectomized female SD rats, 6 weeks of oral exposure to OMC significantly induced thickening of the uterine endometrium, epithelium, and myometrium (LOEC <57.5 mg/kg/day) (Seidlova´-Wuttke et al. 2006a). Additionally, in uterotrophic assays conducted on immature female Long Evans and Sprague–Dawley rats, acute (4-5 days) and chronic (12 weeks) oral exposure to OMC elicited significant increases in uterine weight at doses as low as 278.9 mg/kg/day (Klammer et al. 2005; Schlumpf et al. 2001; Seidlova´-Wuttke et al. 2006b), while AVB and HS had no effects at the maximum doses tested (Schlumpf et al. 2001).

Several studies have reported thyroid-disrupting effects of OMC. In female SD rats, 5 days and 12 weeks of oral exposure to OMC significantly reduced serum thyroid stimulating hormone (TSH) levels (Klammer et al. 2007; Seidlova´-Wuttke et al. 2006b), but in ovariectomized SD rats, the same treatment increased serum TSH levels (Schmutzler et al. 2004). None of the

test organisms showed alterations in serum T4 levels. Furthermore, OMC was shown to perturb serum T4 levels in both male and female Swiss Webster mice (Ferraris et al. 2020) and increase thyroid tissue weight in male and female Wistar rats (Axelstad et al. 2011).

Topical application of HS at 2 mg/cm^2 has been demonstrated to cause both sex and thyroid hormone disruption in Wistar Hannover rats, but the related phenotypic observations varied by sex and life stage (Erol et al. 2017). Female rats treated in the prenatal period showed a significant increase in LH and estradiol (E2), but those treated during lactation and infancy exhibited decreased E2 levels. TSH levels were also significantly reduced in both male and female rats treated during prenatal and lactational periods but increased after treatment during infancy only in the male group (Erol et al. 2017). Reproductive toxicity was evaluated only for OC in New Zealand white rabbits and Charles River CD-1 mice, but an effect was not observed (Odio et al. 1994).

Experimental *in vivo* studies also suggested potential neurotoxicity of AVB, BP-3, OC, and OMC, but most observations were made for endpoints related to oxidative stress and apoptosis (Table 6). According to Krzyżanowska et al. (2018), SD rats that dermally received BP-3 showed increased apoptosis in the brain. A similar conclusion was reported by the same group, who also demonstrated neuronal damage caused by a substantial increase in extracellular glutamate levels and lipid peroxidation in the brains of SD rats (Skórkowska et al. 2020). In addition, a significant increase in BP-

3 concentration was noted in the exposure group, suggesting that BP-3 can cross the blood–brain barrier (BBB) (Skórkowska et al. 2020). The same group also demonstrated significant induction of caspase-3 activity associated with decreased spatial memory ability (Pomirny et al. 2019).

Table 6. Endocrine disrupting activities of UV filters reported in studies using rodents and rabbits

Comp-ound	Hormonal activity	Experimental animals (male/female)	Exposure route/duration	Endpoints	Observa-tion	Toxicity value	Reference
AVB	Estrogenic (uterotrophic assay)	Long Evans rat (F)	Oral, (4 days)	Uterine weight, ED50	(-)	Inactive	Schlumpf et al. 2001
				Uterine weight, LOEC	(-)	>636 mg/kg/day	
BP-3	Androgenic	Sprague-Dawley rat (M, F)	Dermal (22-23 days)	Hypothalamic *Ar* expression	(↓)	100 mg/kg/day	Krzyżanowska et al. 2018
				Testosterone	(↑)		
	Estrogenic	Sprague-Dawley rat (M, F)	Dermal (22-23 days)	Hypothalamic *Erα, Erβ* expression	(↓)	100 mg/kg/day	
	Reproduction	C57BL/6J mouse (F)	Dermal (7 days)	Placenta weight	(↓)	50 mg/kg/day	Santamaria et al. 2020
				Offspring female sex ratio	(↑)	50 mg/kg/day	
				Fetal weight	(↓)	50 mg/kg/day	
	Thyroid	Sprague-Dawley rat (M, F)	Dermal (22-23 days)	TSH, fT4, fT3	(↑)	100 mg/kg/day	Krzyżanowska et al. 2018
				TSH	(↓)	100 mg/kg/day	Skórkowska et al. 2020

				fT4, fT3	(↑)	100 mg/kg/day	
	Neurotoxic	Sprague-Dawley rat (M, F)	Dermal (22-23 days)	Apoptosis (%)	(↑)	100 mg/kg/day	Krzyżanowska et al. 2018
				Caspase-3, -9 activity	(↑)	100 mg/kg/day	
				Caspase-3 acvitity	(↑)	100 mg/kg/day	Pomirny et al. 2019
				Glutamate (extracellular)	(↑)	100 mg/kg/day	
				Lipid peroxidation (MDA)	(↑)	100 mg/kg/day	Skórkowska et al. 2020
				Spatial memory	(↓)	100 mg/kg/day	Pomirny et al. 2019
				Spatial memory	(↓)	100 mg/kg/day	Skórkowska et al. 2020
				BP-3 concentration in brain	(↑)	100 mg/kg/day	
HS	Androgenic	Wistar Hannover rat (M)	Dermal, (GD 1 to GD 22)	FSH	(-)	2 mg/cm^2	Erol et al. 2017
			Dermal, (PND 2 to PND 21)		(-)	2 mg/cm^2	
			Dermal, (PND 21 to PND 26)		(↑)	2 mg/cm^2	
			Dermal, (GD 1	LH	(↑)	2 mg/cm^2	

		to GD 22)			
		Dermal, (PND 2 to PND 21)		(-)	2 mg/cm^2
		Dermal, (PND 21 to PND 26)		(↑)	2 mg/cm^2
		Dermal, (GD 1 to GD 22)	Testosterone	(↓)	2 mg/cm^2
		Dermal, (PND 2 to PND 21)		(-)	2 mg/cm^2
		Dermal, (PND 21 to PND 26)		(-)	2 mg/cm^2
Estrogenic	Wistar Hannover rat (F)	Dermal, (GD 1 to GD 22)	Estradiol	(↑)	2 mg/cm^2
		Dermal, (PND 2 to PND 21)		(↓)	2 mg/cm^2
		Dermal, (PND 21 to PND 26)		(↓)	2 mg/cm^2
		Dermal, (GD 1 to GD 22)	FSH	(-)	2 mg/cm^2
		Dermal, (PND 2 to PND 21)		(-)	2 mg/cm^2
		Dermal, (PND 21 to PND 26)		(-)	2 mg/cm^2
		Dermal, (GD 1 to GD 22)	Graffin follicle number	(↑)	2 mg/cm^2
		Dermal, (PND 2 to PND 21)		(-)	2 mg/cm^2
		Dermal, (PND 21 to PND 26)		(-)	2 mg/cm^2
		Dermal, (GD 1 to GD 22)	LH	(↑)	2 mg/cm^2
		Dermal, (PND 2		(-)	2 mg/cm^2

		to PND 21)				
		Dermal, (PND 21 to PND 26)		(-)	2 mg/cm^2	
Estrogenic (uterotrophic assay)	Long Evans rat (F)	Oral, (4 days)	Uterine weight, ED50	(-)	Inactive	Schlumpf et al. 2001
			Uterine weight, LOEC	(-)	>892 mg/kg/day	
Thyroid	Wistar Hannover rat (F)	Dermal, (GD 1 to GD 22)	Thyroid gland weight	(↓)	2 mg/cm^2	Erol et al. 2017
		Dermal, (PND 2 to PND 21)		(-)	2 mg/cm^2	
		Dermal, (PND 21 to PND 26)		(↑)	2 mg/cm^2	
		Dermal, (GD 1 to GD 22)	TSH	(↓)	2 mg/cm^2	
		Dermal, (PND 2 to PND 21)		(-)	2 mg/cm^2	
		Dermal, (PND 21 to PND 26)		(-)	2 mg/cm^2	
	Wistar Hannover rat (M)	Dermal, (GD 1 to GD 22)	Thyroid gland weight	(-)	2 mg/cm^2	
		Dermal, (PND 2 to PND 21)		(-)	2 mg/cm^2	
		Dermal, (PND 21 to PND 26)		(-)	2 mg/cm^2	
		Dermal, (PND 21 to PND 26)	Total T4	(↑)	2 mg/cm^2	
		Dermal, (GD 1 to GD 22)	TSH	(-)	2 mg/cm^2	
		Dermal, (PND 2 to PND 21)		(↓)	2 mg/cm^2	

			Dermal, (PND 21 to PND 26)		(↑)	2 mg/cm^2	
OC	Reproduction	New Zealand white rabbit (M/F)	Dermal, (GD 6 to GD 18)	Reproduction, LOEC	(-)	>267 mg/kg/day	Odio et al. 1994
		Virgin Charles River CD-1 mouse (F)	Oral (GD 8 to GD 12)	Reproduction, LOEC	(-)	>1000 mg/kg/day	
	Others	New Zealand white rabbit (F)	Dermal, (91 days)	Liver weight, LOEC	(↑)	264 mg/kg/day	
				Liver weight, NOEC		130 mg/kg/day	
		New Zealand white rabbit (M)		Liver weight, LOEC	(↑)	<130 mg/kg/day	
OMC	Androgenic	Wistar rat (M)	Oral (GD 7 to PND 17)	Prostate weight, LOEC	(↓)	1000 mg/kg/day	Axelstad et al. 2011
				Prostate weight, NOEC		750 mg/kg/day	
				Serum testosterone, LOEC	(↓)	<500 mg/kg/day	
				Sperm cound	(↓)	<500 mg/kg/day	
				Testes weight, LOEC	(↓)	<500 mg/kg/day	
	Estrogenic	Sprague-Dawley rat (F)	Oral (12 weeks)	Serum LH, LOEC	(↑)	278.9 mg/kg/day	Seidlova´-Wuttke et al. 2006b
				Serum LH, NOEC		52.5 mg/kg/day	
				Uterine weight, LOEC	(↑)	278.9 mg/kg/day	

			Uterine weight, NOEC		52.5 mg/kg/day	
	Wistar rat (F)	Oral (GD 7 to PND 17)	Serum estradiol, LOEC	(↓)	<500 mg/kg/day	Axelstad et al. 2011
Estrogenic (uterotrophic assay)	Long Evans rat (F)	Oral, (4 days)	Uterine weight, ED50	(↑)	934 mg/kg/day	Schlumpf et al. 2001
			Uterine weight, LOEC	(↑)	1035 mg/kg/day	
			Uterine weight, NOEC	(↑)	522 mg/kg/day	
	Sprague-Dawley rat (F)	Oral (5 days)	Pituitary, *Terp1* expression, LOEC	(↑)	333 mg/kg/day	Klammer et al. 2005
			Pituitary, *Terp1* expression, NOEC		100 mg/kg/day	
	Sprague-Dawley rat (F)	Oral (6 weeks)	Thickness of endometrium, LOEC	(↑)	<57.5 mg/kg/day	Seidlova´-Wuttke et al. 2006a
			Thickness of epithelium, LOEC	(↑)	<57.5 mg/kg/day	
			Thickness of myometrium, LOEC	(↑)	<57.5 mg/kg/day	
			Uterine weight, LOEC	(-)	>275 mg/kg/day	
		Oral (5 days)	Uterine wet weight, LOEC	(↑)	333 mg/kg/day	Klammer et al. 2005
			Uterine wet weight, NOEC		100 mg/kg/day	

			Uterus *C3* expression, LOEC	(↑)	1000 mg/kg/day	
			Uterus *C3* expression, NOEC		333 mg/kg/day	
			Uterus *Erb* expression, LOEC	(↑)	1000 mg/kg/day	
			Uterus, *Erb* expression, NOEC		333 mg/kg/day	
Thyroid	Sprague-Dawley rat (F)	Oral (5 days)	Hepatic Dio 1 activity, LOEC	(↓)	333 mg/kg/day	Klammer et al. 2007
		Oral (12 weeks)*	Hepatic Dio 1 activity, LOEC	(↓)	<54 mg/kg/day	Schmutzler et al. 2004
			Hepatic Dio 1 activity, LOEC	(↓)	<266 mg/kg/day	
		Oral (5 days)	Hepatic Dio 1 activity, NOEC		100 mg/kg/day	Klammer et al. 2007
			Serum TSH, LOEC	(↓)	333 mg/kg/day	
		Oral (12 weeks)*	Serum TSH, LOEC	(-)	>285 mg/kg/day	Schmutzler et al. 2004
		Oral (12 weeks)**	Serum TSH, LOEC	(↑)	<266 mg/kg/day	
		Oral (12 weeks)	Serum TSH, LOEC	(↓)	>278.9 mg/kg/day	Seidlova´-Wuttke et al. 2006b
		Oral (5 days)	Serum TSH, NOEC		100 mg/kg/day	Klammer et al. 2007

		Serum tT3, LOEC	(↓)	1000 mg/kg/day	
	Oral (12 weeks)*	Serum tT3, LOEC	(-)	>285 mg/kg/day	Schmutzler et al. 2004
	Oral (12 weeks)**	Serum tT3, LOEC	(↑)	<266 mg/kg/day	
	Oral (5 days)	Serum tT3, NOEC		333 mg/kg/day	Klammer et al. 2007
		Serum tT4, LOEC	(↓)	333 mg/kg/day	
	Oral (12 weeks)	Serum tT4, LOEC	(↓)	<54 mg/kg/day	Schmutzler et al. 2004
		Serum tT4, LOEC	(↓)	<52.5 mg/kg/day	Seidlova´-Wuttke et al. 2006b
		Serum tT4, LOEC	(-)	<278.9 mg/kg/day	
	Oral (5 days)	Serum tT4, NOEC		100 mg/kg/day	Klammer et al. 2007
		TPO activity, LOEC	(-)	>1000 mg/kg/day	
		TSH receptor protein induction (%), LOEC	(↑)	1000 mg/kg/day	
Swiss Webster mouse (F)	Oral (PND 1 to PND 22)	Serum tT4, LOEC	(↓)	1000 mg/kg/day	Ferraris et al. 2020
		Serum tT4, NOEC		500 mg/kg/day	

	Swiss Webster mouse (M)	Oral (PND 1 to PND 22)	Serum tT4, LOEC	(↓)	1000 mg/kg/day	
			Serum tT4, NOEC		500 mg/kg/day	
	Wistar rat (M/F)	Oral (GD 7 to PND 17)	Serum tT4, LOEC	(↓)	<500 mg/kg/day	Axelstad et al. 2011
			Thyroid gland weight, LOEC	(↑)	750 mg/kg/day	
			Thyroid gland weight, NOEC		750 mg/kg/day	

NOEC: no observed effect concentration; LOEC: lowest observed effect concentration; TSH: thyroid stimulating hormone; C3: complement component 3; *ER*: estrogen receptor; *Dio1*: deiodinase 1; tT4: total T4; M: male, F: female. Arrows indicate the direction of positive (↑), negative (↓) effects.

[a] indicate maximal uterine weight increase (% of EE2) of 2.01, 3.79, 22.21 for AVB, HS and OMC, respectively.

MDA: malondialdehyde.

Currently, information on endocrine-disrupting activities in aquatic organisms is mostly limited to BP-3, OC, and OMC (Table 7). The effective concentrations are between <0.001 (i.e., effective at the lowest experimental concentration of 0.001 mg/L) and 0.5 mg/L, which overlap with the range of concentrations that have been detected in the water environment, i.e., up to 10,400 ng/L, 3,730 ng/L and 640 ng/L for BP-3, OC, and OMC, respectively (Lee et al. 2018; Allinson et al. 2018; Bratkovics et al. 2015). Exposure to 9.8 mg/L BP-3 significantly upregulated whole-body levels of *erα, erβ,* and *vtg* mRNA expression in 50 hpf zebrafish embryo-larvae (Meng et al. 2020). As noted in a rodent study, long-term exposure to BP-3 significantly increased the female sex ratio at 0.39 mg/L along with an increased concentration of vtg (Kinnberg et al. 2015). This study also reported a significant increase in zebrafish ovarian and testicular maturity (Kinnberg et al. 2015). Exposure to 0.001 mg/L OMC decreased visceral E2 and vtg contents in adult zebrafish (*Danio rerio*) exposed at 120 days postfertilization (Zhou et al. 2019). However, different directions of estrogenic responses were also reported depending on species or developmental stage. For example, OMC exposure demonstrated a significant increase in vitellogenic oocytes in fathead minnow (*Pimephales promelas*) (Cristen et al. 2011) and an increase in liver vitellogenin (vtg) concentration in *D. rerio* (Zhang et al. 2016). For OC, plasma concentrations of E2, 11-ketotestosterone (11-KT), and vtg were increased in Japanese medaka (*Oryzias latipes*) after 28 days of exposure (Yan et al. 2020). In *O. latipes*, agonistic activities toward estrogen receptor (*er*) and androgen receptor (*ar*), as well as enhanced brain aromatase activity

(*cyp19b*) and downregulated hepatic *vtg* gene expression, were observed in both male and female fish (Zhang et al. 2016). In *D. rerio*, significant upregulation of the *er* and *cyp19b* genes along with downregulation of the *ar* gene were observed in gonads, whereas significant upregulation of the hepatic *vtg* gene was observed in both male and female fish. Moreover, exposure to a high concentration of OC stimulated ovary development in *D. rerio* (Zhang et al. 2016). Taken together, these findings suggest that OC and OMC disrupt sex hormone balances and gonadal development in several freshwater fish species.

Thyroid hormone effects have been reported only for BP-3 and OMC. Lee et al. (2018) concluded that exposure to BP-3 significantly decreased whole-body T3 but not T4 content at environmentally relevant concentrations, i.e., <0.03 mg/L. Exposure to OMC decreased the whole-body T3 content in *O. latipes* at 154 days postfertilization, suggesting its thyroid disruption potential (Lee et al. 2019). Supporting the thyroid-disrupting potential of OMC, decreased whole-body T3 and T4 and downregulation of thyroid-related genes were recently detected in zebrafish larvae following 120 hours of exposure to OMC (Chu et al. 2021).

Studies on invertebrate endocrine disruption have rarely been reported. In *Chironomus riparius* (*C. riparius*), a freshwater benthic invertebrate, OMC significantly upregulated the *ecdysone receptor* gene (Ozáez et al. 2013), while OC downregulated the same gene (Mu˜niz-Gonz´alez et al. 2020). In *Daphnia magna*, chronic exposure to OMC significantly increased brood size

and mean neonates per daphnid (LOEC: 0.02 mg/L) (Boyd et al. 2021).

Several animal studies have shown the neurotoxic effects of AVB, BP-3, OC, and OMC in aquatic organisms. Specifically, in the most recent study, exposure to AVB significantly increased whole-body acetylcholinesterase (AChE) activities and decreased mean velocity (mm/s) in zebrafish larva at 144 hpf (Liu et al. 2022). These authors also suggested that oxidative stress caused by AVB exposure might be associated with decreased zebrafish behavior. Campos and others (2017) exposed the aquatic invertebrate *C. riparius* to BP-3 or OC and found that this exposure caused no significant changes in the whole-body level of AchE. Other research groups have examined neurobehavioral changes following exposure to BP-3 in freshwater fish. For example, waterborne exposure to BP-3 significantly increased the mean swimming velocity (mm/s) and induced apoptosis-related gene expression changes (*bcl-2, bax, caspase-3*) and delayed axonal growth in zebrafish larvae (Tao et al. 2020). Studies from Moreira et al. (2022) and Chen et al. (2018) showed that BP-3 caused hypoactivity of adult zebrafish and larvae, respectively, as well as decreased shoal preference, which is an indicator of social behavior. Only one study has been conducted to assess the toxic effects of UV filters on the kidney. Chu et al. 2021 evaluated the effects of OMC in embryo-larval and adult male zebrafish, showing significant modulations of nephrogenesis-related (*wt1a*, *podocin*, *etv4*) gene expression.

Table 7. Endocrine disrupting activities of UV filters reported in studies using freshwater aquatic organism

Compound	Taxonomic group	Hormonal activity	Experimental animals	Exposure route/duration	Endpoints	Observation	Toxicity value (mg/L)	Reference
AVB	Invertebrate (crustacea)	Reproduction	*Daphnia magna*	Waterborne, 21 d	Mean neonates per daphnid, LOEC	(↑)	0.02	Boyd et al. 2021
					Number of molts, LOEC	(-)	>0.02	
					Time to first brood (days), LOEC	(-)	>0.02	
					Total brood size per daphnids, LOEC	(↑)	0.02	
	Fish	Neurotoxicity	*Danio rerio*	Waterborne, 144 hpf	Acetylcholineestrase activity (AChE), LOEC	(↑)	<0.01	Liu et al. 2022
					Mean velocity (mm/s), LOEC	(↓)	<0.01	
					Superoxide dismuatase activity (SOD), LOEC	(↑)	<0.01	
BP-3	Invertebrate (benthic)	Neurotoxicity	*Chironomus riparius*	Waterborne, 48 h	Whole body AChE acvitity, LOEC	(-)	>18.23 mg/kg sediment	Campos et al. 2017
	Fish	Sex hormone/reproduction	*Danio rerio*	Waterborne, 50 hpf	*erα, erβ, vtg* mRNA expressions	(↑)	9.8 (=43.3 μM)	Meng et al. 2020
				Waterborne, 50 hpf	Steroid synthesis related gene expression (*11β-hsd*)	(↓)	9.8 (=43.3 μM)	
				Waterborne, 60 dph	Sex differentiation (female ratio), LOEC	(↑)	0.39	Kinnberg et al. 2015

		Waterborne, 60 dph	Ovarian maturation stage, LOEC	(↑)	0.39	
		Waterborne, 60 dph	Testicular maturation stage, LOEC	(↑)	0.47	Kinnberg et al. 2015
		Waterborne, 12 d	Vitellogenin concentration, LOEC	(↑)	0.27	
Neurotoxicity	*Danio rerio*	Waterborne, 120 dpf	Mean velocity (mm/s) during dark phases, LOEC	(↑)	<0.01	Tao et al. 2020
	Betta splenders	Waterborne, 28 d	Maximum velocity (cm/s), LOEC	(↓)	<0.01	Chen et al. 2016
	Danio rerio	Waterborne, 15 d	Total distance traveled (m)	(↓)	<0.01	Moreira et al. 2022
			Mean velocity (m/s)	(↓)	<0.01	
			Spatial memory	(↓)	<0.01	
			Agonistic behavior (aggressiveness)	(↓)	0.1	
		Waterborne, 120 dpf	Touch response rate (%), LOEC	(↓)	<0.01	Tao et al. 2020
			Apoptosis related gene expression (*bcl-2, bax, caspase-3*), LOEC	(↑)	<0.01	
			Axonal growth	(↓)	<0.01	
			Neurogenesis gene expression (*mbp, gap43*), LOEC	(↑)	<0.01	

			Amphiprion ocellaris	Diet, 90 d	Social behavior, LOEC	(-)	>0.01	Chen et al. 2018
		Thyroid disruption	*Danio rerio*	Waterrborne, 144 dpf	Whole body T3 level, LOEC	(↓)	<0.03	Lee et al. 2018
					Whole body T4 level, LOEC	(-)	>0.3	
					Gene expression of HPT axis (*tg, dio1, ugt1ab*)	(↑)	0.1	
OC	Invertebrate (crustacea)	Reproduction	*Daphnia magna*	Waterborne, 21 d	Mean neonates per daphnid, LOEC	(-)	>0.0005	Boyd et al. 2021
					Number of molts, LOEC	(-)	>0.0005	
					Time to first brood (days), LOEC	(-)	>0.0005	
					Total brood size per daphnids, LOEC	(-)	>0.0005	
	Invertebrate (benthic)	Neurotoxicity	*Chironomus riparius*	Waterborne, 48 h	Whole body AChE acvitity, LOEC	(-)	>18.23 mg/kg sediment	Campos et al. 2017
		Sex hormone/reproduction		Waterborne, 96 h	Ecdysone receptor induction, LOEC	(↓)	<0.1	Mu˜niz-Gonz´alez et al. 2020
				Waterborne, 24 h	Ecdysone receptor induction, LOEC	(-)	>10	Ozáez et al. 2013
					Estrogen-related receptor induction, LOEC	(-)	>10	
	Fish	Sex hormone/reproduction	*Danio rerio* (female)	Waterborne, 28 d	Androgen receptor (*ar*) induction (hypothalamus-pituitary), LOEC	(-)	>3	Zhang et al. 2016
					Androgen receptor (*ar*)	(↓)	1	

induction (ovary), LOEC		
Androgen receptor (*ar*) induction (ovary), NOEC		0.1
Aromatase P450 (*cyp19b*) induction (hypothalamus-pituitary), LOEC	(-)	>3
Aromatase P450 (*cyp19b*) induction (ovary), LOEC	(↑)	1
Aromatase P450 (*cyp19b*) induction (ovary), NOEC		0.1
Estrogen receptor (*esr1*) induction (hypothalamus-pituitary), LOEC	(-)	>3
Estrogen receptor (*esr1*) induction (testis), LOEC	(↑)	1
Estrogen receptor (*esr1*) induction (testis), NOEC		0.1
Gonadosomatic index (GSI), LOEC	(↑)	3
Gonadosomatic index (GSI), NOEC		1
Oogenesis (previtellogenic oocytes) (%), LOEC	(-)	>3
Oogenesis (primary oocytes), (%) LOEC	(↓)	3
Oogenesis (primary oocytes), (%) NOEC		3
Oogenesis (vitellogenin	(↑)	3

				oocytes) (%), LOEC		
				Oogenesis (vitellogenin oocytes) (%), NOEC		3
				Retinol binding protein (*rbp2a*) (liver), LOEC	(↑)	<0.1
				Vitellogenin (*vtg1*) induction (liver), LOEC	(↑)	3
				Vitellogenin (*vtg1*) induction (liver), NOEC		1
				Androgen receptor (*ar*) induction (hypothalamus-pituitary), LOEC	(-)	>3
Fish	Sex hormone/reproduction	*Danio rerio* (male)	Waterborne, 28 d	Androgen receptor (*ar*) induction, (testis) LOEC	(↓)	1
				Androgen receptor (*ar*) induction, (testis) LOEC		0.1
				Aromatase P450 (*cyp19b*) induction (hypothalamus-pituitary), LOEC	(↑)	1
				Aromatase P450 (*cyp19b*) induction (hypothalamus-pituitary), NOEC		0.1
				Aromatase P450 (*cyp19b*) induction (ovary), LOEC	(↑)	3
				Aromatase P450 (*cyp19b*) induction (ovary), NOEC		1

		Estrogen receptor (*esr1*) induction (hypothalamus-pituitary), LOEC	(↓)	1	
		Estrogen receptor (esr1) induction (hypothalamus-pituitary), NOEC		0.1	
		Estrogen receptor (*esr1*) induction (testis), LOEC	(↑)	3	
		Estrogen receptor (*esr1*) induction (testis), NOEC		1	
		Gonadosomatic index (GSI), LOEC	(-)	>3	
		Retinol binding protein (*rbp2a*) (liver), NOEC	(↑)	<0.1	
		Vitellogenin (*vtg1*) induction (liver), LOEC	(↑)	3	
		Vitellogenin (*vtg1*) induction (liver), NOEC		1	
Oryzias latipes (female)	Waterborne, 28 d	F0 generation gonadosomatic index (GSI), LOEC	(↑)	0.5	Yan et al. 2020
Oryzias latipes (female)	Waterborne, 28 d	F0 generation gonadosomatic index (GSI), NOEC		0.05	
		F0 generation hepatic somatic index (HSI), LOEC	(↑)	0.5	
		F0 generation hepatic somatic index (HSI), NOEC		0.05	
		F0 generation plasma 11-KT concentration	(↑)	<0.005	

					(ng/mL), LOEC			
					F0 generation plasma E2 concentration (pg/mL), LOEC	(↑)	0.05	
					F0 generation plasma E2 concentration (pg/mL), NOEC		0.005	
					F0 generation plasma Vtg concentration (ng/mL), LOEC	(↑)	0.05	
					F0 generation plasma Vtg concentration (ng/mL), NOEC		0.005	
					F0 generation gonadosomatic index (GSI), LOEC	(-)	>0.5	
					F0 generation hepatic somatic index (HSI), LOEC	(-)	>0.5	
					F0 generation plasma 11-KT concentration (ng/mL), LOEC	(↑)	0.05	
					F0 generation plasma E2 concentration (pg/mL), LOEC	(↑)	<0.005	
					F0 generation plasma Vtg concentration (ng/mL), LOEC	(↑)	<0.005	
OMC	Invertebrate (benthic)	Sex hormone/reproduction	*Chironomus riparius*	Waterborne, 24 h	Ecdysone receptor induction, LOEC	(↑)	1	Ozáez et al. 2013

				Ecdysone receptor induction, NOEC		0.1	
				Estrogen-related receptor induction, LOEC	(-)	>10	
		Chironomus riparius (embryo)		Ecdysone receptor induction at 1 mg/L, LOEC	(↑)	1	
		Chironomus riparius (larva)		Ecdysone receptor induction at 1 mg/L, LOEC	(-)	>1	
Fish	Nephrotoxicity	*Danio rerio*	Waterborne, 120 dpf	Nephrogenesis related genes (*wt1a, etv4, sim1a*), LOEC	(↓)	8.7 (=10 μM)	Chu et al. 2021
				Nephrotoxicity related genes (*podocin, kim-1, nephrin*) (*p* for trend, p<0.05)	(↑)	Dose range 0.3-8.7 (1-30 μM)	
		Danio rerio	Waterborne, 21 d	Nephrogenesis related genes (*wt1a, etv4, sim1a*), LOEC	(↓)	2.9 (=10 μM)	
				Nephrotoxicity related genes (podocin, kim-1, nephrin), LOEC	(↓)	2.9 (=10 μM)	
	Neurotoxicity	*Danio rerio*	Waterborne, 120 dpf	F0 generation AChE activity, LOEC	(↑)	<0.001	Zhou et al. 2019
			Waterborne, 5 dpf	F1 generation whole body AChE activity, LOEC	(↑)	<0.001 (w/o OMC)	
				F1 generation whole body AChE activity, LOEC	(↑)	<0.1 (w/ OMC)	

		Waterborne, 120 dpf	Neurogenesis related gene (*mbp, gap43, syn2a*)	(↓)	0.3 (= 1 μM)	Chu et al. 2021
			Neurotoxic related gene (*gfap, c-fos*) (*p* for trend, p<0.05)	(↑)	Dose range 0.3-8.7 (1-30 μM)	
		Waterborne, 21 d	Neurogenesis related gene (*mbp, gap43, syn2a*)	(-)	>8.7 (=30 μM)	
			Neurotoxic related gene (*gfap, c-fos*)	(-)	>8.7 (=30 μM)	
Sex hormone/reproduction	*Danio rerio*	Waterborne, 120 dpf	F0 generation aromatase activity, LOEC	(-)	>0.1	Zhou et al. 2019
			F0 generation visceral E2 content, LOEC	(↓)	<0.001	
			F0 generation visceral Vtg content, LOEC	(↓)	<0.001	
	Pimephales promelas (female)	Waterborne, 14 d	3β-Hydroxysteroid dehydrogenase (*3β-HSD*) induction (hypothalamus-pituitary), LOEC	(-)	>0.394	Christen et al. 2011
			3β-Hydroxysteroid dehydrogenase (3β-HSD) induction (liver), LOEC	(↓)	0.245	
			3β-Hydroxysteroid dehydrogenase (3β-HSD) induction (liver), NOEC		0.0375	
			3β-Hydroxysteroid dehydrogenase (3β-HSD) induction (ovary), LOEC	(-)	>0.394	
			Androgen receptor (*ar*)	(-)	>0.394	

induction (hypothalamus-pituitary), LOEC		
Androgen receptor (ar) induction (liver), LOEC	(↓)	0.0375
Androgen receptor (ar) induction (liver), NOEC		0.0054
Androgen receptor (ar) induction (ovary), LOEC	(-)	>0.394
Estrogen receptor (*esr1*) induction (hypothalamus-pituitary), LOEC	(-)	>0.394
Estrogen receptor (esr1) induction (liver), LOEC	(-)	>0.394
Estrogen receptor (esr1) induction (ovary), LOEC	(-)	>0.394
Gonadosomatic index (GSI), LOEC	(-)	>0.394
Oogenesis (atretic follicles) (%), LOEC	(-)	>0.394
Oogenesis (previtellogenic oocytes) (%), LOEC	(-)	>0.394
Oogenesis (primary oocytes) (%), LOEC	(-)	>0.394
Oogenesis (vitellogenin oocytes) (%), LOEC	(↑)	0.394
Oogenesis (vitellogenin oocytes) (%), NOEC		0.245
Plasma Vtg concentration, LOEC	(-)	>0.394
Vitellogenin (*vtg1*) induction (hypothalamus-pituitary), LOEC	(-)	>0.394

Pimephales promelas (male)	Waterborne, 14 d	Vitellogenin (*vtg1*) induction (liver), LOEC	(-)	>0.394
		Vitellogenin (vtg1) induction (ovary), LOEC	(-)	>0.394
		3β-Hydroxysteroid dehydrogenase (*3β-HSD*) induction (hypothalamus-pituitary), LOEC	(-)	>0.394
		3β-Hydroxysteroid dehydrogenase (3β-HSD) induction (liver), LOEC	(↓)	0.0375
		3β-Hydroxysteroid dehydrogenase (3β-HSD) induction (liver), NOEC		0.0054
		3β-Hydroxysteroid dehydrogenase (3β-HSD) induction (testis), LOEC	(-)	>0.394
		Androgen receptor (*ar*) induction (hypothalamus-pituitary), LOEC	(-)	>0.394
		Androgen receptor (ar) induction (liver), LOEC	(-)	>0.394
		Androgen receptor (ar) induction (testis), LOEC	(-)	>0.394
		Estrogen receptor (*esr1*) induction (hypothalamus-pituitary), LOEC	(-)	>0.394
		Estrogen receptor (esr1) induction (liver), LOEC	(-)	>0.394
		Estrogen receptor (esr1) induction (testis), LOEC	(-)	>0.394
		Gonadosomatic index (GSI), LOEC	(-)	>0.394

			Nuptial tubercles score, LOEC	(-)	>0.394	
			Plasma Vtg concentration, LOEC	(↑)	0.245	
			Plasma Vtg concentration, NOEC		0.0375	
			Spermatogenesis (spermatocytes) (%), LOEC	(-)	>0.394	
			Spermatogenesis (spermatogonia) (%), LOEC	(-)	>0.394	
			Spermatogenesis (spermatotides) (%), LOEC	(↓)	0.394	
			Spermatogenesis (spermatotides) (%), NOEC		0.245	
			Vitellogenin (*vtg1*) induction (hypothalamus-pituitary), LOEC	(-)	>0.394	
			Vitellogenin (*vtg1*) induction (liver), LOEC	(-)	>0.394	
			Vitellogenin (*vtg1*) induction (testis), LOEC	(-)	>0.394	
Thyroid disruption	*Danio rerio* (larva)	Waterborne, 21 d	Plasma T3, LOEC	(↓)	0.29 (=1 µM)	Chu et al. 2021
			Plasma T3, NOEC		<0.29 (=1 µM)	
			Plasma T4, LOEC	(↓)	0.87 (=3 µM)	

		Plasma T4, NOEC		0.29 (=1 μM)	
Danio rerio (male)	Waterborne, 21 d	Plasma T3 (*p* for trend, $p<0.05$)	(↓)	Dose range 0.3-8.7 (1-30 μM)	
Oryzias latipes	Waterborne, 154 dpf	Whole body T3 content, LOEC	(↓)	0.5	Lee et al. 2019
		Whole body T3 content, NOEC		0.158	
		Whole body T4 content, LOEC	(-)	>5	

AChE: acetylcholinesterase; dph: day post hatch; E2: estradiol; 11-KT: 11-ketotestosterone; NOEC: no observed effect concentration; LOEC: lowest observed effect concentration. Arrows indicate the direction of positive (↑), negative (↓), or no (-) effects.

1.4.3 Epidemiological support

Epidemiological evidence associated with the endocrine disruption potentials of organic UV filters is thin and mostly based on studies in the US and Chinese populations (Table 8). In the US population, several studies reported a significant association between urinary concentrations of BP-3 and female infertility (n=789) (Arya et al. 2020) and a negative association with reproductive hormones (E2, FSH) in healthy adult women (n=509) (Pollack et al. 2018). In addition, an elevated urinary concentration of BP-3 was significantly associated with decreased serum fT4 and T4 in the NHANES study (Kim et al. 2017); however, no association between urinary levels of BP-3 and thyroid hormones was observed in Aker et al. (2018) and Przybyla et al. (2018).

In a prospective follow-up study on elementary and high school students in Shanghai, China, urinary concentrations of OMC (geometric mean of 1.77 and 2.28 ng/mL in baseline and follow-up groups, respectively) showed significant negative associations with testicular volume, anogenital development, and later onset of pubic hair development (Huang et al. 2020). In a case–control study on Chinese women with no history of pregnancy (n= 123), urinary levels of OC (range: 0.09-42.8 ng/mL) showed a significant association with polycystic ovary syndrome (PCOS) among those with BMI ≥ 24 (adjusted OR=1.512, 95% CI 1.043, 2.191), but HS did not show such an association (Guo et al. 2020).

Observations of outcomes related to neurotoxicity in humans have been made, but only for BP-3 (Fig. A4). Human population studies regarding neurodevelopmental dysfunction are also limited to BP-3. An article by Philippat and others (2017) reported neurodevelopmental dysfunction caused by BP-3 exposure in boys aged 3 and 5, specifically, behavioral disorders in response to parental exposure to BP-3. Moreover, another mother–child group study demonstrated a correlation between maternal and childhood urinary BP-3 concentrations and poorer prosocial behaviors at 10 years of age, which showed a stronger association in boys than in girls (Guo et al. 2020). On the other hand, Nakiwala et al. (2018) found no significant association between prenatal BP-3 exposure and verbal or performance IQ in boys aged 5 and 6.

The only related epidemiological studies on kidney function have been conducted by Kang and colleagues (Kang et al. 2019). These authors found a significant association between the urinary concentration of BP-3 and the albumin-to-creatinine (ACR) ratio in a healthy adult female population.

Table 8. Endocrine disruption associated with UV filters in epidemiological studies

Compounds	Country (region)	Population characteristics	N (M/F)	Median (Urinary concentration, ng/mL)	Range	Endocrine disruption	Reference
BP-3	United States	NHANSE (cross-sectional), women aged between 18-45 yr)	789 (F)	32.5	8.7-129.8	Self-reported infertility was significantly associated with urinary BP-3 concentration	Arya et al. 2020
		Healthy adult women aged 18-44 yr (prospective cohort)	509 (F)	3.8	<LOD-1422.1	Significant negative association between BP-3 and reproductive hormones (estradiol, FSH) in multichemical exposure model	Pollack et al. 2018
		Pregnant women (case-control study)	439 (F)	-	51.62-61.66	No significant relationship between BP-3 and thyroid hormone levels however, inverse trent with T3 and T4 was shown	Aker et al. 2018
		NHANSE (cross-sectional)	850/710	Male: 11.73[a]	NA	No significant association between BP-3 and thyroid hormone levels	Przybyla et al. 2018
				Female: 27.83[a]	NA	No significant association between BP-3 and thyroid hormone levels	
		National Health and Nutrition Examination Survey (NHANSE, retrospective cohort)	1829 (F)	9.49[d]	3.28-48135[d]	High urinary BP-3 concentration was associated with decreased fT4 and T4	Kim et al. 2017
	China (Jiangsu)	Mother-child cohort	386	0.60	Mother: 0.07-432	Associatioon between urinary BP-3 concentration and prosocial behavior	Guo et al. 2020
				1.76	Child: <LOD-783		
	France (Poitiers)	Mother-child cohort (mother-son pairs)	546	2.32	1.39-3.96	Associations with decreased emotional symtomp score at 5 yr old boy	Philippat et al. 2017

	France (Poitiers)	Mother-child cohort (mother-son pairs)	452	2.20	<LOD-63.7	No significant relationship between BP-3 concentration verbal, and performance IQ	Nakiwala et al. 2018
	South Korea	Healthy adult females (general population, cross-sectional study)	441 (F)	2.1	1.0-4.6	Positive association between BP-3 and albumin-to-creatinine ratio (ACR)	Kang et al. 2019
OMC	China (Shanghai)	521 elementary and high school students from suburban area (prospective follow-up study)	227/244	Baseline: 1.77[a]	Baseline: 1.599-1.956[b]	Negative association with stages of testicular volume and genital development, later pubertal onset of pubic hair (p<0.05)	Huang et al. 2020
	China (Shanghai)	521 elementary and high school students from suburban area (prospective follow-up study)	227/244	Follow-up: 2.28[a]	Follow-up: 1.985-2.622[b]		
OC	China (Shanghai)	521 elementary and high school students from suburban area (prospective follow-up study)	227/244	Baseline: 1.29[a]	Baseline: 1.080-1.548[b]	Associations were not measured due to low detection level (%>LOD: 0~11.9)	
	China (Shanghai)	521 elementary and high school students from suburban area (prospective follow-up study)	227/244	Follow-up: 0.50[a]	Follow-up: 0.221-1.110[b]		
HS	China (Shandong, Zhejiang province and Shanghai)	123 women with no history of pregnancy (age of 20-41) (case-control study)	123 (F)	NA	0.09-42.8	Significant association with polycystic ovary syndrome (PCOS) in women with BMI ≥24 (adjusted OR=1.512, 95%CI: 1.043, 2.191)	Gu et al. 2020a
	China (Shandong, Zhejiang province	123 women with no history of pregnancy (age of 20-41) (case-control study)	123 (F)	NA	0.04-8.21	No significant relationship between HS concentrations and PCOS	

	and Shanghai)

[a] : Geometric mean of urinary UV filter levels detected. (Initial study was conducted in October to November 2011; the follow-up study, in April to May 2013).
[b] : 95% confidence interval.
[c] : Creatinine adjusted value (μg/g creatinine)
NA: Not available.

1.5 Summary

The six major organic UV filters chosen in the present study, i.e., AVB, BP-3, HS, OMC, OS, and OC, have been increasingly used in many consumer and home products worldwide. These organic UV filters are frequently detected at high concentrations, especially in marine waters near recreational beaches and water bodies downstream of WWTPs, indicating their major sources of environmental release. While the available evidence is fragmented, with many information gaps, the current body of knowledge generally supports that the study UV filters AVB, BP-3, HS, OC, and OMC can disrupt sex or thyroid hormone balances and could impair the reproduction of both aquatic organisms and humans. Toxicological information, however, is mostly limited to sex hormone disruption and the reproductive effects of BP-3. Considering the production and usage, environmental exposure and ecological significance, and lack of toxicological knowledge, AVB, BP-3, OC and OMC were selected as test UV filters for further toxicological evaluation.

Based on this review, three main areas that warrant further studies were identified, including environmental occurrences and behavior of frequently used UV filters; ecotoxicological consequences and mechanisms of action related to endocrine disruption following long-term exposure at the levels found in the environment; and sensitive biomarkers that can be used for the risk assessment of UV filters. The information gleaned through this review will help better formulate directions for integrated risk assessment and management for this emerging group of environmental chemicals, aimed

toward the protection of both humans and aquatic ecosystems.

1.5 Research objectives and study design

The aim of the study was to investigate the potential thyroid disruption, neurotoxicity and kidney toxicity of major organic UV filters and the related mechanisms. The results of this study may contribute to our understanding of the pathways through which UV filters cause adverse outcomes at multiple life stages. The present study utilized zebrafish of different life stages, as zebrafish is a powerful model organism for modeling human disease and investigating the mechanisms of action of environmental pollutants.

The outline for the present study is shown in Fig. 4. In Chapter 2, we evaluate the TH-disrupting potential of AVB, BP-3, OC, and OMC in zebrafish of different life stages. Analysis of mRNA transcriptional changes in the hypothalamus-pituitary-thyroid axis (HPT axis) and genes involved in hepatic deiodination and TH metabolism provided insight into the underlying mechanisms of TH disruption. In Chapter 3, the neurotoxic potentials of the study UV filters are examined in zebrafish at multiple life stages. In Chapter 4, aiming to investigate the potential kidney toxic effects of the study UV filters, zebrafish at 3 different life stages are employed.

Taken together, our results demonstrate that commonly used UV filters have the potential to cause TH disruption and neuro- and kidney toxicity. Our findings can be considered basic toxicological information that could be considered in integrated risk assessment and management for major organic

UV filters and contribute to the current understanding of the potential health implications of these chemicals in both humans and aquatic animals.

This study was conducted with the approval of the Institutional Animal Care and Use Committee of Seoul National University, Seoul, Korea (protocols IACUC/SNU-190114-5-1 and SNU-210505-1).

