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Joint Effects of Multiple UV Filters on Zebrafish Embryo Development

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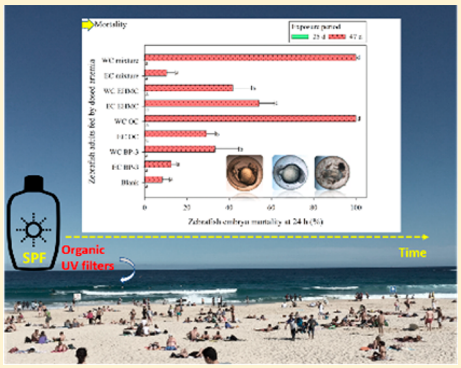
Supporting Information

ABSTRACT: The widespread use of UV filters has resulted in significant amounts of these chemicals appearing not only in the environment but also in organisms. This study first assessed the levels of nine UV filters in waters along the coast of Shenzhen, China, in tapwater, and in a nearby reservoir. UV filters were found to be high, in both winter and summer at most locations. Then, using zebrafish as a model, the influence of a UV filter mixture after dietary and aqueous exposure was assessed. After exposing artemia to three dominant UV filters at two levels and then feeding these artemia to zebrafish adults, concentrations in both were up to 4 times higher when exposed to the mixtures than when exposed to only a single UV filter. A short-term 25-day dietary exposure to the zebrafish adults did not appear to significantly influence early life stage development of the second generation; however, relatively long exposure over 47 days had significant adverse effects on embryo development. Aqueous exposure of fish embryos to mixtures of the three UV filters demonstrated a general trend of decreased heart/hatching rate as doses increased, coupled with significant changes in activities of catalase and malate dehydrogenase.



INTRODUCTION

Exposure to UV radiation, a known carcinogen, is highly associated with skin cancers and melanomas;[1] organic UV filters constitute a heterogeneous group of chemicals that, when applied to the skin, can block some of the sun's damaging UV radiation. Hence, various types of personal care products (PCPs) containing organic UV filters, such as sunscreens, skin lotions, and makeup products,[2] are widely and consistently consumed.[3] Apart from PCPs, UV filters are also present in textiles, plastics, and paints as protection against photodegradation and discoloring.[4,5]

The massive production and use of UV filters have resulted in the appearance of significant amounts of these chemicals in the environment (~6812 ng/L in water; ~10 400 ng/L in wastewater influent; ~41 610 ng/g-dw in sewage sludge)[6−10] with potential bioaccumulation in both animals (~242 ng/g-dw in fish; ~782 ng/g lipid weight in dolphin; ~3348 ng/g-dw in bird egg) and humans (~15.7 ng/mL in urine).[11−14] These chemicals are referred to as endocrine disruptors with effects reported in several in vitro and in vivo studies: median effective concentrations of benzophenone-3 (BP-3) and ethylhexyl methoxycinnamate (EHMC) were in the mg/L range for immobility of *Daphnia magna* and growth inhibition of *Raphidocelis subcapitata*.[15] BP-3 at 26 μg/L significantly affected endocrine balance and reproduction performance in Japanese medaka (*Oryzias latipes*).[16] EHMC at 394 μg/L induced significant histological changes in testes and ovaries of fathead minnows (*Pimephales promelas*).[17] Octocrylene (OC) at 383 μg/L induced alteration of 628 and 136 transcription of genes in zebrafish brain and liver, respectively, indicating influence on developmental processes, organ development, and metabolism.[18] Given the endocrine disrupting capacity of UV filters, risk assessments have been conducted using worst-case scenarios for single compounds and have concluded that the current levels of organic UV filters pose no/low risk.[7,15,19,20] Such studies, however, fail to take into consideration the possibilities (a) that interactions of environmentally relevant

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mixtures can induce additional problems not observed when compounds are tested individually and (b) that these interactions develop over time, becoming increasingly dangerous.

Shenzhen, situated on the east side of the Pearl River Delta in South China, is one of the world's most rapidly urbanized cities. The Delta's marine environment is reportedly influenced by contaminants from both domestic and industrial wastewater; these contaminants include UV filters, phenols,[3] artificial sweeteners,[21] organophosphate flame retardants, cationic surfactants, and polycyclic aromatic hydrocarbons.[22,23] Meanwhile, the southeast coastline of Shenzhen includes more than 20 popular recreational beaches where products containing organic UV filters are consumed in large quantities, adding to any contaminants from water treatment plant discharges. No studies of the effects of these contaminants with regard to the safety of the waters have been done.

