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Impact of Egg Exposure to UV Filter-Spiked Sediment on the Survival, Hatching Success, Cardiac Frequency and Metabolic Scope of Zebrafish Embryos

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Abstract: Chemical UV filters are increasingly used in cosmetics to protect skin from UV radiation. As a consequence, they are released into the aquatic environment via recreational activities and wastewaters. In aquatic ecosystems, fish eggs in contact with sediment can be affected by organic and lipophilic pollutants such as UV filters. The present study aims to evaluate the toxicity of six individual UV filters, diethylhexyl butamido triazone (DBT), diethylamino hydroxybenzoyl hexyl benzoate (DHBB), ethylhexyl triazone (ET), 2-ethylhexyl salicylate (ES), homosalate (HS), and octocrylene (OC), in embryo-larval stages of zebrafish *Danio rerio*. Contamination of fish eggs and larvae with UV filters occurred by contact with spiked sediment for 96 h at a concentration of 10 µg g⁻¹. Among the six UV filters tested, OC delayed hatching success whereas ES significantly increased the heartbeat rate of embryo-larvae after sediment exposure probably as a stress response.

Keywords: *Danio rerio*; artificial sediment; metabolic rate; heart rate; sublethal concentration; sunscreen

1. Introduction

Organic UV filters are used in a wide range of products and plastics to protect human skin and materials from the deleterious effects of UV radiation [1,2]. These compounds are found in many cosmetics, especially sunscreens and environmental contamination by UV filters may occur via direct input from recreational activities and wastewaters [1,3–5]. As a consequence, UV filters are found in all aquatic compartments of freshwater and seawater and in biota (rivers, lakes, coastal oceans, surface waters and sediments). Their environmental concentrations were summarized in recent reviews [1,3,5–10].

This study selected six compounds of interest: diethylhexyl butamido triazone (DBT), diethylamino hydroxybenzoyl hexyl benzoate (DHBB), ethylhexyl triazone (ET), 2-ethylhexyl salicylate (ES), homosalate (HS), and octocrylene (OC).

UV filters have low solubility and tend to accumulate in organisms and sediment. Sediment contact is therefore an important way of contamination that was chosen in this study [11]. Among the six UV filters studied, OC is one of the most widely and increasingly used UV filters in cosmetics. Due to the high lipophilicity of OC and its low biodegradability [12], OC has a considerable tendency for bioaccumulation. This is especially true since we have demonstrated that OC can bioaccumulate in the form of analogues [13,14]. Previous studies reported OC concentrations ranging from 0.4 ng/g in Japanese river sediment to a concentration of 652 ng/g in lake sediment [15–20]. ES, which is also widely used, was detected at concentrations from 1.35 ng/g to maximal concentrations of 13.3 ng/g and 13.7 ng/g in Spanish and Chinese river sediment, respec-

tively. Kameda et al. (2011) measured an HS concentration of 26 ng/g in Japanese stream sediment. Lower concentrations of 0.6 ng/g [15], 0.89 ng/g and 1.2 ng/g dw were also found. Few studies have measured DBT and ET concentrations in the environment due to their more recent use in cosmetics. The maximum DBT concentration of 629 ng/g was measured in lake sediment [17]. DBT was recently detected at concentrations ranging from 22 to 210 ng/g in Banyuls Bay and Villeneuve-de-la-Raho Lake sediments [11]. Apel et al. (2018) measured 0.31 ng/g and 2 ng/g ET in surface sediment of German lakes. Unfortunately, no measurement of the DHHB concentration in sediment is available. The Table 1 provide a summary of the concentrations of the six selected UV filters measured in environmental sediment. In the present study, the tested concentration of 10 µg/g was 15 times higher than the OC and DBT environmental concentrations and higher than the highest concentrations of DBT, DHHB, HS and ES measured in aquatic sediment. Previous studies reported the bioaccumulation and toxicity of these UV filters in several aquatic organisms (see for a review), mainly on algae and coral [14,23], but also on crustaceans [22,24] and fishes [25,26]. Among the 60 UV-filters find in the global market, only 15 appear in literature with described toxicity on biota. Most studies investigate mortality, oxidative stress or growth [9]. As UV-filters are emerging pollutants, more toxicological data are needed to develop biomarker-based assay to evaluate UV filter toxicity.