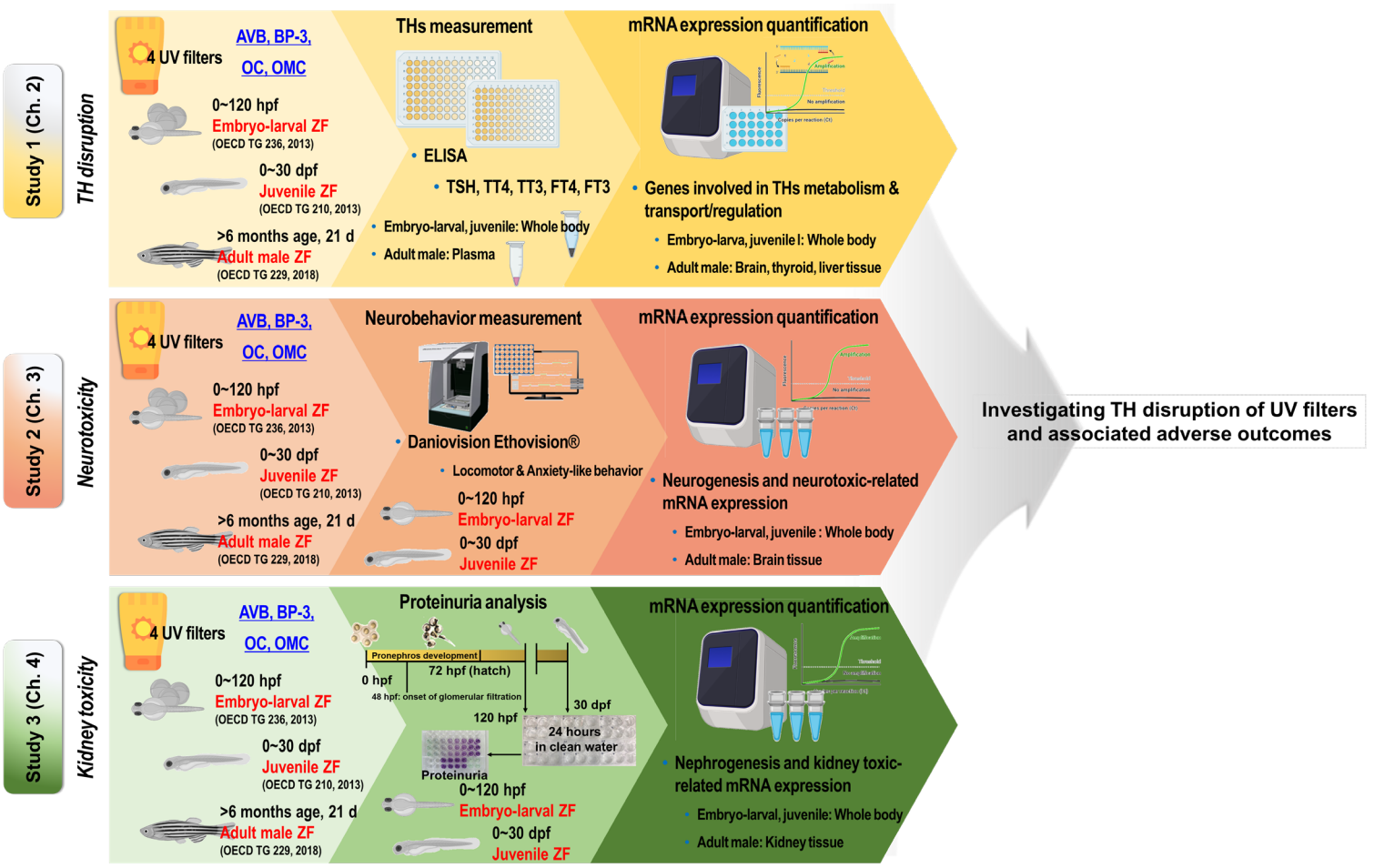

Study 1 (Ch. 2)
TH disruption
4 UV filters
AVB, BP-3,
OC, OMC
0~120 hpf
Embryo-larval ZF
(OECD TG 236, 2013)
0~30 dpf
Juvenile ZF
(OECD TG 210, 2013)
>6 months age, 21 d
Adult male ZF
(OECD TG 229, 2018)
THs measurement
ELISA
TSH, TT4, TT3, FT4, FT3
Embryo-larval, juvenile: Whole body
Adult male: Plasma
mRNA expression quantification
Genes involved in THs metabolism & transport/regulation
Embryo-larva, juvenile l: Whole body
Adult male: Brain, thyroid, liver tissue
Study 2 (Ch. 3)
Neurotoxicity
4 UV filters
AVB, BP-3,
OC, OMC
0~120 hpf
Embryo-larval ZF
(OECD TG 236, 2013)
0~30 dpf
Juvenile ZF
(OECD TG 210, 2013)
>6 months age, 21 d
Adult male ZF
(OECD TG 229, 2018)
Neurobehavior measurement
Daniovision Ethovision®
Locomotor & Anxiety-like behavior
0~120 hpf
Embryo-larval ZF
0~30 dpf
Juvenile ZF
mRNA expression quantification
Neurogenesis and neurotoxic-related mRNA expression
Embryo-larval, juvenile : Whole body
Adult male: Brain tissue
Study 3 (Ch. 4)
Kidney toxicity
4 UV filters
AVB, BP-3,
OC, OMC
0~120 hpf
Embryo-larval ZF
(OECD TG 236, 2013)
0~30 dpf
Juvenile ZF
(OECD TG 210, 2013)
>6 months age, 21 d
Adult male ZF
(OECD TG 229, 2018)
Proteinuria analysis
Pronephros development
0 hpf
72 hpf (hatch)
48 hpf: onset of glomerular filtration
30 dpf
120 hpf
24 hours
in clean water
Proteinuria
0~120 hpf
Embryo-larval ZF
0~30 dpf
Juvenile ZF
mRNA expression quantification
Nephrogenesis and kidney toxic-related mRNA expression
Embryo-larval, juvenile: Whole body
Adult male: Kidney tissue
Investigating TH disruption of UV filters and associated adverse outcomes

Fig. 4. Study design for the investigation of the thyroid hormone disrupting effects and associated toxicity of UV filters.

Chapter 2 Thyroid disruption of organic UV filters in zebrafish (*Danio rerio*) of different life stages

2.1 Introduction

The thyroid endocrine system is affected by many classes of environmental chemicals that alter the central regulation of hormonal feedback of thyroid hormones (THs) via the HPT axis, TH synthesis and secretion by the thyroid gland, the systemic transport and distribution of THs through serum binding proteins, the local cellular responses of TH transporters and peripheral deiodinases, the systemic metabolism and excretion of THs from the liver, or direct action on thyroid receptors (TRs) (Murk et al. 2013). Environmental chemicals are known to adversely impact the thyroid system and are defined as thyroid-disrupting chemicals (TDCs) (Crofton 2008).

The endocrine disrupting potential of UV filters is an emerging public health issue. Previous studies have reported potential health outcomes after exposure to UV filters. BP-3 is a well-known endocrine-disrupting chemical (EDC) that adversely affects reproductive functions and sex hormone homeostasis. The TH disruption potential of BP-3 has been suggested in several epidemiologic studies. For example, among the general adult population of the USA who participated in the 2007-2008 U.S. National

Health and Nutrition Examination Survey (NHANES), urinary BP-3 concentration was significantly associated with decreased serum total T4 in males and free T4 and total T4 in females, while no significant association with serum thyroid stimulating hormone (TSH) levels was observed (Kim et al. 2017). A similar negative association was observed for free T3 levels in a small cohort of pregnant women in Puerto Rico (Aker et al. 2016). The TH-disrupting effects of BP-3 have been demonstrated in several experimental models. In rat pituitary (GH3) and thyroid follicle (FRTL-5) cell lines, BP-3 exposure induced significant transcriptional changes in thyroid-regulating genes (Lee et al. 2018). In larval zebrafish, a 144 h exposure to BP-3 resulted in reduced TH levels and altered HTP-axis gene regulation (Lee et al. 2018). While the direction of the change was different, dermal exposure of female rats to BP-3 during the prenatal and adulthood periods significantly reduced plasma TSH concentrations and increased free T3 and free T4 (Skórkowska et al. 2020). AVB and OMC have also been investigated for their TH disrupting potentials. For example, AVB showed anti-thyroid activity in rat pituitary cells (GH3-TRE-Luc) cotreated with T3 (Klopci et al. 2017). In addition, exposure to OMC decreased whole-body T3 concentrations in Japanese medaka (*O. latipes*) after exposure at 154 days post-fertilization (dpf) and T4 and T3 levels in embryo larval zebrafish (*D. rerio*) at 120 hours post-fertilization. (Lee et al. 2019; Chu et al. 2021) In addition, plasma T4 levels were significantly reduced and genes in the HPT axis were downregulated after 21 days of exposure, suggesting TH disruption potential (Chu et al. 2021). While relatively comprehensive information is available on the TH-

disrupting effects of BP-3 and OMC, the toxicological properties of other commonly used organic UV filters, such as AVB and OC, have not yet been investigated. In addition, studies to demonstrate transcriptional level changes are mostly restricted to *in vitro* evaluations or studies of a specific life stage. Therefore, alterations of THs due to major organic UV filter exposure at multiple life stages need further investigation.

In the present study, embryo-larval (~120 hpf), early-life (~30 hpf) and adult male zebrafish (~21 d) were used to evaluate the TH-disrupting effects of 4 organic UV filters, AVB, BP-3, OC, and OMC, at the protein and gene transcriptional levels. We also examined thyroid histological changes in adult male zebrafish. The results from this study provide basic information to identify the most suitable organic UV filters in terms of TH-disrupting potential.

2.2 Materials and methods

2.2.1 Test chemicals

AVB (avobenzone; butyl methoxydibenzoylmethane), BP-3 (benzophenone-3; 2-hydroxy-4-methoxybenzophenone) OC (octocrylene; octocrylene), and OMC (octyl methoxycinnamate; ethyl hexyl methoxycinnamate (EHMC)) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO) was used as the solvent for the preparation of fish test solutions and was purchased from Junsei Chemical Co. (99.8%, Tokyo, Japan). The solvent concentration was set at 0.01% for AVB, OC and OMC and 0.005% for BP-3.

2.2.2 Zebrafish maintenance and exposure

Wild-type adult male zebrafish (*D. rerio*) were acclimated in a 45×35×40 cm aquarium containing charcoal-filtered dechlorinated tap water for at least two weeks prior to the experiment. The fish were maintained at 26±2 °C under a 16:8 h light/dark photoperiod in the Environmental Toxicology Laboratory at Seoul National University in Korea. The fish were fed *ad libitum* with commercially available mosquito larvae and Artemia (Green Fish, Seoul, Korea) twice daily. The remaining food and feces were removed one hour after feeding. The newly fertilized zebrafish embryos used in the present study were acquired by breeding sexually mature adult fish pairs (>6 months). The embryos were harvested in the early morning (8:00-9:00 am) of the day of exposure.

Embryo-larval exposure (120 hpf) was conducted following OECD test guideline 236 (OECD, 2013) with minor modifications (i.e., no. of fish used, test duration, and test concentration dilution factor). For each replicate, a total of 300 fertilized eggs were randomly placed into 1 L glass beakers with 500 mL exposure media. The test concentrations, i.e., 1, 3, 10 and 30 μM AVB, were determined using preliminary range-finding tests; 0.14, 0.44 and 1.40 μM BP-3; and 1, 3, 10, and 30 μM OMC were selected based on previous studies (Lee et al. 2018; Chu et al. 2021). Four replicates per treatment and control were used, and the exposure solution was renewed every day. Water chemistry was checked before and after water renewal. During the exposure,

embryo and larval survival, hatching rate, time to hatch and body weight (wet weight of 220 larvae) were recorded.

Early-life stage exposure (~30 dpf) was slightly modified from OCED test guideline 210 (OECD, 2013) with minor modifications (i.e., no. of fish used and observation endpoints). Briefly, 50 fertilized embryos (<4 hpf) were exposed to 0.1, 0.3, 1, or 3 μM AVB, 0.14, 0.44 or 1.40 μM BP-3, or 0.1, 0.3, 1, or 3 μM OMC based on our preliminary range-finding tests. Each group had four replicates, and water quality parameters (i.e., pH, conductivity, temperature, dissolved oxygen) were monitored at every water renewal. During the exposure period, embryo and larval survival, hatching rate, and time to hatching were recorded daily. From 5 dpf, fish were fed Gemma micro 75 (ZEBCARE, and *Artemia nauplii* (Greenfish, Korea). At the end of exposure, body weight (wet weight of 15 fish) was recorded for each group.

In the adult fish experiment, wild-type adult male zebrafish (>6 months) were exposed to the test UV filters or control treatment for 21 days. Tests were conducted according to OECD test guideline 229 (OECD, 2012) with some modifications (i.e., no. of fish used, no. of replicates, and observation endpoints). The test concentrations were 1, 3, 10, and 30 μM AVB and 0.14, 0.44, 1.40, and 4.44 μM BP-3. For OMC, the concentration range (1, 3, 10, 30 μM) was chosen. Four replicates with eight fish per replicate (5 L volume tanks with 4 L exposure media) were prepared for each control and treatment group. During the 21 d exposure, fish were fed twice daily. The exposure medium was replaced daily with newly prepared test solution. The pH,

conductivity, temperature, and dissolved oxygen concentration of the exposure media were routinely measured. No mortality was observed during the exposure period.

At the end of the exposure period (i.e., 120 hpf for embryo-larvae, 30 dpf for juvenile, and 21 d for adult), all surviving fish were euthanized using ice. Whole embryo-larval (120 hpf) and early life juvenile (30 dpf) zebrafish and plasma and tissues from adult male zebrafish were collected and kept frozen at 80 °C for further analysis.

This study was conducted with the approval of the Institutional Animal Care and Use Committee of Seoul National University, Seoul, Korea (IACUC/SNU-190114-5-1, SNU-210505-1).

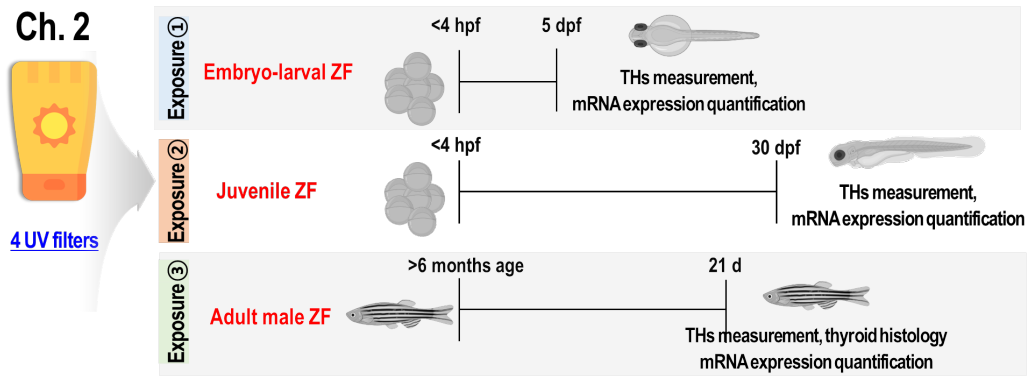


Fig. 5. Experimental design of Chapter 2.

2.2.3 Thyroid hormone measurement

For thyroid hormone measurement in embryo-larval and juvenile zebrafish, a total of 220 larvae and 15 juveniles were pooled. Samples were then rinsed with 1×PBS twice and homogenized with a tissue grinder in 1×PBS (250 and 200 μL for embryo-larval and juvenile fish, respectively). Homogenates were then disrupted by sonication for 15 min on ice and centrifuged at 10000×g for 15 min. The supernatants were transferred to new 1.7 mL tubes. For adult male zebrafish, a blood sample was collected from the caudal vein using a microcapillary tube. Blood samples from eight fish from each replicate were pooled for one measurement (n=4 per treatment or control). Plasma was separated by centrifugation (5000×g for 10 min) and stored at -80 °C until further analysis.

On the day of analysis, TSH (TSH; Cat No. CSB-EQ027261FI, Cusabio), total thyroxine (TT4; Cat No., LDN), total triiodothyronine (TT3; Cat No., LDN), free T4 (fT4; Cat No., LDN), and free T3 (fT3; Cat No., LDN) were quantified for embryo-larval fish (120 hpf), and TSH, TT4, TT3, and fT3 were measured for juvenile and adult zebrafish using enzyme-linked immunosorbent assay (ELISA) kits following the manufacturer's recommendations.

2.2.4 Gene transcription analysis

The transcription of genes related to the HPT axis and hepatic metabolism was quantified (Table 9).

NucleoSpin® RNA mini-kits (Macherey-Nagel, Germany) were used for total RNA isolation from embryo-larval (30 larvae/replicate), juvenile (10 larva/replicate) and adult male zebrafish (5 tissues/replicate). The RNA quality of all samples was checked by absorbance measurements (260/280 ratio 1.8-2.2) by using an EpochTM-Take 3 microplate spectrophotometer (BioTek Instruments Inc., Winooski, VT, USA). RNA (80-100 ng/μL) samples were used for reverse transcription using an IScript cDNA synthesis kit (Bio–Rad, Hercules, CA, USA).

Quantitative real-time polymerase chain reaction (qPCR) was performed on an Applied Biosystems QuantStudio 3 (Applied Biosystems, Foster City, CA, USA). A qRT–PCR mixture of 10 μL of 2×SYBR Green^{TM} PCR master mix (Applied Biosystems), 0.1 μL of each 10 pmol forward and reverse PCR primer, 7.8 μL of nuclease free water (NFR), and 2 μL of cDNA sample was used. The reaction mixtures were then preincubated at 95 °C for 10 min, followed by 40 cycles of amplification at 95 °C for 10 s, 60 °C for 20 s, and 72 °C for 20 s. Normalized values were expressed as the “fold difference”. For quantification of the results, the threshold cycle (Ct) values for each gene of interest were normalized to those of reference genes using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Among eight candidate housekeeping

genes *(18S rRNA, β-actin, b2m, ef1a, ef1al1, rpl8, rpl13,* and *rpl13a)*, *rpl8*, *rpl13* and *rpl13a* were identified as the most stable genes in the brain, thyroid and liver, respectively, by our geNorm analysis for each adult tissue (data not shown). Therefore, *rpl8* was used as the reference gene for the embryo-larval and juvenile stages and for normalization.

Table 9. Primer sequences for qRT–PCR analysis used in this study

Gene	Protein name	Primer sequence (5'-3')	Accession no.
Reference gene			
rpl8[a*]	60S ribosomal protein L8	F: ttgttggtgttgttgctggt R: ggatgctcaacagggttcat	NM_200713.1
rpl13[b]	60S ribosomal protein L13	F: accgcaccaaactcatc R: cgggccttctctttctt	NM_198143.1
rpl13a[c]	60S ribosomal protein L13a	F: agctcaagatggcaacacag R: aagttcttctcgtcctcc	NM_212784
Target gene			
trh	Thyrotropin-releasing hormone	F: gctctctccgtcggtctgtt R: gcgagatccgtgctgatga	NM_001012365
trhr	Thyrotropin-releasing hormone receptor	F: cagtgccatcaaccctctga R: ggcagcgcggaacttct	NM_001114688
tshb	Thyrotropin subunit beta	F: gcagatcctcacttcacctacc R: gcacaggtttggagcatctca	NM_181494.2
tra	Thyroid hormone receptor alpha	F: gccgcttcctgcacatg R: agcggcgggaacagttc	NM_131396
trb	Thyroid hormone receptor beta	F: tggcatggctacagacttggt R: tcagcttccgcttggctaa	NM_131340
tshr	Thyroid-stimulating hormone receptor	F: gcgagaagggagaggaggtt R: tcctcgcaagggttgaactc	NM_001145763
nis	Sodium-iodide symporter	F: ggtggcatgaaggctgtaat R: gatacgggatccattgttgg	NM_001089391
tpo	Thyroid peroxidase	F: gttcggtctgccaggacact R: tccaagcgcttcagcagagt	EU267076
tg	Thyroglobulin	F: gtctcttgagtgttcgaatgacaag R: aaaggcgggccattaagg	XM_689200
dio1	Type I iodothyronine deiodinase	F: aacttggaggagaggcttgct R: agggcatggagggtcttctt	BC076008
dio2	Type II iodothyronine deiodinase	F: cgcgaaatgggcttgct R: ccaggcaaaatctgcaaagtta	NM_212789
dio3[d]	Type III iodothyronine deiodinase	F: tcgcacctgtattctccgtg R: gcgcgtacggagcttacttc	NM_001256003
dio3[e]	Type III iodothyronine deiodinase	F: tcctgaccgcccttcatgac R: tgcgcctcctcgatgtacac	NM_001177935
ugt1ab	UDP-glucuronosyltransferase	F: gccagctttgatgaacttgcc R: aactcctccagttccttggtt	NM_213422.2
sult1 st5	Sulfotransferase	F: cccatccaacttttgcctcg R: ggatccccataccaattgtcct	NM_001199903.1
ttr	Transthyretin	F: cgggtggagtttgacacttt R: gctcagaaggagagccagtg	BC081488

Reference genes used in the brain[a], thyroid and liver[b], kidneys[c], and the embryo-larval and juvenile stage[*]; primer sequence for *dio3*, used in the brain, thyroid, kidney[d] and liver[e].

2.2.6 Statistical analysis

Statistical analysis was performed by using SPSS® version 22.0 for Windows® (SPSS, Chicago, IL, USA). Differences between the solvent control and exposure groups were investigated using one-way of variance (ANOVA) with Dunnett's test. Spearman's rank correlation was used to determine the dose–response relationship. A value of p less than 0.01 was considered statistically significant. All data are expressed as the mean values and standard deviation (SD). Spearman correlation analysis was conducted to determine relationships between developmental endpoints (time to hatch and body weight) and molecular endpoints (THs and THs regulating genes) were analyzed using R studio (Boston, MA, USA).

2.3 Results

2.3.1 Fish survival and developmental effects

2.3.1.1 Embryo-larval

The survival and developmental effects of test UV filters in embryo-larval zebrafish are demonstrated in Fig 6A. Hatchability (%) was significantly decreased when exposed to 0.14 and 0.44 μM BP-3, however no effect was observed at the highest concentration. Time to hatch was significantly increased when zebrafish embryos at ≥3 μM AVB and ≥0.44 μM BP-3. The relationship between time to hatch and body weight were determined by using Spearman correlation analysis (Fig 7A-D). Time to hatch was significantly correlated with *dio3* gene expression in embryo-larval zebrafish exposed to AVB (rho > 0.5 or rho < -0.5, p<0.05). For BP-3, *dio1* gene and TSH, TT4, TT3, and FT3 were highly correlated with time to hatch. mRNA expressions of *trhr*, *tpo*, and *dio1* were significantly correlated with time to hatch after exposure to OC and OMC, respectively (Fig 7C-D) (rho > 0.5 or rho < -0.5, p<0.05).

2.3.1.2 Juvenile

The survival and developmental effects of test UV filters in juvenile zebrafish are shown in Fig 6B. Fish survival (%) was significantly decreased when exposed to 3 μM OMC. Time to hatch was significantly increased when zebrafish embryos were exposed to AVB and BP-3. The relationship between time to hatch and body weight were determined by using Spearman correlation analysis (Fig 8A-D). Following exposure to AVB, BW was significantly correlated with *tshr* and *tpo* genes, and time to hatch was significantly correlated with *trβ*, *ugt1ab*, *sult1 st5*, *ttr* and *dio1* genes. For BP-3, time to hatch was negatively correlated with *trh*, *trhr*, *tshβ*, and *ttr* genes (rho > 0.5 or rho < -0.5, $p<0.05$). Upon exposure to OC, BW was significantly correlated with whole body TT4 level and time to hatch was significantly associated with *tshβ*, *sult1 st5* genes, and whole body TSH and FT3 levels. No significant relationship was observed for OMC.

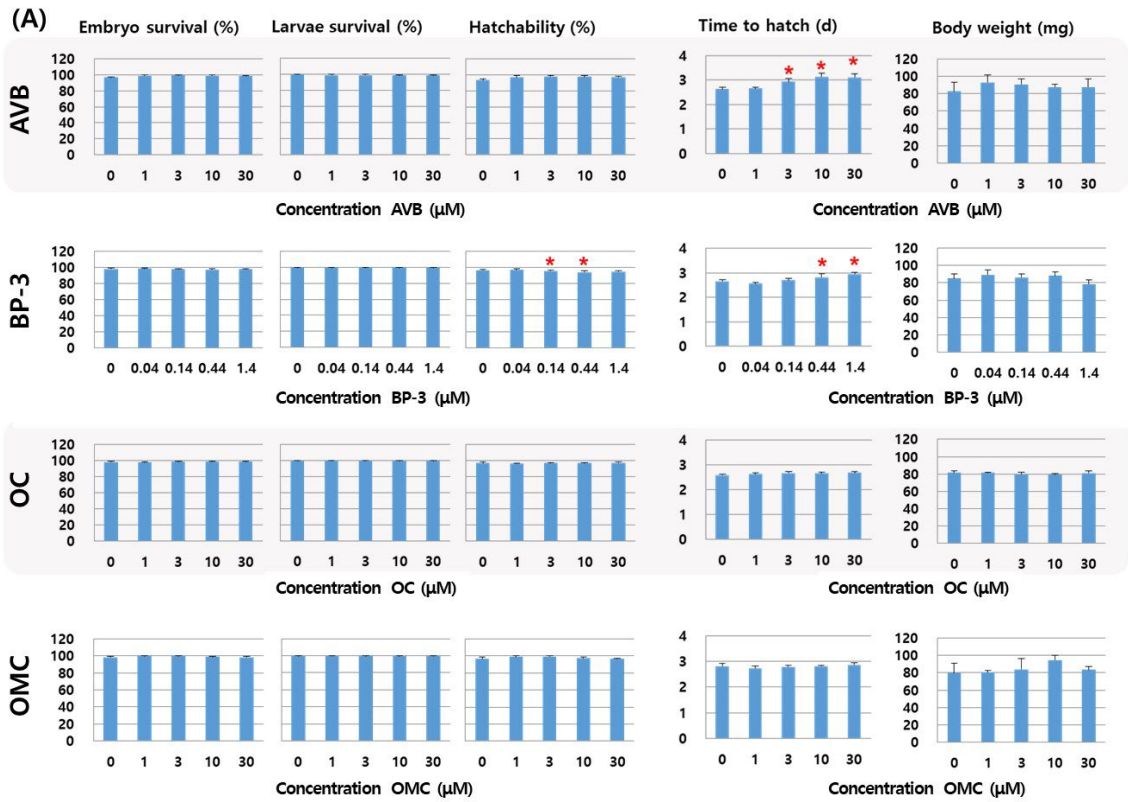

(A)
Embryo survival (%)
Larvae survival (%)
Hatchability (%)
Time to hatch (d)
Body weight (mg)
AVB
BP-3
OC
OMC
Concentration AVB (μM)
Concentration BP-3 (μM)
Concentration OC (μM)
Concentration OMC (μM)

Fig. 6. Survival and developmental effects in embryo-larval (A) and juvenile (B) zebrafish after exposure to UV filters. Survival and hatchability (%), hatchability (%), time to hatch (day), body weight (mg) are shown. Values represent mean ± standard deviation of four replicates. *Asterisks indicate significant differences from the solvent control (p<0.05).

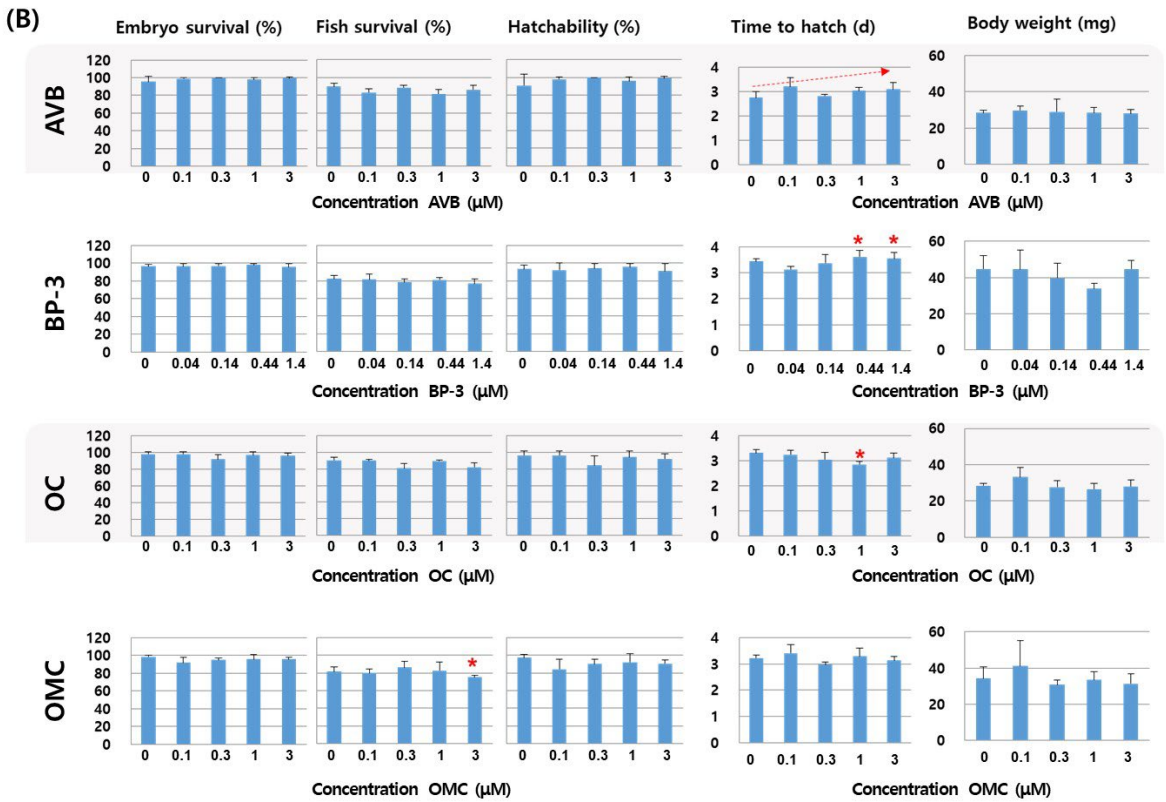


Fig. 6. Continued.

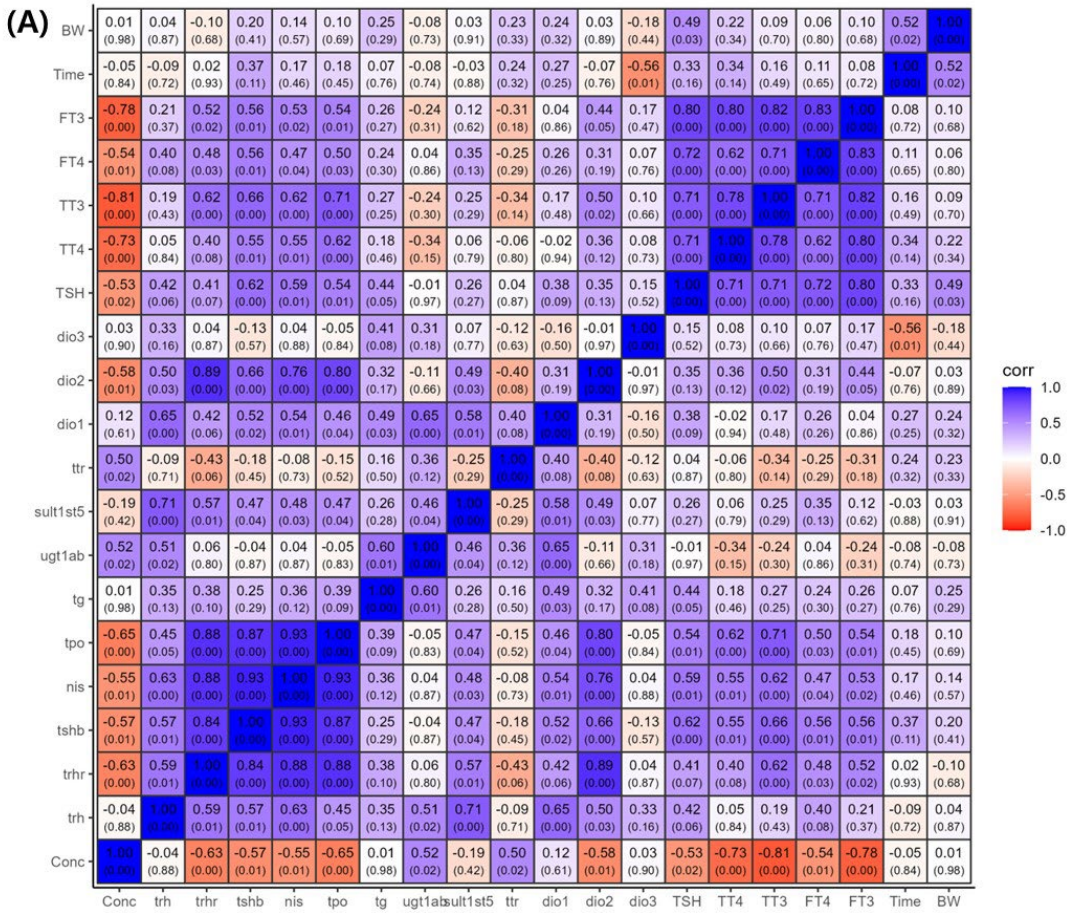

(A)
BW 0.01 (0.98) 0.04 (0.87) -0.10 (0.68) 0.20 (0.41) 0.14 (0.57) 0.10 (0.69) 0.25 (0.29) -0.08 (0.73) 0.03 (0.91) 0.23 (0.33) 0.24 (0.32) 0.03 (0.89) -0.18 (0.44) 0.49 (0.03) 0.22 (0.34) 0.09 (0.70) 0.06 (0.80) 0.10 (0.68) 0.52 (0.02) 1.00 (0.00)
Time -0.05 (0.84) -0.09 (0.72) 0.02 (0.93) 0.37 (0.11) 0.17 (0.46) 0.18 (0.45) 0.07 (0.76) -0.08 (0.74) -0.03 (0.88) 0.24 (0.32) 0.27 (0.25) -0.07 (0.76) -0.56 (0.01) 0.33 (0.16) 0.34 (0.14) 0.16 (0.49) 0.11 (0.65) 0.08 (0.72) 1.00 (0.00) 0.52 (0.02)
FT3 -0.78 (0.00) 0.21 (0.37) 0.52 (0.02) 0.56 (0.01) 0.53 (0.02) 0.54 (0.01) 0.26 (0.27) -0.24 (0.31) 0.12 (0.62) -0.31 (0.18) 0.04 (0.86) 0.44 (0.05) 0.17 (0.47) 0.80 (0.00) 0.80 (0.00) 0.82 (0.00) 0.83 (0.00) 1.00 (0.00) 0.08 (0.72) 0.10 (0.68)
FT4 -0.54 (0.01) 0.40 (0.08) 0.48 (0.03) 0.56 (0.01) 0.47 (0.04) 0.50 (0.03) 0.24 (0.30) 0.04 (0.86) 0.35 (0.13) -0.25 (0.29) 0.26 (0.26) 0.31 (0.19) 0.07 (0.76) 0.72 (0.00) 0.62 (0.00) 0.71 (0.00) 1.00 (0.00) 0.83 (0.00) 0.11 (0.65) 0.06 (0.80)
TT3 -0.81 (0.00) 0.19 (0.43) 0.62 (0.00) 0.66 (0.00) 0.62 (0.00) 0.71 (0.00) 0.27 (0.25) -0.24 (0.30) 0.25 (0.29) -0.34 (0.14) 0.17 (0.48) 0.50 (0.02) 0.10 (0.66) 0.71 (0.00) 0.78 (0.00) 1.00 (0.00) 0.71 (0.00) 0.82 (0.00) 0.16 (0.49) 0.09 (0.70)
TT4 -0.73 (0.00) 0.05 (0.84) 0.40 (0.08) 0.55 (0.01) 0.55 (0.01) 0.62 (0.00) 0.18 (0.46) -0.34 (0.15) 0.06 (0.79) -0.06 (0.80) -0.02 (0.94) 0.36 (0.12) 0.08 (0.73) 0.71 (0.00) 1.00 (0.00) 0.78 (0.00) 0.62 (0.00) 0.80 (0.00) 0.34 (0.14) 0.22 (0.34)
TSH -0.53 (0.02) 0.42 (0.06) 0.41 (0.07) 0.62 (0.00) 0.59 (0.01) 0.54 (0.01) 0.44 (0.05) -0.01 (0.97) 0.26 (0.27) 0.04 (0.87) 0.38 (0.09) 0.35 (0.13) 0.15 (0.52) 1.00 (0.00) 0.71 (0.00) 0.71 (0.00) 0.72 (0.00) 0.80 (0.00) 0.33 (0.16) 0.49 (0.03)
dio3 0.03 (0.90) 0.33 (0.16) 0.04 (0.87) -0.13 (0.57) 0.04 (0.88) -0.05 (0.84) 0.41 (0.08) 0.31 (0.18) 0.07 (0.77) -0.12 (0.63) -0.16 (0.50) -0.01 (0.97) 1.00 (0.00) 0.15 (0.52) 0.08 (0.73) 0.10 (0.66) 0.07 (0.76) 0.17 (0.47) -0.56 (0.01) -0.18 (0.44)
dio2 -0.58 (0.01) 0.50 (0.03) 0.89 (0.00) 0.66 (0.00) 0.76 (0.00) 0.80 (0.00) 0.32 (0.17) -0.11 (0.66) 0.49 (0.03) -0.40 (0.08) 0.31 (0.19) 1.00 (0.00) -0.01 (0.97) 0.35 (0.13) 0.36 (0.12) 0.50 (0.02) 0.31 (0.19) 0.44 (0.05) -0.07 (0.76) 0.03 (0.89)
dio1 0.12 (0.61) 0.65 (0.00) 0.42 (0.06) 0.52 (0.02) 0.54 (0.01) 0.46 (0.04) 0.49 (0.03) 0.65 (0.00) 0.58 (0.01) 0.40 (0.08) 1.00 (0.00) 0.31 (0.19) -0.16 (0.50) 0.38 (0.09) -0.02 (0.94) 0.17 (0.48) 0.26 (0.26) 0.04 (0.86) 0.27 (0.25) 0.24 (0.32)
ttr 0.50 (0.02) -0.09 (0.71) -0.43 (0.06) -0.18 (0.45) -0.08 (0.73) -0.15 (0.52) 0.16 (0.50) 0.36 (0.12) -0.25 (0.29) 1.00 (0.00) 0.40 (0.08) -0.40 (0.08) -0.12 (0.63) 0.04 (0.87) -0.06 (0.80) -0.34 (0.14) -0.25 (0.29) -0.31 (0.18) 0.24 (0.32) 0.23 (0.33)
sult1st5 -0.19 (0.42) 0.71 (0.00) 0.57 (0.01) 0.47 (0.04) 0.48 (0.03) 0.47 (0.04) 0.26 (0.28) 0.46 (0.04) 1.00 (0.00) -0.25 (0.29) 0.58 (0.01) 0.49 (0.03) 0.07 (0.77) 0.26 (0.27) 0.06 (0.79) 0.25 (0.29) 0.35 (0.13) 0.12 (0.62) -0.03 (0.88) 0.03 (0.91)
ugt1ab 0.52 (0.02) 0.51 (0.02) 0.06 (0.80) -0.04 (0.87) 0.04 (0.87) -0.05 (0.83) 0.60 (0.01) 1.00 (0.00) 0.46 (0.04) 0.36 (0.12) 0.65 (0.00) -0.11 (0.66) 0.31 (0.18) -0.01 (0.97) -0.34 (0.15) -0.24 (0.30) 0.04 (0.86) -0.24 (0.31) -0.08 (0.74) -0.08 (0.73)
tg 0.01 (0.98) 0.35 (0.13) 0.38 (0.10) 0.25 (0.29) 0.36 (0.12) 0.39 (0.09) 1.00 (0.00) 0.60 (0.01) 0.26 (0.28) 0.16 (0.50) 0.49 (0.03) 0.32 (0.17) 0.41 (0.08) 0.44 (0.05) 0.18 (0.46) 0.27 (0.25) 0.24 (0.30) 0.26 (0.27) 0.07 (0.76) 0.25 (0.29)
tpo -0.65 (0.00) 0.45 (0.05) 0.88 (0.00) 0.87 (0.00) 0.93 (0.00) 1.00 (0.00) 0.39 (0.09) -0.05 (0.83) 0.47 (0.04) -0.15 (0.52) 0.46 (0.04) 0.80 (0.00) -0.05 (0.84) 0.54 (0.01) 0.62 (0.00) 0.71 (0.00) 0.50 (0.03) 0.54 (0.01) 0.18 (0.45) 0.10 (0.69)
nis -0.55 (0.01) 0.63 (0.00) 0.88 (0.00) 0.93 (0.00) 1.00 (0.00) 0.93 (0.00) 0.36 (0.12) 0.04 (0.87) 0.48 (0.03) -0.08 (0.73) 0.54 (0.01) 0.76 (0.00) 0.04 (0.88) 0.59 (0.01) 0.55 (0.01) 0.62 (0.00) 0.47 (0.04) 0.53 (0.02) 0.17 (0.46) 0.14 (0.57)
tshb -0.57 (0.01) 0.57 (0.01) 0.84 (0.00) 1.00 (0.00) 0.93 (0.00) 0.87 (0.00) 0.25 (0.29) -0.04 (0.87) 0.47 (0.04) -0.18 (0.45) 0.52 (0.02) 0.66 (0.00) -0.13 (0.57) 0.62 (0.00) 0.55 (0.01) 0.66 (0.00) 0.56 (0.01) 0.56 (0.01) 0.37 (0.11) 0.20 (0.41)
trhr -0.63 (0.00) 0.59 (0.01) 1.00 (0.00) 0.84 (0.00) 0.88 (0.00) 0.88 (0.00) 0.38 (0.10) 0.06 (0.80) 0.57 (0.01) -0.43 (0.06) 0.42 (0.06) 0.89 (0.00) 0.04 (0.87) 0.41 (0.07) 0.40 (0.08) 0.62 (0.00) 0.48 (0.03) 0.52 (0.02) 0.02 (0.93) -0.10 (0.68)
trh -0.04 (0.88) 1.00 (0.00) 0.59 (0.01) 0.57 (0.01) 0.63 (0.00) 0.45 (0.05) 0.35 (0.13) 0.51 (0.02) 0.71 (0.00) -0.09 (0.71) 0.65 (0.00) 0.50 (0.03) 0.33 (0.16) 0.42 (0.06) 0.05 (0.84) 0.19 (0.43) 0.40 (0.08) 0.21 (0.37) -0.09 (0.72) 0.04 (0.87)
Conc 1.00 (0.00) -0.04 (0.88) -0.63 (0.00) -0.57 (0.01) -0.55 (0.01) -0.65 (0.00) 0.01 (0.98) 0.52 (0.02) -0.19 (0.42) 0.50 (0.02) 0.12 (0.61) -0.58 (0.01) 0.03 (0.90) -0.53 (0.02) -0.73 (0.00) -0.81 (0.00) -0.54 (0.01) -0.78 (0.00) -0.05 (0.84) 0.01 (0.98)
Conc trh trhr tshb nis tpo tg ugt1ab sult1st5 ttr dio1 dio2 dio3 TSH TT4 TT3 FT4 FT3 Time BW
corr
1.0
0.5
0.0
-0.5
-1.0

Fig. 7. Spearman correlation coefficients (r) between developmental endpoints (time to hatch and body weight) and thyroid related molecular endpoints (thyroid hormones and genes) in embryo-larval zebrafish after AVB (A), BP-3 (B), OC (C), and OMC (D) exposure.

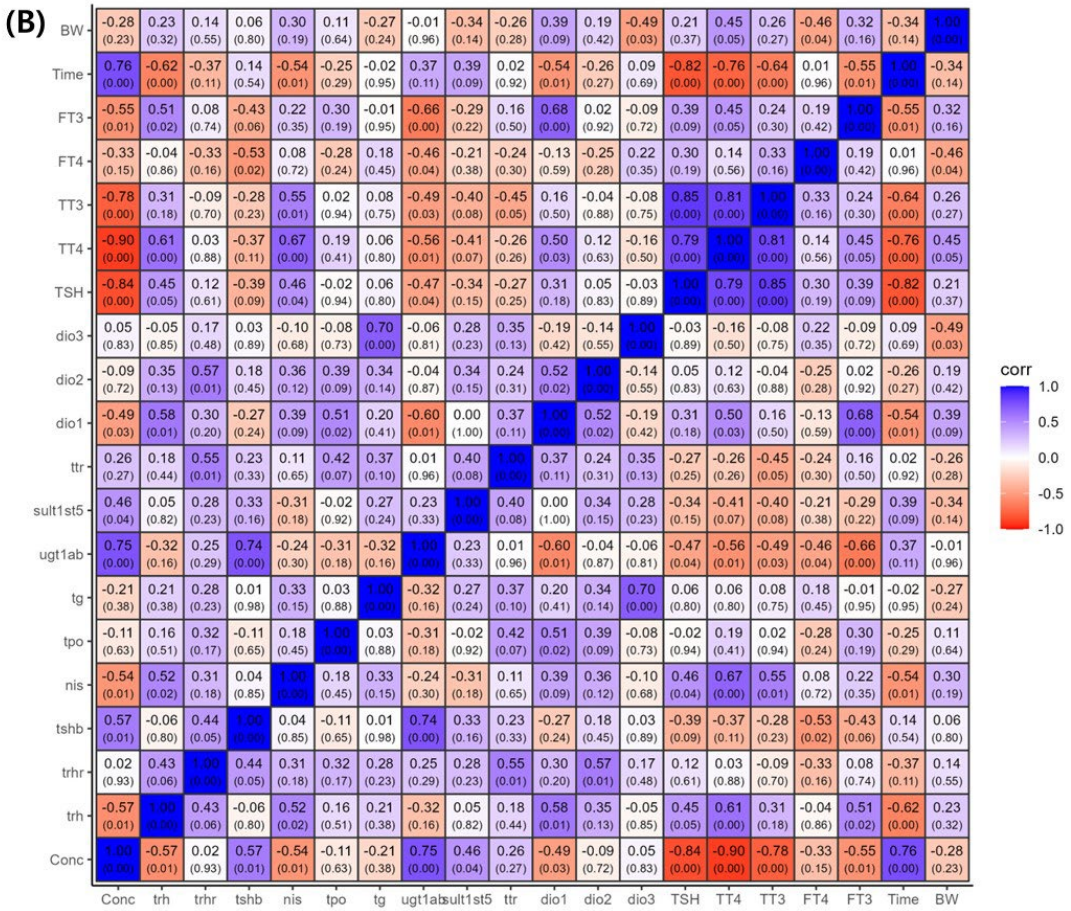


Fig. 7. Continued.