The objectives of this study are threefold: (1) to determine the occurrence and distribution of nine commonly used organic UV filters, benzophenone-1 (BP-1), BP-3, benzophenone-8 (BP-8), EHMC, octyl dimethyl-*p*-aminobenzoic acid (OD-PABA), OC, 4-hydroxybenzophenone (4-HB), 4-methylbenzylidene camphor (4-MBC), and 3-benzylidene camphor (3-BC) in surface waters of Shenzhen, China (Figure S1); (2) to test the three most dominant UV filters' absorption (i.e., BP-3, EHMC and OC) in adult zebrafish *D. rerio* through dietary uptake of *Artemia franciscana nauplii* dosed at environmental-case (EC) and worst-case levels (WC: level similar to toxic concentrations used in studies of mode of action),[16−18] both singly and in mixtures; (3) to evaluate embryonic development of the fish's second generations via both maternal dietary exposure and direct aqueous exposure to the three UV filters mixture before the onset of exogenous feeding. To our knowledge, this is the first report of the effect of multiple organic UV filters on successive generations of fish.

MATERIALS AND METHODS

Standards and Reagents. Standards for BP-1 (99%, China), BP-3 (100%, USA), BP-8 (98%, USA), EHMC (100%, USA), OC (100%, USA), and 4-HB (98%, China) were ordered from Sigma-Aldrich. Standards for OD-PABA (>95%, China), 4-MBC (>99%, China), and 3-BC (90−100%, USA) were purchased from TCI, Alfa Aesar, and MP Biomedicals, respectively. Stock solutions of each analyte were prepared in pure methanol at 10 g/L and stored in amber glass vessels at 4 °C. Solvents including Milli-Q water (18.2 MΩ cm, Millipore, USA), LC-MS grade methanol (Duksan Pure Chemicals, Korea), and formic acid (98−100%, International Laboratory, USA) were utilized.

Water Sampling. Seawater samples were collected from 21 locations along the southeast coast of Shenzhen in the winter and summer of 2016 (Figure S1; Table S1). The sampling sites covered the locations where almost all recreational activities at popular/developed and undeveloped beaches occur. In addition, water from Shenzhen Reservoir, a water source conservation zone, and city tap water were collected. Surface water was stored in precleaned opaque polyethylene bottles at −20 °C prior to analysis. Water was filtered through 0.45 μm membranes before extraction.

Animal Husbandry. Zebrafish *D. rerio* adults (aged 3 months), cultured in dechlorinated tap water at the Pearl River Fisheries Research Institute, were fed *Artemia franciscana nauplii* twice a day (Aquamaster, China). The fish were maintained at 26 ± 1 °C in 14 h/10 h light/dark cycles. The procedures for fish maintenance, experimentation, and sacrifice were approved by the Ethics Committee of the Pearl River Fisheries Research Institute, Chinese Academy of Fishery Sciences.

Dietary Exposure to UV Filters. To facilitate direct comparison within the mixture−interaction study, selected doses of BP-3, EHMC, and OC were individually administered in parallel with the mixture. Stock solutions of each analyte were diluted by dechlorinated tap water for further use. Artemia cysts were exposed to BP-3 (5.5 and 550 μg/L), EHMC (4 and 400 μg/L), OC (7 and 700 μg/L), and two mixtures (environmental-case (EC): 5.5 μg/L BP-3, 4 μg/L EHMC, and 7 μg/L OC; worst-case (WC): 550 μg/L BP-3, 400 μg/L EHMC, and 700 μg/L OC) in saline water (35−40 ppt) for 24 h, respectively. The hatched artemia were rinsed by dechlorinated tap water, frozen at −20 °C, and fed to zebrafish adults twice a day for 60 consecutive days. Zebrafish were fed ad libitum, and artemia residues were removed after 1 h of food provision. In order to keep up the water quality during zebrafish dietary exposure and to avoid free UV filters associated with artemia, half of the water was changed daily, and the beaker was washed twice per week. Each treatment had three replicates, and each replicate had four fish in a 2 L beaker.

Breeding. After a period of dietary exposure, the zebrafish in a ratio of 2:2 females/males were kept in a tank with a board separating females from males in fresh dechlorinated tap water, overnight. The next morning, the board was removed, and when the light came on, the fish began to breed. Embryos were collected after 1 h. Apical end points of embryos were checked under an inverted microscope (Nikon Eclipse MA100, Japan).