Table 1. UV filters tested and their environmental occurrence. ^a USA: United States of America, EU: European Union, Aus.: Australia. n.a.: not approved.

Abbr.	COSING Name	Alternative Names	CAS #	Formula	Maximum Concentration in Final Product ^a			Higher Environmental Concentration Measured in Sediment (in ng/g)	References
					USA	EU	Aus.		
DBT	Diethylhexyl butamido triazone	Iscotrizinol Uvasorb HEB	154702-15-5	C ₄₄ H ₅₉ N ₇ O ₅	n.a.	10%	n.a.	629	[11,17]
DHHB	Diethylamino hydroxybenzoyl hexyl benzoate	Uvinul A+	302776-68-7	C ₂₄ H ₃₁ NO ₄	n.a.	10%	10%	-	-
ET	Ethylhexyl triazone	Uvinul T150	88122-99-0	C ₄₈ H ₆₆ N ₆ O ₆	n.a.	5%	n.a.	2	[20]
ES	2-Ethylhexyl salicylate		118-60-5	C ₁₅ H ₂₂ O ₃	n.a.	5%	n.a.	13.7	[19–21]
HS	Homosalate	Homomenthyl salicylate Sunobel®HMS	118-56-9	C ₁₆ H ₂₂ O ₃	15%	10%	15%	26	[15,19,20]
OC	Octocrylene		6197-30-4	C ₂₄ H ₂₇ NO ₂	10%	10%	10%	652	[15–20]

Fishes are suitable organisms that can be used to monitor the toxicity of persistent lipophilic contaminants. In this study, the fish *Danio rerio* was selected as a model species recommended for ecotoxicity testing [27–31] due to its many biological advantages. In fish, several studies found that UV filters are reprotoxic as endocrine disruptors [1], genotoxic and can impair the development of fish [33]. As UV filters are emerging pollutants, a major effort must be made to develop UV filter risk assessment and understand their toxicity in the aquatic environment. In this context, this study investigated the effect of six UV filter-spiked sediment on the early life stages of zebrafish. Toxic responses of exposed embryos and larvae were examined through physiological parameters such as survival, hatching success, cardiac frequency, and aerobic metabolism.

2. Materials and Methods

The protocols used in the present study were described in detail in [34]. The preparation of UV filter-spiked sediments and the exposure processes were performed using a procedure adapted from Le Bihanic et al. [35,36].

2.1. Chemicals Used and the Preparation of Spiked Artificial Sediment

The UV filters used in this study are listed in Table 1 and were kindly provided by Pierre Fabre Laboratories.

The reconstituted sediment was composed of sand measuring 0.2–0.5 mm (sable de Loire SCALARE, Aquastore, France), 5% kaolin clay (Merck, Darmstadt, Germany), and Sphagnum blond peat (Florentaise, SaintMars-du-Désert, France). Briefly, peat was dried for 48 h and sieved (0.5 mm). Milli-Q water was added to the peat (12:1, *v/v*) in glass bottles and shaken for 48 h at 180 rpm at room temperature. Sand and 5% kaolin clay were then mixed to humid peat and shaken for 24 h. pH was adjusted to 6.5 with 10% CaCO₃ solution. After 7 days of stabilization at room temperature, 1:4 *v/v* of Milli-Q water was added to the sediment. After a 24-h equilibration period, supernatant water was removed and the sediment was dried at 105 °C for 14 h. After cooling, 4 g artificial sediment added in glass Petri dishes was spiked with one of the UV filters at 10 µg g⁻¹ dry weight sediment each as follows: a solution of the UV filter in dichloromethane (10 µL, 4 mg mL⁻¹) was mixed to sediment and then solvent was left to evaporated overnight in the dark at room temperature under a fume hood. This ensures complete residual solvent removal. A control with solvent (dichloromethane without UV filters) and sediment was prepared in the same manner. A negative control was tested too (no solvent, only artificial water E3 and sediment).