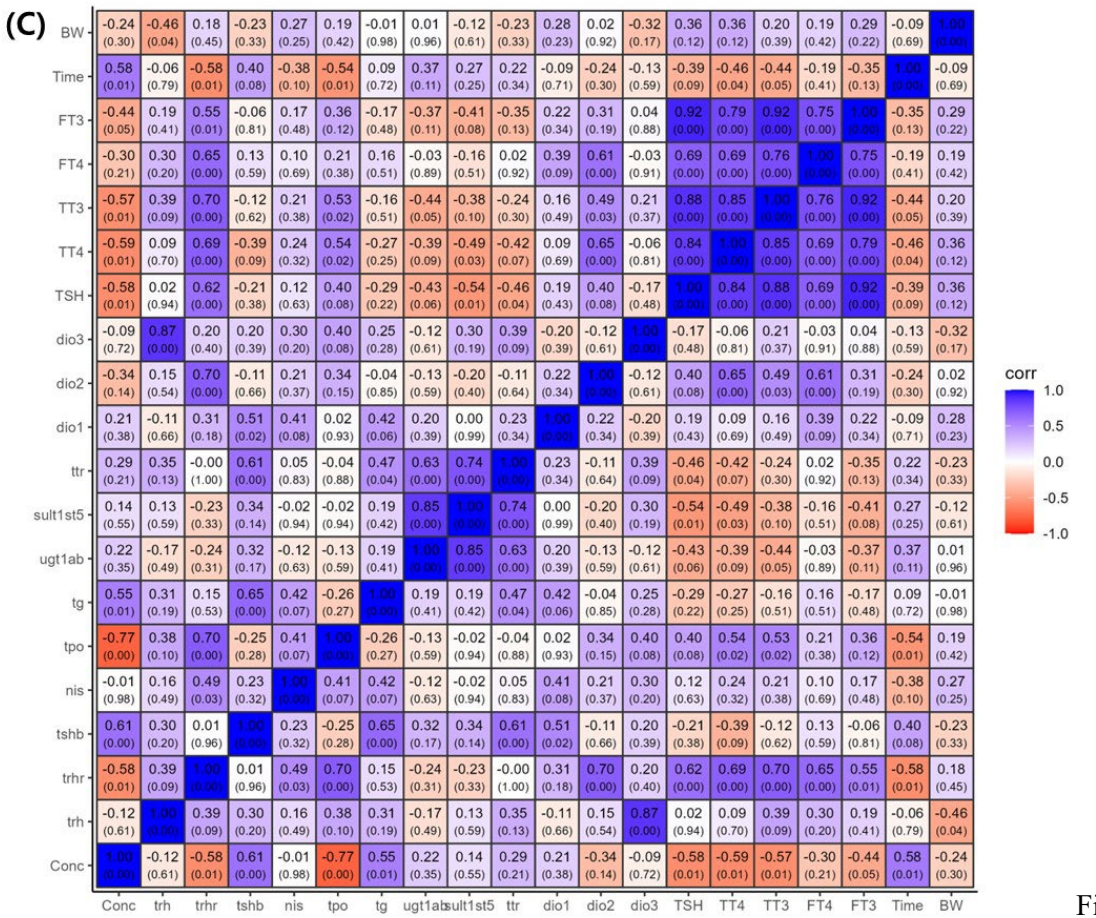
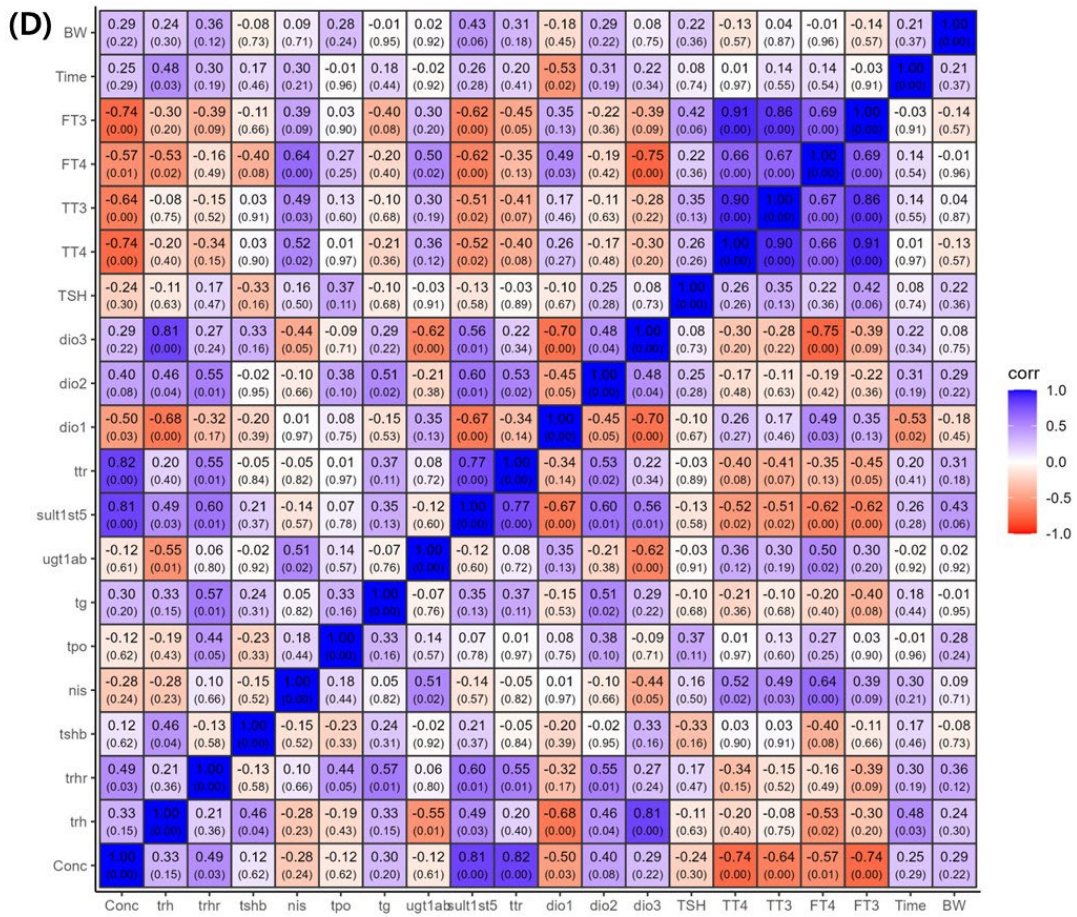


Fig. 7. Continued.



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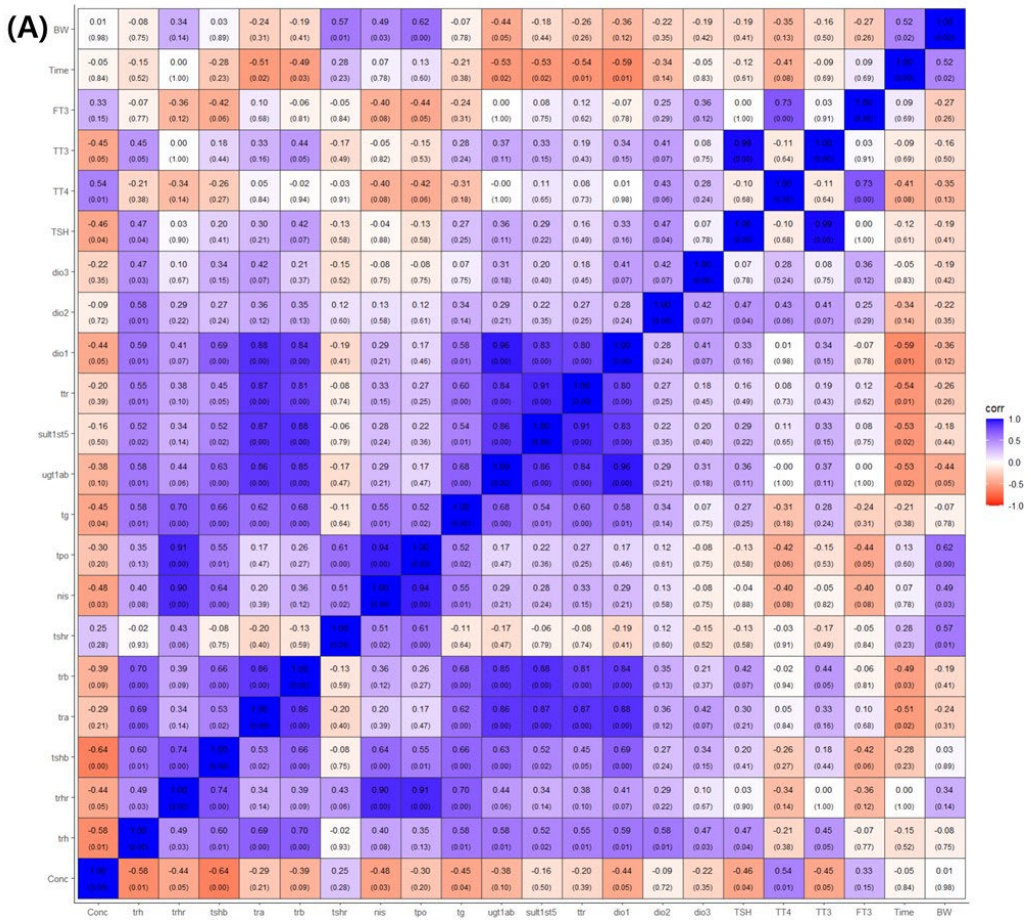

(A)
BW
Time
FT3
TT3
TT4
TSH
dio3
dio2
dio1
ttr
sult1st5
ugt1ab
tg
tpo
nis
tshr
trb
tra
tshb
trhr
trh
Conc
Conc
trh
trhr
tshb
tra
trb
tshr
nis
tpo
tg
ugt1ab
sult1st5
ttr
dio1
dio2
dio3
TSH
TT4
TT3
FT3
Time
BW
corr
1.0
0.5
0.0
-0.5
-1.0

Fig. 8. Spearman correlation coefficients (r) between morphological endpoints (time to hatch and body weight) and molecular endpoints (thyroid hormones and genes) in juvenile zebrafish after AVB (A), BP-3 (B), OC (C), and OMC (D) exposure.

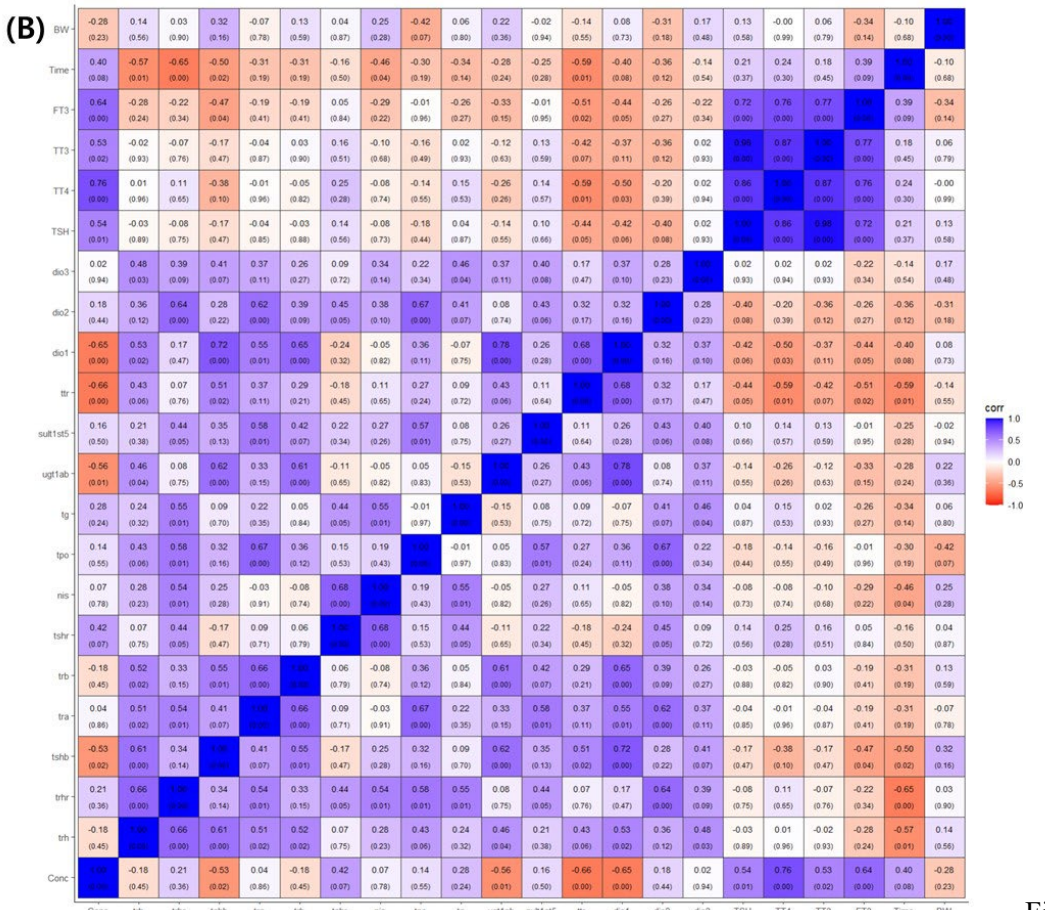


Fig. 8. Continued.

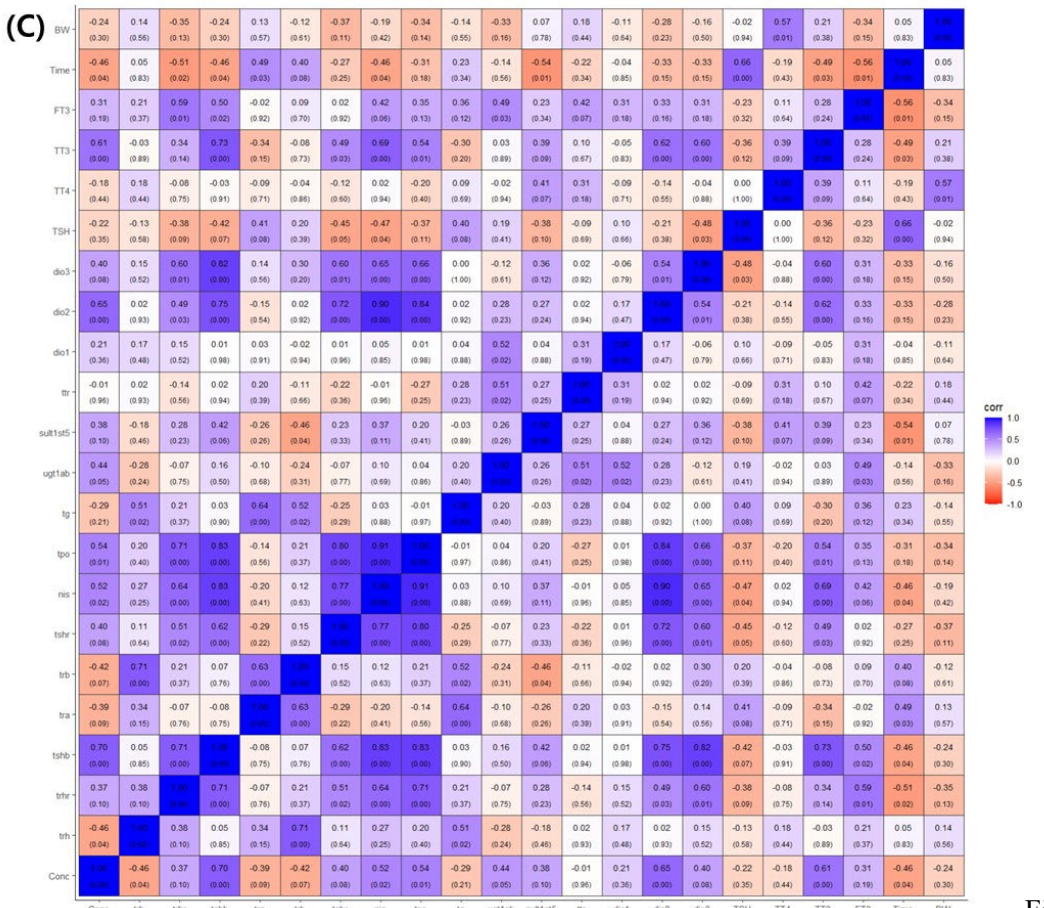


Fig. 8. Continued.

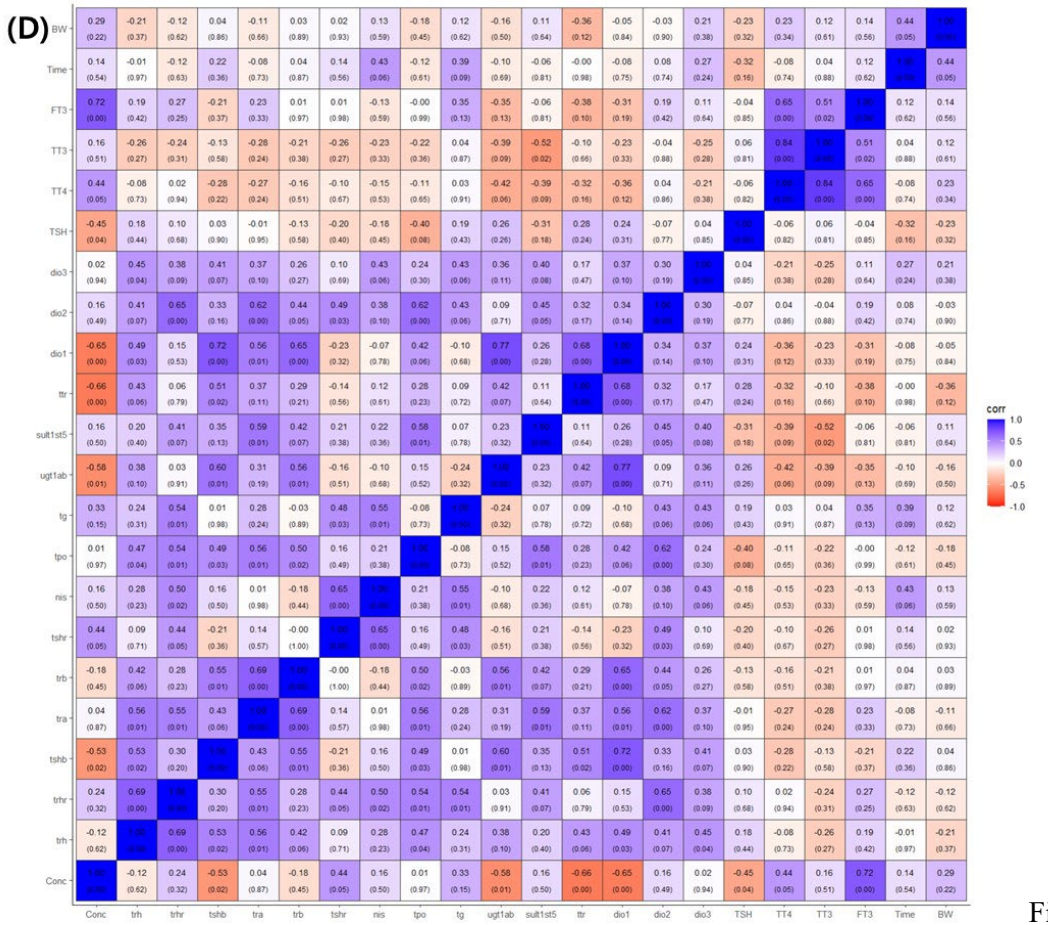


Fig. 8. Continued.

2.4.1 Thyroid hormone level changes

2.4.1.1 Embryo-larval stage

In embryo-larval zebrafish, all measured thyroid-related hormones were significantly altered. AVB and OC exposure significantly reduced whole-body TSH, TT4, TT3, FT4, and FT3 levels (Fig. 9). BP-3 treatment also significantly decreased whole-body TSH, TT4, TT3, and FT3 levels. Similarly, OMC induced dose-dependent decreases in whole-body TT4, TT3, FT4, and FT3 levels.

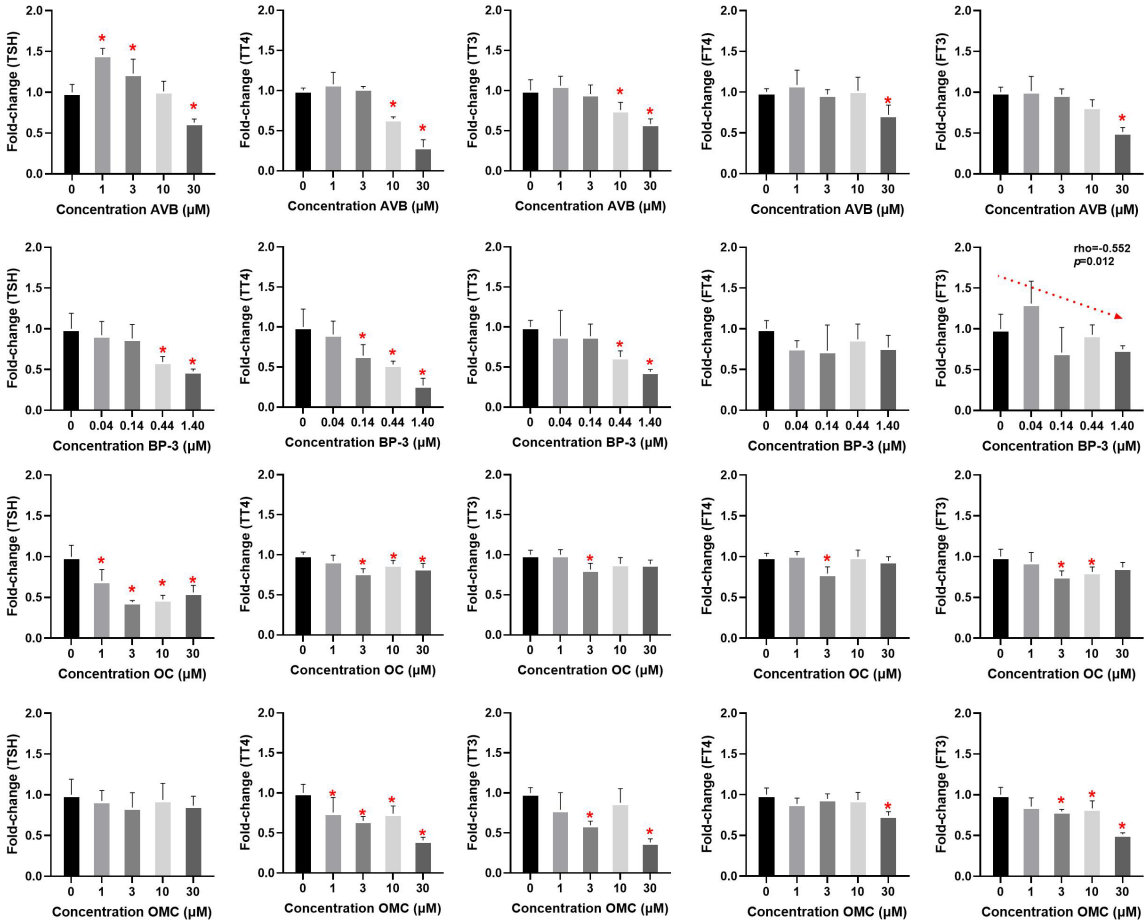

Fold-change (TSH)
Fold-change (TT4)
Fold-change (TT3)
Fold-change (FT4)
Fold-change (FT3)
Concentration AVB (μM)
Concentration BP-3 (μM)
Concentration OC (μM)
Concentration OMC (μM)
0 1 3 10 30
0 0.04 0.14 0.44 1.40
rho=-0.552
p=0.012

Fig. 9. Thyroid hormone level changes in embryo-larval zebrafish (120 hpf) after exposure to UV filters. *Asterisks indicate significant differences from the solvent control ($p<0.05$). The dotted red line indicates a significant correlation ($p<0.05$).

2.4.1.2 Juvenile

In juvenile zebrafish, the whole-body thyroid hormone changes displayed different profiles. Thirty days of UV filter exposure (AVB, BP-3, OC, OMC) resulted in a significant increase in whole-body TT4 levels. AVB and OMC significantly reduced TSH levels, whereas BP-3 increased TSH levels. In addition, BP-3 and OMC exposure caused significantly elevated FT3 levels (Fig. 10).

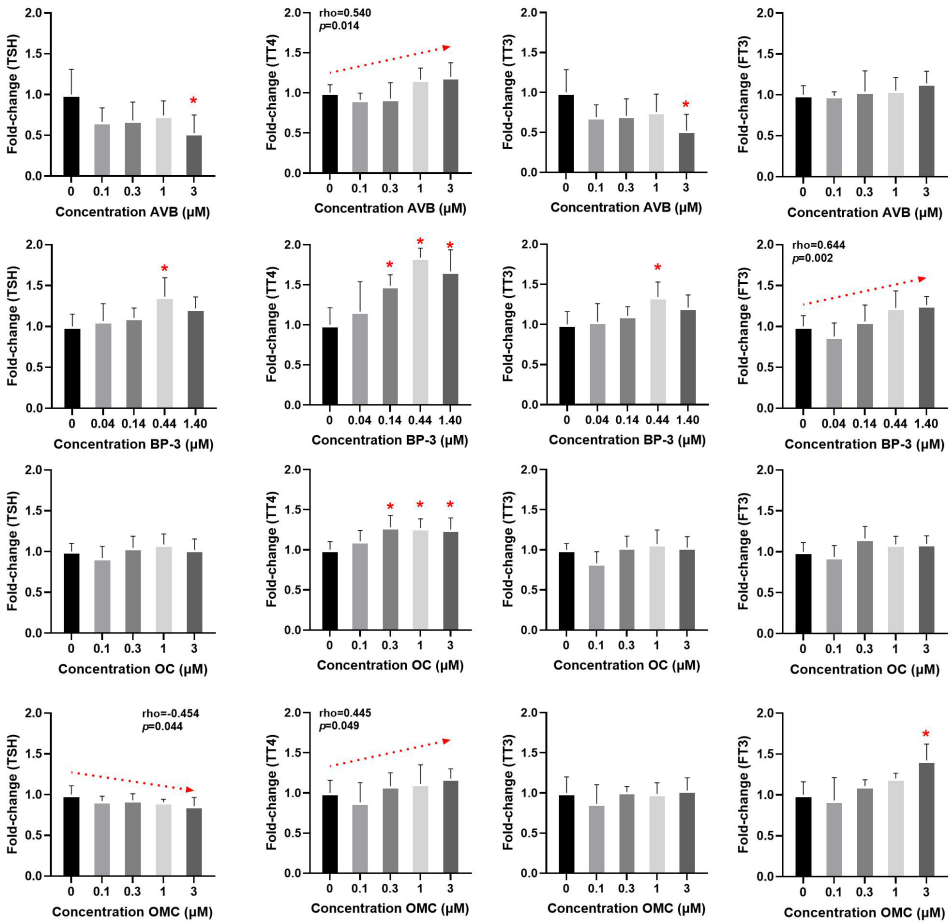

Fold-change (TSH)
Fold-change (TT4)
Fold-change (TT3)
Fold-change (FT3)
Concentration AVB (μM)
Concentration BP-3 (μM)
Concentration OC (μM)
Concentration OMC (μM)
0 0.1 0.3 1 3
0 0.04 0.14 0.44 1.40
rho=0.540
p=0.014
rho=0.644
p=0.002
rho=-0.454
p=0.044
rho=0.445
p=0.049

Fig. 10. Thyroid hormone level changes in juvenile zebrafish (30 dpf) after exposure to UV filters. *Asterisks indicate significant differences from the solvent control ($p<0.05$). Dotted red line indicates significant correlation ($p<0.05$).

2.3.1.3 Adult male

In adult male zebrafish, AVB induced a marked increase in plasma TT4 and a decrease in plasma TT3. BP-3 and OC caused significant increases in plasma TT3 levels. Increasing trends in plasma TSH and FT3 were observed in OMC-exposed adult male zebrafish; however, the changes were relatively smaller (Fig. 11).

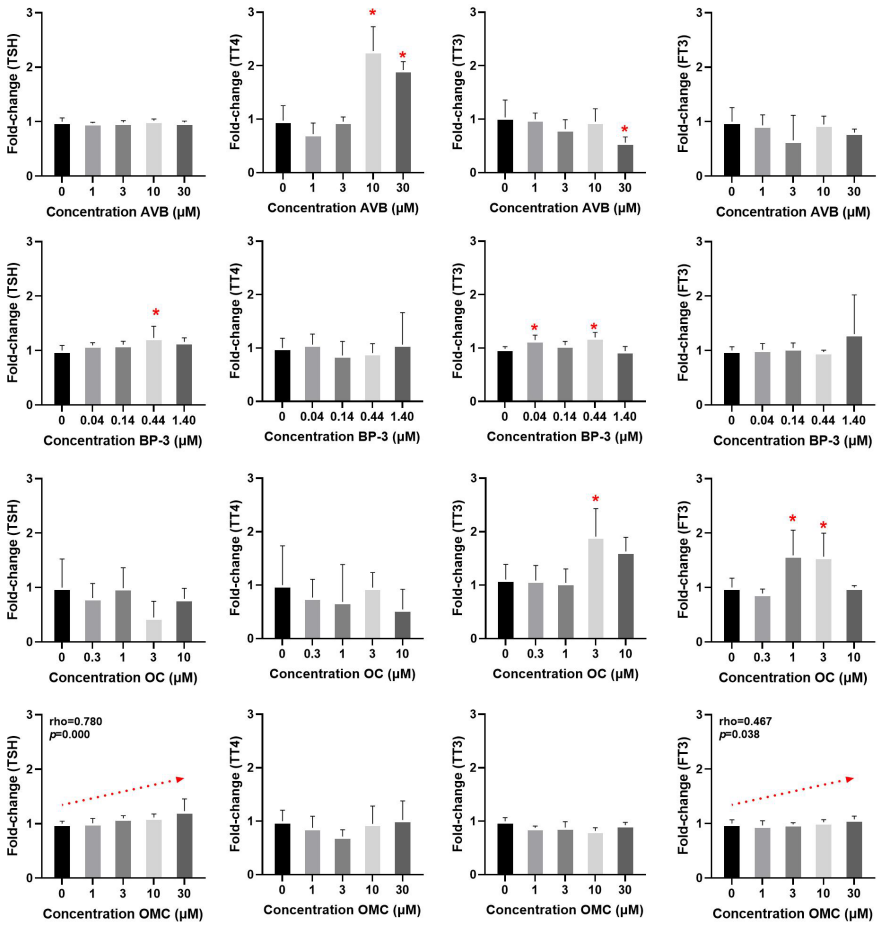

Fold-change (TSH)
Fold-change (TT4)
Fold-change (TT3)
Fold-change (FT3)
Concentration AVB (μM)
0 1 3 10 30
Concentration BP-3 (μM)
0 0.04 0.14 0.44 1.40
Concentration OC (μM)
0 0.3 1 3 10
Concentration OMC (μM)
0 1 3 10 30
rho=0.780
p=0.000
rho=0.467
p=0.038
*

Fig. 11. Changes in thyroid hormone levels in adult male zebrafish after 21 d of exposure to UV filters. *Asterisks indicate significant differences from the solvent control (p<0.05). The dotted red line indicates a significant correlation (p<0.05).

2.3.2 Gene transcription level changes

2.3.2.1 Embryo-larval zebrafish mRNA expressions changes related to THs regulation

To elucidate the molecular mechanisms of the thyroid-disrupting activities of UV filters in zebrafish early development, we performed qPCR analysis of genes related to the central regulation, synthesis, transport, deiodination and metabolism of THs. Significant transcriptional changes in genes related to the HPT axis were generally observed in whole-body embryo-larval fish at the highest concentrations (Fig. 12). After exposure to AVB, transcripts of *trhr* and *tshb* were significantly downregulated. In addition, BP-3 exposure significantly downregulated *trh* and upregulated *tshb* gene expression. Significant upregulation of *tshb* and *tg* mRNA expression was observed following exposure to OC. In OMC-exposed embryo-larval fish, increasing patterns of *trh* and *trhr* gene expression were observed.

Moreover, genes involved in TH deiodination and metabolism were analyzed in embryo-larval zebrafish. qPCR data revealed that *dio1* gene transcription was significantly upregulated by exposure to AVB and OMC but downregulated by BP-3 and OMC. The expression of dio2 mRNA was significantly induced by OMC exposure. Following exposure to AVB, BP-3, OC, and OMC, significant alterations were observed in *ugt1ab* and *sult1 st5* mRNA expression. The expression of the *ugt1ab* gene was significantly greater in the AVB- and BP-3-exposed groups and lower in the OMC-exposed

groups. Although not statistically significant, a >1.5-fold change in *ugt1ab* gene expression was induced by OC exposure. Furthermore, BP-3, OC and OMC significantly increased the transcription of the *sult1 st5* gene. Exposure to AVB, OC and OMC significantly upregulated *ttr* gene transcription.

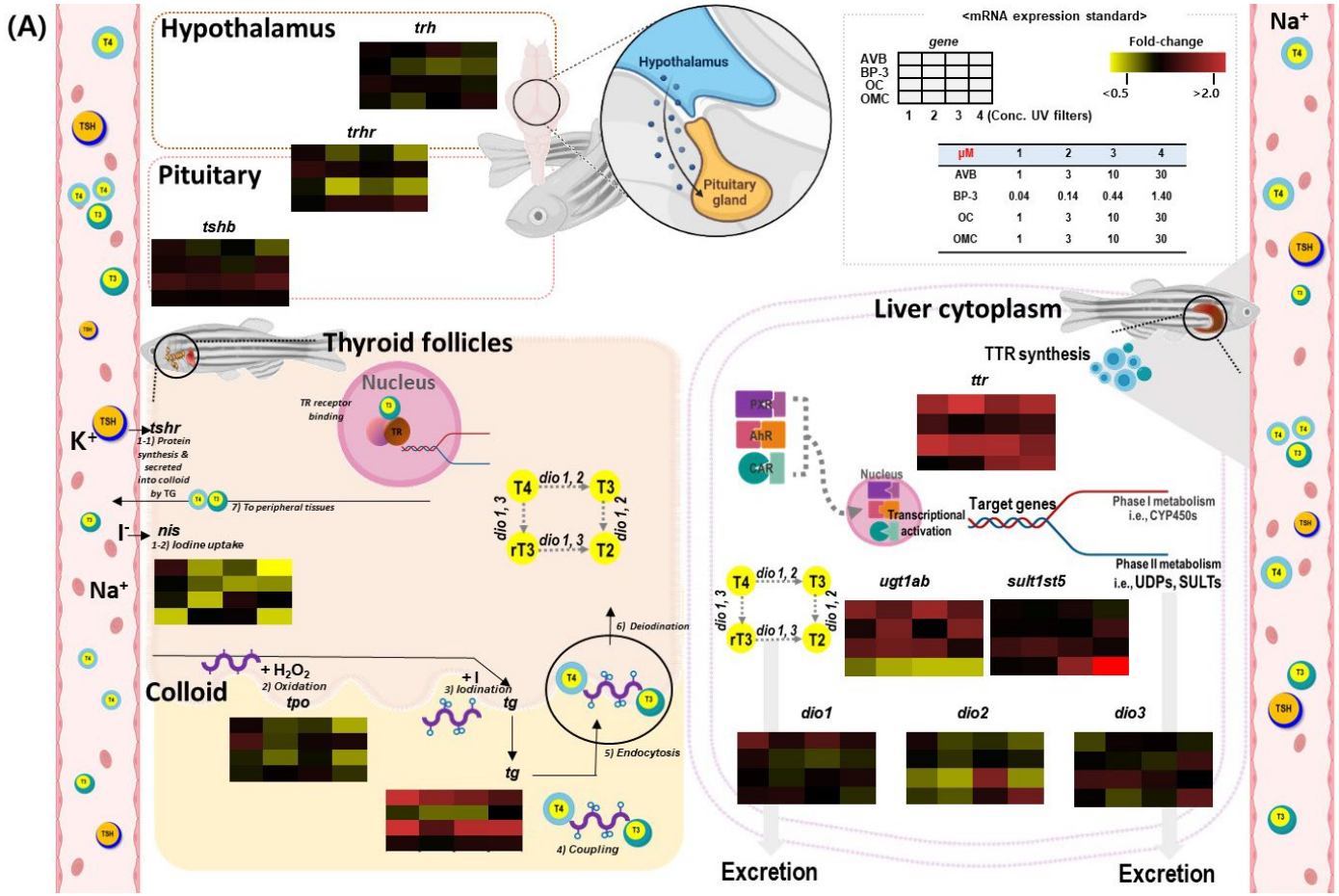

(A)
Hypothalamus
trh
trhr
Pituitary
tshb
Hypothalamus
Pituitary gland
<mRNA expression standard>
gene
Fold-change
AVB
BP-3
OC
OMC
<0.5
>2.0
1 2 3 4 (Conc. UV filters)
μM 1 2 3 4
AVB 1 3 10 30
BP-3 0.04 0.14 0.44 1.40
OC 1 3 10 30
OMC 1 3 10 30
Na+
Thyroid follicles
Nucleus
TR receptor binding
K+
tshr
1-1) Protein synthesis & secreted into colloid by TG
7) To peripheral tissues
I-
nis
1-2) Iodine uptake
Na+
T4
dio 1, 2
T3
dio 1, 3
dio 1, 2
rT3
dio 1, 3
T2
Colloid
+ H2O2
2) Oxidation
tpo
+ I
3) Iodination
tg
tg
6) Deiodination
5) Endocytosis
4) Coupling
Liver cytoplasm
TTR synthesis
ttr
PXR
AhR
CAR
Nucleus
Transcriptional activation
Target genes
Phase I metabolism i.e., CYP450s
Phase II metabolism i.e., UDPs, SULTs
ugt1ab
sult1st5
dio1
dio2
dio3
Excretion
Excretion

Fig. 12. Overview of thyroid hormone regulation related mRNA expression changes (A) in embryo-larval zebrafish (120 hpf) after exposure to UV filters. Concentration response of gene expression changes (bar graphs) related to HPT-axis (B), deodination and metabolism (C) are described. *Asterisks indicate significant differences from the solvent control ($p<0.05$). Dotted red line indicates significant correlation ($p<0.05$).

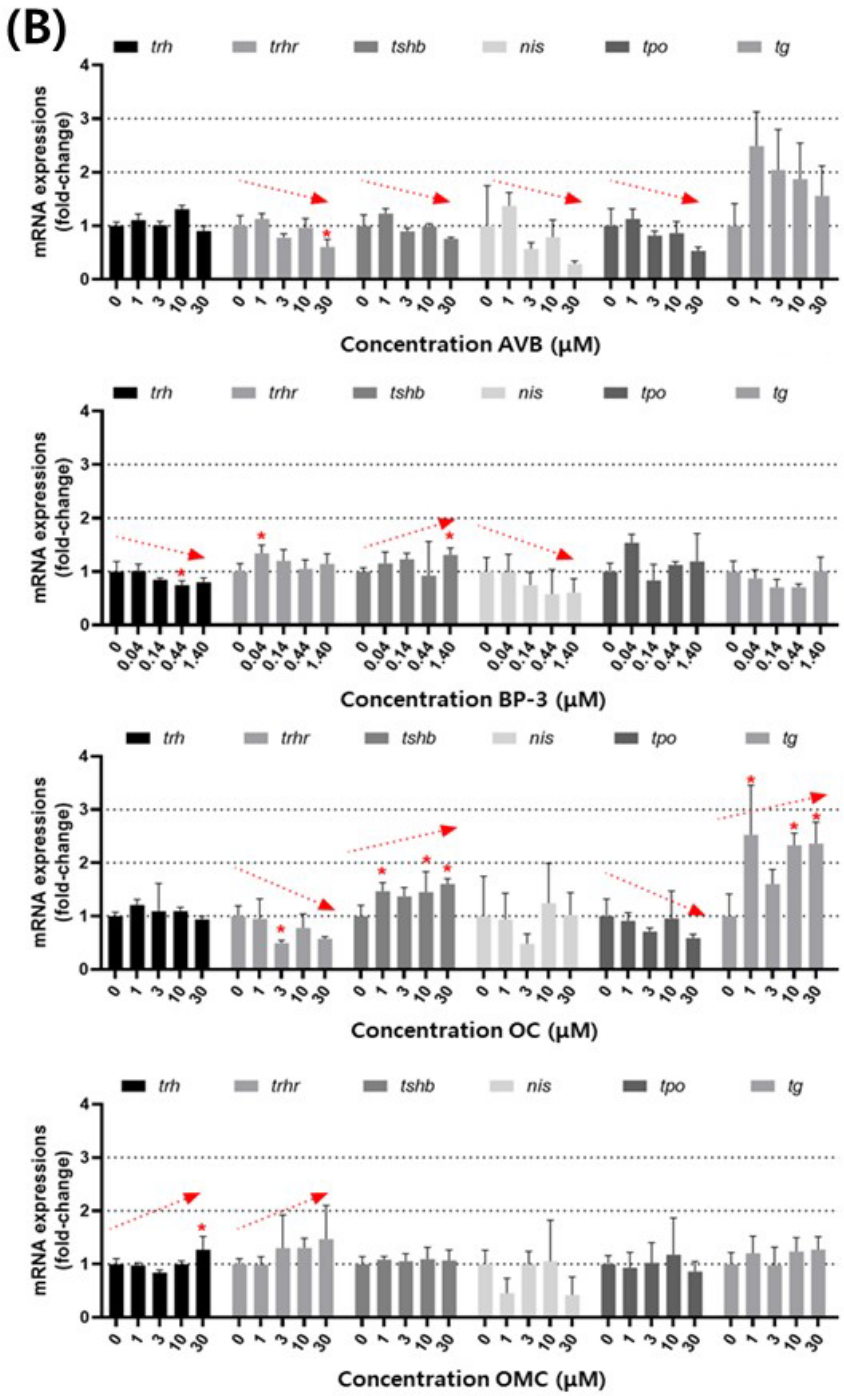


Fig 12. Continued.

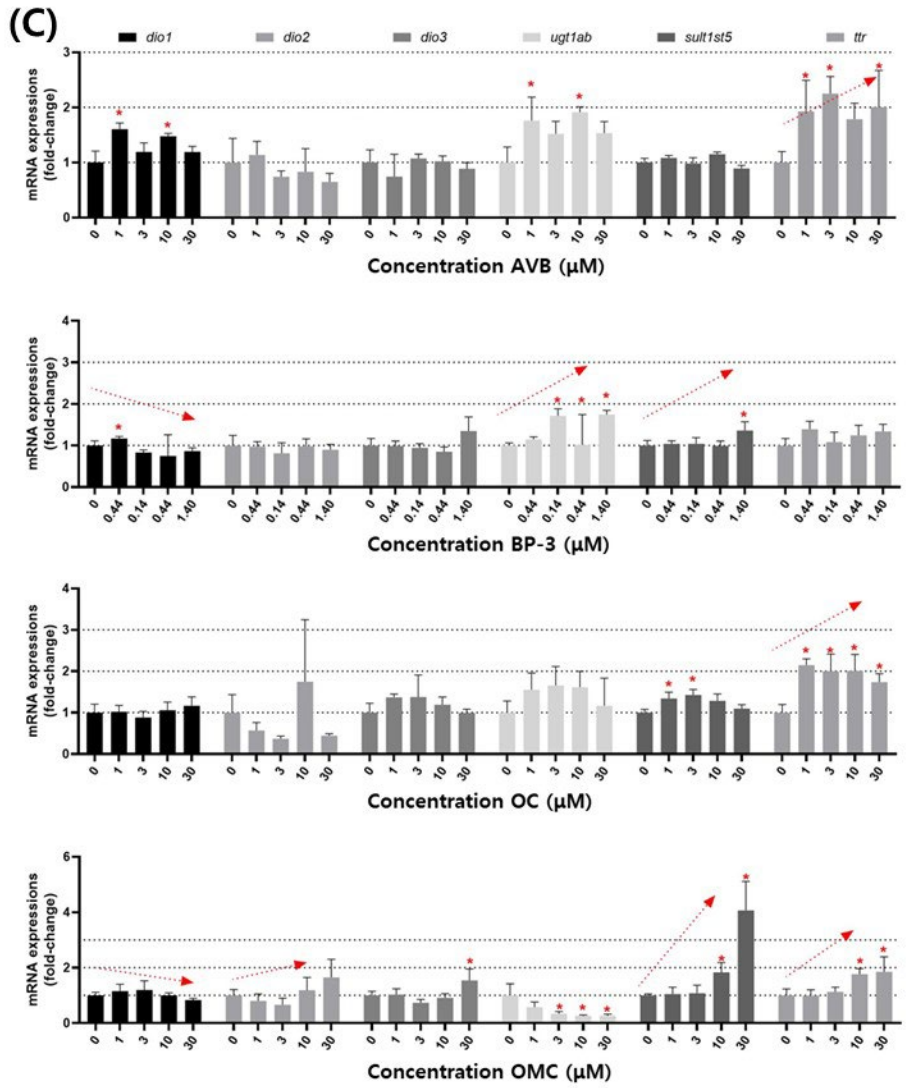


Fig. 12. Continued.

2.3.2.2 mRNA expression changes related to TH regulation in juvenile zebrafish

The same target genes were evaluated in juvenile zebrafish (Fig. 13). The *trh* and *tshb* transcripts exhibited downregulation, whereas *tshr* demonstrated significant upregulation, at 0.1 μM AVB. Exposure to BP-3 led to marked increases in *trhr*, *tshb*, *tshr*, *nis*, *tpo*, and *tg* mRNA expression. In addition, the expression levels of the *trhr*, *tshb*, *tshr*, *nis* and *tpo* genes were significantly elevated, and *tg* mRNA was significantly downregulated, following exposure to OC. mRNA expression changes were also observed in the OMC-treated groups, with significant upregulation of *trhr* and *nis* gene transcription at 0.1 μM OMC. The downregulation of *tshb* mRNA was significantly correlated with OMC exposure. The expression of mRNAs related to TH transport showed different patterns after exposure to the tested UV filters.

Genes involved in TH deiodination and metabolism were also evaluated in juvenile fish. In AVB-exposed juvenile fish, significant downregulation of the *dio1* and *ugt1ab* genes was observed. BP-3 exposure elevated *dio2* mRNA expression but significantly downregulated *ugt1ab* and *sult1 st5* gene expression. Exposure to OC also significantly induced *dio2* gene expression. Marked upregulation of the *ugt1ab* gene and significant downregulation of *dio1* and *ugt1ab* mRNA expression was observed in juvenile fish exposed to OC..