Aqueous Exposure to UV Filters. Using the same breeding procedure, embryos were collected from unexposed adult zebrafish and then dosed directly with the UV filters by aqueous exposure. The percentage of solvent used was controlled at <0.01% for all treatments. There were four replicates for each treatment, and sample size was 12. The test lasted for 7 days with end points of mortality at 24 h, heart rate at 48 h, hatching rate at 72 h, and mortality at 7 days. At the end of the exposure, live fish larvae were flash-frozen and stored at −80 °C for further analysis. The exposure was repeated in order to collect enough larvae (≥50 larvae/sample) for measurement of enzyme activities. The method to measure enzyme activities is detailed elsewhere.[24]

Sample Treatment. After spiking by internal standards of D10-BP-3 (Sigma-Aldrich, USA) and D15-EHMC (Sigma-Aldrich, Swizerland), water samples of 100 mL were loaded onto an LC-18 solid phase extraction cartridge (500 mg, Supelco Inc., USA). The target compounds were eluted with 8 mL of acetone/ethyl acetate (v/v 1:1) and 4 mL of methanol/dichloromethane (v/v 1:1). After replacing solvent with methanol, the extracts were analyzed using UHPLC (Agilent 1290 series) coupled with MS/MS (Agilent 6460 series) (Agilent Technologies, Santa Clara, CA, USA). Samples of freeze-dried artemia and zebrafish adult (100 mg) in acetonitrile were sonicated for 30 min (Branson 3510-DTH, USA). After centrifugation, the supernatant was collected for further nitrogen concentration and acetonitrile reconstitution. Finally, the extracts were analyzed by UHPLC-MS/MS.

Chemical Analytical Method. For water sample analysis, chromatographic separation was performed on an ACQUITY UPLC BEH C8 column (100 × 2.1 mm, 1.7 μm, Waters,

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Ireland) in the isocratic elution mode for 10 min. The mobile phases were composed of 28% Milli-Q water and 72% methanol, both containing 0.1% formic acid (v/v). The flow rate was set to 0.4 mL/min with an injection volume of 10 μL. Agilent Jet Stream (AJS)-ESI-MS/MS ion source was operated in positive ionization mode by multiple reaction monitoring (MRM). Details of MRM transitions of each analyte are shown in Table S2. The dry gas of nitrogen flow was set at 8 L/min with 325 °C and the nebulizer, at 40 psi, while sheath gas of nitrogen was at 8 L/min with 350 °C. Capillary voltage and nozzle voltage were set to 4 kV and 500 V, respectively. The method performance of UV filters in the water sample is detailed in Table S3.

For biological samples, chromatographic separation was performed on the same column as for water samples. The mobile phases were set at 20% Milli-Q water and 80% methanol for 5 min, both containing 0.1% formic acid (v/v). The flow rate was 0.35 mL/min with an injection volume of 5 μL. The QqQ-MS/MS mode was applied with an ion source of Dual AJS ESI in positive ionization mode. The method performance of UV filters in artemia and zebrafish adults is shown in Table S4.

Statistical Analysis. Data are expressed as mean ± standard deviation or mean ± standard error. Statistical analyses were performed using SPSS version 19 (SPSS Inc., Chicago). One-way analysis of variance (ANOVA) using UV filters treatment as a fixed factor was used to infer the difference among chemical treatment groups. If the null hypothesis was not accepted by ANOVA, different means were identified using the Student−Newman−Keuls (SNK) test. A p value <0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Multiple UV Filters Present in Shenzhen Waters. The current method with sample cleanup via a SPE cartridge was validated for the simultaneous analysis of nine UV filters in the water matrix. The limit of detection (LOD) and limit of quantification (LOQ) were as low as 0.83 and 2.77 ng/L, respectively (Table S3). Seven out of nine UV filters were detected in Shenzhen waters, with higher levels in summer than in winter, similar to previous studies (Figure 1; Table S5).[6,8,25] Generally speaking, BP derivatives including BP-3, BP-8, BP-1, and 4-HB as well as EHMC were the most extensively found, with >50% detection rate (Table S5). The total concentrations of UV filters were relatively high in Sites 1, 14, 16 (popular public beaches), and 11 (a harbor) with $\sum_9$ UV filters ranging from 192 to 645 ng/L in the summer (Table S5a). Surprisingly, Shenzhen Reservoir showed UV filter pollution in both seasons ($\sum_9$ UV filters: 97−161 ng/L), even though it is a water resource conservation zone. Tap water in Shenzhen was contaminated by BP-3 (Table S5).