2.2. Zebrafish Embryo-Larval Assay

The Institute of Functional Genomics of Lyon (IGFL) provided adult wild-type AB-TU strain (Tübingen, Germany) zebrafish (*Danio rerio*). Fish were maintained in groups of 10 individuals in ten liters' aquaria were filled with a mix of 1/3 reverse osmosis-treated water and 2/3 tap water without chlorine, both filtered beforehand with dust and charcoal filters. The average pH measured was 7.8 ± 0.5 and the conductivity of 450 ± 50 µS cm⁻¹. Oxygen level was maintained ≥80% thanks to a rack aeration. Fish were maintained in groups of 10 individuals per tank at a water temperature of 28 ± 1 °C and under a photoperiod of 14-h light/10-h dark. Ammonia, nitrite, and nitrate were monitored weekly and remained within recommended ranges [36]. The fish were fed twice a day with commercial pellets (Ocean Nutrition Europe, Belgique) and *Artemia* sp. nauplii (*Artemia* EG > 225,000 nauplii/g). Occasionally, food was supply by red sludge worms (Boschetto-frozen fish food). Eggs were obtained from this culture by random pairwise mating of zebrafish. One adult male and one female were placed together in spawning boxes (AquaSchwarz, Germany) the evening before eggs were required. Spawning and fertilization took place within 30 min after the onset of light in the morning. Fertilized and normally developed eggs were selected (This time was T₀) to test the effect of the 6 selected UV-filters. In order to do so groups of 30 selected eggs were placed in contact with 4 g of spiked artificial sediment and 8 mL of artificial water (E3) in glass Petri dishes (35 mm diameter). Exposure to the UV filters spiked sediment lasted 96 h at 28 ± 0.5 °C, with the same photoperiod as the rearing room. At 96 h, the larvae were transferred into freshly prepared E3 medium for an additional 6 days period (total of 10 days testing) for analysis. Larvae were not fed during the experiment.

2.3. Survival and Hatching Success

Embryonic and larval survival was recorded daily until 10 dpf (days post fertilization). For each condition tested, four replicates of 30 individuals were observed. Dead individuals were removed. The survival rate (SR, %) was estimated as:

$$SR = 100 \times (SN_{tx}/SN_{t0}) \quad (1)$$

where SN_{tx} and SN_{t0} are the number of live individuals at time t_x (time of the measure, x hours post fertilization—hpf) and t₀ (beginning of the experiment, 0 hpf).

Similarly, hatched individuals were counted between 48 and 72 hpf. At the end of exposure (96 hpf), hatching success (HS, %) was calculated as:

$$HS = 100 \times (HI_{t96h}/SN_{t96h}) \quad (2)$$

where HI_{t96h} is the number of hatched and living larvae.

2.4. Cardiac Frequency

To assess cardiac frequency (f_H), a total of 301 larvae were tested individually ($n_{\text{solvent control}}=33$, $n_{\text{negative control}}=35$, $n_{\text{OC}}=41$, $n_{\text{ES}}=39$, $n_{\text{HS}}=40$, $n_{\text{UV}}=38$, $n_{\text{UA}+}=41$, $n_{\text{UT150}}=35$). The larvae were placed in a lateral position into 3% methylcellulose on Petri dishes (i.e., 6–7 mL) in order to see the heart by transparency and to measure f_H . Two hours acclimation were needed to recover from the transfer [42]. Videos of 20 s were recorded three times (Olympus DP71) to observe the in vivo beating heart of each larva.

2.5. Static Respirometry

This study used intermittent flow respirometry to record the oxygen consumption of fish. The setup was composed of eight independent glass microrespirometer chambers (diameter $d = 9$ mm, volume $V = 0.6$ mL; Loligo Systems, Denmark) submerged into buffer tanks (depth \times length \times height $14 \times 35 \times 13$ cm) and filled with oxygenated E3 solution at a constant temperature of 28 °C. Dissolved oxygen levels were measured by an optic fiber system (PreSens, Witrox 4) during the phase of 30 min oxygen consumption alternating with phases of 15 min oxygen renewal. Ten groups of 5 larvae were tested for each trial and each group of larvae was tested once. As described in Lucas et al. [34], two metabolic rates were measured: active metabolic rate (AMR) represents the maximum oxygen transport capacity of an individual estimated as the maximum energy expenditure reached after having chased the larvae, and standard metabolic rate (SMR) correspond to the minimal maintenance metabolic rate estimated at rest for 48 h. Aerobic metabolic scope (AMS) was calculated as the difference between AMR and SMR.