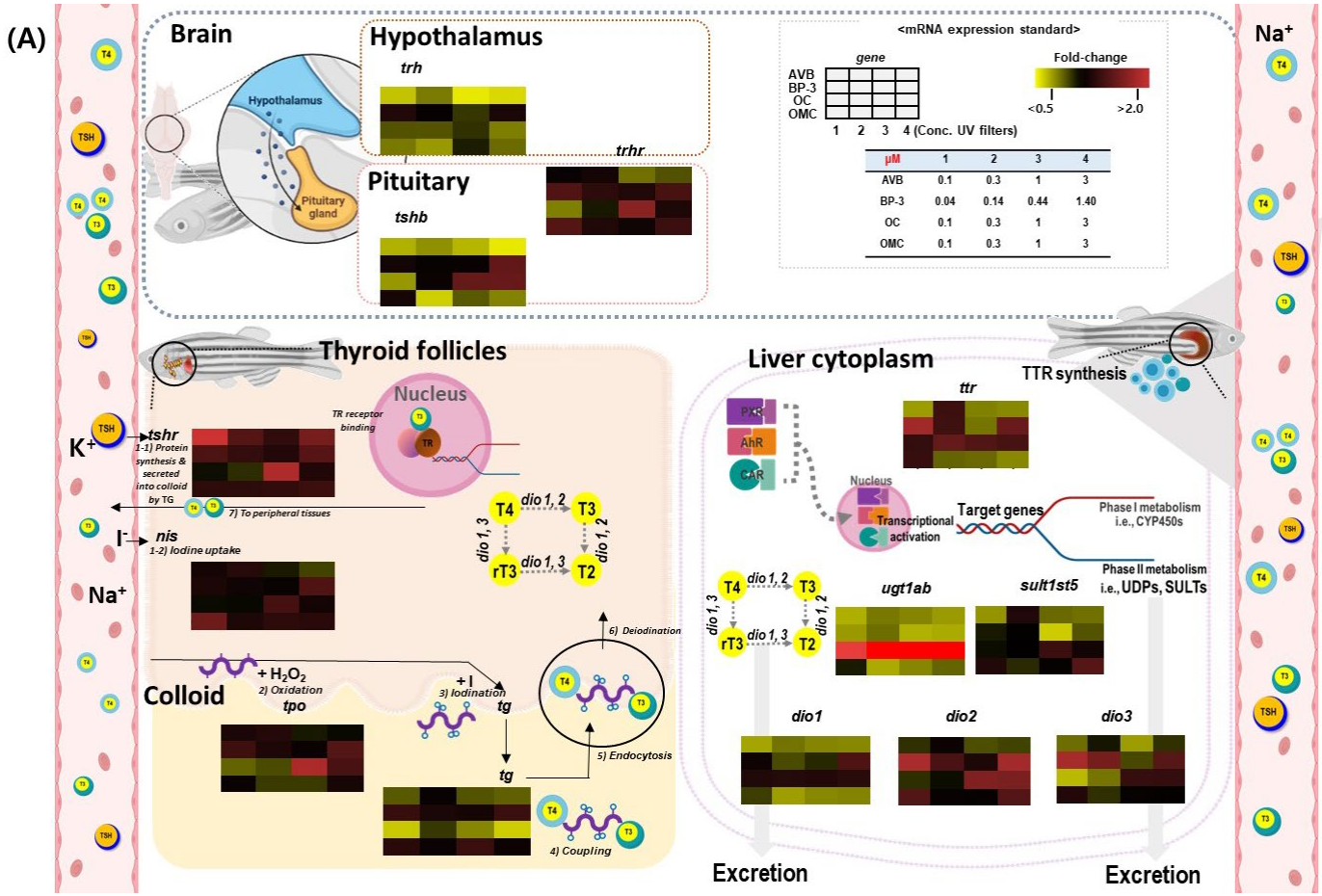

(A)
Brain
Hypothalamus
Pituitary gland
Hypothalamus
trh
trhr
Pituitary
tshb
<mRNA expression standard>
gene
Fold-change
AVB
BP-3
OC
OMC
1 2 3 4 (Conc. UV filters)
<0.5
>2.0
μM 1 2 3 4
AVB 0.1 0.3 1 3
BP-3 0.04 0.14 0.44 1.40
OC 0.1 0.3 1 3
OMC 0.1 0.3 1 3
Na+
Thyroid follicles
Nucleus
TR receptor binding
K+
tshr
1-1) Protein synthesis & secreted into colloid by TG
7) To peripheral tissues
I-
nis
1-2) Iodine uptake
Na+
dio 1, 2
dio 1, 3
T4
T3
rT3
T2
Colloid
+ H2O2
2) Oxidation
tpo
+ I
3) Iodination
tg
6) Deiodination
5) Endocytosis
4) Coupling
Liver cytoplasm
TTR synthesis
ttr
PXR
AhR
CAR
Nucleus
Transcriptional activation
Target genes
Phase I metabolism i.e., CYP450s
Phase II metabolism i.e., UDPs, SULTs
ugt1ab
sult1st5
dio1
dio2
dio3
Excretion
Excretion

Fig. 13. Overview of thyroid hormone regulation related mRNA expression changes (A) in juvenile zebrafish (30 dpf) after exposure to UV filters. Concentration response of gene expression changes (bar graphs) related to HPT-axis (B), deodination and metabolism (C) are described. *Asterisks indicate significant differences from the solvent control ($p<0.05$). Dotted red line indicates significant correlation ($p<0.05$).

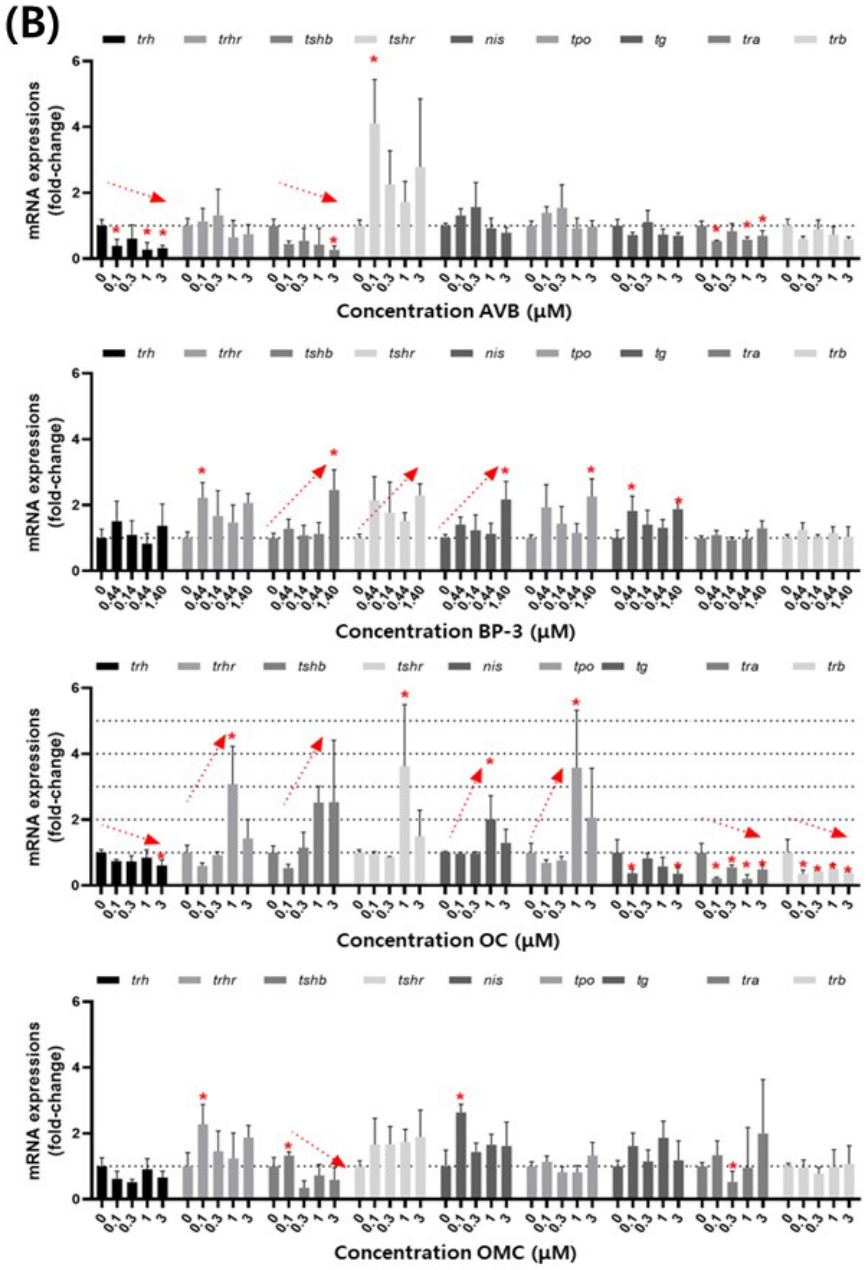


Fig. 13. Continued.

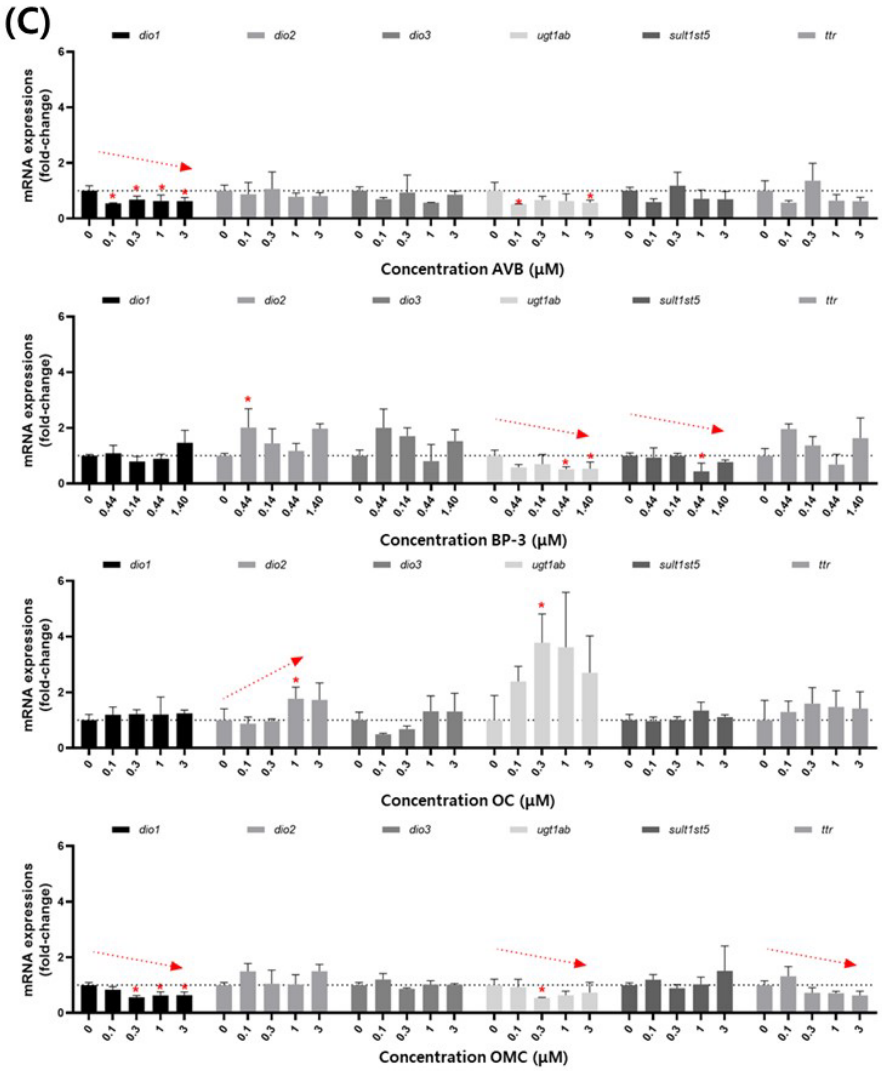


Fig. 13. Continued.

2.3.2.3 Adult male zebrafish mRNA expressions changes related to THs regulation

To investigate the effects of adult life UV filter exposure on thyroid endocrine systems, adult male zebrafish were used to characterize tissue-specific responses of genes in the hypothalamus and pituitary, thyroid and liver. Several genes regulating the HPT axis were influenced by UV filter exposure in adult male zebrafish (Fig. 14).

In the brains of AVB-exposed fish, a concentration response was observed with downregulation of the *trh* and *tshb* genes. In BP-3-exposed adult male brains, rapid upregulation of *tshb* was observed. The expression of *dio2* tended to decrease. In addition, BP-3 exposure led to significant induction of *tra* and *trb* gene transcription. In the brains of male zebrafish exposed to OC, concentration-related and parallel increases in *tshb*, *dio1*, *tra* and *trb* transcripts occurred. Exposure to OMC also significantly affected the *trhr* and *dio3* genes, which were significantly downregulated in the exposed compared to the control group.

In adult male zebrafish thyroid tissues, similar profiles were observed for genes regulating THs. Following exposure to AVB, significant upregulation of the *tshr*, *nis*, *tpo*, *dio1*, *dio2*, *tra* and *trb* genes was observed. In the BP-3- and OC-exposed groups, rapid upregulation of *trhr*, *nis*, *tpo*, *tg*, *dio1*, and *dio2* mRNA was observed. After exposure to 30 μM OMC, overall upregulation of

genes in thyroid tissues was observed, specifically, significant upregulation of *tshr*, *nis*, *tpo*, *tg*, *dio1*, *dio2*, *dio3*, *tra* and *trb*.

In adult male zebrafish livers, significant transcriptional changes were observed for genes related to hepatic deiodination and TH metabolism. After exposure to AVB, the *dio1* and *dio2* genes were significantly downregulated, while the *dio3* and *sult1 st5* genes were upregulated. In BP-3-exposed adult male liver, transcript levels of *dio1*, *dio2* and *ttr* were significantly reduced, whereas those of the *ugt1ab* and *sult1 st5* genes were significantly upregulated. OC resulted in significant upregulation of *ugt1ab* and *sult1 st5* in the adult male zebrafish liver. Moreover, *dio1* and *sult1 st5* mRNA expression changes were significantly correlated with OMC concentrations (down- and upregulated, respectively).

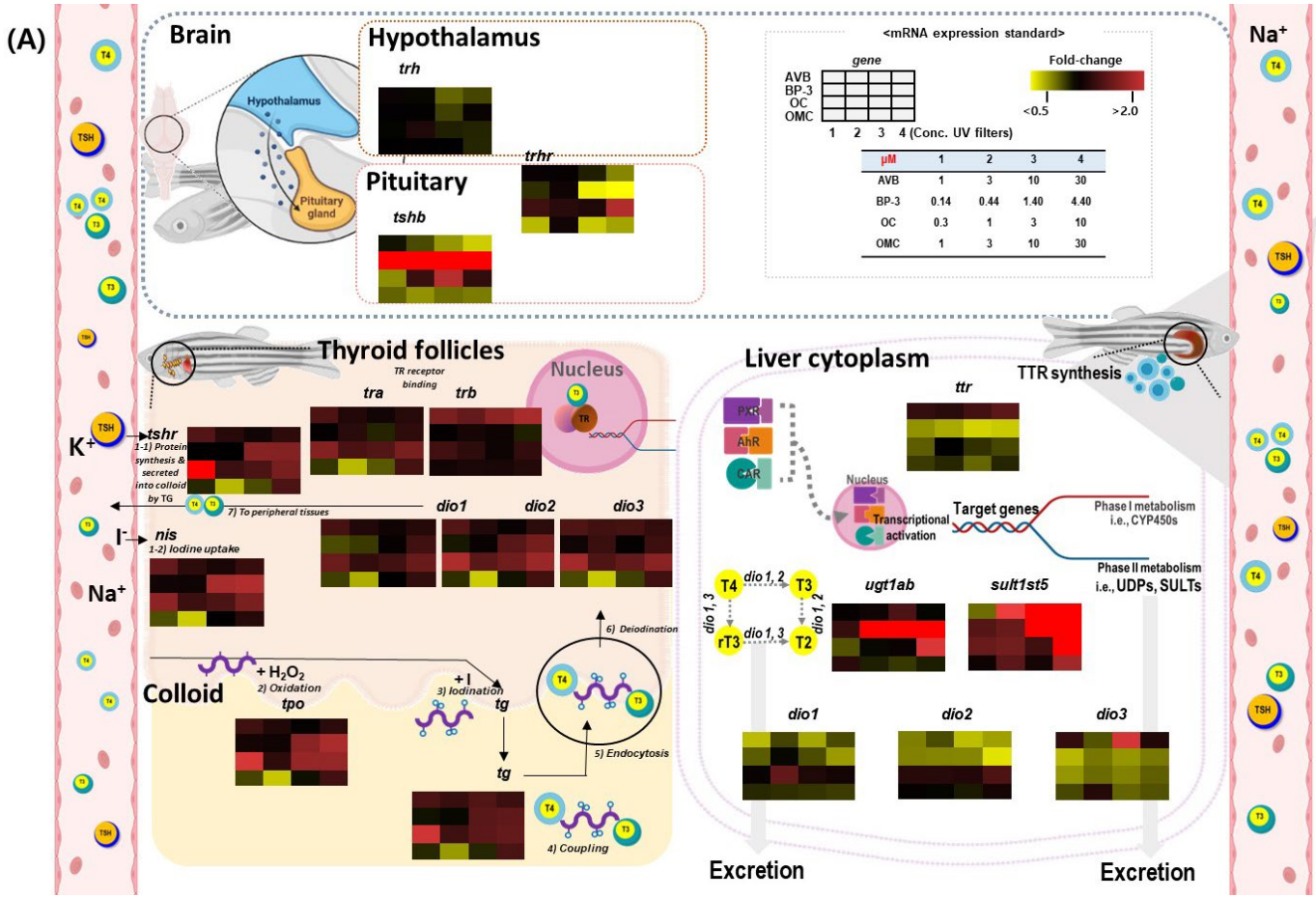

(A)
Brain
Hypothalamus
Pituitary gland
Hypothalamus
trh
Pituitary
trhr
tshb
<mRNA expression standard>
gene
Fold-change
AVB
BP-3
OC
OMC
1 2 3 4 (Conc. UV filters)
<0.5
>2.0
μM 1 2 3 4
AVB 1 3 10 30
BP-3 0.14 0.44 1.40 4.40
OC 0.3 1 3 10
OMC 1 3 10 30
Na+
Thyroid follicles
TR receptor binding
Nucleus
tra
trb
K+
tshr
1-1) Protein synthesis & secreted into colloid by TG
7) To peripheral tissues
dio1
dio2
dio3
I-
nis
1-2) Iodine uptake
Na+
Colloid
+ H2O2
2) Oxidation
tpo
+ I
3) Iodination
tg
6) Deiodination
5) Endocytosis
4) Coupling
Liver cytoplasm
TTR synthesis
ttr
PXR
AhR
CAR
Nucleus
Transcriptional activation
Target genes
Phase I metabolism i.e., CYP450s
Phase II metabolism i.e., UDPs, SULTs
T4
T3
rT3
T2
dio 1, 2
dio 1, 3
ugt1ab
sult1st5
dio1
dio2
dio3
Excretion
Excretion

Fig. 14. Overview of thyroid hormone regulation related mRNA expression changes (A) in adult male zebrafish after 21 d exposure to UV filters. Concentration response of gene expression changes (bar graphs) belong to hypothalamus and pituitary (B), thyroid tissues (C), hepatic metabolisms (D) are described. *Asterisks indicate significant differences from the solvent control ($p<0.05$). Dotted red line indicates significant correlation ($p<0.05$).

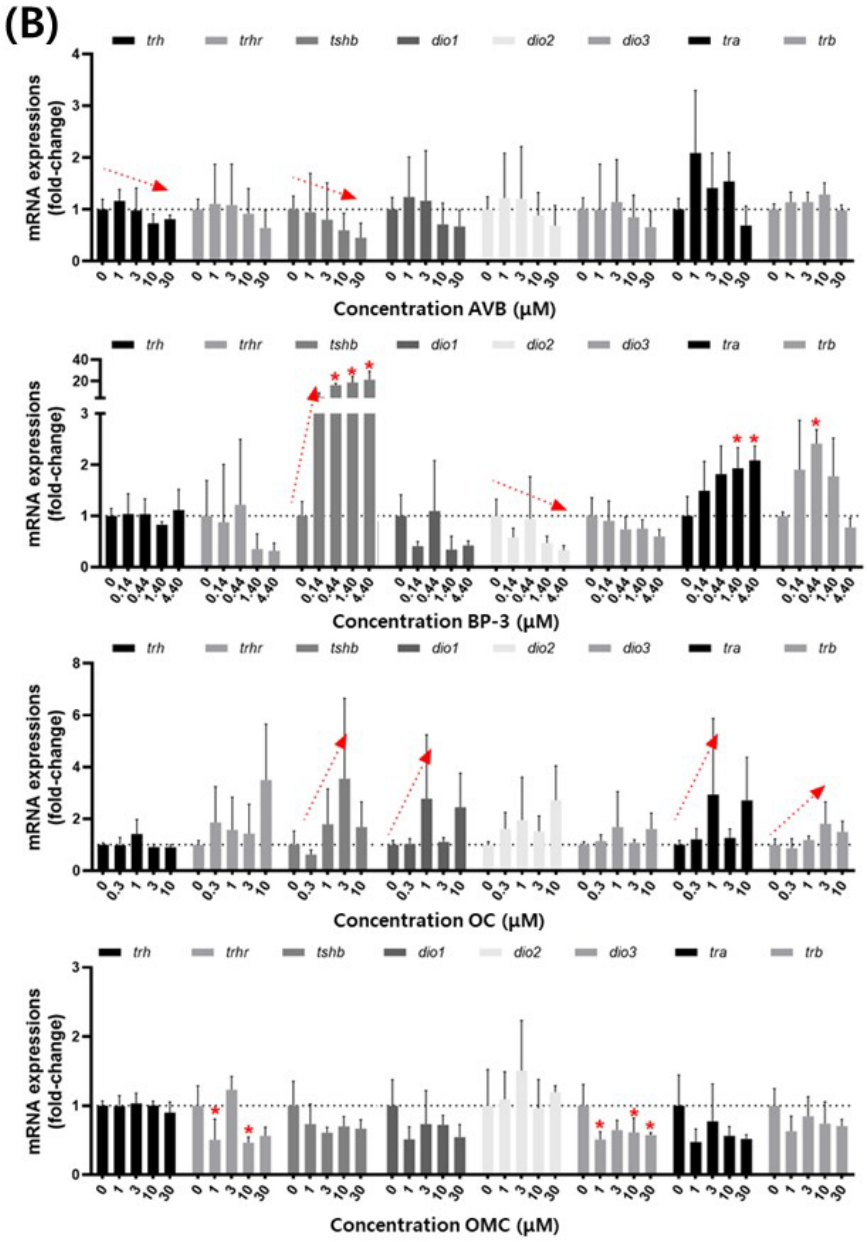


Fig 14. Continued.

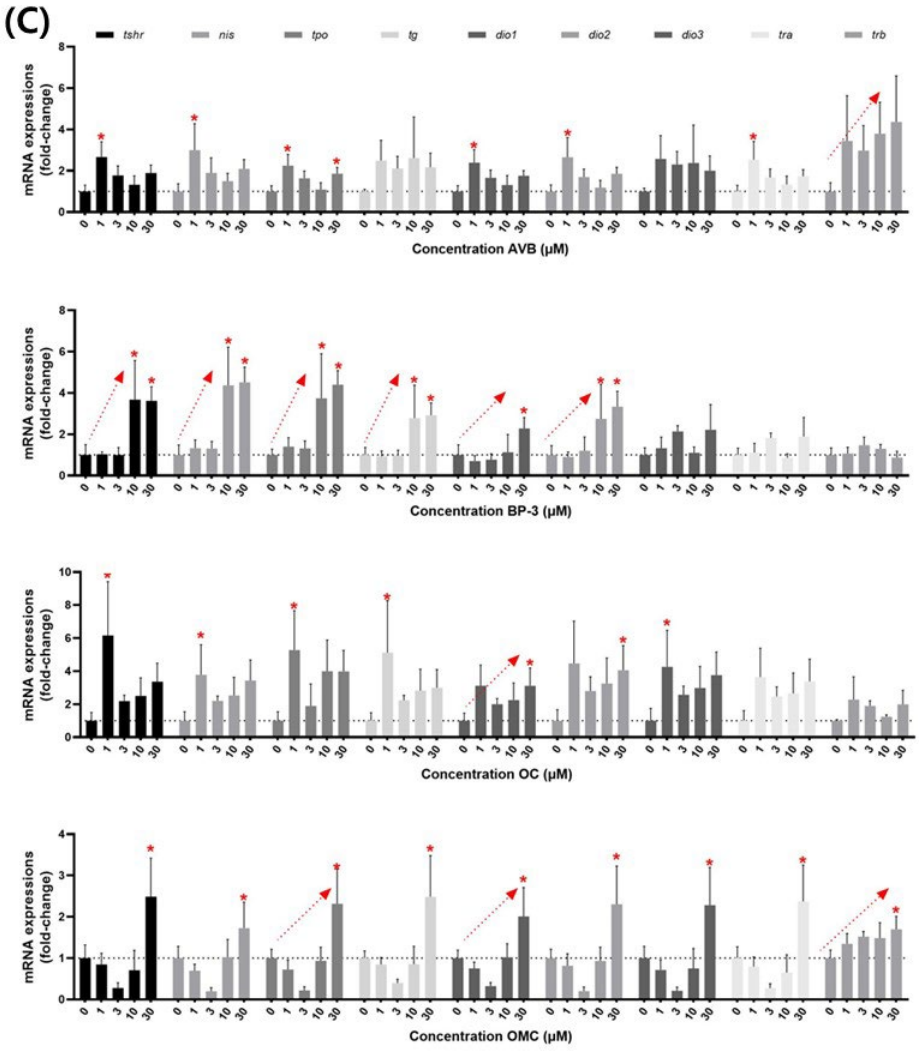


Fig 14. Continued.

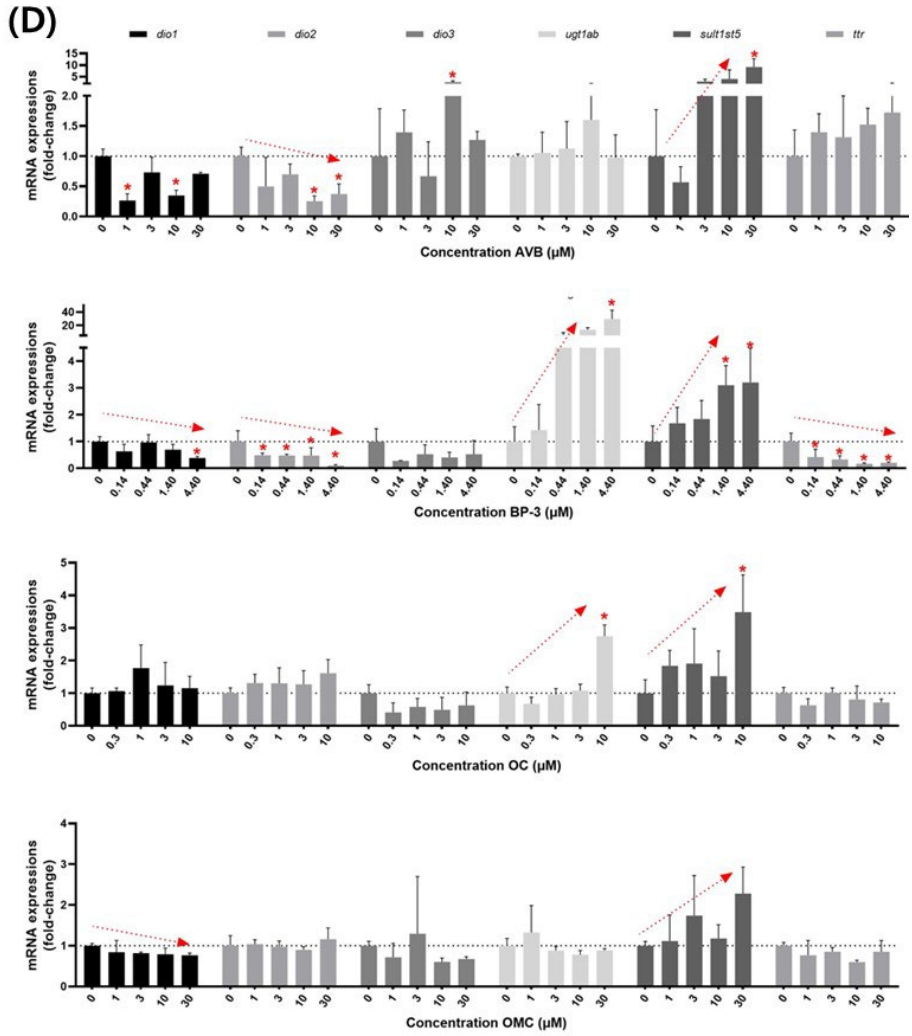


Fig. 14. Continued.

2.4 Discussion

In the present study, exposure to the tested UV filters clearly demonstrated an influence on the HPT axis and liver genes that regulate thyroid hormone balance. The changes in thyroid hormone levels in embryo-larval, juvenile and adult male fish following exposure to four UV filters, i.e., AVB, BP-3, OC, and OMC, also provide evidence of the thyroid endocrine disruption abilities of these chemicals. The results of our study suggest that the most commonly used UV filters could imbalance the thyroid system in a life stage-specific manner. In addition, the results of this study imply that the early-life period specifically is more vulnerable to exposure to UV filters.

THs play significant role in the development, growth and metabolism in teleost fish. Previous studies observed effects on organism levels such as embryo-larval survival, hatchability, time to hatch, and malformation rate along with changes of THs status (Zhang et al. 2017; Lee et al. 2022). In our study, we analyzed TH levels and regulatory changes of THs related mRNA expressions. In addition, effects on embryo-larval survival, hatchability, time to hatch, and body weights were determined. We found that exposure to AVB and BP-3 significantly increased time to hatch. However, other effects such as survival rate, hatchability, body wight were observed. Lee et al. (2018) reported no effects on survival and hatchability in BP-3 exposed embryo-larval zebrafish. Other study from Ka and Ji (2022) found significant increase in time to hatch after exposure to AVB and OMC in wild-type, and trα$a^{-/-}$

embryo-larval zebrafish. Such difference between experimental studies could rise from different intrinsic (genotypic and epigenetic differences) (Guryev et al. 2006) or extrinsic factors such as laboratory conditions such as temperature and light and dark cycle. Spearman correlation analysis to determine relationships between time to hatch or body weight with THs and related genes have revealed that time to hatch was significantly influenced by THs status after exposure to UV filters, suggesting the importance of THs during embryo-larval transition (Ka and Ji 2022).

The decreases in thyroid hormones observed in embryo-larval zebrafish following exposure to AVB, BP-3, OC, and OMC can be explained by regulatory changes in the genes that are responsible for TH balance. In the present study, several genes related to the central regulation of THs in the brain, TH synthesis in the thyroid, and deiodination and TH metabolism were significantly altered following UV filter exposure. The profiles observed in embryo-larval zebrafish were similar to those reported in previous studies. Experimental evidence demonstrated the anti-thyroid hormone-like activities of AVB in GH3 cells transfected with human thyroid receptor element (GH3. TRE-Luc) (Klopčič and Dolenc, 2017).

Similar to previous studies (Lee et al. 2018), our results showed that BP-3 caused a significant decrease in TH levels. Epidemiological studies have also reported similar observations (Aker et al. 2016; Kim et al. 2017). Specifically, Kim et al. (2017) demonstrated a significant negative correlation of free T3 levels with urinary levels of BP-3 in pregnant women (n=106). Aker et al.

(2016) showed a negative association of T4 levels and BP-3 concentrations in the general U.S. population (n=1,829).

Moreover, the decreased T4 and T3 levels in OMC-exposed embryo-larval zebrafish coincided with the results of previous studies that reported decreased T4 and/or T3 levels in embryo-larval zebrafish and juvenile Japanese medaka (*O. latipes*) (Lee et al. 2019; Chu et al. 2021). Similar thyroid profiles were observed in female SD rats (Klammer et al. 2007) and Swiss Webster mice (Ferraris et al. 2020).

In the present study, however, OC caused a significant reduction in TH levels. These results are distinct from those of a previous rodent study (unpublished study as cited by ECHA, 2019), which reported a significant induction of TSH in orally administered Wistar rats (age of 70±1 days) exposed to OC for 13 weeks. As described in Capen 1997, rats are more sensitive than humans to thyroid disruption caused by drugs, chemical, and physiologic perturbations due to the shorter plasma half-life of rat T4 (12-24 hours) and the considerable differences in TH transport proteins. Siglin et al. (2000) also reported a half-life of approximately 6 hours for T3 in rats. Fish have an even shorter T3 half-life. According to Geyten et al. (2005), the plasma half-life of intravenously injected T3 was determined to be 70~80 min in tilapia (*Oreochromis niloticus*). In addition, different observations were made regarding TH level changes in fish exposed to tetrabromodiphenyl ether (PBDE-47 or DE-47). In short, exposure to DE-47 significantly increased plasma T4 levels in embryo-larval zebrafish (Kuiper et al. 2008) but

decreased T4 in juvenile lake trout (*Salvelinus namaycush*) and in gavaged Wistar rats (Stoker et al. 2004; Tomy et al. 2004).

While thyroid-disrupting effects of UV filters have been reported in fish previously (Lee et al. 2018; Lee et al. 2019; Chu et al. 2021), long-term effects on thyroid disruption have rarely been investigated. In the present study, in juvenile and adult life stages, the patterns of TH level change varied among the tested UV filters. The differential response of THs across life stages is not clear. However, one research group showed that age and sex could influence thyroid hormone levels in a large Chinese population (n=83,643) and showed that TSH and fT3 levels were significantly increased with age (Chen et al. 2020). Moreover, because zebrafish embryos are characterized by external fertilization and development, THs are first obtained from maternal transfer to the yolk, and endogenous TH production increases gradually beginning at approximately 72 hpf (Porazzi et al. 2009; Power et al. 2001). In addition, Vergauwen et al. (2018) demonstrated that maternal *trhr* and *tshr* transcripts, which are crucial in feedback mechanisms for TH regulation, are absent from zebrafish embryos at 1.5 hpf; the transcription of these factors starts after 1.5 hpf and is stopped by 7 dpf. In the present study, although the changes were moderate, treatment with OMC increased plasma TSH and fT3 in adult male zebrafish, and the levels of these factors were significantly correlated with the OMC concentrations. However, apart from the indicated mild increase in TSH and fT3, no significant differences were observed in T3, as found in a previous study by Chu et al. (2021), and the

increases compared to the control were relatively small (approximately <1.3-fold change). Similar contradictory results were also found in juvenile lake trout upon DE-71 exposure (Tomy et al. 2004; Kuiper et al. 2008). For instance, DE-71 exposure caused a significant decrease in the plasma T4 level of juvenile lake trout (Tomy et al. 2014); however, no significant change in juvenile lake trout was noted in Kuiper et al. (2008). Moreover, Christian et al. (2003) suggested that thyroid hormone levels are influenced by factors including animal strain, age, cardiac rhythms, stress associated with blood collection techniques or animal handling, and the relative activity of the animal; these factors should be considered when evaluating thyroid function in rodent models.

THs play crucial roles in the development, growth and metabolism of teleosts. In vertebrates, the secretion of THs is centrally regulated by the HPT axis. TSH released from the pituitary acts as a major regulator of THs for the thyroid gland (Chu et al. 2021). It has been suggested that assessment of *TSHb* gene transcription could be a valuable determinant for thyroid dysfunction caused by environmental pollutants (Yu et al. 2013; Zhai et al. 2014). TSH acts on the thyroid gland to stimulate TH synthesis. This process involves several sequential steps, including absorption of iodide by thyroid follicular cells (NIS), oxidation of iodide (TPO), and iodination of thyroglobulin (TG) (Manchado et al. 2008; Porazzi et al. 2009).

In our study, exposure to the tested UV filter significantly altered the gene transcription of the HPT axis. The reduced TSH, T4, and T3 levels in AVB-

exposed embryo-larval zebrafish can be explained by the downregulation of the *trhr*, *tshb*, *nis*, and *tpo* genes. Since the *nis* and *tpo* genes are involved in TH synthesis, the downregulation of these genes in embryo-larval fish might contribute to the decrease in THs. Long-term exposure to AVB in juvenile fish also led to decreased levels of TSH and T3. The reduced TSH and T3 levels might be attributed to significant downregulation of the *trh* and *tshb* genes. In adult male zebrafish exposed to AVB, significant upregulation of *tshr*, *nis*, *tpo*, and *tg* gene transcription might reflect efforts to compensate for the decreased plasma T3 level.

Exposure to BP-3 also significantly modulated the levels of genes in the HPT axis. For instance, significant downregulation of *trh* and *nis* was observed. The decreased TH levels could be explained by reduced stimulation of the hypothalamus and reduced synthesis in thyroid tissue. Although *tshb* gene transcription was slightly upregulated, thyrotropin-releasing hormone (TRH) is the main stimulator of TSH release in vertebrates, which explains the decreased TSH level in embryo-larval zebrafish. Chronic exposure to BP-3 significantly upregulated genes related to thyroid stimulation in the hypothalamus and pituitary and to synthesis in the thyroid tissue. The substantial increase in TH levels in juvenile fish could be explained by the upregulation of the *tshb*, *trhr*, *nis*, *tpo*, and *tg* genes, which were also found by Lee et al. (2018). In adult male zebrafish, exposure to BP-3 also significantly elevated the levels of THs, which is attributed to the upregulation of the *tshb*, *tshr*, *nis*, *tpo*, and *tg* genes in the brain and thyroid tissues.

For OC, the downregulation of the *trhr* gene in embryo-larval zebrafish following exposure to OC might contribute to decreased TSH. Upregulation of *tg*, which is the precursor of TH, is well known for its important role in TH synthesis. Elevated transcription of the *tg* gene might reflect a compensatory response to lowered TH levels. In juvenile and adult fish exposed to OC, the greater production of THs was accompanied by increases in *trhr*, *tshb*, *trhr*, *nis*, and *tpo* and *tshr*, *nis*, *tpo*, and *tg*, respectively.

In the present study, the upregulation of *trh* and *trhr* gene transcription caused by OMC exposure could be a compensatory effect for negative feedback regulation in zebrafish embryo-larvae. Chu et al. (2021) also found significant upregulation of *tshr* in embryo-larval zebrafish, suggesting compensatory efforts to facilitate TH synthesis. In juvenile fish, the concentrations of OMC demonstrated significant negative and positive correlations with TSH and T4, respectively, which could be explained by mild downregulation of *tshb* and upregulation of *nis*. Although the gene expression profiles in the hypothalamus-pituitary of adult male zebrafish were similar, transcription patterns were different in the thyroid tissues, as observed by Chu et al. (2021). According to Chu et al. (2021), significant downregulation of the *tshr*, *nis*, *tpo*, and *tg* genes was observed in adult male zebrafish thyroid; however, these genes were significantly induced in the present study. There are several possible reasons for this observation. Harding et al. (2019) found that genetic and epigenetic diversity play a fundamental role in determining the basic traits of individuals that account for intraspecific variation in

response to anthropogenic stress. One study explored interstrain genetic variation in wild-type zebrafish ranging from 7% (inbred) to 37% (wild-derived), which may affect experimental design and interpretation (Guryev et al. 2006). Another study also reviewed how the gut microbiota interacts with the thyroid by regulating iodine uptake, degradation, and enterohepatic circulation (Fröhlich and Wahl, 2019). A study on the intestinal microbiota in zebrafish found differences in the intestinal fungal communities between wild-caught and laboratory-reared zebrafish (Siriyappagouder et al. 2018).

In fish, the concentrations of T3 in peripheral tissues are primarily regulated by deiodinase activity, which converts T4 to biologically active T3 (Kim et al. 2015). Deiodinase type 1 (*dio1*) is responsible for both the production and clearance of T3 and reverse T3 (rT3). Deiodinase type 2 (*dio2*) plays a major role in the activation of outer ring deiodination by converting T4 to active T3 (Johnson et al. 2011). Deiodinase type 3 (*dio3*) removes iodine from the inner ring of T4 and T3 to terminate these hormones into inactive forms, such as rT3 and diiodothyronine (T2) (Johnson et al. 2011).

In the current study, exposure to AVB significantly induced the *dio1* gene levels in embryo-larval zebrafish but significantly downregulated this gene in juvenile bodies and adult male zebrafish livers. Induction of *dio1* in the embryo-larval stage could be a compensatory effort to increase T3 levels. In tilapia, upregulation of *dio1* mRNA transcription in the liver was noted under the hypothyroid state (Van der Geyten et al. 2001). In addition, marked

downregulation of the hepatic *dio2* gene and upregulation of the *dio3* gene also support the hypothesis that plasma T3 levels are decreased in adult male zebrafish.

Upon BP-3 exposure, significant induction of the *dio2* gene in juvenile fish coincided with that in OC-exposed juvenile fish, to promote the degradation of increased T4. A similar observation was made by Lee et al. (2018), who demonstrated significant upregulation of the *dio1* gene in the hypothyroid state of embryo-larval zebrafish after exposure to BP-3. The significant upregulation of the *dio1* and *dio2* genes in adult male zebrafish thyroid tissue after exposure to BP-3 or OC could be interpreted as an effort to degrade excess THs. The reason for the downregulation of the *dio2* gene in zebrafish adult male liver after exposure to BP-3 likely lies in the possibility that ubiquitination could reduce *dio2* activity under hyperthyroid conditions (Kim et al. 2015).

For OMC, significant upregulation of the *dio2* and *dio3* genes was observed in embryo-larval zebrafish, which is in agreement with a previous study (Chu et al. 2021). After 5 days of exposure to OMC, significant upregulation of the *dio2* gene was observed, along with decreased TH levels; however, the expression changes in the *dio3* gene were not measured. In juvenile fish, downregulation of the *dio1* gene was found. Another previous study by Lee et al. (2019) observed significant downregulation of the *dio2* gene in juvenile Japanese medaka exposed to OMC for 38 days; however, transcriptional changes in the dio1 gene were not evaluated. After 21 days of

exposure to OMC, significant upregulation of the *dio1*, *dio2*, and *dio3* genes was observed in the adult male zebrafish thyroid, which disagrees with previous findings (Chu et al. 2021). This seemingly discrepant observation may be explained by the influence of genetic and epigenetic diversity that is partially responsible for intraspecific variation, as discussed above (Harding et al. 2019).

We also examined genes involved in the glucuronidation and sulfation of THs. These modifications are catalyzed by uridine diphosphate glucuronosyltransferase (UDPGT) and sulfotransferase (SULT), respectively, both of which are phase II enzymes involved in TH excretion (Yanagiba et al. 2008). Trt is a carrier protein for THs in fish and plays a role in supplying THs to target tissues (Power et al. 2000). Simultaneously, induction of *ttr* can delay the metabolic elimination of free THs from the circulation (Kim et al. 2016). The reduction of *ttr* gene transcription eventually reduces the ability of *ttr* to bind free THs, causing the extra unbound THs to more easily undergo hepatic metabolic processing, resulting in significant declines in circulating TH levels (Zhang et al. 2018).

As shown in our study, AVB, BP-3, and OC significantly increased *ugt1ab* and/or *sult1 st5* gene expression in embryo-larval zebrafish, resulting in diminished TH levels due to enhanced excretion. For OMC, downregulation of *ugt1ab* but upregulation of the *sult1 st5* gene was observed in embryo-larval fish. Despite a significant reduction in *ugt1ab* gene levels to attempt to decrease TH elimination, the decreased levels of THs could not be restored in

larval fish. Yanagiba and colleagues (2008) reported that downregulation of *Ahr* and its target gene *Ugt1a1* is associated with an increase in total T4 levels, which is consistent with our results in juvenile zebrafish exposed to AVB, BP-3, and OMC, which showed elevated levels of T4 and reduced expression of *ugt1ab*. For OC, significant upregulation of the *ugt1ab* gene was observed, which could be interpreted as an adaptive mechanism to eliminate excessive T4 levels. This means that elevated transcription of *ugt1ab* and/or *sult1 st5* may be related to facilitated excretion of THs in adult male zebrafish liver exposed to AVB, BP-3, OC, and OMC.

The upregulation of the *ttr* gene may also be associated with lowered TH levels and compensatory efforts to increase TH levels in embryo-larval zebrafish exposed to AVB, OC, and OMC. In BP-3-exposed embryo-larval fish, a slight upregulation in *ttr* was noted; however, the change was not large enough to achieve statistical significance. The significant decrease in *ttr* gene expression in adult male zebrafish liver exposed to BP-3 suggests a secondary response to elevated T3, facilitating its elimination.

2.5 Summary

Overall, we observed thyroid endocrine disrupting effects of UV filter compounds on endpoints regulated by or relevant to the HPT axis during different life stages. The effects of the tested UV filters seem to more severe in early life stages. In addition, each UV filter compound exhibited a distinct spectrum of TH alterations, providing evidence that both early- and adult-stage life could be influenced by UV filter exposure.

The embryo-larval fish demonstrated more severe effects in terms of thyroid disruption. A number of studies have also shown that the embryonic stage is particularly susceptible to anthropogenic stress compared with later life stages (Abe et al. 2001; Oliveira et al. 2009; Domingues et al. 2010; Sisman, 2011; Ton et al. 2012; Cao et al. 2016), suggesting that this most sensitive period should be given special attention. In view of the importance of THs as growth factors in early life, animals deprived of THs at this stage of life may be more susceptible to developing thyroid-related endocrine dysfunction over time (Segni, 2000). Additionally, our results highlight that *in vivo* biotoxicity assays performed for a single life stage may not be adequate for evaluating external toxic harm. Therefore, to determine the sensitivity of species to potentially toxic chemicals, systematic tests should be performed at different life stages.