Finding multiple UV filters in a natural water environment is not surprising because broad-spectrum PCPs often contain several active ingredients to enhance sun radiation blockage[1] with their larger consumption during the summer. Seasonal variations of UV filters occurrence have been previously reported, and this trend is likely to continue as coastal populations increase.[6,25,26] The relatively high levels of UV filters found in waters at coastal recreation areas are linked with direct (recreational activities) and indirect anthropogenic inputs (sewage discharge). Our Site 1, for example, is the most popular beach in Shenzhen because of its superior maintenance and convenient access as well as it being free.

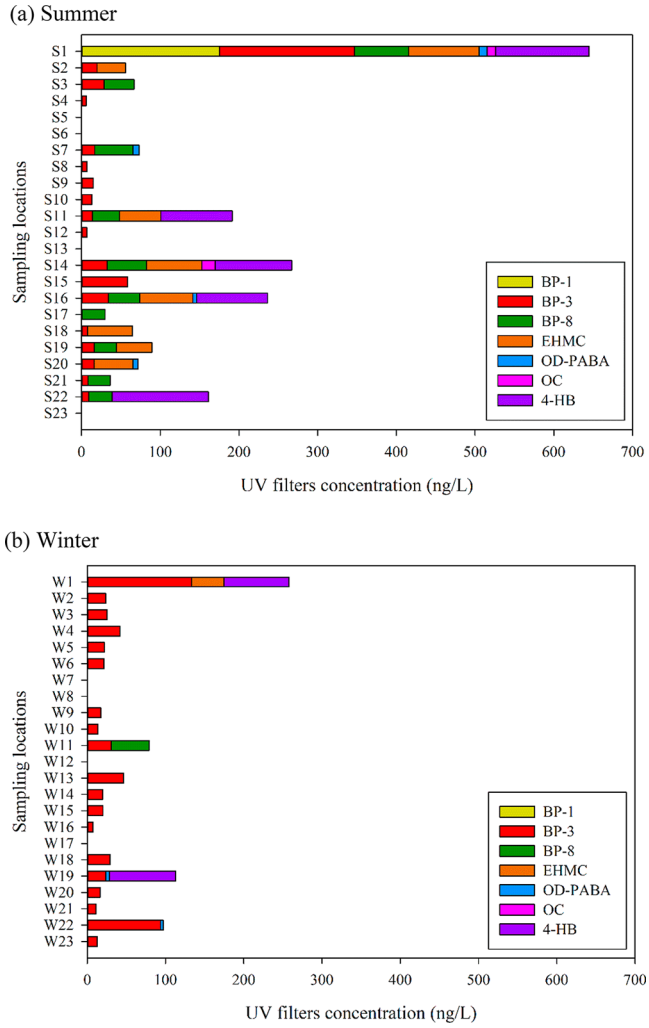

Figure 1. Spatial distribution of UV filters detected at 23 coastal locations near Shenzhen during summer (a) and winter (b) 2016.

Thus, finding high levels for this site was expected. What we did not expect was to find UV filters in a drinking water source (Site 22) and in tap water (Site 23). This result showed us another possible exposure pathway for humans besides direct dermal application and, considering the association between the human exposure of BP-3 and health outcomes, it raises concern. If it reflects the inefficiency of current water treatment processes, then further study of ways to remove these organic UV filters may be advisable in order to protect human health in the region.

BP-3 and EHMC have already been detected as the most extensively occurring organic UV filters all over the world, including Hong Kong, European countries, and the U.S.[8,27] Although BP-8, 4-HB, and BP-1 are not the permitted additives used in cosmetics in China, the EU, and the U.S., they are the metabolites of benzophenone or BP-3 and finding high detection frequency was expected. Although OC was only detected in several sampling points in this study, it has been previously massively detected in Hong Kong, Tokyo, New York, and Los Angeles with nearly 100% detection rate and up to 6000 ng/L concentration.[8] Being present in the water, these UV filters (BP-3, EHMC, OC, etc.) released from sunscreens may accumulate in diverse species.[13,19,28,29] Exposure of humans, including infants,[5] kindergarten children,[30] and

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Table 1. Measured Concentrations (Mean ± Standard Deviation, ng/g of Dry Weight, n = 3) of BP-3, EHMC, and OC in Artemia and Zebrafish at Environmental Cases (EC, 5.5 μg/L BP-3, 4 μg/L EHMC, and 7 μg/L OC), at Worst-Case (WC, 550 μg/L BP-3, 400 μg/L EHMC, and 700 μg/L OC), and after Single/Mixture Exposures[a]

	measured concentrations (ng/g of dry weight)		
exposures	BP-3	EHMC	OC
		Blank Control	
artemia	n.d.	n.d.	n.d.
zebrafish	n.d.	n.d.	n.d.
		EC Single	
artemia	27.4 ± 5.6 a	126.9 ± 2.7 a	87.4 ± 11.6 a
zebrafish	17.0 ± 1.8 a	23.5 ± 0.7 a	23.0 ± 3.3 a
		EC Mixture	
artemia	28.7 ± 3.6 a	161.0 ± 52.5 a	142.8 ± 32.2 a
zebrafish	18.0 ± 4.3 a	26.2 ± 2.7 a	30.8 ± 4.2 a
		WC Single	
artemia	4873.7 ± 2200.0 b	35 382.4 ± 1503.2 b	23 655.3 ± 1919.8 b
zebrafish	130.3 ± 14.9 b	94.5 ± 24.3 a	147.7 ± 3.1 b
		WC Mixture	
artemia	9415.4 ± 188.8 c	92 378.0 ± 351.8 c	35 187.3 ± 2451.2 c
zebrafish	191.6 ± 52.8 c	400.4 ± 130.0 b	496.2 ± 41.0 c

[a]Values with different letters denote significantly different means (p < 0.05, Student-Newman-Keuls test) in artemia and zebrafish. n.d.: <LOD (signal-to-noise ratio of 3, 0.3−6.0 ng/g dry weight).

adults,[31,32] to UV filters has been reported. Given the fact of the UV filters' extensive application, wide environmental/human detection, and multiple hormonal activities, it is critical to evaluate their long-term and mixture influence on the ecosystem.

Influence of Dietary Exposure to UV Filters on the Next Generation of Zebrafish. Multiple UV filters are commonly applied in PCPs and are, consequently, detected in the environment.[7,33] On the other hand, the influence of UV filters is rarely evaluated as a mixture at environmentally relevant levels. On the basis of the data in the literature and findings of the current work, BP-3, EHMC, and OC were found to be dominant in surface water, sediment, wastewater treatment plant effluent, and biota as mentioned.[3,6,7,10,27] After aqueous exposure to doses at both EC and WC of these three UV filters, artemia were found to accumulate them, and these compounds were then transferred to adult zebrafish by dietary uptake. The level of UV filters detected in fish exposed to mixtures was 1.5−4.2 times higher than in fish exposed to single compounds (Table 1). This offers a potential for a toxicologically synergistic effect that increases the overall toxicity to the ecosystem. One possible explanation could be the effect of "solute−solute interaction", which normally happens between trace organic contaminants and bulk organic matter.[34] Although no common bulk organic matter such as humic acid was added during our experiment, the interaction between these three chemicals is difficult to predict, which may have implications for biological uptake.[34] Considering the complex formulas of most cosmetics, encountering multi-organic UV filters reflects the real conditions in aquatic ecosystems. In this study, co-occurrence of BP-3, EHMC, and OC enhances the bioaccumulation of these species in organisms compared to their individual constituents. For artemia, the primary consumer in this food chain, the enhancement factor was approximately 1- to 2-fold for co-occurrence of the three UV filters while, for zebrafish, the enhancement factor could reach 4.2 (Table 1). Therefore, the influence of co-occurring multiple organic UV filters may also increase through the food chain. If so, then previous estimates based on single compounds underestimate the ecotoxicity of these compounds in the real aquatic environment.

Further testing of the embryos produced by zebrafish that had been fed the three UV filters revealed that the impact on their apical end points was both time and dose dependent (Figure 2). After 25 days of continuous exposure to the two dosed levels of either a single compound or a mixture, the zebrafish displayed no differences in embryo mortality (Figure 2a) or hatching rate (Figure 2c). When Japanese medaka *O. latipes* was exposed to 26 μg/L of BP-3 for 28 days, embryo hatchability of the second generation was also not affected.[16] However, after 47 days, embryo mortality increased dramatically for WC BP-3, EC/WC OC, EC/WC EHMC, and the WC mixture, and it reached 100% for the WC OC (single compound) and the WC mixture (a mixture of the three compounds) (Figure 2a). Hatching rate was significantly decreased for exposures to the EC/WC OC, EC/WC EHMC, and WC mixtures (Figure 2c). The embryos after 48 h of fertilization had a significant decrease in heart rate at all doses relative to the blanks, with an exception of no statistical difference between the treatments at WC BP-3, WC OC, and EC/WC EHMC and the blank on day 25. (Figure 2b).