Chapter 3 Neurotoxicity and related mechanisms of organic UV filters in zebrafish (*Danio rerio*) of different life stages

3.1 Introduction

EDCs are environmental toxicants that interfere with endogenous hormone signaling pathways. To date, EDCs have been extensively studied for their disruptive activities against reproduction, development, and sex and thyroid hormones. Over the past few decades, a large body of research has confirmed that endogenous hormones play a decisive role in the developing brain, influencing neural cell proliferation, apoptosis, differentiation, migration and myelination (Schug et al. 2015; Calamandrei and Ricceri, 2018; Iturbide et al. 2021; Cediel-Ullo et al. 2022). These findings raise concerns about the health consequences associated with early exposure to EDCs. According to a recent study, Bellanger et al. (2015) concluded that EDCs contribute significantly to neurobehavioral deficits and disease in Europe with costs of up to €150 billion annually.

Among the many EDCs known, bisphenol A (BPA), phthalates, and perfluoroalkyl substances (PFAS) are best studied in terms of neurotoxic potentials (Braun 2016; Street et al. 2018). In addition, the Horizon 2020 research project ENDpoiNTs (no. 825759) predicted BPA, phthalates, PFAS and synthetic pyrethroids (4,4'-DDE, cypermethrin, endosulfan, permethrin)

as neurodevelopmental toxicants induced by endocrine disruption (Lupu et al. 2020). The authors stated that these groups of chemicals can act by perturbing endocrine signaling pathways, such as the estrogen, androgen, thyroid, glucocorticoid and retinoid signaling pathways, which are key drivers of sexual and social aggression, cognition, anxiety and depression, by modulating neurotrophic factors (Küppers et al. 2001; Hines, 2006; McCarthy, 2008; Bayless et al. 2016; Bakker, 2019). Moreover, a comprehensive review of laboratory animals and human populations suggested that these substances are strongly associated with neurodevelopmental toxicity and related diseases such as autism spectrum disorder (ASD), attention deficit–hyperactivity disorder (ADHD), and intellectual disabilities (Street et al. 2018; Cediel-Ullo et al. 2022).

Despite the longstanding evidence that the developing brain is particularly vulnerable to early-life exposure to certain EDCs (Lee and Freeman 2014), other studies have implied that the adult life stage is also a critical window for neurotoxicant exposure (Della Seta et al. 2005; Johnson et al. 2015; Catanese and Vanderberg, 2017; Keller et al. 2019). Specifically, exposure to several EDCs, such as BPA, bisphenol S, and ethinyl estradiol (EE), significantly reduced maternal nursing behavior (time licking and grooming pups, etc.) in different species of pregnant rodent models. Strikingly, studies of parental exposure to polychlorinated biphenyls (PCBs) and vinclozolin in SD rats have revealed that these chemicals could induce transgenerational behavioral

deficits, such as social preferences, in their offspring (F_1-F_6 generations, n=1200) (Gillette et al. 2022).

There is an increasing amount of evidence from several *in vitro* and *in vivo* models suggesting the neurotoxic potentials of organic UV filters, as described in Chapter 1 (Table 5-7). However, toxicological information related to neurodevelopmental or neurobehavioral effects is mostly limited to BP-3 (Table 5-7). The findings from these previous studies suggested growth delay, apoptosis and sex hormone disruption as possible mechanisms of neuronal dysfunction. However, the direct or indirect neurotoxic effects of other commonly used UV filters, i.e., AVB, OC, and OMC, are rarely covered. Apart from sex hormones, THs are another important factor that could impair the normal function of nervous systems (Chatonnet et al. 2013; Jansen et al. 2019). Hence, it is important to address the impact of EDC exposure on THs and the mechanisms involved in their disruption of neurological function.

Zebrafish is a vertebrate model organism that has recently emerged as a key model in various aspects of neuroscience research (de Esch et al. 2012; Schmidt et al. 2013; Nishmura et al. 2015). Zebrafish have high fecundity, generating large numbers of embryos that are relatively transparent. In addition, zebrafish can be used as a neurotoxicity screening model as early as 30 hpf, while it is still in the embryonic stage. Due to their small sizes at the early life stage, 96-well plates can be used for toxicity testing of zebrafish, allowing high-throughput screening of toxic chemicals (d'Amora and Giordani, 2018). Zebrafish have a fully sequenced genome and share a high

(>70%) genetic homology to humans. In addition, their CNSs have been shown to resemble the relevant areas of the human brain (de Esch et al. 2012; Schmidt et al. 2013).

The purpose of this study was to evaluate the neurotoxic potentials of organic UV filters in embryo-larval, early-life and adult zebrafish. For this purpose, we examined neurobehavioral changes and the transcript levels of several genes related to the CNS.

3.2 Materials and methods

3.2.1 Test chemicals

AVB (avobenzone; butyl methoxydibenzoylmethane), BP-3 (benzophenone-3; 2-hydroxy-4-methoxybenzophenone) OC (octocrylene; octocrylene), and OMC (octyl methoxycinnamate; ethyl hexyl methoxycinnamate (EHMC)) were obtained from Sigma–Aldrich (St. Louis, MO, USA). All test chemicals were dissolved in dimethyl sulfoxide (DMSO), and the solvent concentration did not exceed 0.01% for AVB, OC, OMC, and 0.005% for BP-3. The purity of all chemicals used in the present study was ≥97%.

3.2.2 Zebrafish maintenance and exposure design

Wild-type adult zebrafish were maintained in a temperature-controlled room under a 16:8 h light/dark photoperiod in the Environmental Toxicology Laboratory at Seoul National University in Korea. The fish were fed *Artemia nauplii* and mosquito larvae (Green Fish, Seoul, Korea) twice per day *ad libitum*. Zebrafish embryos were obtained from approximately 6-month-old adult zebrafish mating pairs and exposed to test solutions within 4 hours on the day of exposure.

Embryo-larval exposure (120 hpf) was slightly modified from OECD test guideline 236 (OECD, 2013). Fertilized eggs were equally distributed into test solutions. Test concentrations, i.e., 1, 3, 10 and 30 μM AVB, were determined using preliminary range-finding tests; 0.14, 0.44 and 1.40 μM BP-3 and 1, 3, 10, and 30 μM OMC were selected based on previous studies (Lee et al. 2018; Chu et al. 2021). Each treatment group had four replicates, and renewal of the test solution was conducted every 24 hours.

An early-life stage experiment (~30 dpf) was conducted according to OCED test guidelines with minor modification. Fifty fertilized embryos (<4 hpf) were exposed to each treatment in four replicates through semistatic renewal exposure until 30 dpf. Exposure concentrations, i.e., 0.1, 0.3, 1, and 3 μM AVB; 0.14, 0.44 and 1.40 μM BP-3; and 0.1, 0.3, 1, and 3 μM OMC, were determined based on preliminary range-finding tests. The water quality parameters, including pH, conductivity, temperature, and dissolved oxygen,

were monitored before and after water renewal. Fish were fed twice per day with commercially available Gemma micro 75 (ZEBCARE, Netherlands) from Day 5 to 15 after fertilization and freshly hatched *Artemia nauplii* (Greenfish, Korea) from 16 to 29 days after fertilization.

For adult fish exposure, 8 wild-type adult male zebrafish (>6 months) were exposed to the test UV filters and control groups for 21 days. All tests were performed with reference to OECD test guideline 229 (OECD, 2012). Test concentrations, i.e., 1, 3, 10, and 30 μM AVB and 0.14, 0.44, 1.40, and 4.44 μM BP-3, were determined following our preliminary range-finding tests. For OMC, the concentration range (1, 3, 10, 30 μM) was determined in a previous study (Chu et al. 2021). The exposure medium was renewed every day. Mortality was recorded daily until experiment termination.

After each exposure, the fish were sacrificed on ice, and whole embryo-larval (120 hpf) and early-life juvenile (30 dpf) fish and plasma and tissues from adult male zebrafish were collected and kept frozen at 80 °C until analysis.

This study was conducted with the approval of the Institutional Animal Care and Use Committee of Seoul National University, Seoul, Korea (IACUC/SNU-190114-5-1, SNU-210505-1).

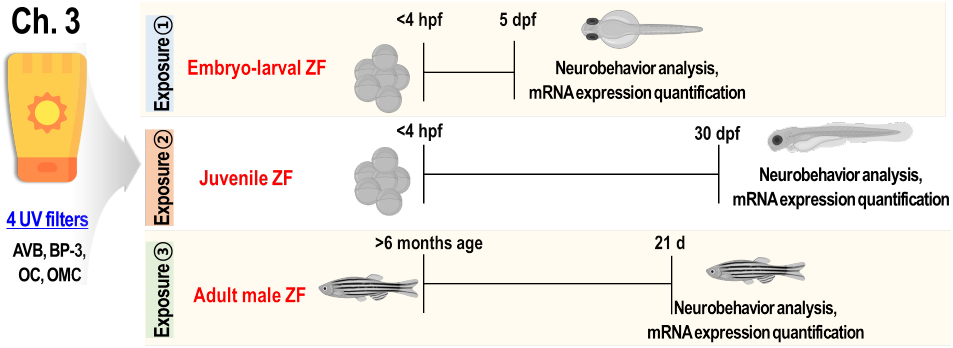


Fig. 15. Experimental degisn of chapter 3.

3.2.3 Neurobehavior measurement

Neurobehavioral tests were perform on embryo-larval and juveniles zebrafish.

Following exposure, three larvae (120 hpf) from each replicate (total n=12 fish per group) and two juveniles (30 dpf) from each replicate (total n=8 fish per group) were randomly selected and deposited in 96-well and 48-well plates, respectively. Plates were then placed in a prewarmed (26.5 °C) Daniovision chamber (Noldus, Netherlands) equipped with a live video tracking instrument. For neurobehavior evaluation in zebrafish embryo-larvae, the larval fish were first exposed to a 10 min light state for acclimation, followed by 20 min of alternating light and dark states (Light 1: 20 min, Dark 1: 20 min, Light 2: 20 min). For juvenile fish behavior analysis, fish were acclimated for 10 min in light conditions and challenged with 15 min of light and dark cycles (Light 1: 15 min, Dark 1: 15 min, Light 2: 15 min, Dark 2: 15 min). The mean velocity (mm/sec) and total distance traveled (mm) under light or dark conditions were evaluated as indicators of hyper or hypoactivity. Moreover, thigmotaxis or 'anxiety-like' behavior (i.e., frequency of entering center zone, time spent in center zone, latency to enter center zone) of the larval and juvenile fish was assessed in dark phases, along with locomotor behavior changes (mean velocity (mm/sec) and total distance traveled (mm)), by Ethovision (Nodulus).

3.2.4 Gene transcription analysis

The transcription of genes related to neurotoxicity was measured (Table 10).

Total RNA was extracted from whole embryo-larval (n=30 per replicate) and juvenile (n=10 per replicate) fish and adult male zebrafish brains (n=5 per replicate) using a NucleoSpin® RNA mini-kit (Macherey-Nagel, Germany) according to the manufacturer's instructions (n=4). Then, the total RNA concentration and quality was evaluated using an EpochTM-Take 3 microplate spectrophotometer (BioTek Instruments Inc., Winooski, VT, USA). RNA (80-100 ng/µL) samples were used for reverse transcription using an IScript cDNA synthesis kit (Bio–Rad, Hercules, CA, USA).

For qPCR analysis, 20 µL qPCR reactions containing 10 µL of 2×SYBR Green^{TM} PCR master mix (Applied Biosystems), 0.1 µL of each 10 pmol forward and reverse PCR primer, 7.8 µL of nuclease free water (NFR), and 2 µL of cDNA sample were used. The real-time qPCR conditions were as follows: preincubation at 95 °C for 10 min, followed by 40 cycles of amplification at 95 °C for 10 s, 60 °C for 20 s, and 72 °C for 20 s. The data were analyzed and expressed as the relative gene transcription level using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The transcription level of each target gene was normalized to that of the most stable housekeeping gene (*rpl8*) identified by geNorm analysis.

Table 10. Primer sequences for qRT-PCR analysis used in this study

Gene	Protein name	Primer sequence (5'-3')	Accession no.
Reference gene			
rpl8[a]	60S ribosomal protein L8	F: ttgttggtgttgttgctggt R: ggatgctcaacagggttcat	NM_200713.1
Target gene			
mbp	Myelin basic protein	F: cagcaggttcttcggaggag R: acgaggagaggacacaaagc	XM_001340280.4
gap43	Axonal membrane protein GAP-43	F: aaatagacaaaccagacgctgc R: cgaacataaagcaggctgtcg	NM_131341.1
gfap	Glial fibrillary acidic protein	F: ggatgcagccaatcgtaat R: ttccaggtcacaggtcag	NM_131373
c-fos	Fos proto-oncogene	F: tgcagcacggcttcaccgag R: cgggcatgaagagatcgccgt	NM_205569.1
syn2a	Synapsin IIa	F: gttctgatccggcaacatgc R: cagacatgcaaatgcccagg	NM_001002597.2
syt1a	Synaptotagmin	F: ggctcagcagagaatagcga R: ggtttttccttttgtgctctctgt	XM_017354995.2
stxbp1b	Syntaxin-binding protein 1b	F: gaccagtgcccgttatggaa R: tgtgtggagccgataagagc	NM_001089376.1

[a]Reference genes used in embryo-larval and juvenile fish, adult male brain.

3.2.5 Statistical analysis

The statistical analysis was the same as that described in Chapter 2.

3.3 Results

3.3.1 Locomotor behavior changes

3.3.1.1 Effects on locomotor behavior of embryo-larval zebrafish (120 hpf)

To evaluate the potential neurotoxicity of UV filters on zebrafish early development, basic motor responses were evaluated in free-swimming embryo-larval zebrafish. UV filters had similar profiles in the embryo-larval stage. Exposure to a series of concentrations of AVB, BP-3, OC and OMC resulted in hypoactivity during both light and dark transitions (Fig. 16) which was reflected by the reduced total distance moved (mm) and mean velocity (mm/s).

3.3.1.2 Effects on locomotor behavior of juvenile zebrafish (30 dpf)

Next, we investigated chronic exposure (0~30 dpf) to environmentally relevant concentrations of UV filters to provide evidence of neurodevelopmental impairment triggered by UV filter exposure. Interestingly, similar locomotor patterns were demonstrated by juvenile zebrafish after 30 days of exposure to each chemical. Upon AVB, BP-3, OC and OMC exposure, the juvenile fish showed a significant decrease in locomotion parameters such as total distance moved (mm) and mean velocity (mm/s) during both the light and dark phases (Fig. 17).

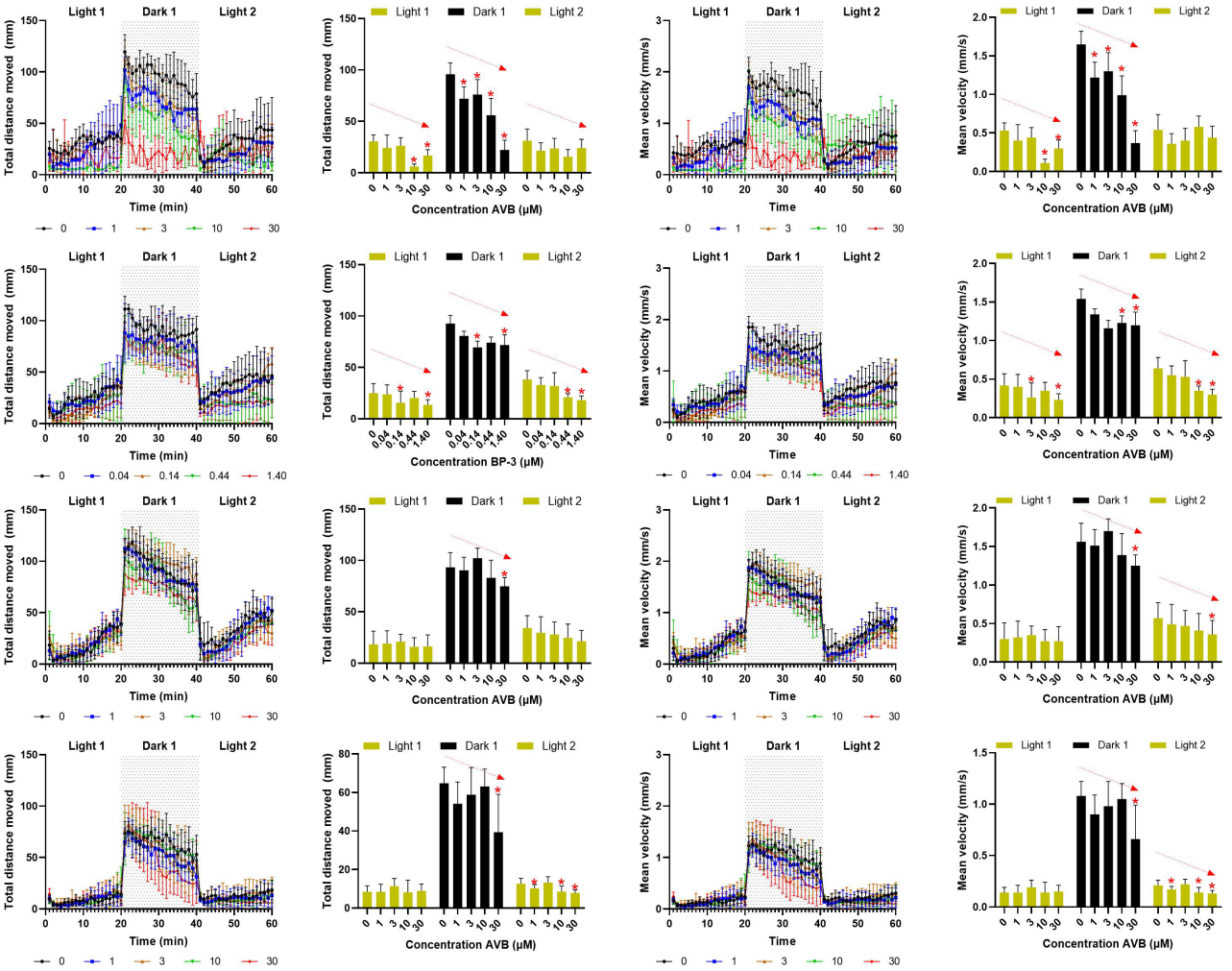

Light 1
Dark 1
Light 2
Total distance moved (mm)
Time (min)
Concentration AVB (μM)
Concentration BP-3 (μM)
Mean velocity (mm/s)
Time
0
1
3
10
30
0.04
0.14
0.44
1.40

Fig. 16. Locomotor changes in embryo-larval zebrafish after exposure to UV filters. *Asterisks indicate significant differences from the solvent control (p<0.05). Dotted red line indicates significant correlation (p<0.05).

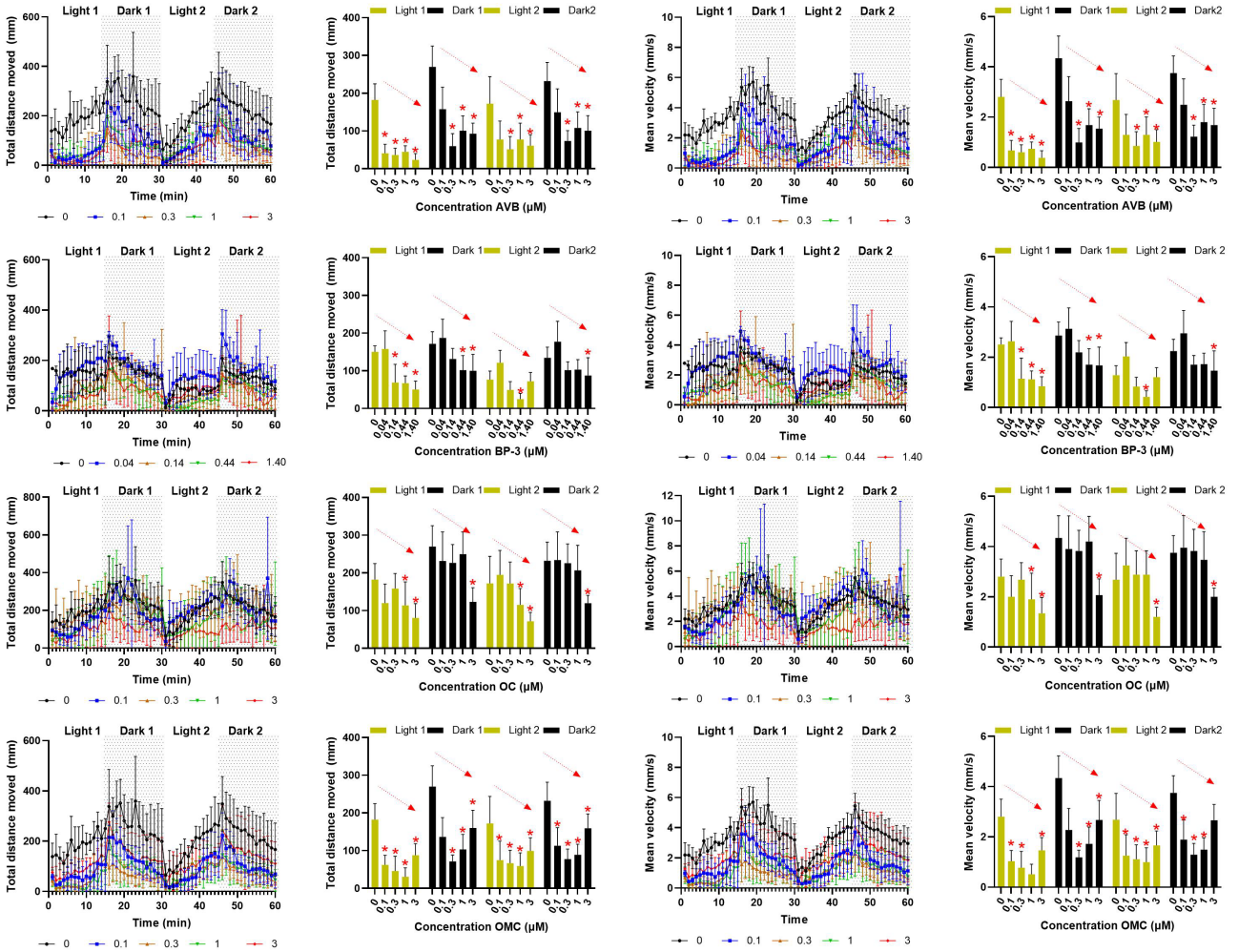

Light 1
Dark 1
Light 2
Dark 2
Dark2
Total distance moved (mm)
Mean velocity (mm/s)
Time (min)
Time
Concentration AVB (μM)
Concentration BP-3 (μM)
Concentration OC (μM)
Concentration OMC (μM)
0
0.1
0.3
1
3
0.04
0.14
0.44
1.40

Fig. 17. Locomotor changes in juvenile zebrafish after exposure to UV filters. *Asterisks indicate significant differences from the solvent control ($p<0.05$). The dotted red line indicates a significant correlation ($p<0.05$).

3.3.2 Anxiety evaluation

3.3.2.1 Anxiety-like behavior caused by UV filter exposure in embryo-larval zebrafish (120 hpf)

The open-field test was used to evaluate anxiety-like behavior patterns in early developing zebrafish. After exposure to AVB, BP-3, OC and OMC, embryo-larval zebrafish showed significant differences in neurobehaviors (Fig. 18). Following AVB and OMC exposure, behavior parameters such as total distance moved (mm) and mean velocity (mm/s) in both peripheral and center areas showed significant decreases. In addition, anxiety parameters such as "in zone frequency" (entries into the center area), were significantly reduced after AVB and OMC exposure, and significant changes in total distance moved and mean velocity demonstrated hypoactivity; however, no differences were observed in "in zone frequency" or "latency to first entry" into the center area.

3.3.2.2 Anxiety-like behavior caused by UV filter exposure in juvenile zebrafish (30 dpf)

Next, we questioned whether early exposure to UV filters might result in similar anxiety behavior patterns in juvenile zebrafish. Fish were raised with constant exposure to a series of concentrations of UV filters, including AVB, BP-3, OC and OMC, for 1 month, and then anxiety-like neurobehavior analysis was conducted in the juvenile fish (Fig. 19). Interestingly, juvenile zebrafish revealed different responses compared to controls. Specifically, in the dark phases, we observed a significant decrease in the total and mean values of both traveling distance and velocity in AVB-, BP-3- and OC-treated groups in peripheral and center zones. OMC-treated fish only displayed reduced mean velocity in the peripheral area. Moreover, juvenile zebrafish subjected to early AVB and BP-3 exposure showed a significant reduction in the frequency of entry into the center area; however, no differences in this behavioral measure were found for OC and OMC exposures. During the test, AVB-treated groups showed significant reduction time spent in the center area, whereas notable induction of time spent at the center upon BP-3 exposure, pointing to a long-term effect on general neurobehavior impairment induced by early-life exposure to UV filters.

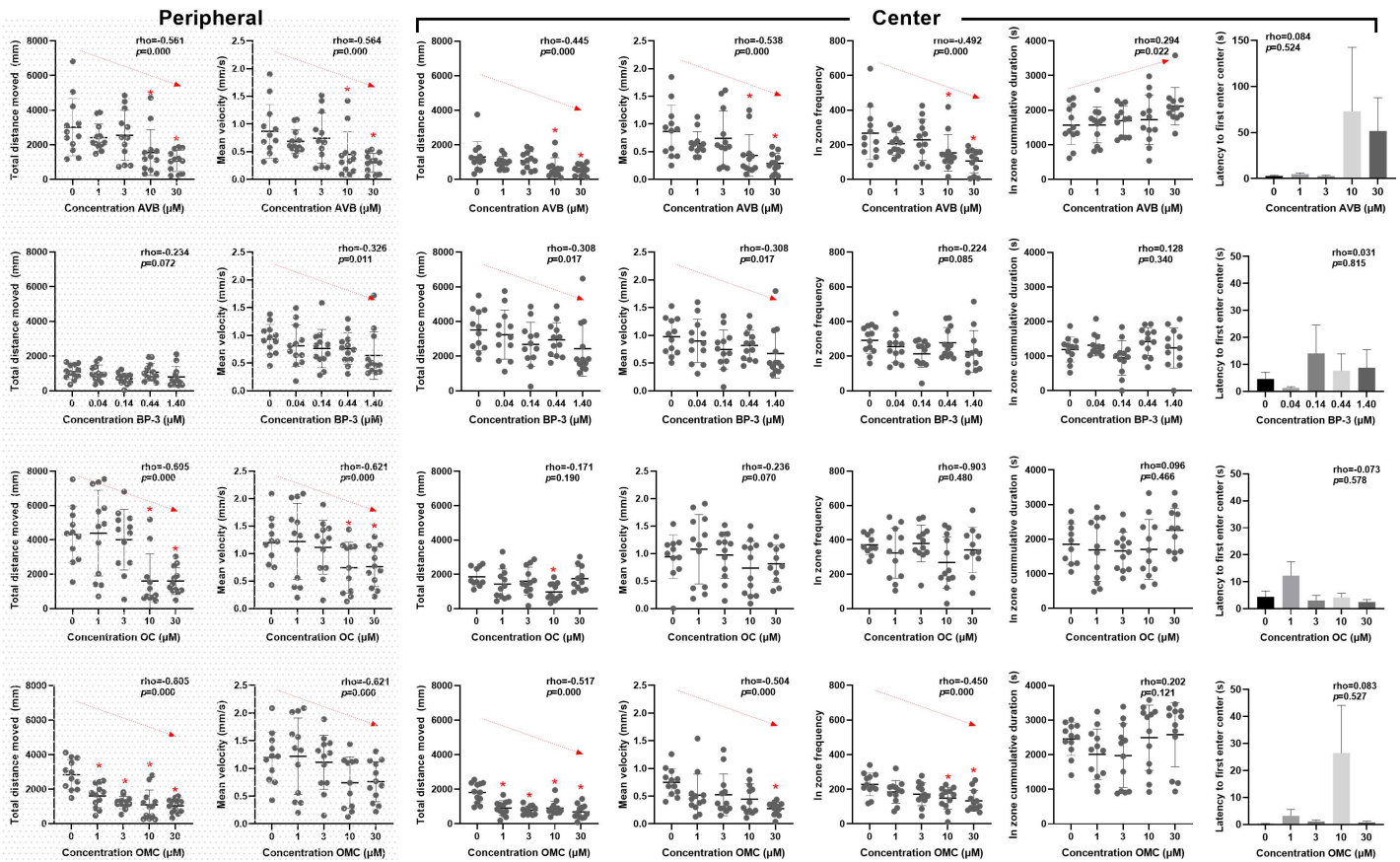

Peripheral
Center
Total distance moved (mm)
Mean velocity (mm/s)
In zone frequency
In zone cummulative duration (s)
Latency to first enter center (s)
Concentration AVB (μM)
Concentration BP-3 (μM)
Concentration OC (μM)
Concentration OMC (μM)
rho=-0.561 p=0.000
rho=-0.564 p=0.000
rho=-0.445 p=0.000
rho=-0.538 p=0.000
rho=-0.492 p=0.000
rho=0.294 p=0.022
rho=0.084 p=0.524
rho=-0.234 p=0.072
rho=-0.326 p=0.011
rho=-0.308 p=0.017
rho=-0.308 p=0.017
rho=-0.224 p=0.085
rho=0.128 p=0.340
rho=0.031 p=0.815
rho=-0.605 p=0.000
rho=-0.621 p=0.000
rho=-0.171 p=0.190
rho=-0.236 p=0.070
rho=-0.903 p=0.480
rho=0.096 p=0.466
rho=-0.073 p=0.578
rho=-0.605 p=0.000
rho=-0.621 p=0.000
rho=-0.517 p=0.000
rho=-0.504 p=0.000
rho=-0.450 p=0.000
rho=0.202 p=0.121
rho=0.083 p=0.527

Fig. 18. Anxiety-like behavior changes in embryo-larval zebrafish after exposure to UV filters. Gray dots indicate individual values. *Asterisks indicate significant differences from the solvent control ($p<0.05$). The dotted red line indicates a significant correlation ($p<0.05$).

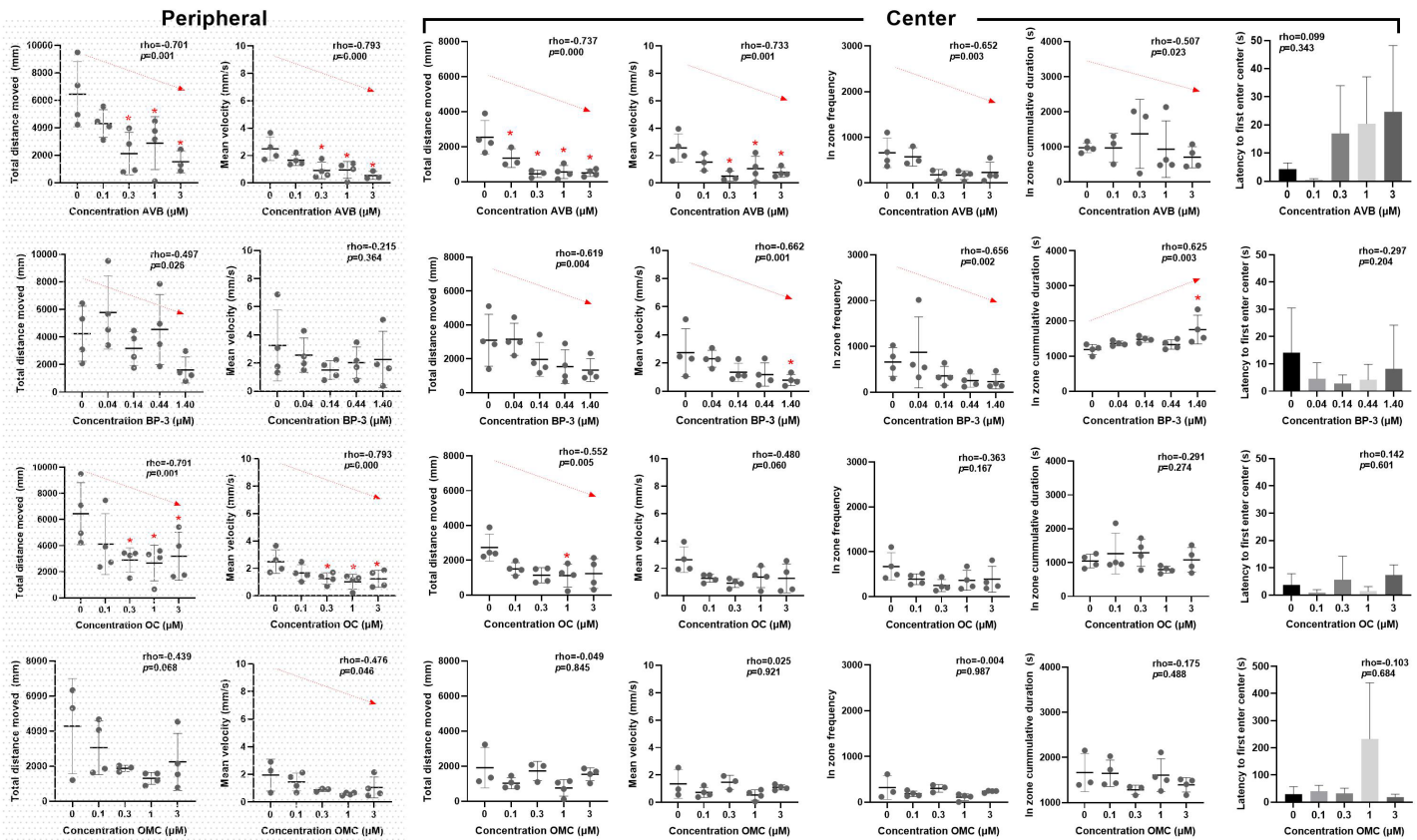

Peripheral
Center
Total distance moved (mm)
Mean velocity (mm/s)
In zone frequency
In zone cummulative duration (s)
Latency to first enter center (s)
Concentration AVB (μM)
Concentration BP-3 (μM)
Concentration OC (μM)
Concentration OMC (μM)
rho=-0.701 p=0.001
rho=-0.793 p=0.000
rho=-0.737 p=0.000
rho=-0.733 p=0.001
rho=-0.652 p=0.003
rho=-0.507 p=0.023
rho=0.099 p=0.343
rho=-0.497 p=0.026
rho=-0.215 p=0.364
rho=-0.619 p=0.004
rho=-0.662 p=0.001
rho=-0.656 p=0.002
rho=0.625 p=0.003
rho=-0.297 p=0.204
rho=-0.791 p=0.001
rho=-0.793 p=0.000
rho=-0.552 p=0.005
rho=-0.480 p=0.060
rho=-0.363 p=0.167
rho=-0.291 p=0.274
rho=0.142 p=0.601
rho=-0.439 p=0.068
rho=-0.476 p=0.046
rho=-0.049 p=0.845
rho=0.025 p=0.921
rho=-0.004 p=0.987
rho=-0.175 p=0.488
rho=-0.103 p=0.684

Fig. 19. Anxiety-like behavior changes in juvenile zebrafish after exposure to UV filters. Gray dots indicate individual values. *Asterisks indicate significant differences from the solvent control (p<0.05). The dotted red line indicates a significant correlation (p<0.05).

3.3.2 Gene transcription level changes

To answer our questions in more detail, we evaluated genes involved in neurogenesis (*mbp*, *gap43*) and related to neurotoxicity (*gfap*, *c-fos*). We also investigated genes that are primarily involved in CNS development and neurotransmitter systems (*syn2a*, *syt1a*, *stxbp1b*).

In detail, exposure to AVB significantly reduced the mRNA expression of *mbp* in embryo-larval and adult male zebrafish; however, no significant change was observed in juveniles. The *gfap* and *gap43* genes were significantly decreased in embryo-larval and juvenile fish. For the *c-fos* gene, significant upregulation was observed in embryo-larval and adult zebrafish. Juvenile fish also showed >1.5-fold upregulation; however, the difference was not statistically significant (Fig. 20). Significant decreases in the levels of the *syn2a*, *syt1a*, and *stxbp1b* genes were observed in embryo-larval zebrafish. However, in juvenile and adult fish, statistical significance was only determined for the *syn2a* and *syt1a* genes, respectively.

The results of gene expression changes after BP-3 exposure are described in Fig. 20. The expression of *mbp* was significantly downregulated in embryo-larval, juvenile, and adult male zebrafish. The *gfap* gene was significantly upregulated in embryo-larval fish but downregulated in juvenile fish. The *gap43* gene was only significantly induced in the juvenile stage. The expression level of *c-fos* following BP-3 exposure differed by life stage. Specifically, in embryo-larval *c-fos,* mRNA expression was significantly

downregulated; however, it was significantly upregulated in juvenile and adult fish. No effects on the *syn2a*, *syt1a*, and *stxbp1b* genes were noted.

The central nervous systems of zebrafish treated with OC were significantly modulated (Fig. 20). The mRNA levels of all measured genes, i.e., *mbp*, *gfap*, *gap43*, *c-fos*, *syn2a*, *syt1a*, and *stxbp1b,* were significantly decreased in the embryo-larval stage. In juvenile fish, the transcription levels of *gap43* and *c-fos* were significantly upregulated, distinct from the embryo-larval observations. In adult male zebrafish brains, the mRNA level of *c-fos* was affected only by OC exposure.

For OMC, a significant reduction in *mbp* mRNA levels was observed in embryo-larvae and juveniles. The *gap43*, *syn2a* and *stxbp1b* genes were significantly downregulated in both embryo-larval and juvenile fish. The downregulation of *syt1a* mRNA expression was significant only in juvenile fish. In the adult male zebrafish brain, the *gfap* and *c-fos* genes were affected and were significantly downregulated and upregulated, respectively (Fig. 20).

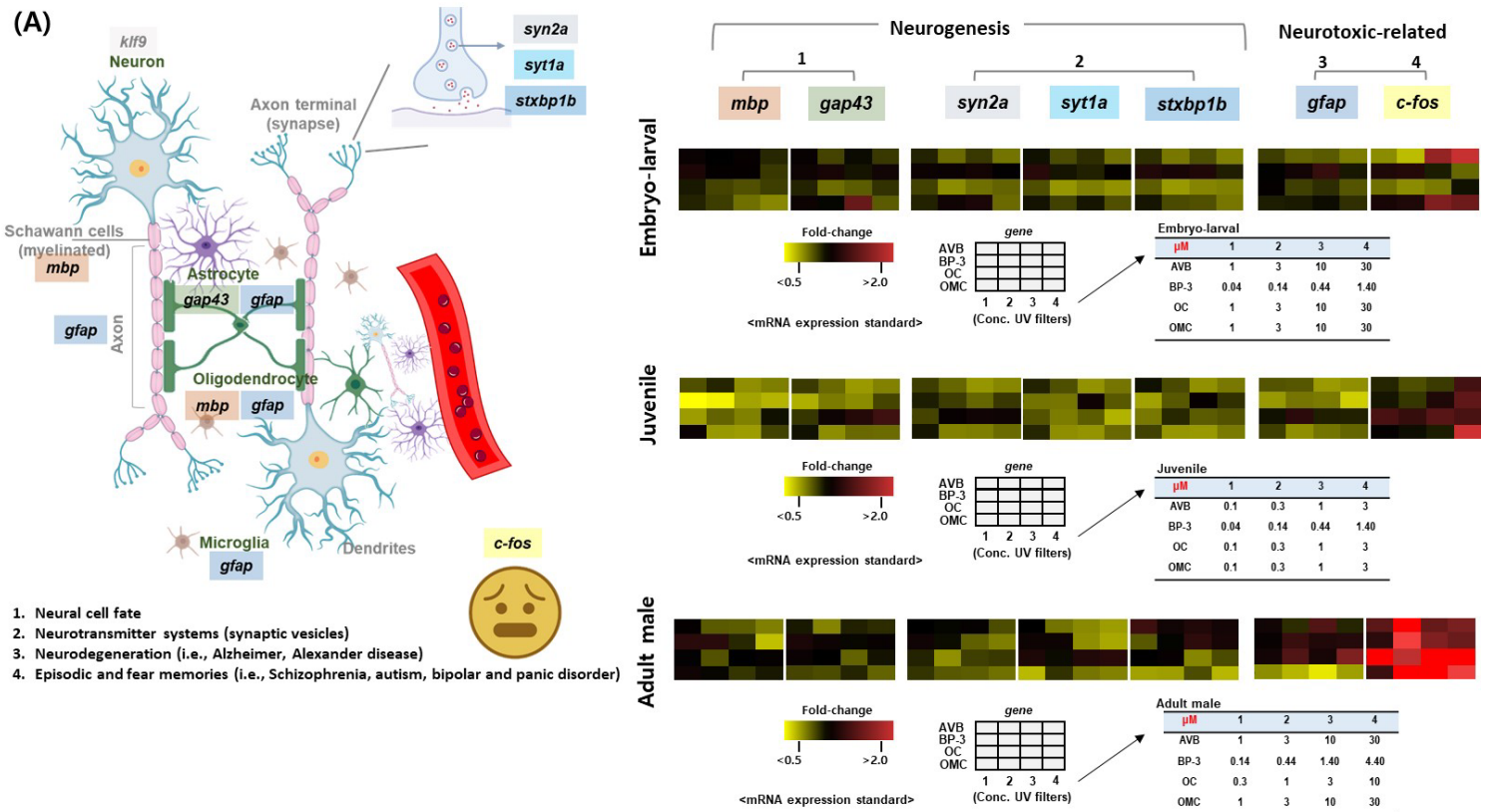


Fig. 20. Overview of neurogenesis and toxicity-related gene transcription changes in whole body of embryo-larval, juvenile and adult male brain (A) after exposure to UV filters. Concentration response of gene expression changes (bar graphs) in

embryo-larval (B), juvenile (C), and adult male (D) fish are described. *Asterisks indicate significant differences from the solvent control ($p<0.05$). Dotted red line indicates significant correlation ($p<0.05$).

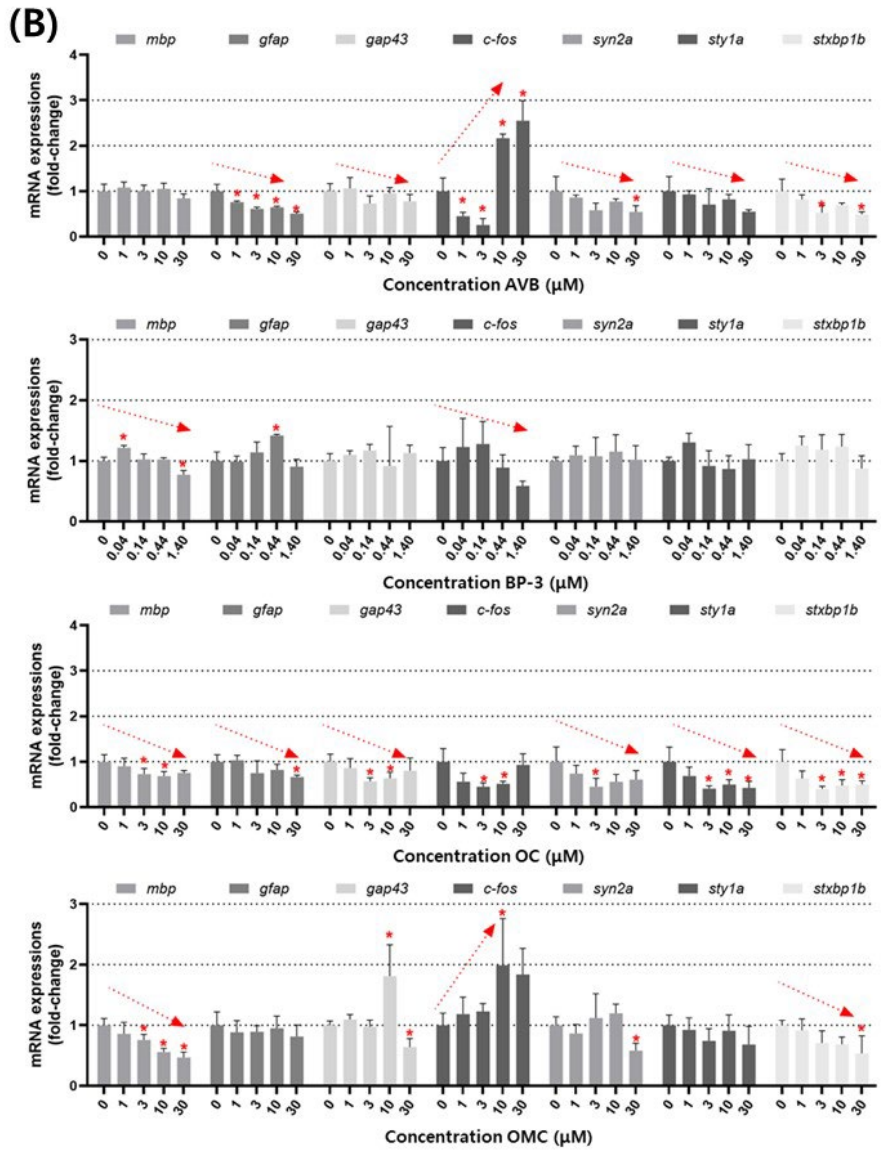


Fig. 20. Continued.