After a 47-day exposure of zebrafish adults to UV filters as single compounds and as mixtures in their food, mortality, heart rate, and hatchability of the second generation were significantly compromised. One possibility is that these UV filters were maternally transferred to their progeny and thus affected early development of the embryos. Another possibility is that the UV filters, known to disrupt the endocrine system, may have damaged the reproductive system of the parents leading to unhealthy progeny. For the zebrafish exposed to the mixtures at environmental-case doses, no significantly negative effects were noted in either 24 h mortality or 72 h hatching rate in the second generation; however, at 48 h, heart rate displayed a significant decrease compared with the blanks. Our results seem to contradict a statement from the European Commission that interactions either are unlikely to occur or are toxicologically insignificant at low levels of exposure.[35] We propose that whether interactions occur or are toxicologically

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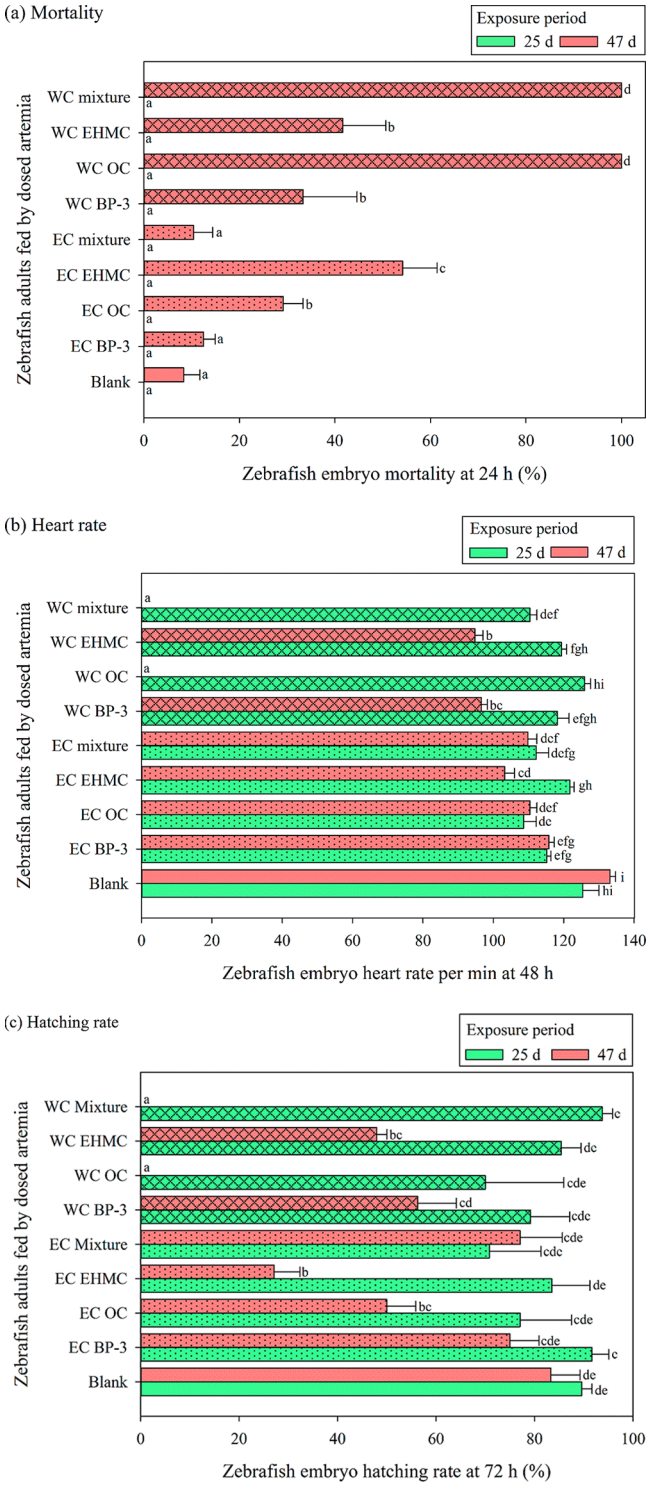

Figure 2. Apical end points (mean ± standard error) of (a) mortality at 24 h (n = 4), (b) heart rate at 48 h (n = 5), and (c) hatching rate (n = 4) at 72 h, of zebrafish *Danio rerio* embryos produced from zebrafish adults fed artemia dosed with different UV filters at environmental-case (EC) and worst-case (WC) single/mixture levels. Bars with different letters denote significantly different means (p < 0.05, Student−Newman−Keuls test).

significant depends on end points of evaluation, exposure duration/route, and biological target(s). It is worth noting that the three tested UV filters may interact with each other at the environmental level, resulting in reduced toxic effects on embryo development compared with the single compound effect (For example, after exposure for 47 days, mortality of the EC mixture showed no difference with the blank while that for EC EHMC and EC OC significantly increased (Figure 2a)). A similar situation was also observed for hatching rate, suggesting the antagonistic effect. However, this effect was not observed for the WC mixture. Accordingly, different types of toxicological interactions at low and high mixture concentration were previously reported. For instance, exposure to mixtures of the pesticides imidacloprid and thiacloprid resulted in synergism at low concentrations and antagonism at high concentrations in *Caenorhabditis elegans*.[36] Also, in *D. magna*, exposure to triclosan and carbendazim suggested both synergism and antagonism for different end points, showing that chemicals can also interact with each other in the organisms.[37] At the level of the worst-case dose, the adults exposed for 25 days produced embryos with no significantly negative changes in mortality or hatching rate, with the exception of heart rate; however, when the adults were exposed for 47 days, virtually all animals (100%) of the second generation suffered mortality after 24 h of fertilization. Such interactions at medium or high dose levels have been reported to cause external sex organ malformation in Wistar rats exposed to a mixture of four antiandrogens after postnatal day 16.[38] Any evaluation of the effect of UV filter mixtures, thus, should take both factors of exposed dose and duration into consideration in order to achieve an accurate understanding of the chemical interactions. Although functionally correlated and generally assumed to have similar impacts, all UV filters do not elicit the same apical responses, as shown in this study.

The UV filter OC at a single WC exposure caused 100% zebrafish embryo mortality, as did the WC mixture after 47 days of dietary exposure. OC is reported to be the most toxic for midge embryos[39] and to alter transcription of genes related to developmental and metabolic processes in the zebrafish adult.[18] Formulations containing OC have superior performance in preventing erythema and melanoma and, therefore, are preferably used. However, the applications of these formulations might need further consideration, as OC compromises itself as revealed in the predominantly negative effects in fish egg development at the environmentally relevant level. EHMC is another UV filter to which we need to give more attention because its EC dose presented higher mortality (p < 0.05) and similar hatching rate (p > 0.05) as the WC dose after 47 days of exposure. EHMC has been on the European Commission's UV filter "Watch List" since 2015.[40] In our study, BP-3, having the highest solubility,[6] showed the lowest toxicity in terms of effect on zebrafish embryo development.

Our study suggests that the choice of biological targets is critical for accurate ecotoxicological assessment of organic UV filters. Previous reports mainly focused on the direct exposure of test organisms to a contaminated environment. EHMC, OC, and BP-3 do not show any significant effect in environmentally relevant concentrations on either zebrafish or other aquatic organisms, which result in a high NOEC of organic UV filters.[41,42] In this study, none of the zebrafish adults appeared to suffer lethal effects; the negative impact was shown by the next generation: embryos. Even after exposure to the environmentally related concentration after 47 days, the mortality and hatching rate were significantly impacted. A two-generation study of rats showed that EHMC would reduce T_4 levels in dams and have trans-generation effects on offspring, for example, reducing testosterone or progesterone

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levels and thereby compromising their reproductive system.[43] Maternal transfer could be possible through milk during lactation since EHMC has been detected in 78% of human milk with up to 79 ng/g concentration,[44] as well as OC and BP-3.[44,45] BP-3 can also be maternally transferred directly through umbilical cord blood.[46] If organic UV filters can reside inside the body for a longer period of time due to their high lipophilicity, if they can be transferred to offspring, and if they have endocrine disrupting properties, it is very likely that exposure to them can have trans-generation effects. The evidence from our work with zebrafish and from studies with rats suggests this could be a vital issue for human beings. Although trans-generation effects are difficult to evaluate in humans, given the significance of the hormone system in regulating virtually all metabolic processes, including reproduction, if UV filters can negatively impact hormones/the reproductive system, they must be evaluated carefully. The health of future generations could depend on it.

Aqueous Exposure of Zebrafish Embryos to UV Filters. Once it was confirmed that dietary exposure of adult zebrafish to UV filters activated apical end points in the next generation, it was of interest to analyze the effects of aqueous exposure to these chemicals on embryo development. Within 7 days after zebrafish embryos were exposed to the three UV filter mixtures, (a) mortality rate was the same for fish in all treatment groups; (b) hatching rate was negatively dose dependent except for those exposed to the T4 treatment; (c) heart rate was decreased significantly by all treatments (Figure 3). It is worth noting that an environmental-case mixture of T4