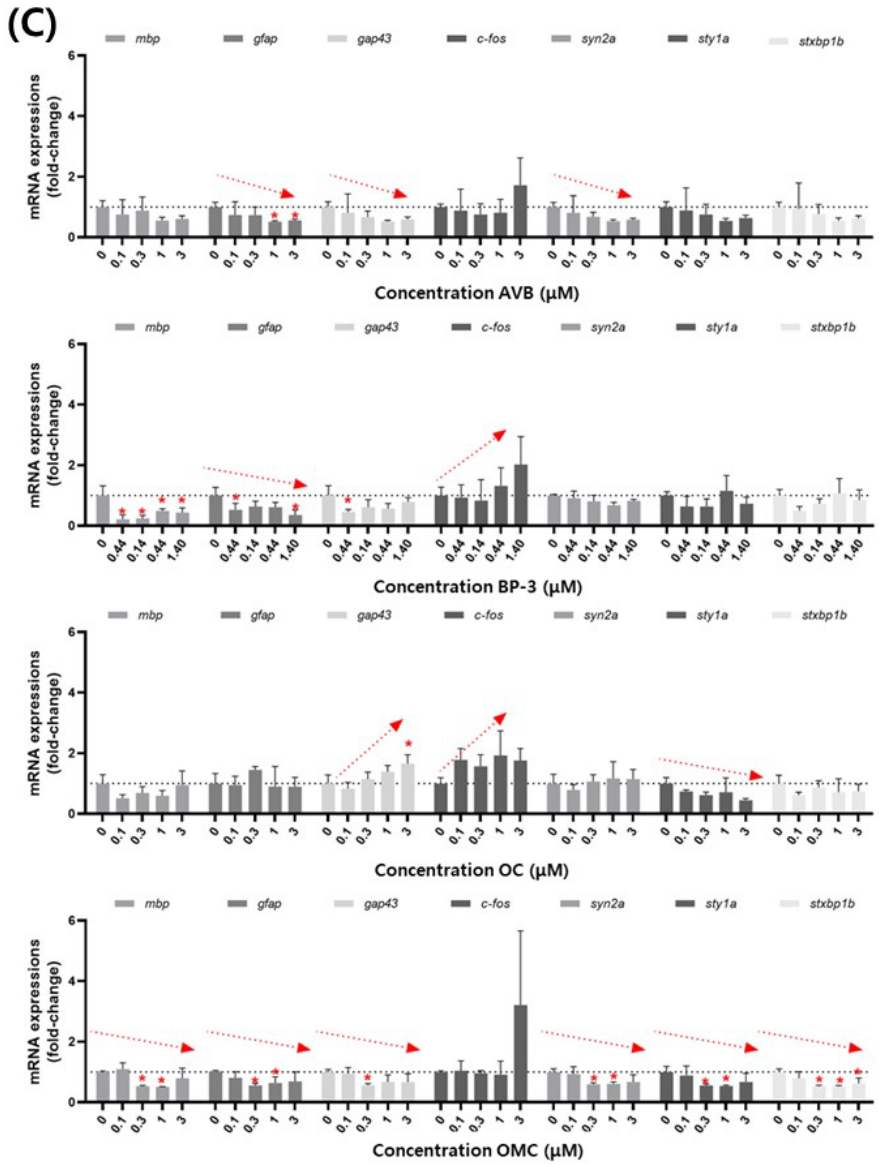


Fig. 20. Continued.

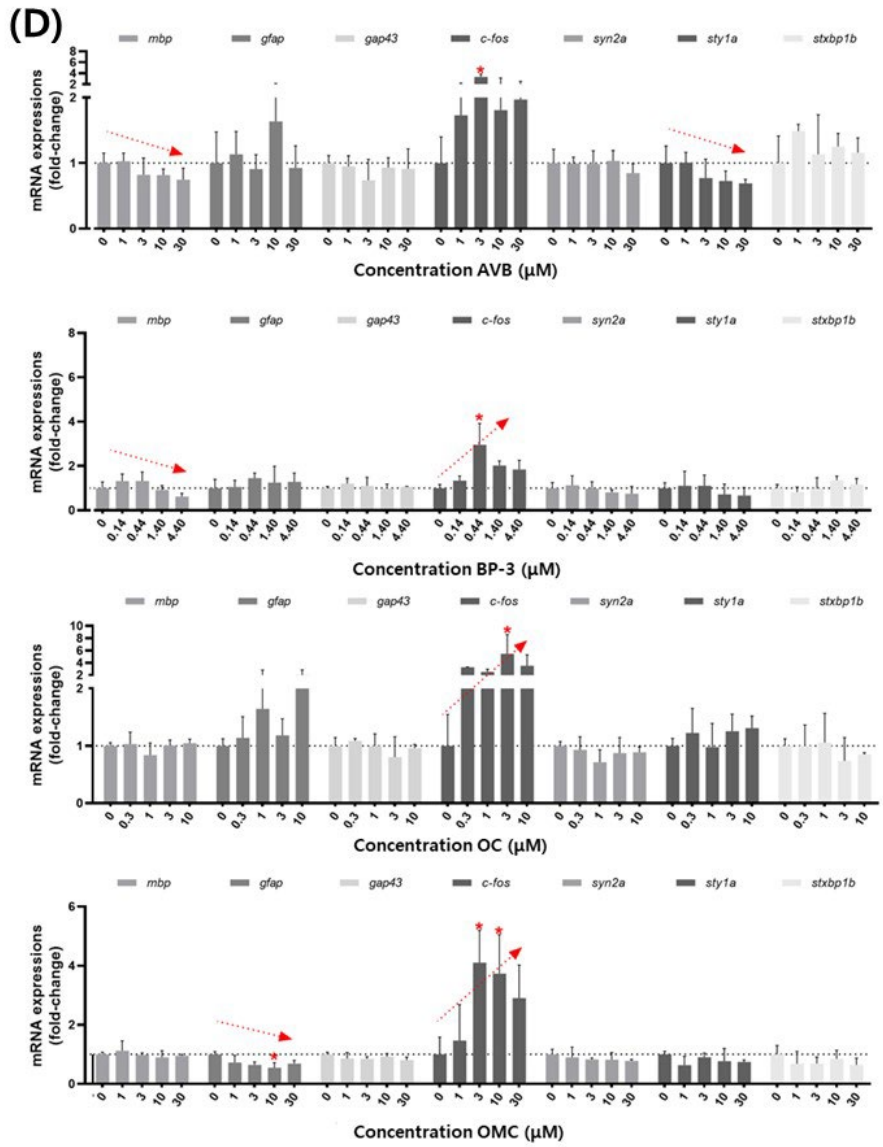


Fig. 20. Continued.

3.4 Discussion

In this chapter, the neurotoxicity of four major organic UV filters were examined with respect to the expression of selected genes and neurobehavioral parameters. The results from this chapter highlight that locomotion and anxiety-like behavior changes are likely the result of altered gene expression in the CNS and/or direct neurotoxic effects on neuronal cells.

In evaluations of neurotoxicity, behavioral phenotypes provide some of the most compelling evidence of whether neuronal communication has been disrupted. Behavior tests are widely used in neurotoxicity testing of pharmaceuticals and environmental pollutants (Moser, 2011). The behavior of an organism can serve as an indicator of emotional state, such as fear, anxiety, or aggression; the open-field test is frequently used to examine such behavioral endpoints. Test parameters measured in zebrafish using open fields are similar to those examined in rodent studies (Audria et al. 2018). Therefore, the locomotor and anxiety behavior of zebrafish were observed to reflect the potential neurotoxicity of UV filters in our study.

Locomotor activity refers to the movement from one location to another, which includes parameters such as total distance moved and mean velocity (Ji et al. 2017). Zebrafish larvae prefer areas with lighter conditions, which affects their locomotion (Basnet et al., 2019). In the presence of sudden changes in light and dark stimuli, zebrafish larvae display an increased movement pattern in the dark followed by resting in the light (Burgess and Granato, 2007). In general, our study showed that embryo-larval zebrafish

displayed more sensitivity to sudden dark stimuli than juvenile zebrafish during light and dark transitions. Although neurobehavioral traits of juvenile zebrafish at the age of 30 dpf have not been described, the effect of developmental stage on light-dark stimuli was previously evaluated between 4~10 dpf in zebrafish. The authors revealed that the response to the light and dark transition was significantly pronounced between 4 and 8 dpf, whereas only a mild effect was observed at 10 dpf (Kristofco et al. 2016). As a consequence, our observations demonstrate that all four tested UV filters (AVB, BP-3, OC, and OMC) resulted in significant hypoactivity during the light-dark transition in the embryo-larval and juvenile stages, indicating that their neurodevelopmental processes were compromised. A previous study also showed hypoactivity caused by AVB exposure in zebrafish larvae at 144 hpf (Liu et al. 2022). A number of studies have been conducted to evaluate neurobehavioral changes following exposure to BP-3 in fish, which varied by study. For example, waterborne exposure to BP-3 significantly increased mean velocity (mm/s) in zebrafish larvae (Tao et al. 2020). Findings from Moreira et al. (2022) and Chen et al. (2018) demonstrated hypoactivity induced by BP-3 in adult zebrafish and larvae, respectively, as well as decreased shoal preference, which is an indicator of social behavior. The inconsistency may be related to laboratory conditions, context differences (i.e., exposure concentration, duration and behavior parameters), age of the animal, and individual personality and sociability (Demin et al. 2019; Buenhombre et al. 2021)

Thigmotaxis describes the tendency of animals to favor the edges of the arena. It has been well established as a result of anxiety in larval and adult zebrafish, as well as rodents (Bencan et al. 2009). A number of anxiolytic and anxiogenic drugs, such as diazepam and caffeine, have been shown to attenuate and enhance thigmotaxis in larval zebrafish. Increased thigmotaxis is correlated with higher levels of anxiety in zebrafish larvae (Schnörr et al. 2012; Liu et al. 2016). To the best of our knowledge, our study is the first to examine the anxiety-like behavior induced by UV filter exposure in zebrafish, especially during different life stages. Anxiety behavior tests revealed that concentrations of AVB and OMC significantly correlated with the frequency of entering the center area in embryo-larval zebrafish. In juvenile fish, the frequency of center entry was significantly decreased by AVB and BP-3. To date, the effects of UV filters on anxiety behavior have not been addressed, unlike those of other environmental pollutants, such as lead, BPS, and PCBs. For example, long-term exposure to low levels of lead chloride in adult zebrafish significantly elevated freezing behavior and reduced exploratory behaviors (Thi et al. 2020). Chronic exposure to environmentally relevant concentrations of BPS significantly induced anxiety behavior, as indicated by longer latency to enter and less time spent in the upper area in adult zebrafish (Wei et al. 2020). Moreover, zebrafish with embryonic exposure to PCBs exhibited enhanced thigmotaxis, which is a ‘wall hugging’ behavior that is considered an anxiety-like phenotype in laboratory models (Gonzalez et al. 2016).

Myelin basic protein (*mbp*) is expressed in oligodendrocytes in both the central and peripheral nervous systems and is widely used as a biomarker of axon myelination (Wang et al. 2015). Therefore, disruption of *mbp* gene expression can lead to impairment of neuronal function. In our study, we observed downregulation of *mbp* gene expression during different life stages following UV filter exposure. The effect on *mbp* gene expression was especially pronounced during the developmental stage. Clinically, the absence of *MBP* protein results in the accumulation of amyloid plaques, a neuropathological hallmark of Alzheimer's disease (AD), increasing the risk of neurodegenerative disease onset later in life (Chakrabarty et al. 2010; Furman et al. 2012; Ou-Yang and Nostrand, 2013).

Axonal membrane protein GAP-43 (*gap43*) often serves as a marker for the process of reinducing axonal regeneration following injury (Fan et al. 2010), which contributes to plasticity and the growth of the presynaptic terminal (Holahan, 2017). Therefore, the upregulation of the *gap43* gene may be a response to maintain overall brain growth, which is necessary to offset the direct effects of neurotoxicant exposure (Wang et al. 2015). However, our results demonstrated downregulation of the *gap43* gene in most cases, except for juvenile fish exposed to OC, in which it was significantly upregulated. Downregulation of the *Gap43* gene has been reported to delay axonal regrowth after laser axotomy in mice (Mascaro et al. 2012). In addition, *GAP43* knockout mice showed impaired memory (Rekart et al. 2005).

Glial fibrillary acidic protein (*gfap*) is an intermediate filament protein that is highly expressed in astrocytes and in the radial glial cells of the CNS and is a marker for neurotoxicity (Fan et al. 2010). Downregulation of the *gfap* gene indicates impaired glial cell integrity and function (Wang et al. 2022). In our study, exposure to UV filters generally decreased the mRNA expression level of *gfap.* In humans, a decreased level of *GFAP* is associated with Alexander disease, a neurodegenerative disorder that primarily affects the white matter of the CNS (Lee et al. 2017). In addition, *GFAP* expression is highly associated with amyloid plaque load, as it limits amyloid deposition, and decreased *GFAP* may result in Alzheimer's disease (Chakrabarty et al. 2010; Furman et al. 2012). Our observations on the transcription level of *gfap* are in line with those associated with early-life zebrafish exposure to lead, in which *gfap* was significantly downregulated (Wang et al. 2022). In contrast, in embryo-larval fish exposed to BP-3, significant upregulation of the *gfap* gene was noted. The upregulation of the *gfap* gene was also observed in 4-month-old adult zebrafish exposed to 1% ethanol for 4 weeks, but this finding has not been discussed in detail (Kily et al. 2008).

The expression of the early immediate gene (IEC) *c-fos* is considered a reliable marker for measuring neuronal activity (Chatterjee et al. 2015). Furthermore, it has been shown that *c-fos* gene expression changes in psychiatric conditions such as anxiety and in association with fear memories in the brain (Gallo et al. 2018). We observed significant upregulation of the *c-fos* gene during all life stages, except in embryo-larval zebrafish exposed to

BP-3 and OC. The upregulation of *c-fos* is considered an innate response to avoid fear, which in turn increases anxiety-like behavior (Butler et al. 2011). Therefore, the decreased frequency of entering the center area in embryo-larval zebrafish treated with AVB and OMC and juvenile fish exposed to AVB and BP-3, suggestive of anxiety, may be partly attributable to increased *c-fos* expression. In contrast, the anxiety parameters in embryo-larval zebrafish exposed to BP-3 and OC were not affected as they were in zebrafish exposed to AVB and OMC. As mentioned earlier, significant downregulation of the *c-fos* gene was noted. Anxiety-fear memory persistence requires *c-Fos* expression, and functional loss of *c-Fos* has been reported to cause deficits in the consolidation and persistence of this type of memory (Katche et al. 2010; Katche et al. 2013; Katche and Medina, 2017). Thus, it is tempting to speculate that downregulation of *c-fos* impairs the fear learning process, which eventually impairs the ability to anticipate environmental threats.

Synapsin IIa (*syn2a*), synaptotagmin I (*syt1a*), and syntaxin binding protein I (*stxbp1b*) are important components of presynaptic proteins that facilitate both synaptogenesis and neurotransmitter release in zebrafish (Roy-Carson et al. 2017). We observed significant downregulation of the aforementioned gene transcription mostly in developing zebrafish, suggesting that the early life stage of zebrafish is uniquely vulnerable to UV filter exposure in terms of synaptogenesis. The downregulation of genes associated with synaptogenesis suggests severe impairment of presynaptic vesicle docking, which is necessary for neurotransmission. Clinically, mutations in

SYN2 have been implicated in ASD (Corradi et al. 2013). The *Syn2* knockout mouse model induced ASD-like phenotypes (i.e., reduced social sniffing and increased repetitive self-grooming behavior). (Michetti et al. 2017). As previously reported, *Syt1* is critical for sensing the Ca^{2+} necessary for action potential induction when serious motor delay and cognitive disorder are disrupted (Baker et al. 2018). Moreover, mutations in the human *STXBP1* gene are associated with early infantile epileptic encephalopathy (Grone et al. 2016).

Our results clearly indicate that locomotion and anxiety-like behavior changes resulted from alterations in gene expression in the central nervous system and/or through direct neurotoxic effects on neuronal cells. To date, there is limited knowledge on the effects of UV filters on neurotoxicity and neurobehavior, despite their increasing production and use. Therefore, it is necessary to understand the potential health effects posed by UV filters to both aquatic ecosystems and humans.

3.5 Summary

In light of the obtained results, it could be concluded that the tested UV filters may adversely affect the structure and function of nerve cells and may contribute to the deterioration of neurobehavioral health. Our data indicate that disengagement of neural cell communications by perturbing neurogenesis and direct toxic effects on myelination, oligodendrocytes and astrocytes could potentially play a key role in the pathogenesis of neurodevelopmental, neurodegenerative and emotional disorders. Furthermore, we determined the most critical period of UV filter exposure with respect to neurotoxicity. Moreover, disruption of normal behavior has important ecological implications, as it can affect fish reproduction, resource acquisition, and hierarchy formation, reducing fitness (Chen et al. 2016). This study provides new information on the neurotoxic effects of UV filters during different life stages, from the transcriptional level to behavioral effects. Considering the lifelong exposure to UV filters, longer-term studies and evaluations of the effects of mixtures are needed to characterize the consequences of chronic exposure.

Chapter 4 Kidney toxicity and related mechanisms of organic UV filters in zebrafish (*Danio rerio*) of different life stages

4.1 Introduction

Chronic kidney disease (CKD) is an emerging global health problem (GBD Chronic Kidney Disease Collaboration, 2020). Globally, CKD is the major contributor to catastrophic health expenditures exceeding 40% of household income and is expected to be the world's fifth leading cause of death by 2040 (Luyckx et al. 2021). Among the 17 Sustainable Development Goals (SDGs) adopted by the United Nations, SDG 3.9 aims to reduce environmental exposures linked to CKD by 2030, addressing the global burden of kidney disease (Luyckx et al. 2018). Although chronic diseases such as metabolic syndrome and diabetes mellitus are major risk factors for CKD, chemical exposure has also been implicated in CKD development (Wimalawansa, 2016; Kulathunga et al. 2019).

Previous human studies of EDC exposure and health consequences have primarily focused on endocrine-related dysfunction (Kabir et al. 2015; Darbre et al. 2017; Benvenga et al. 2020; You et al. 2021). However, there is growing evidence that links exposure to EDCs such as bisphenol A, dioxins, phthalates, and heavy metals (i.e., arsenic, cadmium, lead, etc.) with early progression to end-stage renal disease (ESRD) in human population studies (Kataria et al.

2015; Kang et al. 2019; Kulathunga et al. 2019; Hsu and Tain, 2021; Singh et al. 2021). For instance, a study of Chinese adults (n=3,055) (Li et al. 2012) and children who participated in the NHANES study (2009-2010, n=710) (Trasande et al. 2013) found that an increase in BPA exposure resulted in an increase in albuminuria. The NHANES (2009-2010, n=667) study also found a significant association between urinary di-(2-ethyl-hexyl) phthalate (DEHP) metabolites and elevated ACR, a marker of kidney function, in urine (Trasande et al. 2014). In a study of 1,531 adults from Taiwan, exposure to polychlorinated dibenzo-p-dioxins (PCDD) at the highest quartile was strongly associated with a reduced estimated glomerular filtration rate (eGFR), which is another indicator of kidney function (Chang et al. 2013).

A number of studies have investigated the possible underlying mechanisms of EDCs in experimental animal models. Bosch-Panadero and colleagues (2017) evaluated the nephrotoxic potential of BPA using human kidney proximal tubule cells (HK-2). They found that exposure to BPA significantly induced mitochondrial injury, oxidative stress and apoptotic death in HK-2 cells, which may contribute to CKD progression. Another study found that maternal exposure to BPA significantly induced glomerular abnormalities and changes in glomerular number and density in the offspring of OF1 mice (Nuñez et al. 2018). Prenatal exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in C51BL/6N mice resulted in hydronephrosis and increased renal fibrosis (Aragon et al. 2008). Furthermore, long-term exposure to low-dose cadmium decreased relative kidney weight in Sprague–Dawley rats (Luo et al.

2015). Hsu and Tain (2021) suggested several nephrotoxic mechanisms induced by environmental chemicals, including increased ROS generation, decreased antioxidant capacity, and low nephron number; these mechanisms mainly involve oxidative stress and apoptosis. Therefore, the molecular mechanisms by which EDCs affect the kidney, including nephrogenesis and nephrotoxic-related markers, need further investigation.

The nephrotoxic potential of UV filters is another major knowledge gap. In an adult female population in Korea, significant associations among urinary concentrations of BP-3, a major metabolite of BP-3, and ACR were observed, suggesting the adverse effects of BP-3 on kidney function (Kang et al. 2019). However, experimental studies investigating nephrotoxicity are rare. One study from Chu and colleagues (2021) demonstrated that exposure to OMC in embryo-larval and adult male zebrafish significantly perturbed nephrogenesis (*etv4*) and nephrotoxicity-related marker (*podocin*) genes. However, current knowledge of the nephrotoxicity of UV filters is insufficient to link these transcriptional changes to adverse outcomes.

The zebrafish has become a popular vertebrate model for studying kidney biology and related medical conditions such as acute kidney injury (AKI) and CKD (McCampbell and Wingert, 2014; McCampbell et al. 2015; Outtandy et al. 2019). Zebrafish have pronephric (embryonic) and mesonephric (juvenile to adult) kidneys. The zebrafish pronephros, which are functional during embryonic and early larval stages, comprise two nephrons sharing a central fused glomerulus (Swanhart et al. 2011). The pronephros of zebrafish begin to

form at approximately 12 hpf and begin blood filtration by 48 hpf (Jerman and Sun, 2017), which allows high-throughput kidney toxicity screening. The pronephros share many features with mammalian nephrons, such as the glomerulus filter and a system of tubules that are functionally segmented into proximal and distal segments (Outtandy et al. 2019). In comparison, the zebrafish mesonephros is a single and relatively flat organ that is present in three characteristic bilaterally symmetric subregions: the head, trunk, and tail (McCampbell et al. 2015). Zebrafish mesonephros continue to accumulate nephrons over the course of their lifetime, and this mesonephric kidney is estimated to comprise approximately 450 nephrons at approximately 6 months of age (Zhou et al. 2010). The complex mesonephric kidneys in zebrafish serve as an excellent model for studying adult renal biology and may be used to uncover genetic components of the nephrotoxic response that can complement research in traditional mammalian models such as mouse and rat (McCampbell and Wingert, 2014).

Therefore, the present study aims to examine the nephrotoxic effects of major UV filters in the embryo-larval, early-life and adult zebrafish. To this end, we evaluated transcriptional level changes in nephrotoxic markers and proteinuria in embryo-larva and early-life zebrafish. The results of this study will help us to understand the potential nephrotoxic effects of UV filters and promote follow-up investigations on the toxicological consequences of endocrine disruption.

4.2 Materials and methods

4.2.1 Test chemicals

AVB (avobenzone; butyl methoxydibenzoylmethane), BP-3 (benzophenone-3; 2-hydroxy-4-methoxybenzophenone) OC (octocrylene; octocrylene), and OMC (octyl methoxycinnamate; ethyl hexyl methoxycinnamate (EHMC)) were purchased from Sigma–Aldrich (St. Louis, MO, USA). All test UV filters were dissolved in dimethyl sulfoxide (DMSO), and the final DMSO concentration in the exposure media was 0.01% for AVB, OC, OMC, and 0.005% for BP-3.

4.2.2 Zebrafish maintenance and exposure design

4.2.2 Zebrafish maintenance and exposure design

Wild-type adult male zebrafish were cultured in charcoal-filtered dechlorinated tap water at 26±2 °C under a 16:8 h light/dark photoperiod in the Environmental Toxicology Laboratory at Seoul National University in Korea. The fish were provided mosquito larvae and Artemia (Green Fish, Seoul, Korea) twice daily *ad libitum*. The remaining food and feces were removed within 1 hour after feeding. The zebrafish embryos used in this study were spawned from adult zebrafish mating pairs (>6 months). The embryos were collected in the early morning (8:00-9:00 am) of the day of exposure.

Embryo-larval exposure (120 hpf) was conducted according to OECD test guideline 236 (OECD, 2013) with minor modifications. For each replicate, a total of 50 fertilized eggs were randomly placed into 250 L glass beakers with 100 mL exposure media. Sublethal concentrations were chosen for AVB (1, 3, 10 and 30 μM), BP-3 (0.14, 0.44 and 1.40 μM), and OMC (1, 3, 10, and 30 μM). The test solution was exchanged daily (>80%) with newly prepared exposure media. Both new and old exposure media were collected for water quality parameter measurement. During the exposure, embryo and larval survival, hatching rate, and time to hatching were recorded.

For the fish early-life stage experiment (~30 dpf), OCED test guideline 210 was followed. Approximately 50 normal embryos (<4 hpf) were randomly selected and distributed into glass beakers containing 100 mL of

test solution. Test concentrations were determined by preliminary range-finding tests: the concentrations were 0, 0.1, 0.3, 1, and 3 μM AVB; 0, 0.14, 0.44 and 1.40 μM BP-3; and 0, 0.1, 0.3, 1, and 3 μM OMC. Four replicates per treatment were employed. During the experiment, >80% of the exposure media was renewed every day. Old and new water quality (i.e., pH, conductivity, temperature, dissolved oxygen) were recorded daily. Embryo and larval survival, hatching rate, and time to hatching were monitored daily. After 5 dpf, fish were fed Gemma micro 75 (ZEBCARE, and *Artemia nauplii* (Greenfish, Korea) twice daily.

In the experiments with adult fish, wild-type adult male zebrafish (>6 months) were exposed to a series of UV filters for 21 days according to OECD test guideline 229 (OECD, 2012). The exposure concentrations of 1, 3, 10, and 30 μM AVB and 0.14, 0.44, 1.40, and 4.44 μM BP-3 were selected based on preliminary tests. For OMC, the concentration range (1, 3, 10, 30 μM) was chosen according to a previous study (Chu et al. 2021). The exposure conditions were maintained at 26±2℃ under a 16:8 h light/dark photoperiod, and freshly hatched *Artemia nauplii* and commercially available mosquito larvae were provided two times per day. The exposure media were exchanged with new test solutions daily, and water quality (pH, conductivity, temperature, dissolved oxygen) was routinely monitored.

After exposure termination (i.e., 120 hpf for embryo-larval, 30 dpf for juvenile, and 21 d for adult fish), the fish were sacrificed on ice. Whole

embryo-larval (120 hpf) and early life juvenile (30 dpf) fish and plasma and tissues isolated from adult male zebrafish were kept frozen at 80 °C until further analysis.

This study was conducted with the approval of the Institutional Animal Care and Use Committee of Seoul National University, Seoul, Korea (IACUC/SNU-190114-5-1, SNU-210505-1).

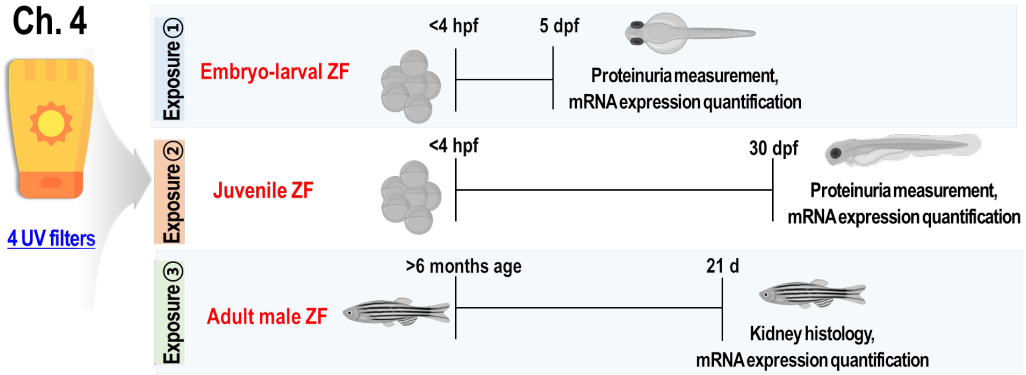


Fig. 21 Experimental design of Chapter 4.

4.2.3 Proteinuria measurement in embryo-larval and early-life stages of zebrafish

The protocols for proteinuria measurement were adopted from Wang et al. (2016) with minor modifications. Briefly, embryo-larval (n=30) and juvenile (n=6) zebrafish from each replicate were randomly collected and carefully transferred to 6-well plates (SPL cell culture plates, nontreated), rinsed twice with clean water and maintained in 3 mL fresh culture media. After 24 h, 1.5 mL of culture media from each replicate was sampled in a 2 mL microcentrifuge tube, and it was ensured that all fish were alive. Then, 300 µL trichloroacetic acid solution (TCA; Sigma–Aldrich, St. Louis, MO, USA) was added, vortexed and incubated for at least 1 hour at 4 °C. Then, the samples were centrifuged in a precooled centrifuge at 13,000 rpm at 4 °C for 5 minutes. After centrifugation, the supernatant was removed, and the pellets were washed twice with ice-cold acetone (-20 °C). The remaining acetone was evaporated thoroughly in a fume hood at room temperature. Pellets were then reconstituted with 160 µL of Nanopure water for further analysis. The micro BCA assay was conducted following the manufacturer's recommendation (Cat No. 23235, Thermo Fisher Scientific). The protein level was normalized to the dry weight. Absorbance was measured at 562 nm using a microplate reader (Spark®, Tecan, Männedorf, Switzerland).

4.2.5 Gene transcription analysis

The transcription of genes related to kidney toxicity was measured (Table 11).

After the exposure period, 30 embryo-larval fish, 10 juvenile fish, and 5 kidney tissues from adult male zebrafish were pooled as one replicate and homogenized using a tissue grinder. Total RNA from each sample was isolated by using a NucleoSpin® RNA mini-kit (Macherey-Nagel, Germany) following the manufacturer's recommendations (n=4). The content and purity of the total RNA were measured using an EpochTM-Take 3 spectrophotometer (BioTek Instruments Inc., Winooski, VT, USA). Then, complementary DNAs (cDNAs) were synthesized using the IScript cDNA synthesis kit (Bio–Rad, Hercules, CA, USA).

qPCR analysis was conducted in 20 µL qPCR reactions. Each qPCR reaction contained 10 µL of 2×SYBR Green^{TM} PCR master mix (Applied Biosystems), 0.1 µL of each 10 pmol forward and reverse PCR primer, 7.8 µL of nuclease free water (NFR), and 2 µL of cDNA sample. qRT PCR was performed using an Applied Biosystems QuantStudio 3 (Applied Biosystems, Foster City, CA, USA). The qPCR thermal cycle profile was as follows: preincubation at 95 °C for 10 min, followed by 40 cycles of amplification at 95 °C for 10 s, 60 °C for 20 s, and 72 °C for 20 s. The comparative Ct method ($2^{-\Delta\Delta Ct}$) was employed to calculate the relative fold change in target gene transcription (Livak and Schmittgen, 2001). The expression levels of the

target genes were compared to solvent control values and normalized to the expression of the most stable reference genes found by our geNorm analysis, *rpl8* (embryo-larval and juvenile fish) and *rpl13a* (adult male zebrafish kidneys).

Table 11. Primer sequences for qRT–PCR analysis used in this study

Gene	Protein name	Primer sequence (5'-3')	Accession no.
Reference gene			
rpl8[a]	60S ribosomal protein L8	F: ttgttggtgttgttgctggt R: ggatgctcaacagggttcat	NM_200713.1
rpl13a[b]	60S ribosomal protein L13a	F: agctcaagatggcaacacag R: aagttcttctcgtcctcc	NM_212784
Target gene			
ppargc1a	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha	F: aatgccagtgatcagagctgtcctt R: gttctgtgccttgccacctgggtat	AY998087.2
tbx2a	T-box transcription factor 2a	F: atgggcatgggacacttgtt R: tggcatggaaataccctgaga	NM_001102384.1
tbxb	T-box transcription factor 2b	F: ttgagtcaccaccatcaggc R: aggtggaaacattctcctgcc	NM_131051.1
etv4	ETS translocation variant 4	F: tgagggttttgtcagactagaga R: ccatgtcacctgggtcttca	XM_021478612.1
etv5	ETS translocation variant 5	F: acgggacttctgcttcgact R: ggaaccacacaggtgtcatc	NM_001126461
sim1a	Single-minded homolog 1-A	F: catagcctgcaagtgagggg R: tggctcggattagggtctca	NM_178222.3
wt1a	Wilms tumor protein homolog A	F: cagcaagccaacctccac R: gcaaccgtgccgtaacct	NM_131046.2
cdh17	Cadherin-17	F: gtcttagacaacgctgggct R: gaacaaaccaatgacgccga	NM_194422.1
kim-1	Kidney injury molecule 1	F: ttgtgtcagactccaccacac R: ccgcagtctgaggaaaagtgc	XM_003200873.5
podocin	Podocin	F: agatataagaccactgcagaaaacc R: ctcttttgcagaaggcgatgg	XM_009296250.3
nephrin	Nephrin	F: gaccagacctccgttactc R: aggatcaccaccacatagac	NM_001040687.1
pax2a	Paired box protein Pax-	F: ccgcgttattaagttcccc	XM_017358685.2

2a	R: tggcgtatccatcttcaatc

Reference genes used for embryo-larval and juvenile zebrafish[a] and adult male kidney[b].

4.2.6 Statistical analysis

The statistical analysis was the same as that in Chapter 2.

4.3 Results

4.3.1 Proteinuria changes in embryo-larval and early-life stages of zebrafish

Proteinuria is among the strongest evidence of kidney toxicity (Wang et al. 2016). The severity of proteinuria can be assessed by comparing the level to that of control groups. In this study, a significant increase in proteinuria was induced by UV filter exposure. In embryo-larval zebrafish, exposure to AVB, BP-3, and OMC led to elevated proteinuria (Fig. 22). In juvenile zebrafish, BP-3 and OMC let to significantly increased proteinuria (Fig. 22).

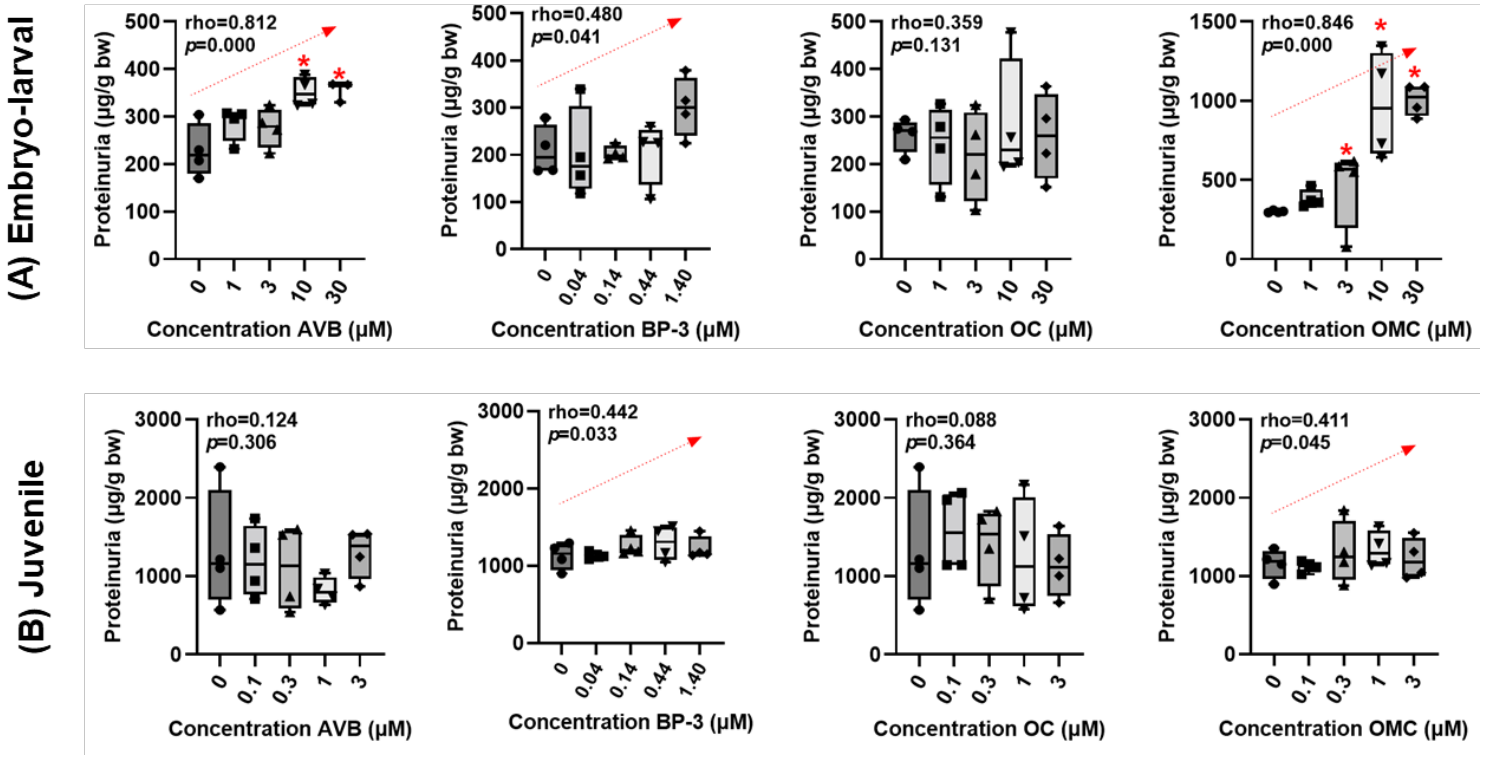


Fig. 22. Proteinuria changes by UV filter exposures in (A) embryo-larval zebrafish (120 hpf), and (B) juvenile zebrafish. Boundaries of box show the interquartile range (IQR); whiskers represent the data range (minimum and maximum values). Black dots indicate each data point (median). *Asterisks indicate significant differences from the solvent control ($p<0.05$). The dotted red line indicates significant correlation ($p<0.05$).

4.3.3 Gene transcription level changes

To explore the molecular mechanisms underlying the observed kidney developmental impacts and toxicity, we characterized UV filter-induced mRNA expression alterations in embryo-larval, juvenile and adult male zebrafish by RT–qPCR analysis. The genes that we evaluated are mainly involved in nephrogenesis or markers of kidney injury (Table 11).

Exposure to AVB significantly downregulated *wt1a*, *sim1a* and *cdh17* gene expression in embryo-larval zebrafish (Fig. 23) Similarly, the mRNA expression of *nephrin*, *sim1a*, and *cdh17* was significantly decreased in juvenile fish (Fig. 24). In adult male zebrafish, following 21 days of BP-3 exposure, the *podocin*, *nephrin*, *etv5*, *ppargc1a*, *tbx2a*, and *cdh17* genes were significantly downregulated, while the *pax2a* gene was significantly upregulated (Fig. 25).

Upon BP-3 exposure, significant induction of the *kim-1* gene and reduction of *sim1a* and *cdh17* gene transcription were observed in embryo-larval zebrafish (Fig. 23). Juvenile zebrafish demonstrated a similar profile, in which the mRNA expression of *kim-1* was significantly increased, whereas the *cdh17* gene was significantly downregulated (Fig. 24). In adult male zebrafish kidneys, the gene transcription of *wt1a*, *podocin*, *nephrin*, *sim1a* and *cdh17* was significantly reduced, and the gene expression of *pax2a*, *etv4*, and *kim-1* was significantly induced (Fig. 25).

Following exposure to OC, significant downregulation of the *wt1a* and *sim1a* genes was noted at 120 hpf (Fig. 23). Additionally, in juvenile fish, the mRNA expression of *wt1a* and *sim1a* was significantly inhibited and that of *kim-1* was significantly up-regulated (Fig. 24). In the adult stage, exposure to OC significantly downregulated *wt1a* and *kim-1* gene expression. However, *pax2a* gene transcription was significantly increased (Fig. 25).

Lastly, embryo-larval zebrafish exposed to OMC demonstrated significant downregulation of *podocin*, *sim1a* and *cdh17* genes (Fig. 23). In juvenile zebrafish, 30 days of OMC exposure significantly reduced the mRNA expression of *wt1a*, *nephrin*, *sim1a*, and *cdh17* genes while *kim-1* was significantly increased (Fig. 24). In adult male zebrafish kidneys, the concentrations of OMC significantly correlated with downregulation of *wt1a*, *podocin*, *pax2a*, *ppargc1a*, *sim1a*, *tbx2a*, and *tbx2b*. A mild induction of the *kim-1* gene was observed however, the change was not large enough to achieve statistical significance (Fig. 25).

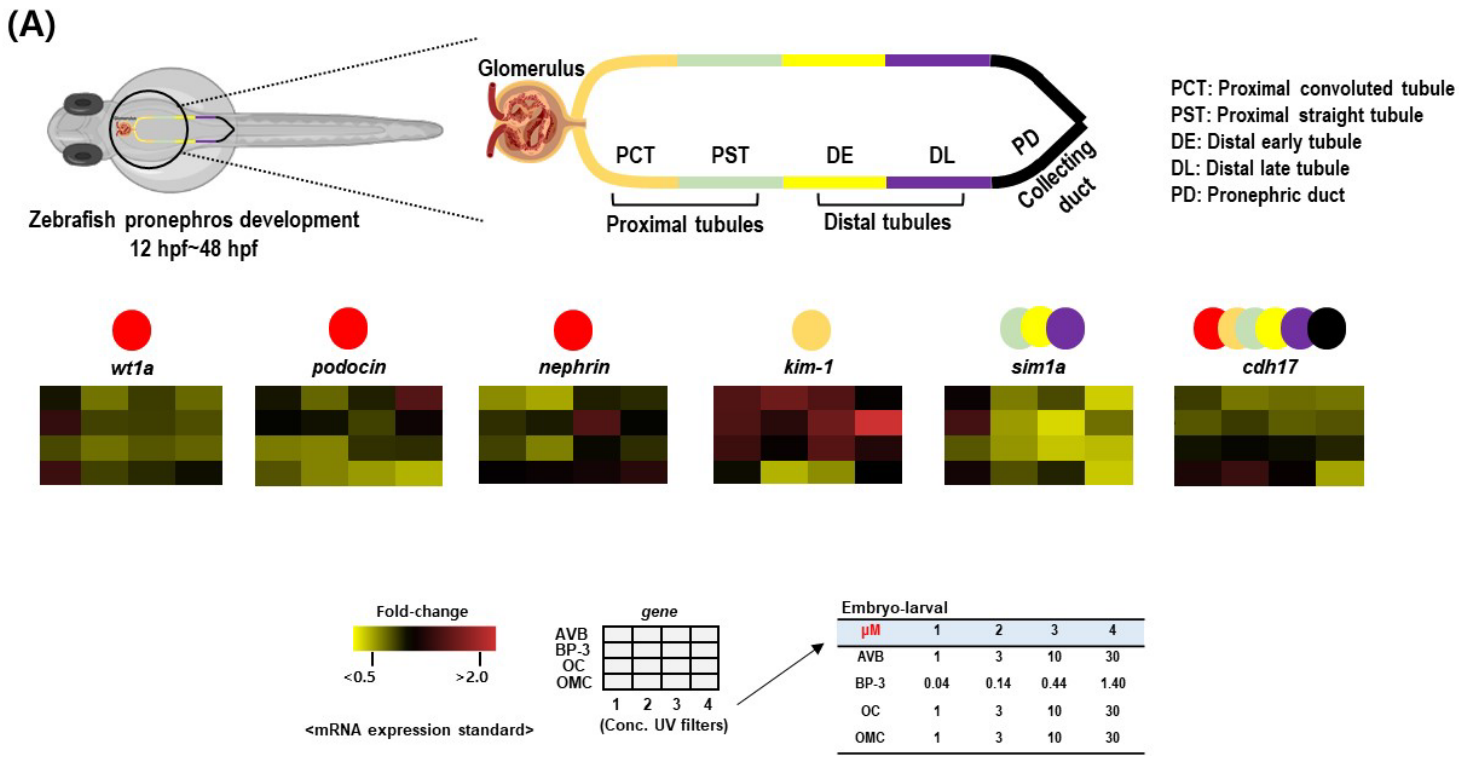


Fig. 23. Overview of nephrogenesis and toxicity-related mRNA transcription changes in embryo-larval zebrafish (A) after exposure to UV filters. Colored dots indicate location of expression, i.e., red: glomerulus, orange and green: proximal tubules; purple: distal tubules; black: collecting ducts. Concentration response of gene expression changes (bar graphs) in embryo-

larval fish (B) is also described. *Asterisks indicate significant differences from the solvent control ($p<0.05$). Dotted red line indicates significant correlation ($p<0.05$).

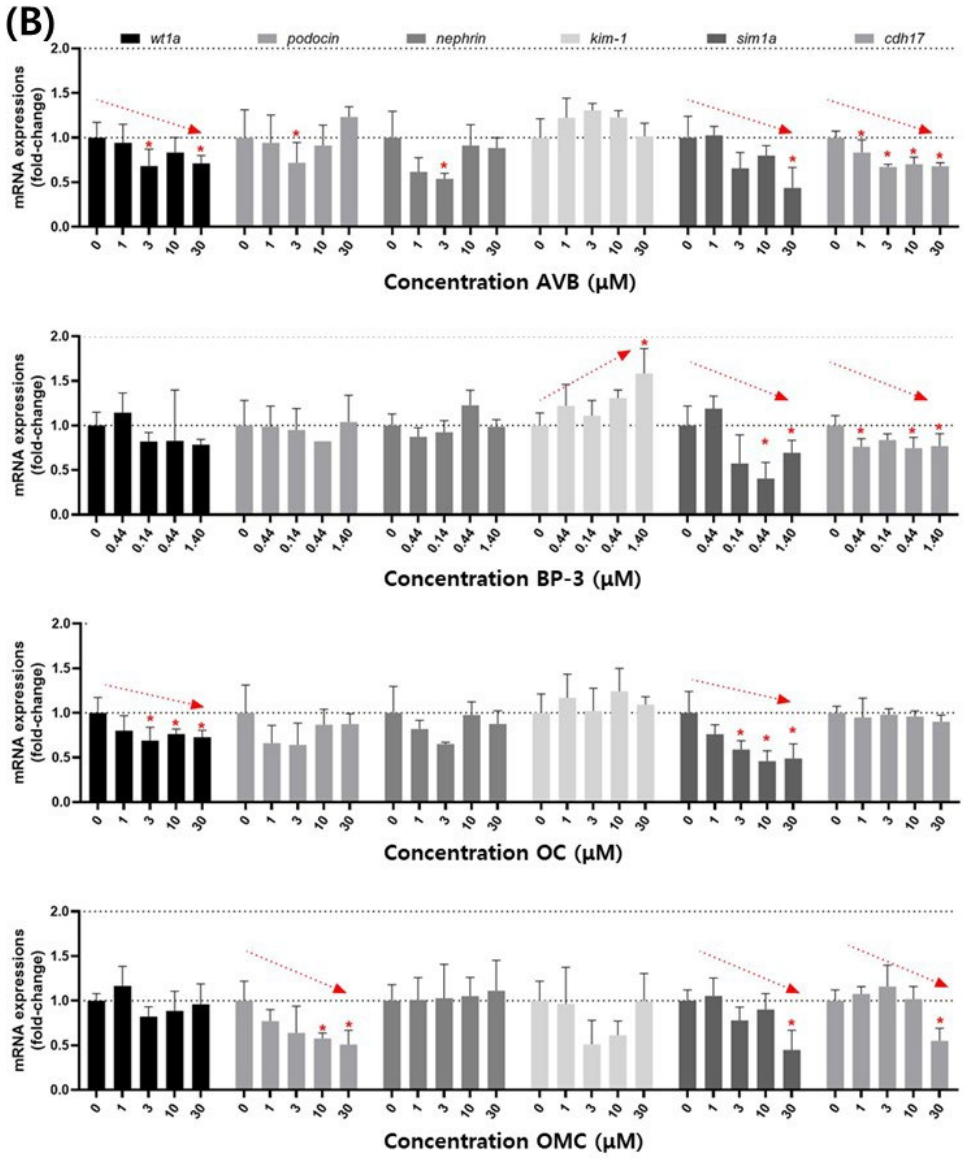


Fig. 23. Continued.

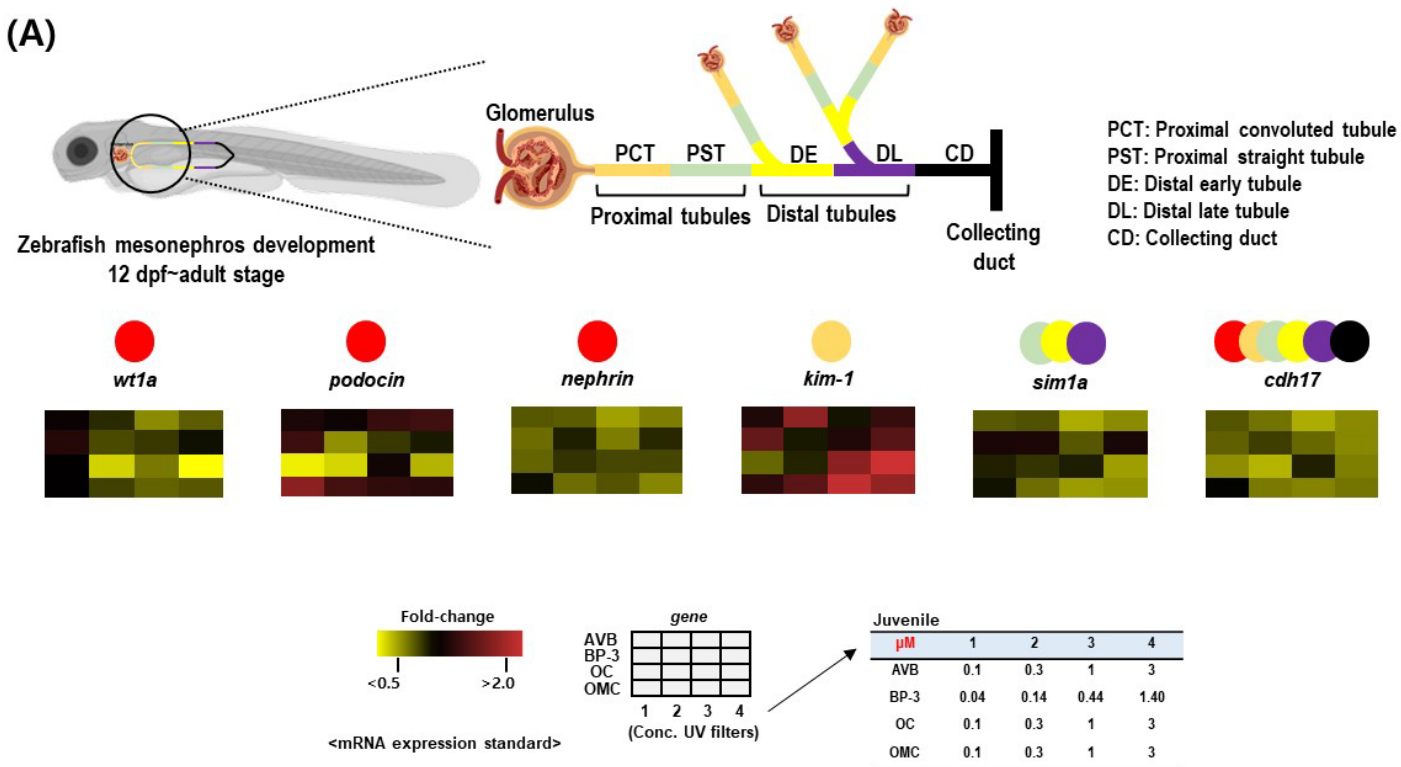


Fig. 24. Overview of nephrogenesis and toxicity-related mRNA transcription changes in juvenile zebrafish (A) after exposure to UV filters. Colored dots indicate location of expression, i.e., red: glomerulus, orange and green: proximal tubules; purple: distal tubules; black: collecting ducts. Concentration response of gene expression changes (bar graphs) in juvenile fish (B) is

also described. *Asterisks indicate significant differences from the solvent control (p<0.05). Dotted red line indicates significant correlation (p<0.05).

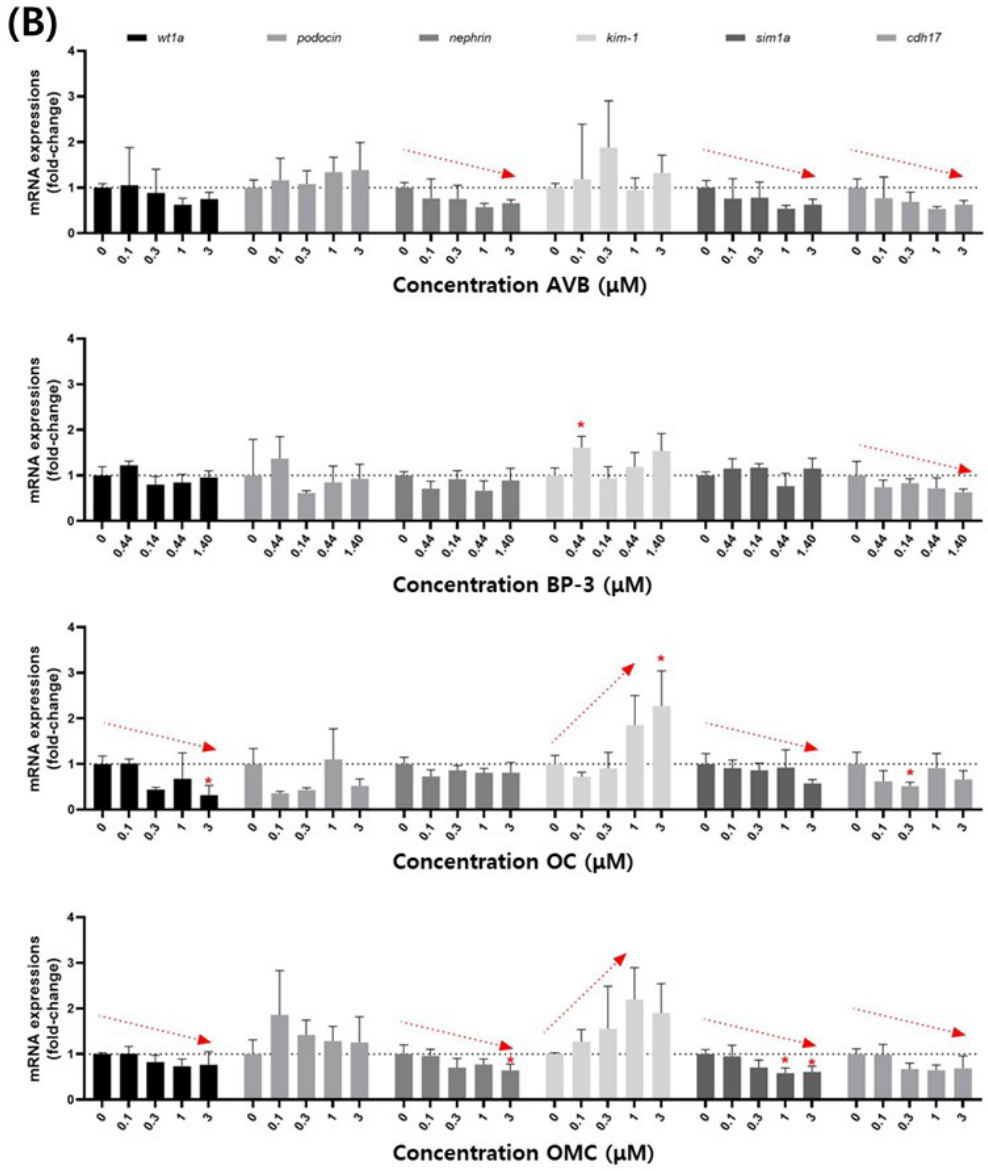


Fig. 24. Continued.

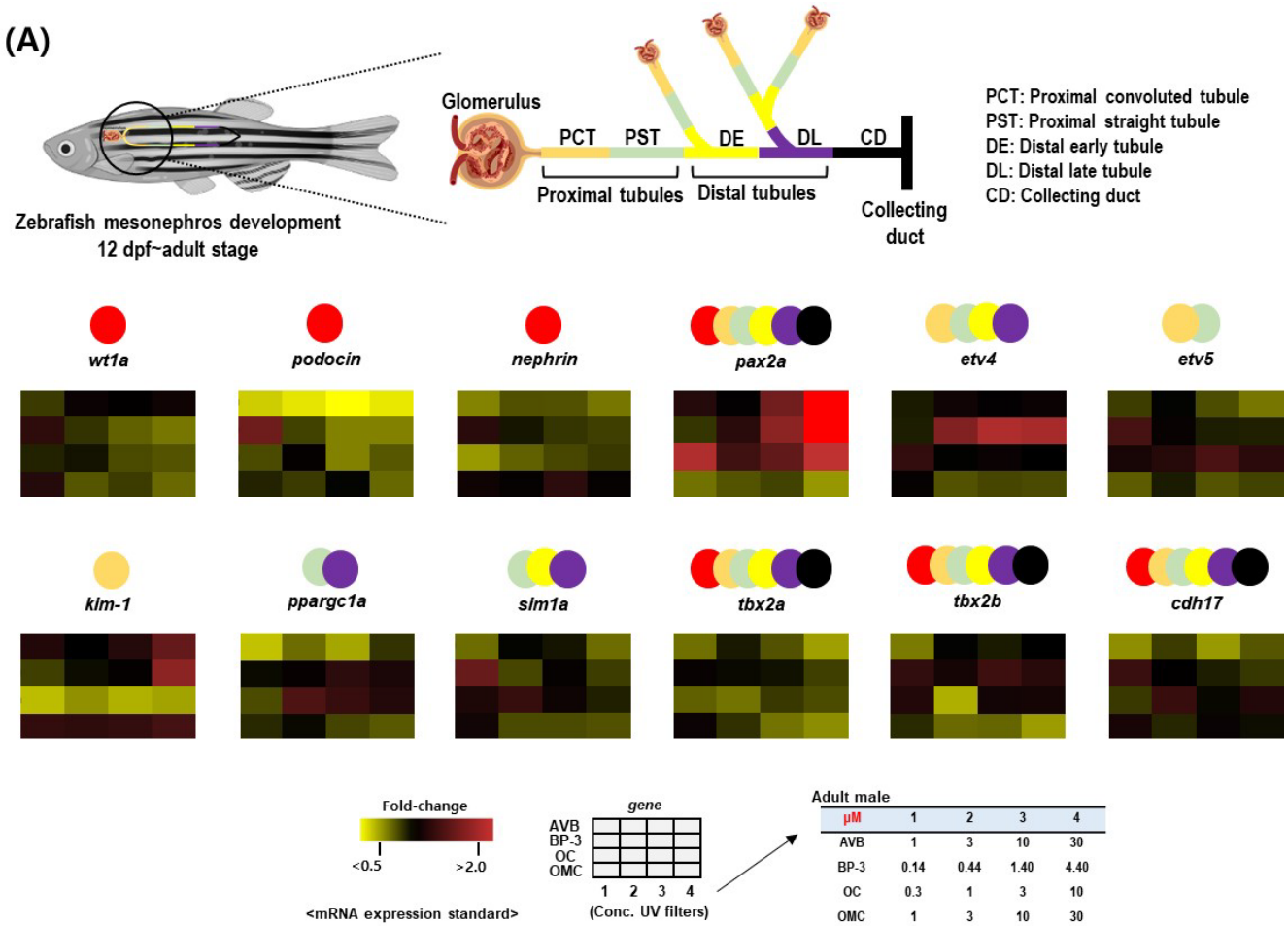

(A)
Zebrafish mesonephros development
12 dpf~adult stage
Glomerulus
PCT
PST
DE
DL
CD
Proximal tubules
Distal tubules
Collecting duct
PCT: Proximal convoluted tubule
PST: Proximal straight tubule
DE: Distal early tubule
DL: Distal late tubule
CD: Collecting duct
wt1a
podocin
nephrin
pax2a
etv4
etv5
kim-1
ppargc1a
sim1a
tbx2a
tbx2b
cdh17
Fold-change
<0.5
>2.0
<mRNA expression standard>
gene
AVB
BP-3
OC
OMC
1 2 3 4
(Conc. UV filters)
Adult male
μM 1 2 3 4
AVB 1 3 10 30
BP-3 0.14 0.44 1.40 4.40
OC 0.3 1 3 10
OMC 1 3 10 30

Fig. 25. Overview of nephrogenesis and toxicity-related mRNA transcription changes in adult male zebrafish (A) after exposure to UV filters. Colored dots indicate location of expression, i.e., red: glomerulus, orange and green: proximal tubules; purple: distal tubules; black: collecting ducts. Concentration response of gene expression changes (bar graphs) in adult male fish (B) is also described. *Asterisks indicate significant differences from the solvent control ($p<0.05$). Dotted red line indicates significant correlation ($p<0.05$).

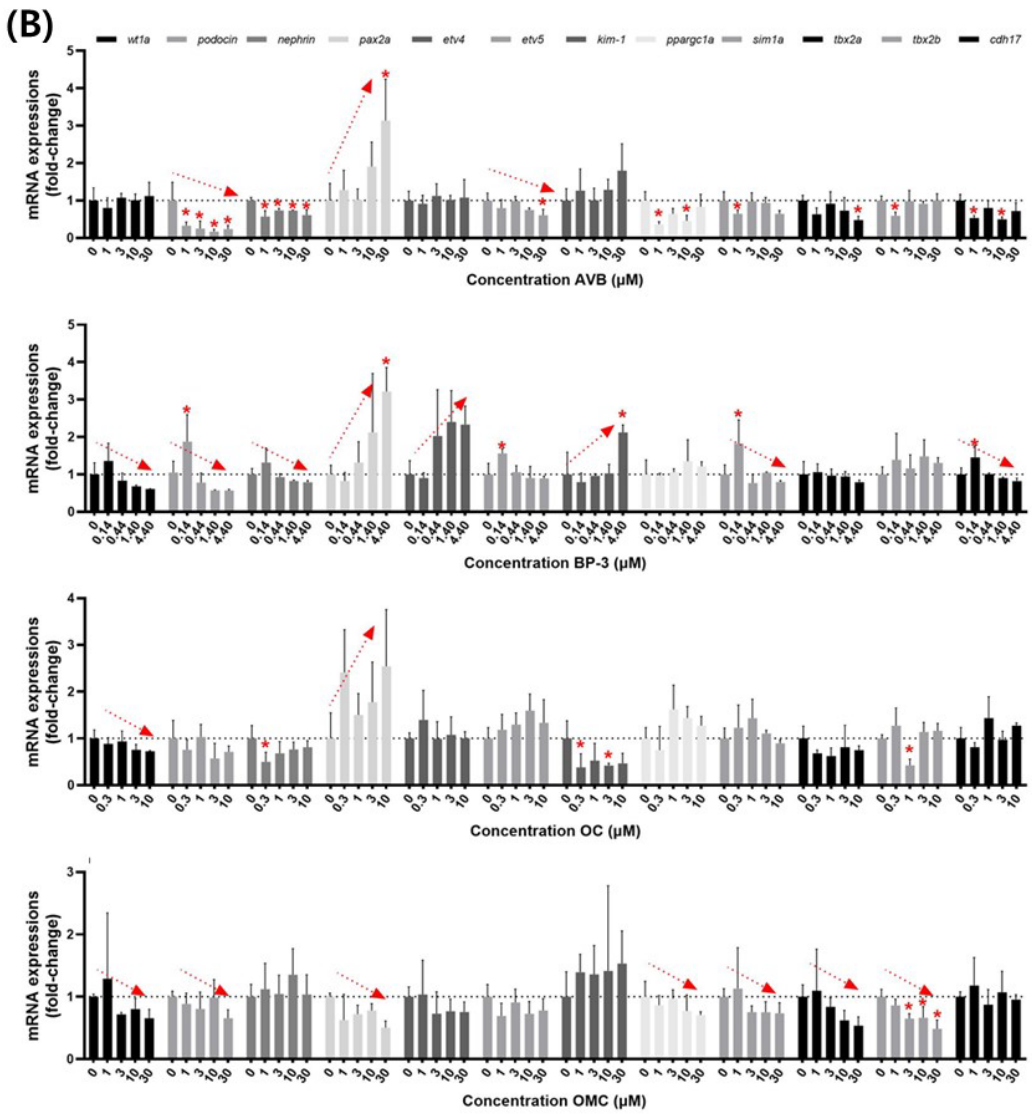


Fig. 25. Continued.

4.4 Discussion

In this chapter, the potential kidney toxic effects of UV filters were investigated. For this, we applied a micro BCA assay to measure protein (albumin) content in zebrafish media and RT–qPCR methods to evaluate transcriptional changes in genes related to nephrogenesis and nephrotoxicity following UV filter exposure. The results of this study clearly indicate a significant concentration-dependent increase in kidney toxicity during different life stages (i.e., embryo-larval, juvenile and adult) due to UV filter exposure. This study provides important insights into kidney health consequences associated with UV filters.

Kidney disease is clinically evidenced by the presence of a high level of proteinuria (National Kidney Foundation, 2002). An evaluation of the size of the proteins in the urine identifies the site of functional deficit in the kidney in humans; large proteins suggest glomerular filter dysfunction, whereas low-molecular-weight proteins suggest proximal tubule injury (Butt et al. 2020). There are three layers of membrane that are responsible for the ultrafiltration of plasma in kidney glomeruli: glomerular epithelial cells, glomerular basement membranes (GBM), and podocyte foot processes that cover the GBM (Wang et al. 2016). When glomeruli are healthy, protein is not found in the plasma filtrate. This is because the filtering barrier in the kidney, which is formed by the adjacent podocytes of each glomerulus, called the podocyte foot process, restricts macromolecule transport on the basis of molecular size,

shape, and charge (Müller and Thomas, 2012). If this podocyte foot process or GBM is disrupted, then large protein molecules will begin to be excreted in urine, which eventually could lead to end-stage renal disease (Chambers and Wingert, 2019).

Zebrafish constantly excrete diluted hypoosmotic urine into the surrounding environment due to the absence of a bladder (Outtandy et al. 2019), and the excretion rate is estimated to be up to one-third the body volume per day (Flik et al. 2009). Additionally, during the first five days of life, zebrafish do not eat or drink, which makes it possible to measure the sole protein content in culture medium (Kersten and Arjona, 2017). Therefore, to assess the potential nephrotoxicity of specific chemical substances, a precise method of quantifying proteinuria in zebrafish would be of great utility. Previous studies have developed several methods to evaluate proteinuria in zebrafish (Hyvärinen et al. 2010; Nishibori et al. 2011; Zhou et al. 2012; Hanke et al. 2013). Hyvärinen and others (2010) first introduced methods for the direct measurement of proteinuria via fluorescence in fish larva microinjected with FITC-dextran into the cardinal vein. Zhou and colleagues (2012) reported a GFP ELISA method for detecting proteinuria in zebrafish medium using *l-Fabp:DBP-eGFP* transgenic zebrafish. Jobst-Schwan and colleagues (2019) generated a podocyte injury zebrafish model by knocking out the *magi2a* gene to induce proteinuria following chemical insults. However, utilizing zebrafish models of proteinuria that require the injection of fluorescently labeled dextrans or genetically modified fish can be both labor

intensive and time-consuming. Recently, Wang et al. (2016) introduced methods for detecting proteinuria in the culture medium using wild-type zebrafish larvae. Briefly, they exposed newly hatched (72 hpf) zebrafish to aristolochic acid, which is well characterized for its nephrotoxicity. The protein in the fish culture water was precipitated by trichloroacetic acid, followed by SDS–PAGE for proteinuria quantification (Wang et al. 2016).

Therefore, in the present study, the methods for detecting proteinuria in zebrafish culture water were modified from Wang et al. (2016) as described in *Section 4.2.3*. The results of our study clearly demonstrated proteinuria induction following UV filter exposure. Significant induction of proteinuria in zebrafish after exposure to known nephrotoxicants, i.e., gentamicin and aristolochic acid, has been reported elsewhere (Nylor et al., 2020; Wang et al. 2020); however, to the best of our knowledge, the present study is the first to evaluate proteinuria changes induced by UV filters, a well-known class of endocrine disruptors.

As mentioned earlier, the functional pronephros and mesonephros of zebrafish are indistinguishable from those of mammalian and human kidneys. As a distinction from human kidneys, zebrafish can undergo *de novo* nephron formation in response to kidney injury throughout their entire lifespan (McCampbell et al. 2015; McKee and Wingert, 2015). As is now known, abnormalities in the podocytes of the glomerulus are major contributing factors to proteinuria development. Therefore, it is crucial to evaluate the potential role of UV filter exposure on nephrogenesis via genes that are

expressed in the glomerulus. In addition, markers of injury to other parts of the kidneys should be considered. For instance, urinary kidney injury molecule (uKIM-1) is a US FDA-approved tubular injury marker currently in drug development (Chen et al. 2018). A recent systematic review on the value of uKIM-1 in individuals diagnosed with AKI has revealed high sensitivity and specificity as an AKI biomarker (Geng et al. 2021). Previous experimental studies have characterized the expression of *kim-1* in the pro- and mesonephros of zebrafish (Yin et al. 2016; Wen et al. 2018).

In the present study, several genes expressed in the glomeruli, including podocytes, proximal and distal tubules, and collecting ducts, i.e., *wt1a*, *podocin*, *nephrin*, *pax2a*, *etv4*, *etv5*, *kim-1*, *ppargc1a*, *sim1a*, *tbx2a*, *tbx2b*, and *cdh17,* were quantified in adult male zebrafish kidneys. In embryo-larval and juvenile zebrafish, only those that are specifically expressed in kidneys, i.e., *wt1a*, *podocin*, *nephrin*, *kim-1*, *sim1a*, and *cdh17,* were measured. As a consequence, the expression of the aforementioned genes was significantly compromised in embryo-larval, juvenile and adult male zebrafish kidneys, which demonstrated similar profiles. In general, UV filter-treated larvae and juvenile and adult male fish exhibited significant downregulation of the *wt1a*, *podocin*, *nephrin*, *sim1a*, and *cdh17* genes, which are important for nephrogenesis, and upregulation of the *kim-1* gene, indicating tubular injury caused by the UV filters. The expression of the *wt1a* gene in the glomerulus of zebrafish is tightly regulated and is necessary for the expression of numerous podocyte genes, such as *nephrin* and *podocin* (Hsu et al. 2003;

Perner et al. 2007; O'Brien et al. 2011). It is important to note that *wt1a* knockout zebrafish embryos do not undergo podocyte differentiation, ultimately failing to express *nephrin* and *podocin*, the main components of the podocyte slit diaphragm (Hsu et al. 2003; Perner et al. 2007; O'Brien et al. 2011). In humans, mutations of WT1 are associated with Wilms tumor, a pediatric kidney cancer (Perner et al. 2007). A prior study was undertaken to analyze the pathogenic role of podocytes in proteinuria using experimental models such as Wistar, SD, and Lewis rats and revealed that the mRNA expression of *podocin* and *nephrin* was significantly reduced during the early stages of nephropathy, prior to the observation of proteinuria (Nakatsue et al. 2005). In another animal study conducted by Luimula et al. (2002), SD rats treated with puromycin aminonucleoside (PA), which is well characterized for its nephrotoxicity, demonstrated a marked reduction *in podocin* and *nephrin* mRNA levels, along with elevated proteinuria. The *sim1a* and *cdh17* genes are essential for maintaining normal nephrogenesis, and these genes are exclusively expressed in kidneys (Horsfield et al. 2002; Chen and Wingert, 2015). Loss of the *sim1a* and *cdh17* genes significantly compromised nephric development, as was reported elsewhere (Horsfield et al. 2002; Chen and Wingert, 2015).

In adult male zebrafish kidneys, the gene expression patterns of *pax2a*, *etv4*, and *etv5* were varied by UV filters. The *pax2a* gene is critical for nephrogenesis and is mainly involved in the differentiation of the podocyte and proximal tubule boundaries (Drummond and Davidson, 2010). Our study

revealed that exposure to AVB, BP-3 and OC significantly induced *pax2a* expression; however, a decreasing trend was observed after exposure to OMC. Previous studies have shown that loss of *pax2a* expression inhibits nephron tubule formation in both zebrafish and mice (Dressier et al. 1993; Majumdar et al. 2000). On the other hand, increased *pax2a* expression has also been reported in AKI-induced adult mice (Imgrund et al. 1999), as well as gentamicin-induced AKI zebrafish embryo-larvae (Cosentino et al. 2013). As a consequence, any deviation in the level of expression from the control, whether up- or downregulated, will likely affect the normal nephrogenesis process in zebrafish. In our study, significant upregulation of the *etv4* gene was noted for BP-3; however, significant downregulation of the *etv5* gene was observed. The *etv4* and *etv5* genes play important roles in nephrogenesis (Marra et al. 2016; Marra et al. 2019). Several developmental processes have been proposed that could be recapitulated by the regeneration process to restore organ and tissue function in animals that have been injured or damaged physically (Bacallao et al. 1989; Wallin et al. 1992; McCambell et al. 2015). Therefore, the upregulation of both *pax2a* and *etv4* in our study seems reasonable. The expression of the *ppargc1a* gene, which is necessary to form proximal tubule segments, was significantly inhibited by AVB and OMC exposure, suggesting impaired nephrogenesis (Marra et al. 2019). The *tbx2a* and *tbx2b* genes are also important candidates with potential roles in nephron segmentation (Drummond et al. 2017) and were significantly downregulated in adult male zebrafish following exposure to AVB and OC. Previous research showed that repression of *Tbx2* expression in *Xenopus* resulted in

enlarged pronephros (Cho et al. 2011) and disrupted formation of distal and proximal tubules in the zebrafish pronephros (Drummond et al. 2017).

Moreover, different observations were made with respect to *kim-1* expression in adult male zebrafish. The expression of *kim-1* was increased by AVB, BP-3, and OMC exposure; however, only BP-3 was associated with a statistically significant increase. When adult zebrafish were treated with OC, *kim-1* gene expression was significantly inhibited. In general, the expression of *kim-1* is elevated upon kidney injury (Han et al. 2002; Hoffmann et al. 2010; Tu et al. 2014; Luo et al. 2016). However, similar to our observation, significant repression of *KIM-1* was reported in HK-2 cell lysates following exposure to cisplatin (Sohn et al. 2013). The mechanism of *kim-1* expression downregulation is not clear and needs further investigation.

4.5 Summary

To summarize, we observed potential nephrotoxic effects of UV filter compounds during different life stages. The tested UV filters seem to induce proteinuria by compromising several genes involved in nephrogenesis, including genes with roles in podocyte differentiation. The nephrotoxic responses generally exhibited similar patterns, suggesting that both early and adult life stages could be influenced by UV filter exposure. In this study, we observed significant regulatory changes in the *wt1a*, *podocin*, *nephrin*, and *pax2a* genes along with elevated levels of proteinuria. These genes encode key proteins that are essential for maintaining the structure and function of the glomerulus. Therefore, changes in their expression could be utilized as an indicator of glomerular filtration impairment. Furthermore, in contrast to genes associated with thyroid hormone regulation and neurotoxicity, the nephrotoxic-related genes measured in this study seemed more sensitive to exposure during the adult stage. It is generally accepted that adult fish are less tolerant to chemical exposure than developing fish; however, the findings from this study imply that fish embryo toxicity tests may not be sufficiently sensitive to replace adult fish in toxicity tests.

Chapter 5 Conclusions

5.1 Summary

To evaluate the TH-disrupting potential and related adverse outcomes of major organic UV filters (i.e., AVB, BP-3, OC, and OMC) and understand their underlying mechanisms during different life stages, a series of studies was carried out. In Chapter 2, the thyroid-disrupting effects of UV filters in zebrafish embryo-larval, juvenile, and adult males were evaluated, and possible TH-disrupting mechanisms were investigated. In Chapter 3, the effects of UV filters on neurobehavior and related mechanisms were explored in zebrafish at different life stages. In Chapter 4, the nephrotoxic effects of UV filters in embryo-larval, juvenile and adult male zebrafish were investigated.

In Chapter 2, the TH-disrupting potential of the UV filters (i.e., AVB, BP-3, OC, and OMC) was examined, and the possible underlying mechanisms were identified using gene transcription analysis of whole-body embryo-larval and juvenile fish and specific tissues (i.e., hypothalamus-pituitary, thyroid, and liver) from adult male zebrafish. The tested UV filters caused significant alterations in gene transcription in different life stages of zebrafish, which indicates that these UV filters may disrupt normal TH homeostasis. UV filters significantly reduced TH levels in embryo-larval however these compounds seemed to increase TH levels in later stage of lives (juvenile and adult fish). The TH-disrupting mechanism of the UV filters may involve direct effects on

the central regulation and liver metabolism of THs. The fish exposed during the embryo-larval stage exhibited greater thyroid disruption, indicating that disruption of THs at this stage of life can cause susceptibility to thyroid-related endocrine dysfunction over time.

In Chapter 3, we evaluated the neurotoxic potentials of organic UV filters in embryo-larval, early-life and adult zebrafish. To evaluate the neurotoxic potentials of UV filters on zebrafish early development, basic motor responses during light-dark transitions and anxiety phenotypes during the dark phase were evaluated in free-swimming embryo-larval and juvenile zebrafish. To investigate these phenotypes in more detail, we evaluated genes involved in neurogenesis (*mbp*, *gap43*), related to neurotoxicity (*gfap*, *c-fos*), and involved in CNS development and neurotransmitter systems (*syn2a*, *syt1a*, *stxbp1b*). Neurotoxicity related gene expression profiles demonstrated similar patterns among different life stages of zebrafish. As a consequence, we concluded that the tested UV filters have a negative impact on the structure and function of nerve cells and may contribute to the deterioration of neurobehavioral health. In addition, the developmental stage was identified as the most critical period for UV filter exposure.

In Chapter 4, we aimed to examine the nephrotoxic effects of major UV filters in embryo-larval, early-life and adult zebrafish. To this end, we evaluated proteinuria in embryo-larval and juvenile zebrafish. To explore the underlying molecular mechanisms of the observed kidney developmental defects and toxicity, we characterized UV filter-induced mRNA expression

alterations in both embryo-larval and juvenile zebrafish. Adult male zebrafish kidneys were evaluated for tissue-specific responses of mRNA transcription. The tested UV filters seem to induce proteinuria by compromising several genes involved in nephrogenesis, including genes with effects on podocyte differentiation. The nephrotoxic responses generally exhibited similar patterns, suggesting that both early and adult zebrafish could be influenced by UV filter exposure.

THs are essential for maintaining homeostasis of our body. For the past few decades, literatures have shown possible implications between THs disruption and adverse health outcomes including neurological disorder and kidney dysfunction. The role of THs action throughout neurodevelopment is well is well documented. For example, there is a link between maternal hypothyroidism and lower IQ scores, as well as ASD and ADHD in different populations (Haddow et al. 1999; Andersn et al. 2018; Getahun et al. 2018). In addition, maternal hypothyroidism can affect the morphology of the brain including changes in volume of the cortex and hippocampus (Wheeler et al. 2011; Wheeler et al. 2015; Korevaar et al. 2016; Jansen et al. 2019). Studies on humans indicate that exposure to TDCs may be implicated in serious health consequences for children (Mughal et al. 2018). Differentiation and maturation of neuronal and oligodendrocyte cells could also be governed by THs (López-Juárez et al. 2012; Remaud et al. 2017). THs are tightly regulated by HPT axis. Upon release, they are primarily transported by serum proteins such as albumin and transthyretin (TTR) (Giannocco et al. 2021). Once they

reach the target tissue, specifically at the cellular level of the brain, T4 is actively transported across the brain-blood barrier (BBB) and taken up by astrocytes by an organic anion-transporting polypeptide 1c1 (OATP1C1) transporter, where it is deiodinated by DIO2 producing T3, which is then able to exit the astrocytes via monocarboxylate transporter 8 (MCT8) (Gilbert et al. 2020; Cediel-Ulloa et al. 2022). After entering the cell, deiodinases (DIO1, DIO2, DIO3) strip TH of iodide according to what the cell needs at that particular moment, specifically DIO1 and DIO2 convert T4 to T3, DIO1 and DIO3 T4 into reverse T3 and DIO1, DIO2 and DIO3 degrade T3 and reverse T3 to T2 (Gilbert et al. 2020; Cediel-Ulloa et al. 2022). Moreover, several neuronal genes such as *mbp*, *gfap* are reported as THs responsive (Kumar et al. 2018; Giannocco et al. 2021). In the present study, we demonstrated hypothyroid activity in embryo-larval zebrafish induced by exposure to UV filters (Fig. 9).

While it is well documented that hypothyroidism is an important factor that modifies kidney structure and function (Mansourian 2012; Benedetti et al. 2019; Kim et al. 2020), few attempts on mechanistic understanding have been made use of THs disruption to make predictions of related kidneys health consequences. A recent experimental study from Agarwal et al. (2021) suggested deiodinase 3 (D3) as an important regulator for podocyte homeostasis. The authors demonstrated healthy podocytes express abundant D3 isoform however, when kidney is injured dramatic decrease in the *Dio3* mRNA and protein levels along with severe proteinuria induction in mouse

model was observed. They also reported down-regulation of *Dio3* would enhance local THs action in podocytes which could increase susceptibility to kidney injury via activating integrin signaling pathways (Agarwal et al. 2021).

Therefore, we performed principal component analysis (PCA) to transform a number of potentially correlated molecular endpoints (mRNA transcriptions) related to THs disruption, neural-, and kidney toxicity into a smaller number of variables called "principal components (PCs)" of 25 genes in embryo-larval fish, 28 genes in juvenile fish, and 42 genes in adult male zebrafish. First two PCs (PC1 and PC2) were then used for regression analysis to determine relationships between THs status.

In embryo-larval zebrafish, the results of PCA revealed that the first principal component (PC1) and second component (PC2) accounted for 44.06% and 18.51% for AVB, 25.38% and 18.06% for BP-3, 43.16% and 18.47% for OC, and 26.43% and 15.27% for OMC, respectively (Table 12). Regression analysis between PC1, PC2 and thyroid hormones showed that PC1 was significantly correlated with TT4, TT3, FT3 levels for AVB, BP-3, OC, and OMC. PC2 was only significantly correlated with TT4 and TT3 of BP-3 exposed groups (Table 13). PC1 of AVB was highly influenced by *trhr*, *tpo*, *dio2*, *gfap*, *syn2a*, *syt1a*, *stxbp1b*, *sim1a*, and *wt1a* genes, PC1 of BP-3 by *trh*, *nis*, *dio1*, *mbp*, *syt1a*, and *wt1a* genes in BP-3, PC1 of OC by *mbp*, *gap43*, *syn2a*, *syt1a*, *stxbp1b*, *sim1a*, and *wt1a* genes, and PC1 of OMC by *trh*, *sult1 st5*, *dio3*, *syn2a*, and *cdh17* genes (Fig. 26-27).

In juvenile zebrafish, the results of PCA showed that the first principal component (PC1) and second component (PC2) accounted for 52.01% and 18.01% for AVB, 22.81% and 18.46% for BP-3, 27.29% and 18.90% for OC, and 42.26% and 16.45% for OMC, respectively (Table 14). Regression analysis between PC1, PC2 and thyroid hormones showed that only PC1 of BP-3 was significantly correlated with TT3 levels (Table 15). PC1 of BP-3 was highly affected by *tshb*, *tshr*, *nis*, *tpo*, *dio2*, *dio3*, and *kim-1* genes (Fig. 28-29).

In adult male zebrafish, the results of PCA demonstrated that the first principal component (PC1) and second component (PC2) accounted for 24.97% and 21.38% for AVB, 33.37% and 18.46% for BP-3, 26.13% and 15.76% for OC, and 24.84% and 16.45% for OMC, respectively (Table 16). Regression analysis between PC1, PC2 and thyroid hormones showed that only PC1 and PC2 of AVB were significantly correlated with TT4 level, and PC2 with FT3 level (Table 17). PC1 of AVB was significantly modulated by tshr, nis, tpo, dio1 (thyroid), dio2 (thyroid), tra (thyroid), and nephrin genes. PC2 of AVB was highly influenced by trh, trhr, tshb, dio1 (brain), dio2 (brain), dio3 (brain), and syt1a genes (Fig. 30-31). Overall, the results of PCA and regression analysis indicate genes which are key components of thyroid, neuro-, and kidney systems could be perturbed simultaneously by UV filter exposures which may imply possible direct or indirect effects of UV filters in thyroid, neuro-, and kidney systems.

Table 12. Result of principal component analysis of gene transcriptions related to THs disruption, neural-, and kidney toxicities in embryo-larval zebrafish.

Classifications	**AVB**		**BP-3**		**OC**		**OMC**	
	PC1	**PC2**	**PC1**	**PC2**	**PC1**	**PC2**	**PC1**	**PC2**
Standard deviation	3.3190	2.1509	2.5192	2.1251	3.2848	2.1491	2.5705	1.9537
Proportion of variance	0.4406	0.1851	0.2539	0.1807	0.4316	0.1847	0.2643	0.1527
Cumulative proportion	0.4406	0.6257	0.2539	0.4345	0.4316	0.6163	0.2643	0.4170
Factor loadings								
trh	0.0652	**-0.3513**	**-0.2889**	-0.0337	0.0588	**0.3034**	**0.3100**	-0.1574
trhr	**0.2755**	-0.1416	-0.2026	**0.2824**	0.2384	0.1682	0.2015	0.3309
tshb	0.2136	-0.2134	0.0674	**0.2828**	-0.0936	**0.3566**	0.1144	-0.2238
nis	0.2330	-0.1316	**-0.2532**	0.0277	0.1479	**0.2600**	-0.1091	0.1181
tpo	**0.2578**	-0.1233	-0.2257	0.1473	0.2016	0.1883	-0.0351	**0.3631**
tg	0.0158	**-0.2931**	-0.0428	**0.2948**	-0.0280	**0.3454**	0.1861	0.2041
ugt1ab	-0.1006	**-0.3420**	0.2203	0.1869	-0.1434	0.0586	-0.1514	0.0697
sult1st5	0.1205	**-0.2995**	0.0647	**0.3227**	-0.1857	0.1661	**0.3570**	0.0472
ttr	-0.1510	-0.1566	-0.1000	**0.2909**	-0.1682	**0.3322**	0.2430	0.2290
dio1	0.0374	-0.**3623**	**-0.3239**	0.0042	0.0227	0.0931	-0.2496	0.0311
dio2	**0.2614**	-0.0656	-0.2379	0.1542	0.0243	0.0404	0.2241	**0.3078**

dio3	-0.0477	0.0013	0.1846	**0.3105**	-0.0196	**0.2662**	**0.3521**	0.0148
mbp	0.2163	-0.0732	**-0.2756**	-0.1717	**0.2683**	-0.0333	-0.1144	-0.0504
gap43	0.2435	0.0542	0.0275	0.1725	**0.2733**	-0.0174	-0.1326	0.2180
gfap	**0.2665**	0.1221	0.0299	**-0.2519**	0.2385	0.1924	-0.0435	0.0552
cfos	-0.0996	0.1598	-0.1938	-0.2208	0.2443	-0.1548	0.0345	**0.2617**
syn2a	**0.2762**	0.1085	-0.1836	0.1492	**0.2999**	0.0140	**-0.2649**	0.2035
syt1a	**0.2635**	0.0650	**-0.3045**	0.2383	**0.2906**	-0.0635	-0.0929	**0.3986**
stxbp1b	**0.2679**	0.1022	-0.2452	-0.0162	**0.2949**	-0.0612	-0.0943	0.1348
sim1a	**0.2816**	-0.0335	-0.2493	0.0279	**0.2509**	-0.1165	-0.2190	0.1748
wt1a	**0.2686**	0.1029	**-0.2801**	-0.0643	**0.2806**	-0.0857	0.0880	0.2014
cdh17	0.2388	0.1526	-0.0835	-0.1452	-0.0751	-0.1691	**-0.3328**	-0.0573
kim1	-0.0040	**-0.3496**	0.1431	**0.2863**	0.0970	**0.4024**	0.2029	-0.0876
podocin	0.0740	**0.2615**	-0.1367	0.1404	0.2489	0.0653	-0.1577	-0.1050
nephrin	0.1237	0.1581	0.0851	-0.0862	0.1988	0.1467	0.0513	-0.1987

Values > 0.25 are shown in bold.

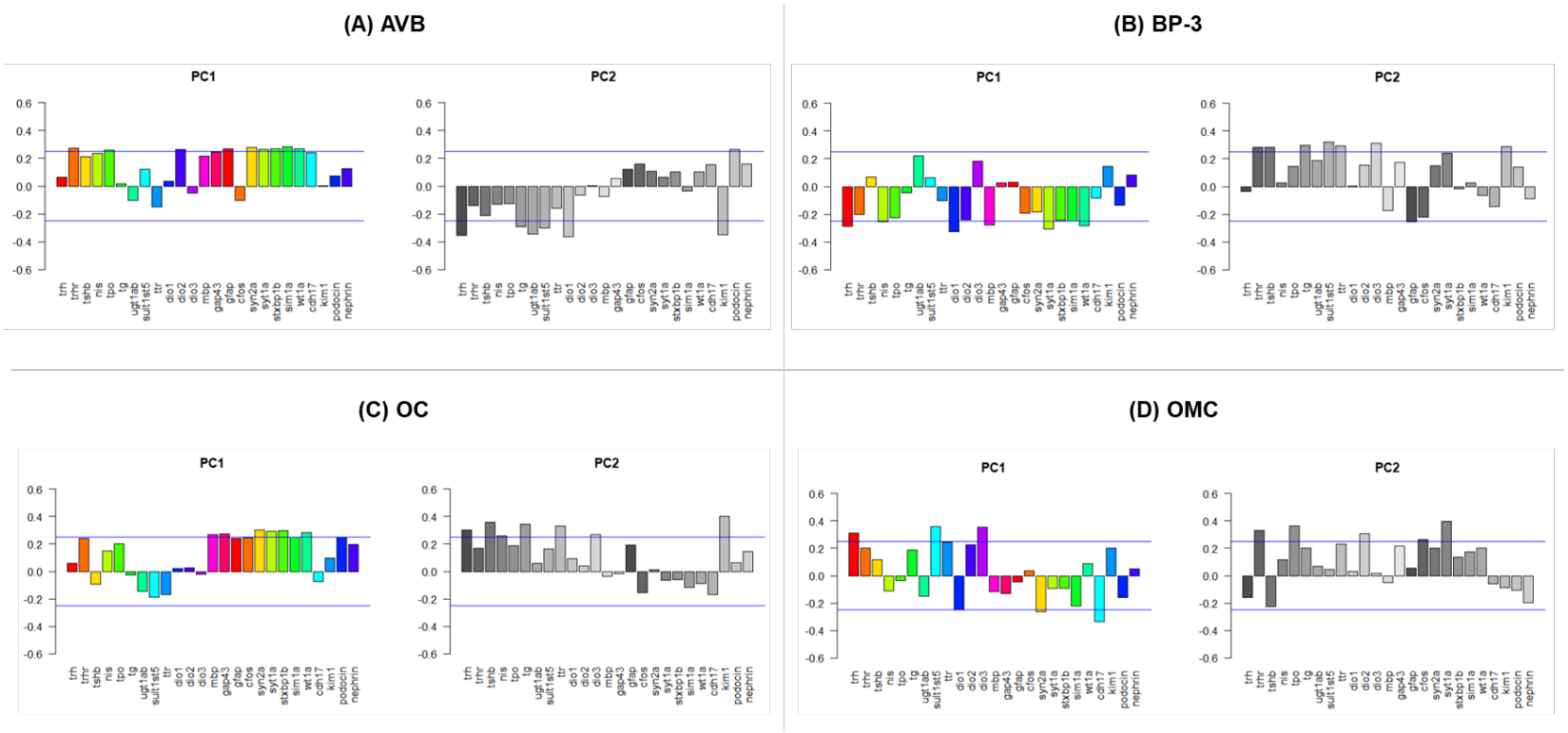


Fig. 26. Factor loading plots of first two PCs of gene transcriptions related to THs disruption, neural-, and kidney toxicities in embryo-larval zebrafish.