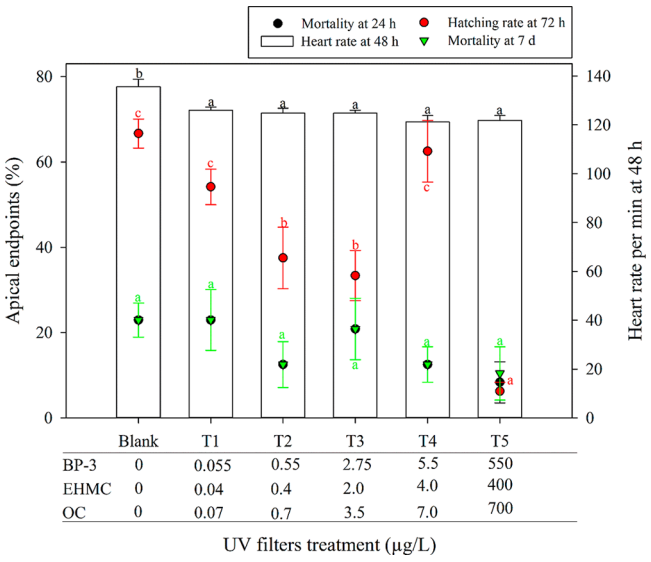

Figure 3. Apical end points (mean ± standard error) of mortality at 24 h (n = 4), heart rate at 48 h (n = 5), hatching rate at 72 h (n = 4), and mortality at 7 days (n = 4) of zebrafish *Danio rerio* embryos exposed to treatment (T) with UV filters BP-3, EHMC, and OC at different concentrations. Bars with different letters denote significantly different means (p < 0.05, Student−Newman−Keuls test).

significantly stimulated hatchability. Considering the antagonistic patterns we observed in the EC mixture for dietary exposure, the same interaction among these mixtures may be the possible explanation for this elevated hatching rate.

Activities of 11 enzymes involved in different metabolic and cellular functions were also studied in embryos exposed to UV filter mixtures, namely, superoxide dismutase (SOD) and catalase (CAT) (oxidative stress), lactate dehydrogenase (LDH; glycolysis), malate dehydrogenase (MDH; citric acid cycle), fructose-1,6-bisphosphatase (FBPase; gluconeogenesis), glucose-6-phosphate dehydrogenase (G6PDH; pentose phosphate pathway), aspartate aminotransferase (AST) and alanine aminotransferase (ALT) (protein metabolism), acid phosphatase (ACP) and alkaline phosphatase (ALP) (phosphate metabolism), and adenosine triphosphatase (ATPase; energy metabolism). Only activities of CAT and MDH displayed statistically significant differences among the UV filters treatments: CAT presented a peak in T2, and MDH increased with an increase in dosage for all treatments (Table S6). The enhanced CAT activity suggested an adaptive response to toxicant stress and a compensatory mechanism to defend against oxidative stress. The subsequent decrease of CAT showed an inhibitive response: mixtures with increasing concentrations of UV filters induced excessive production of reactive oxygen species, but the zebrafish's antioxidant system failed to scavenge these reactive substances.[47] MDH activity showed a pattern of gradual increase with increased doses of the UV filters, suggesting metabolic change to anaerobic respiration in the embryos to mitigate the energy crisis from mitochondrial dysfunction induced by the mixtures.[48]

Our study demonstrates that multiple UV filters occur in natural waters, that these substances are being transmitted through the food chain via food, and that they negatively influence developing progeny, sometimes without apparent effects on adult organisms. Experimental work reveals alterations in the development of fish embryos after continuous exposure of the parents to UV filters in their habitat and corroborates these findings with direct embryo exposure and enzyme analyses. It is very likely that a mixture composed of a larger number of UV filters is producing malformations of subsequent generations in zebrafish with accumulating effects at environmentally influential levels. Laboratory studies are currently being designed to investigate the mechanisms involved and to determine which xenobiotics singly or in combination are causing the observed effects. The results from this study clearly show that UV filters are adversely affecting early life-stage zebrafish. Like the canary in the coal mine, could these relatively small, simple organisms be warning of increasing, cumulative risks and dangers to larger organisms and indeed the ecosystem itself? Comprehensive evaluation of the complex effects that UV filters, and their mixtures, are having on aquatic environments as well as human health should be undertaken. With knowledge, appropriate actions can be taken to curtail their potentially damaging effects.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.8b02418.

Descriptions and locations of water sampling sites, analytical method and its performance, detailed seasonal concentrations of UV filters in 23 locations, 11 enzyme activities of exposed zebrafish embryos in UV filters treatments, figures of sampling sites, and microscope images of toxicity outcome to zebrafish embryo (PDF)

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Notes
The authors declare no competing financial interest.

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