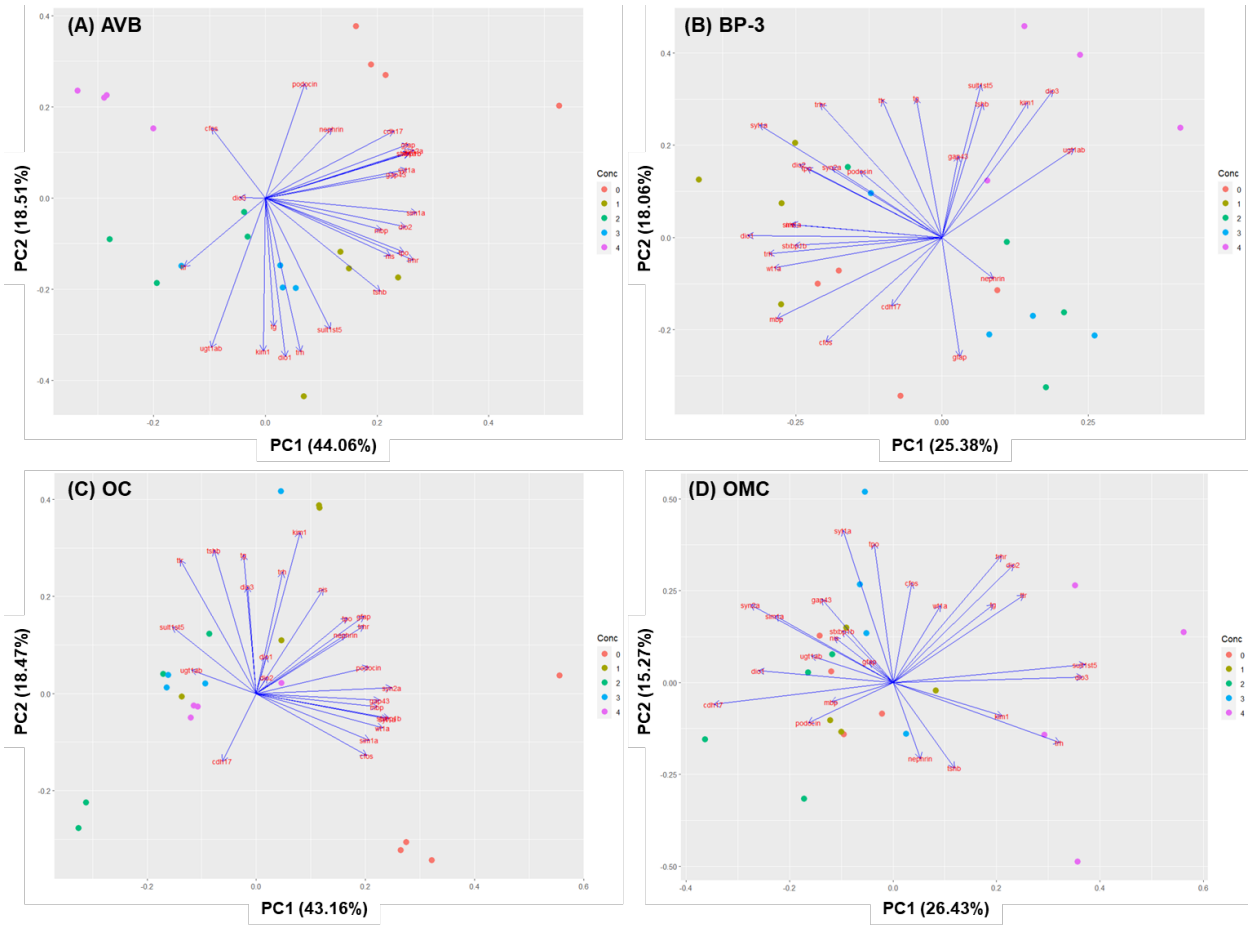

(A) AVB
PC2 (18.51%)
PC1 (44.06%)
Conc
0
1
2
3
4
(B) BP-3
PC2 (18.06%)
PC1 (25.38%)
(C) OC
PC2 (18.47%)
PC1 (43.16%)
(D) OMC
PC2 (15.27%)
PC1 (26.43%)

Fig. 27. Plots of the first two factors of principal component analysis of genes related to THs disruption, neural-, and kidney toxicities in embryo-larval zebrafish.

Table 13. Result of regression analysis of first two PCs and thyroid hormones (TT4, TT3, FT3) in embryo-larval zebrafish.

Classification		PC1				PC2			
		β	**r^2**	***p***		**β**	**r^2**	***p***	
AVB	**TT4**	0.238	0.408	0.001	*	-0.197	0.114	0.080	
	TT3	0.172	0.519	0.000	*	-0.126	0.109	0.085	
	FT3	0.167	0.440	0.001	*	-0.130	0.105	0.089	
BP-3	**TT4**	-0.351	0.430	0.001	*	-0.305	0.195	0.029	*
	TT3	-0.224	0.210	0.024	*	-0.254	0.018	0.035	*
	FT3	-0.246	0.202	0.027	*	-0.124	-0.011	0.384	
OC	**TT4**	0.074	0.520	0.000	*	-0.017	-0.036	0.572	
	TT3	0.072	0.458	0.001	*	0.017	-0.037	0.578	
	FT3	0.089	0.501	0.000	*	-0.009	-0.052	0.811	
OMC	**TT4**	-0.087	0.251	0.014	*	-0.225	-0.049	0.745	
	TT3	-0.090	0.190	0.031	*	0.026	-0.049	0.748	
	FT3	-0.088	0.429	0.001	*	-0.008	-0.054	0.881	

Table 14. Result of principal component analysis of gene transcriptions related to THs disruption, neural-, and kidney toxicities in juvenile zebrafish.

Classifications	**AVB**		**BP-3**		**OC**		**OMC**	
	PC1	**PC2**	**PC1**	**PC2**	**PC1**	**PC2**	**PC1**	**PC2**
Standard deviation	3.8161	2.2509	2.5274	2.2736	2.7642	2.3005	3.4399	2.1464
Proportion of variance	0.5201	0.1809	0.2281	0.1846	0.2729	0.1890	0.4226	0.1645
Cumulative proportion	0.5201	0.7011	0.2281	0.4127	0.2729	0.4619	0.4226	0.5871
Factor loadings								
trh	-0.2146	0.1231	-0.1190	**-0.3275**	**-0.2503**	0.1535	0.1469	**-0.2519**
trhr	-0.1898	0.1085	0.2200	-0.1057	**-0.3448**	-0.0467	0.0618	**-0.4059**
tshb	-0.1731	0.2168	**0.2753**	-0.0773	-0.2135	0.2071	0.2214	-0.1111
tra	-0.1856	0.2253	-0.1680	-0.2934	-0.2092	0.0750	0.0802	**-0.2944**
trb	-0.1779	**0.2549**	-0.1050	-0.3671	-0.1773	0.1824	0.1449	-0.1835
tshr	-0.0512	-0.1625	**0.3104**	-0.1060	-0.1349	-0.1819	-0.0398	-0.1853
nis	-0.1597	0.1177	**0.2539**	-0.1622	**-0.2773**	-0.1450	0.0192	**-0.2688**
tpo	-0.1505	0.1228	**0.3026**	-0.0848	-0.2451	0.2320	0.1674	-0.2075
tg	-0.1960	0.2057	-0.1630	**-0.2556**	-0.1899	-0.2081	-0.0683	**-0.3004**
ugt1ab	-0.1434	0.2261	0.0756	0.1813	-0.0953	**0.3208**	**0.2543**	0.0429
sult1st5	-0.1632	**0.2844**	0.1570	0.0706	-0.1805	0.1689	0.1175	-0.1652

ttr	-0.1808	**0.2624**	-0.0139	0.1136	-0.1222	0.1642	0.1848	-0.0092
dio1	-0.1588	0.2281	-0.0443	0.1134	-0.0559	**0.3652**	**0.2644**	0.0839
dio2	-0.2220	-0.0384	**0.2579**	-0.1354	**-0.2877**	-0.0120	0.0849	**-0.3323**
dio3	-0.1382	0.1132	**0.2925**	-0.1701	**-0.2517**	0.0272	0.0588	**-0.2866**
mbp	-0.2457	-0.0732	-0.0319	-0.1735	0.1675	**0.3253**	0.2465	-0.0444
gap43	-0.2185	-0.1993	0.1957	0.0031	0.1979	0.2214	**0.2745**	0.0187
gfap	-0.2419	-0.1029	-0.1968	0.0458	0.1976	0.2494	**0.2664**	0.0613
cfos	-0.0668	**-0.2669**	0.1538	0.0515	-0.1024	0.1382	0.0744	-0.1753
syn2a	-0.2144	-0.1769	0.0665	-0.2248	0.0025	0.2373	**0.2705**	0.1156
sty1a	-0.2198	-0.2189	-0.1515	**-0.2657**	0.0537	0.1884	**0.2615**	0.0819
stxbp1b	-0.2180	-0.2250	-0.1073	-0.2779	0.0990	0.2140	**0.2603**	0.1489
sim1a	-0.2386	-0.1166	-0.1805	-0.1383	-0.0909	0.0862	0.2332	0.1265
wt1a	-0.2073	-0.2333	-0.2118	-0.1140	-0.1506	0.1347	0.2381	0.0650
cdh17	-0.2267	-0.1500	0.1276	-0.2114	0.2475	0.0612	**0.2610**	0.0894
kim1	-0.1968	-0.1248	**0.3055**	0.0123	-0.2475	0.0095	-0.1602	0.0486
podocin	-0.0343	**-0.2624**	0.1742	**-0.2859**	-0.1046	-0.0486	-0.0283	-0.2106
nephrin	-0.2347	-0.1173	-0.0295	-0.2046	0.0822	0.2075	0.2065	0.1284

Values > 0.25 are shown in bold.

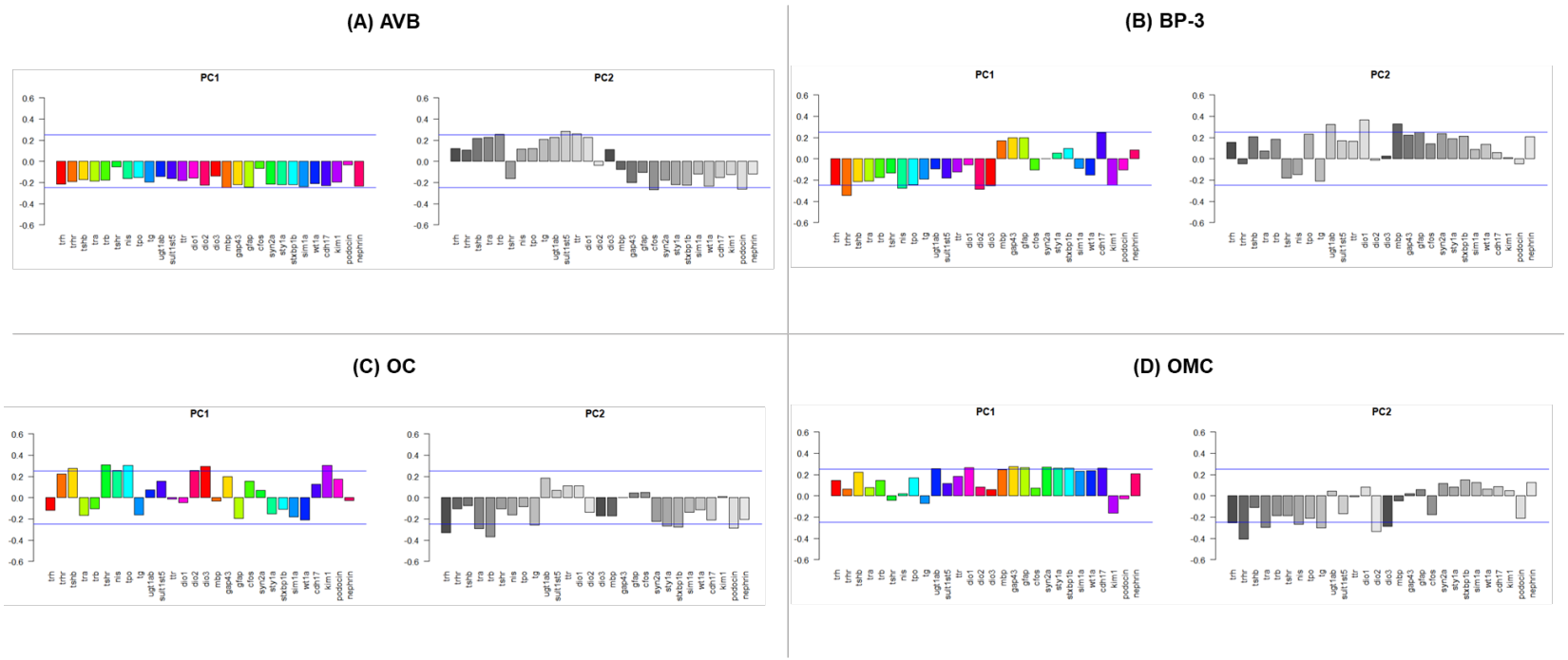


Fig. 28. Factor loading plots of first two PCs of gene transcriptions related to THs disruption, neural-, and kidney toxicities in juvenile zebrafish.

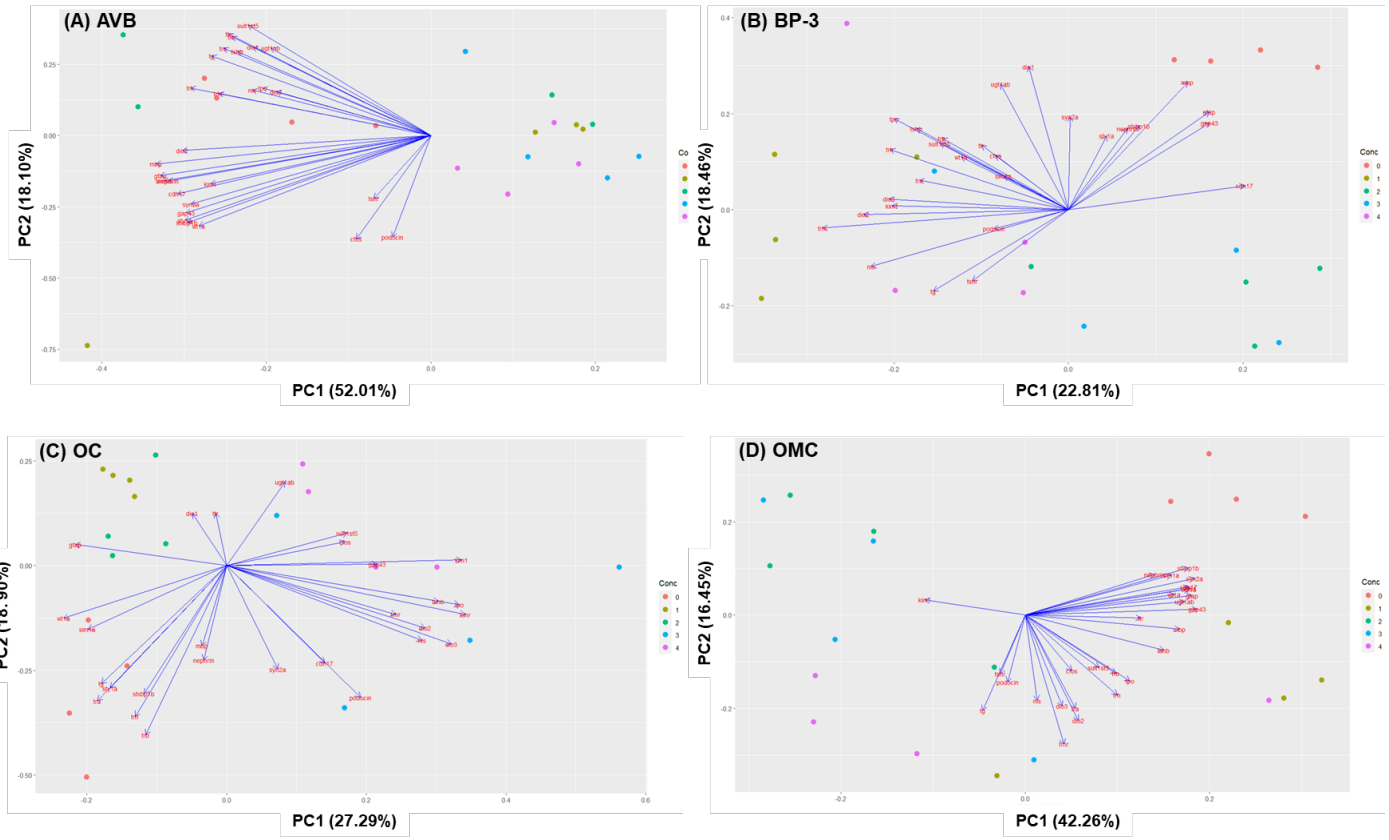

(A) AVB
PC2 (18.10%)
PC1 (52.01%)
(B) BP-3
PC2 (18.46%)
PC1 (22.81%)
(C) OC
PC2 (18. 90%)
PC1 (27.29%)
(D) OMC
PC2 (16.45%)
PC1 (42.26%)
Conc
0
1
2
3
4

Fig. 29. Plots of the first two factors of principal component analysis of genes related to THs disruption, neural-, and kidney toxicities in juvenile zebrafish.

Table 15. Result of regression analysis of first two PCs and thyroid hormones (TT4, TT3, FT3) in juvenile zebrafish.

Classification		PC1				PC2		
		β	**r^2**	***p***		**β**	**r^2**	***p***
AVB	**TT4**	0.000	-0.056	0.990		-0.034	-0.026	0.479
	TT3	-0.060	-0.053	0.167		0.032	-0.043	0.641
	FT3	-0.010	-0.047	0.710		-0.018	-0.044	0.662
BP-3	**TT4**	-0.014	-0.054	0.872		-0.138	0.016	0.267
	TT3	0.018	-0.046	0.690		-0.047	-0.025	0.471
	FT3	0.045	0.001	0.326		0.004	-0.055	0.956
OC	**TT4**	-0.021	-0.047	0.713		0.002	-0.056	0.985
	TT3	0.180	0.547	0.000	*	-0.210	0.140	0.058
	FT3	0.042	-0.013	0.396		0.000	-0.056	0.998
OMC	**TT4**	-0.085	0.122	0.073		0.013	-0.050	0.769
	TT3	-0.06865	0.11999	0.0744		0.035	0.006	0.304
	FT3	-0.092	0.097	0.099		-0.052	0.011	0.286

Table 16. Result of principal component analysis of gene transcriptions related to THs disruption, neural-, and kidney toxicities in adult male zebrafish.

Classifications	AVB		BP-3		OC		OMC	
	PC1	PC2	PC1	PC2	PC1	PC2	PC1	PC2
Standard deviation	3.2386	2.9964	3.7437	2.2610	3.3125	2.5730	3.2297	2.5903
Proportion of variance	0.2497	0.2138	0.3337	0.1217	0.2612	0.1576	0.2483	0.1598
Cummulative proportion	0.2497	0.4635	0.3337	0.4554	0.2612	0.4189	0.2483	0.4081
Factor loadings								
trh	0.0209	**0.2804**	-0.0417	0.1595	0.0261	0.1697	-0.0843	0.1564
trhr	-0.0020	**0.3062**	-0.1370	**0.2660**	0.1080	-0.1457	-0.1636	-0.0741
tshb	-0.0399	**0.3050**	0.2001	-0.0635	0.0184	-0.0904	-0.0866	-0.2014
dio1 (Brain)	0.0204	**0.3092**	-0.1000	0.2398	0.1166	-0.2445	-0.1444	-0.1832
dio2 (Brain)	0.0203	**0.3051**	-0.1456	0.1559	0.1388	**-0.3091**	-0.0940	0.0116
dio3 (Brain)	-0.0187	**0.2962**	-0.1487	**0.2694**	0.1311	-0.2171	-0.1006	-0.1929
tra (Brain)	0.0994	0.1859	0.1808	-0.1929	0.1245	**-0.2657**	-0.1591	-0.1495
trb (Brain)	0.0452	0.1389	-0.0564	**-0.2550**	0.0423	-0.0719	-0.1714	-0.1364
tshr	**0.2720**	0.0393	0.2093	0.1873	**0.2566**	0.1356	**0.2637**	-0.1755
nis	**0.2626**	0.0359	0.2185	0.1735	**0.2856**	0.0335	0.2352	-0.2176
tpo	**0.2648**	0.0222	0.2188	0.1640	**0.2696**	0.0864	**0.2569**	-0.2031

tg	0.2081	-0.0699	0.2111	0.1697	**0.2521**	0.1453	**0.2590**	-0.1740
dio1 (thyroid)	**0.2728**	0.0359	0.1807	0.1460	**0.2852**	0.0444	**0.2550**	-0.2014
dio2 (thyroid)	**0.2659**	0.0353	0.2138	0.1998	**0.2766**	0.0914	**0.2635**	-0.1544
dio3 (thyroid)	0.2102	-0.0425	0.0767	0.0199	**0.2813**	0.0789	**0.2518**	-0.2105
tra (thyroid)	**0.2715**	0.0501	0.0836	-0.0106	**0.2790**	0.0705	**0.2555**	-0.2079
trb (thyroid)	0.2057	-0.0759	-0.0026	-0.2266	0.2127	0.1218	0.0885	0.2328
ttr	0.1030	-0.1392	-0.1674	0.0810	-0.1454	-0.0715	0.0006	0.0213
dio1 (liver)	0.0869	0.0079	-0.1326	-0.1037	0.0215	-0.0080	-0.0254	-0.1016
dio2 (liver)	-0.1586	0.1359	-0.1776	-0.0407	0.2044	-0.0189	0.1504	-0.0775
dio3 (liver)	0.0881	-0.0862	-0.0506	0.0104	-0.0872	-0.1101	-0.0166	0.1139
ugt1ab	-0.0306	-0.0111	0.2227	0.0850	0.1017	-0.2039	-0.0150	0.0162
sult1st5	0.1457	-0.0303	0.1963	-0.0356	0.1278	-0.1349	0.1397	0.0428
mbp	-0.0100	0.1457	-0.1282	-0.1919	0.0022	-0.2097	-0.1131	-0.1780
gap43	-0.0624	0.0884	-0.0708	0.1856	0.0306	-0.1250	-0.1643	-0.1835
gfap	0.0101	0.0709	0.0289	-0.2093	0.1276	-0.3010	-0.1375	-0.1943
cfos	0.0921	-0.0797	0.0946	-0.2418	0.1341	0.0029	0.0013	0.1366
syn2a	-0.0198	0.2079	-0.1631	0.1548	-0.0262	-0.1029	-0.1546	-0.1932
stx1a	-0.0360	**0.2651**	-0.1161	0.2218	0.0549	-0.2170	-0.1641	-0.0813
stxbp1b	0.1237	0.0964	0.0829	0.1934	-0.0873	-0.1529	-0.1641	-0.1325

ppargc1a	-0.2016	-0.0785	0.1324	-0.0551	-0.0341	-0.2182	-0.1074	-0.0160
tbx2a	-0.1398	0.0098	-0.1392	-0.0810	-0.0556	-0.0985	-0.1980	-0.1953
tbx2b	-0.1837	-0.1244	0.0303	-0.0444	0.0981	-0.0375	-0.1732	-0.1361
etv4	-0.0547	-0.2037	0.1909	-0.1988	0.0667	-0.0056	0.0453	0.0758
etv5	-0.1457	0.0465	-0.1520	-0.0637	0.1220	-0.0874	-0.0741	0.0240
sim1a	-0.1846	-0.0670	-0.1126	-0.0835	0.0235	**0.2740**	-0.1026	-0.2352
wt1a	-0.0779	-0.1971	-0.2059	0.1128	-0.0788	-0.0137	-0.1066	-0.1987
cdh17	-0.1880	0.0029	-0.1840	-0.0454	0.0380	-0.2410	-0.0513	-0.2276
kim1	0.1060	-0.1811	0.2086	0.1223	-0.1664	0.0610	-0.0346	0.1773
podocin	-0.2021	0.0812	-0.1692	0.0049	-0.0561	0.1420	-0.1738	-0.0633
nephrin	**-0.2552**	-0.0001	-0.1735	0.0724	-0.1344	-0.2129	-0.0140	0.0383
pax2a	0.0972	-0.1603	0.2012	-0.0175	0.2044	-0.0385	-0.1055	-0.0005

Values > 0.25 are shown in bold.

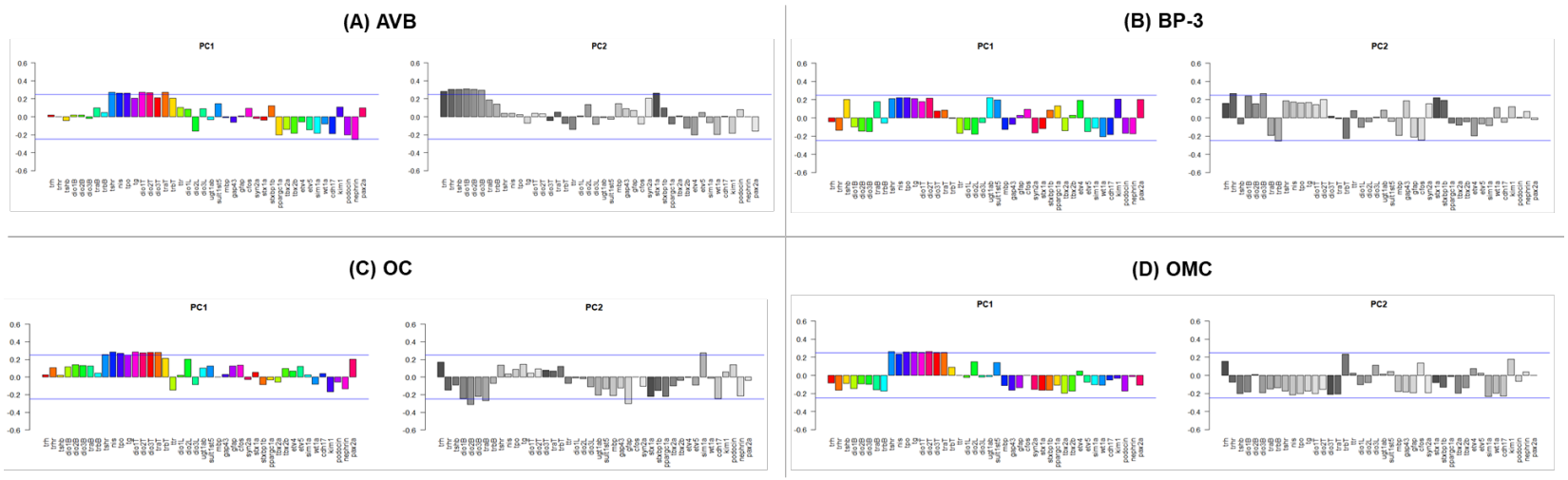
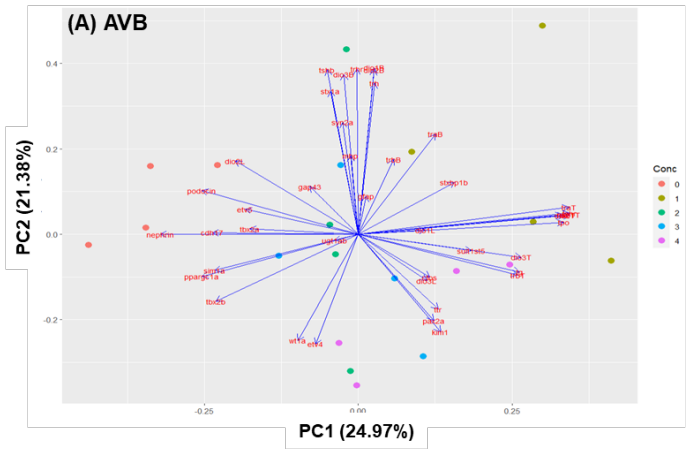
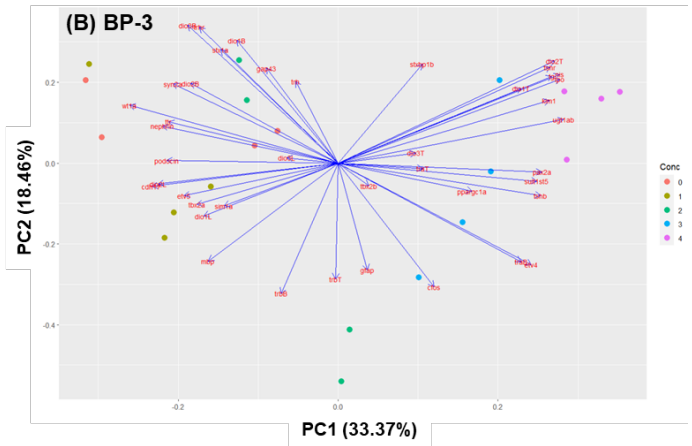
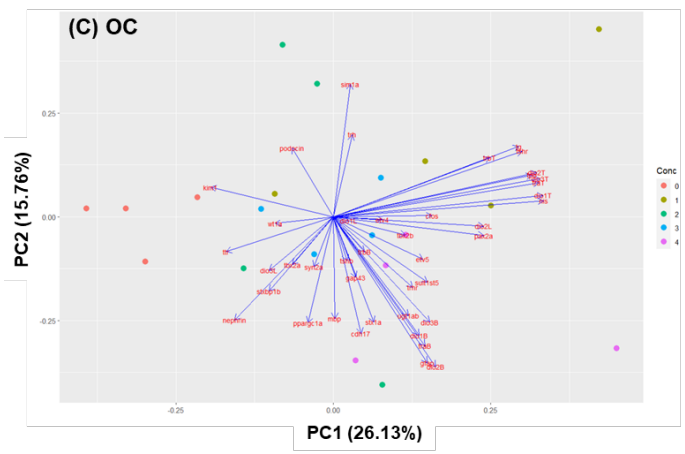


Fig. 30. Factor loading plots of first two PCs of gene transcriptions related to THs disruption, neural-, and kidney toxicities in adult male zebrafish.


(A) AVB
PC2 (21.38%)
PC1 (24.97%)
Conc
0
1
2
3
4


(B) BP-3
PC2 (18.46%)
PC1 (33.37%)
Conc
0
1
2
3
4


(C) OC
PC2 (15.76%)
PC1 (26.13%)
Conc
0
1
2
3
4

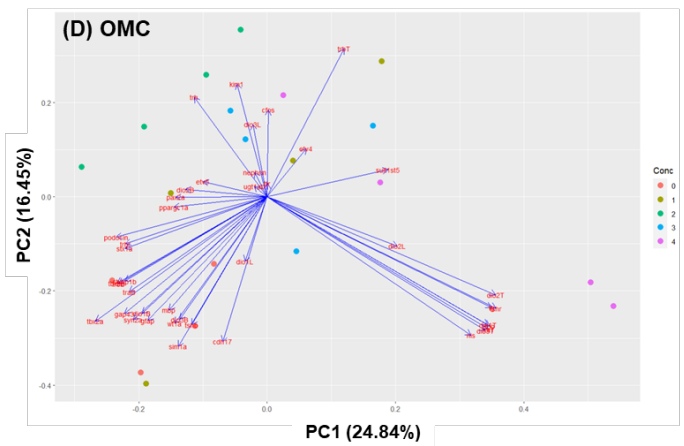

(D) OMC
PC2 (16.45%)
PC1 (24.84%)
Conc
0
1
2
3
4

Fig. 31. Plots of the first two factors of principal component analysis of genes related to THs disruption, neural-, and kidney toxicities in adult male zebrafish.

Table 17. Result of regression analysis of first two PCs and thyroid hormones (TT4, TT3, FT3) in adult male zebrafish

Classification		PC1				PC2			
		β	r^2	*p*		β	r^2	*p*	
AVB	**TT4**	-0.227	0.189	0.032	*	-0.229	0.142	0.038	*
	TT3	-0.050	0.116	0.078		0.071	0.105	0.089	
	FT3	-0.028	0.066	0.143		0.055	0.162	0.042	*
BP-3	**TT4**	0.001	-0.055	0.963		0.011	-0.048	0.724	
	TT3	-0.003	-0.034	0.548		-0.024	0.137	0.060	
	FT3	0.008	-0.025	0.476		0.014	-0.045	0.678	
OC	**TT4**	-0.046	0.131	0.065		-0.049	-0.006	0.358	
	TT3	0.004	-0.054	0.888		-0.044	-0.015	0.409	
	FT3	-0.019	-0.013	0.395		-0.002	-0.055	0.959	
OMC	**TT4**	0.036	0.003	0.320		-0.035	-0.025	0.471	
	TT3	0.006	-0.040	0.612		-0.019	0.021	0.250	
	FT3	0.015	0.092	0.104		-0.003	-0.053	0.842	

There are several limitations of our study. We used relatively high concentrations of UV filters compared to the levels found in the environment. In addition, for embryo-larval and juvenile zebrafish, TH levels and related gene expressions were measured at whole body level. We utilized adult male zebrafish to determine target tissue specific responses, however, adverse outcomes related to thyroid disruption, neurobehavior or kidney function were not determined. Therefore, results from the present study should be interpreted with caution. Although zebrafish share a high genetic similarity and closely resembles human anatomy, zebrafish models cannot fully recapitulate the complexities of human, however, findings from this study provide valuable insights of potential health consequences of UV filters.

5.2 Future research directions

Based on our observations, many UV filters, including AVB, BP-3, OC, and OMC, are currently in use for various applications found in our everyday life. It seems clear that there is a growing body of evidence pointing to the negative impact of organic UV filters. Nevertheless, identifying UV filters that are significant to human health remains challenging. Indeed, our data show that there is a potential risk that UV filters can induce adverse health outcomes in terms of thyroid disruption, neurobehavior and kidney function. Therefore, epidemiological studies on UV filter exposure, particularly for comparing exposure and outcomes that could link molecular and toxicological information to facilitate the assessment of risk and societal impact, are urgently needed. As epidemiological studies rely on associations between exposures and observed effects, animal models are indispensable in elucidating underlying mechanisms in order to support these observations (Gilbert et al. 2020). Even so, there are often disconnects between neurodevelopmental effects and kidney functions influenced by THs disruption in clinical and epidemiological studies, and related outcomes in animal studies. Therefore, further in effort to identify key molecular interactions should complement the observed abnormal phenotypes in epidemiological study as well as the findings from the present study. Furthermore, the tested concentration range of several UV filters (i.e., lowest concentrations of AVB, OC, OMC approximately 30 to 100 μg/L) were higher compared to aquatic environmental concentrations, detected levels up

to 0.32 μg/L). There is need for a better understanding of low-dose and chronic effects of UV filter compounds alone and in combinations. So far, most observations made to date have been acute and simplistic, failing to realistically mimic contemporary sunscreens. UV filters are often applied in combination to provide a broad spectrum of photoprotection. Therefore, it would be advantageous to study the common combination of UV filters that are commercially or environmentally relevant.

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국문 초록(Abstract in Korean)

주요 유기계 자외선 차단 물질이 여러 생애 단계별 제브라피쉬의 갑상선, 신경 및 신장에 미치는 독성과 관련 기전 연구

유기계 자외선 차단물질(organic UV filters)은 자외선차단제, 화장품, 헤어 제품을 포함한 개인위생용품에 널리 사용되고 있는 물질군이다. 최근, 여러 국가에서 널리 사용되고 있는 유기계 자외선 차단물질인 벤조페논-3(benzophenone-3, BP-3) 및 옥틸메톡시신나메이트(octyl methoxycinnamate, OMC)가 함유된 자외선차단제 사용을 금지하였지만, 자외선차단제 외에도 여전히 다양한 용도로 많이 사용되고 있기 때문에 생체시료나 환경 중에서 꾸준히 검출되고 있다. 대부분의 유기계 자외선차단물질은 피부에 도포 후 전신흡수가 가능하기 때문에 주의가 필요하다. 기존 역학 연구들에 따르면, 유기계 자외선 차단물질의 노출이 갑상선 내분비계를 포함하여 다양한 건강 영향들에 대해 보고해왔다. 그러나, 독성학적 정보는 대부분 BP-3의 생식 및 성호르몬 교란에 국한되어 있다. 그러나 유기계 자외선 차단물질들은 구조적 유사성으로 인해 비슷한 수준의 독성영향을 예상할 수 있으며, BP-3를 제외한 나머지 물질에 대한 독성연구는 부족한 수준이다. 특히, 여러 생애주기에 미치는 영향 연구는 매우 부족하다. 따라서, 본 연구는 여러 유기계 자외선 차단물질 노출에 따른 갑상선 내분비계 교란과 갑상선호르몬 교란과 관련된 관련 독성 영향을 파악하고자 한다.

첫번째 연구에서는(Chapter 1) 유기계 자외선 차단물질의 현재 사용 및 생산량, 관련 규제에 대해 조사하고 이 물질들의 독성학적 지식 갭을 확인하기 위해 및 내분비계 교란 가능성에 대해 조사하였다. 환경 중

검출 농도 및 생태학적 중요성을 고려하여 아보벤존(avobenzone, AVB), BP-3, 옥토크릴렌(octocrylene, OC), OMC를 선정하였다.

두 번째 연구(Chapter 2)에서는 글로벌 시장에서 주로 많이 사용되며 환경 중 검출빈도가 높은 AVB, BP-3, OC, 그리고 OMC 4종을 대상으로 제브라피쉬 모형을 이용하여 이들 물질의 갑상선 호르몬 교란 가능성을 배아 발달시기(수정 후 120 시간 노출), 초기 발달단계(수정 후 30일 노출), 성어 시기(6개월령 이상의 수컷, 21일 노출)의 여러 생애주기에서 살펴보았다. 노출 종료 후, 효소 결합 면역 흡착법(ELISA)을 통해 갑상선호르몬 자극 호르몬(TSH) 및 갑상선호르몬(T4, T3)의 변화와 실시간 중합효소 연쇄반응(RT-qPCR)을 이용하여 갑상선호르몬 조절과 관련된 유전자(*trh, trhr, tshβ, tshr, nis, tpo, tg, dio1, dio2, dio3, traα, trβ, ugt1ab, sult1 st5, ttr*)를 분석하였다. 그 결과, 연구 대상 물질 4종 모두(AVB, BP-3, OC, OMC) 제브라피쉬의 모든 생애주기의 시상하부-뇌하수체 갑상선(hypothalamus-pituitary thyroid axis, HPT) 축과 갑상선호르몬의 간 대사와 관련된 유전자의 발현 변화가 관찰되었다. 특히, 배아 발달단계와 유어 시기에 그 영향이 더 크게 나타난 것을 확인하였다. 따라서 두 번째 연구 결과를 종합해 볼 때, 주요 유기계 자외선 차단물질이 여러 생애 단계에 갑상선호르몬의 불균형을 일으키는 것을 확인하였다.

세 번째 연구(Chapter 3)에서는 배아 발달시기(수정 후 120 시간 노출), 초기 발달단계(수정 후 30일 노출), 성어 시기(6개월령 이상의 수컷, 21일 노출)의 여러 생애주기에서 주요 유기계 자외선 차단물질에 대한 신경독성을 평가하기 위해 수행되었다. 제브라피쉬를 배아단계부터 수정 후 120시간 및 30일 동안, 평가 대상 자외선 차단물질에 노출한 후, Daniovision ethovision® (Nodulus)을 이용하여 빛 자극으로 인한 행동변화(hyper-, hypoactivity)와 불안장애 표현형인 신경행동학적 변화와

함께 신경발달 및 신경 독성과 관련된 유전자 마커(*mbp, gap43, gfap, c-fos, syn2a, syt1a, stxbp1b*)를 분석하였다. 또한, 제브라피쉬 수컷 성어를 이용하여 21일간 평가 대상 자외선 차단물질에 노출 후 마찬가지로 신경발달 및 독성과 관련된 유전자의 발현 변화를 확인하였다. 그 결과, 배아 발달 및 초기 발달시기의 행동이 자외선 차단물질의 노출로 인해 대조군에 비해 유의하게 감소하였고, 불안장애의 신경행동학적 변화가 유의하게 나타났다. 본 연구를 통해 신경행동학적 변화는 중추 신경계에 발현되는 주요 유전자 또는 신경 세포에 대한 직간접적인 독성 영향의 결과일 가능성을 시사한다. 신경발달 및 독성과 관련된 유전자의 발현은 모든 생에 주기에서 영향이 나타났지만, 그 중 발달단계가 가장 크게 영향을 받았다. 따라서, 이 연구를 통해 관찰된 신경행동학적 변화는 중추 신경의 주요 유전자의 발현에 직접적인 영향을 미칠 수 있음을 시사한다.

마지막으로, 네 번째 연구(Chapter 4)에서는 배아 발달시기(수정 후 120시간 노출), 초기 발달단계(수정 후 30일 노출), 성어 시기(6개월령 이상의 수컷, 21일 노출)의 여러 생애주기에서 주요 유기계 자외선 차단물질이 신장기능에 미치는 영향에 대해 평가하였다. 노출 종료 후, 제브라피쉬 배아 및 유어를 깨끗한 사육수로 옮긴 후 단백뇨 수준을 관찰하였다. 또한, 신장재생과 구조에 중요한 역할을 하는 핵심 유전자의 발현 변화를 관찰하기 위해 배아 발달 및 유어 시기에서는 신장 특이적으로 발현되는 유전자(*wt1a, podocin, nephrin, kim-1, cdh17, sim1a*)를 분석하였고, 수컷 성어 신장 조직을 이용하여 보다 더 자세한 자외선 차단물질로 인한 신장독성 기전을 파악하기 위해 여러 유전자(*ppargc1a, tbx2a, tbx2b, etv4, etv5, wt1a, podocin, nephrin, kim-1, cdh17, sim1a, pax2a*)를 이용하여 분석하였다. 그 결과, 배아 발달 및 유어 시기의 제브라피쉬의 단백뇨 수준에 변화와 신장 구조 및 기능에 중요한 역할을 하는 주요 유전자의 발현의 변화가 관찰되었다. 본 연구를 통해 자외선 차단물질의 노출이 여

러 생애 단계(배아발달, 유어 및 성어 시기)의 신장에 영향을 미치는 것을 보여주어 자외선 차단물질 노출로 인한 신장 건강 영향에 대한 중요한 실마리를 제공한다.

이상의 세 실험 연구를 종합해보았을 때, 자외선 차단물질(AVB, BP-3, OC, OMC)의 중요한 건강 영향을 보여주었다. 본 연구를 통해 연구 대상 자외선 차단물질이 제브라피쉬의 여러 생애 단계의 갑상선호르몬의 항상성에 영향을 주었다. 더 나아가, 자외선 차단물질에 노출된 제브라피쉬의 신경행동 및 신장의 기능이 손상되었다. 자외선 차단물질은 피부에 오랫동안 머물도록 만들어지고, 피부흡수를 통해 순환계에 도달할 수 있기 때문에 사람의 건강에 미치는 영향은 환경 보건학적으로 매우 중요하다. 많은 자외선 차단물질이 혼합되어 사용되는 특성을 고려하면, 이 물질들의 혼합독성을 이해하는 것이 필요하다.

고농도 노출환경으로 제한되기는 하지만, 주요 유기계 자외선 차단물질은 갑상선호르몬 교란 작용이 있는 것으로 나타났으며, 그 반응이 노출 시기별로 다르게 관찰되었다. 이는 평가 대상 자외선 차단물질이 HPT 축 및 갑상선호르몬의 간 대사에 직접 작용하는 기전에 의한 현상으로 볼 수 있다. 또한, 여러 생애 주기에 신경과 신장의 정상적인 기능을 손상시키는 것으로 보인다. 주요 유기계 자외선 차단 물질이 갑상선호르몬 및 관련 건강에 미치는 영향을 파악하는 것은 환경보건학적인 측면에서 매우 중요하며, 안전한 자외선 차단물질을 선정하기 위한 과학적인 기초자료로도 매우 중요할 것이다. 나아가, 많은 자외선 차단 물질이 혼합되어 사용되는 특성을 고려하면, 이 물질들의 혼합 독성을 이해하는 것이 필요하며, 발달 시기와 같이 민감한 집단에서의 자외선 차단물질로 인한 건강영향과 관련한 실험 및 역학 조사가 필요하다.

표제어: 자외선 차단물질, 내분비계 교란, 갑상선호르몬 교란, 신경독성, 신장독성, 제브라피쉬

학번: 2018-31754

감사의 글

어느덧 긴 대학원 생활을 마무리하며 박사 학위 논문을 제출하게 되었습니다. 지난 시간을 돌이켜보니 그동안 저에게 도움을 주신 분들이 많습니다. 제가 이렇게 성장하기까지 오랜 시간이 걸렸지만, 그 세월 속에서 직간접적으로 힘이 되고 방향을 잡아 주셨던 많은 분들께 감사의 말씀을 전하고자 합니다.

먼저 박사 학위 논문이 완성되기까지 바쁘신 가운데도 많은 격려와 지도로 이끌어주신 최경호 교수님께 진심으로 머리 숙여 감사드립니다. 환경보건학에 대한 뜨거운 열정으로 논문을 지도해주시면서 연구가 갖추어야 할 정확성과 성실성을 깨우쳐주시고, 때로는 제가 숲에서 길을 잃었을 때 나무를 보는 법을 알려주신 덕분에 논문을 잘 마무리할 수 있었습니다. 평소 마음속에 담아두었던 감사함과 존경심을 이번 학위 논문 제출의 감사 글로 소박하게나마 전합니다. 앞으로 훌륭하고 멋진 여성 과학자로서 교수님의 옆에서 자랑스러운 제자로 또, 공동 연구자로 성장하여 그 은혜에 보답하겠습니다.

논문 심사 과정에서 아낌없는 지도로 많은 가르침을 주신 심사 위원장 김성균 교수님, 심사 부위원장 및 '공동 지도교수님'이신 박영주 교수님, 학위 논문에 핵심적으로 필요한 실험 기법을 배울 수 있고 경험할 수 있게 큰 도움을 주신 이정표 교수님, 지금의 저를 있게 해주신 저의 석사 지도 교수님이시자 심사위원이신 지경희 교수님께도 감사드립니다. 제가 건강히 학위 과정을 마칠 수 있도록 믿음을 주시고 심사과정에서 아낌없는 격려와 조언을 주신 덕분에 더 나은 논문이 되었습니다. 교수님의 제자라고 말할 수 있어 행복합니다.

환경독성학 연구실에서 삶의 시간을 함께 나누어 가진 모든 선배님과 후배들께 감사와 사랑을 드립니다. 같은 배움의 터전에서 싶은

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