After 48 h measurements, larvae were removed from the respirometers and slightly anesthetized with benzocaine at a concentration of 50 mg L⁻¹. The body mass of each individual was determined using a microbalance (Sartorius Secura 26-1S). A bacterial measurement was performed before and after each trial without fish to quantify background respiration. Linear changes were assumed in background oxygen consumption over the 48-h experimental trial, and the calculated background was subtracted from the corresponding total oxygen consumption measured.

Oxygen consumption (MO_2) is expressed in $\text{mgO}_2 \cdot \text{g}^{-1} \cdot \text{h}^{-1}$ and calculated using the following formula:

$$\text{MO}_{2\text{meas}} = \Delta[\text{O}_2] \cdot \Delta t^{-1} \cdot V \cdot M_{\text{meas}}^{-1} \quad (3)$$

where $\Delta[\text{O}_2]$ (in $\text{mgO}_2 \cdot \text{L}^{-1}$) is the change in oxygen concentration during the measurement period Δt (in h), V (in L) is the volume of the respirometer minus the volume of the fish and M_{meas} (in g) is the mass of the corresponding group of 5 larvae.

An allometric relationship exists between oxygen consumption and body mass, which encourages correction of $\text{MO}_{2\text{meas}}$ using the following formula:

$$\text{MO}_{2\text{cor}} = \text{MO}_{2\text{meas}} \cdot (M_{\text{meas}} \cdot M_{\text{cor}}^{-1})^{1-b} \quad (4)$$

where $\text{MO}_{2\text{cor}}$ (in $\text{mg O}_2 \cdot \text{g}^{-1} \cdot \text{h}^{-1}$) is the oxygen consumption related to a standard fish of 1 g (M_{cor}), $\text{MO}_{2\text{meas}}$ (in $\text{mg O}_2 \cdot \text{g}^{-1} \cdot \text{h}^{-1}$) is the oxygen consumption estimated with Equation (3) for experimental fish whose mass was M_{meas} (in g), and b is the allometric scaling exponent describing the relationship between oxygen consumption and fish mass. In the case of this study, we used $b_{\text{AMR}} = 0.926$ for the correction of active metabolic rate and $b_{\text{SMR}} = 0.965$ for the correction of standard metabolic rate [39].

2.6. Statistical Analysis

Statistical analyses were performed using GraphPad Prism software. Data are expressed as mean \pm standard error. Results were statistically evaluated with the Shapiro and Bartlett tests to check normality and homoscedasticity. An unpaired *t*-test was then used to determine which chemical treatment differed significantly from the solvent control. A significance level of 5% was used for all analyses.

3. Results and Discussion

The embryo larval stages of fish are particularly sensitive and vulnerable to pollutants in the aquatic environment. Thus, ecotoxicity tests on fish early life stages has been widely developed in the last decades. In the current study, sediment contact was used as relevant test to evaluate the toxicity of six organic UV filters on embryo-larval stages of the zebrafish [35]. This type of test allowed to reproduce the multiple sources of exposure of benthic organisms via sediment, particle, and dissolved phases. Four different physiological endpoints were assessed: survival, hatching success, cardiac frequency and aerobic metabolism. For all parameters, a negative control (sediment) and solvent control (solvent and sediment) were tested to estimate the impact of the solvent. No difference was observed between these two controls regardless of the parameters measured ($p > 0.05$).

Impact on Survival and Hatching

Table 2. Survival and hatching success of zebrafish assessing UV filter toxicity. Mean values \pm standard deviation. * indicates a significant difference from the solvent control experiment (*t*-test, $p < 0.05$).

Treatment	Number of Replicates	Survival Rate (%)		Hatching Success (%)
		96 hpf	10 dpf	96 hpf
Negative control	4	86.7 \pm 6.7	85.6 \pm 5.1	95.75 \pm 5.28
Solvent control	4	84.2 \pm 7.4	84.2 \pm 7.4	92.5 \pm 11.2
OC	3	73.3 \pm 20.0	72.2 \pm 18.4	67.1 \pm 23.5 *
ES	4	78.3 \pm 3.3	75.8 \pm 3.2	93.7 \pm 8.0
HS	6	78.8 \pm 15.6	77.8 \pm 15.0	94.0 \pm 4.1
DBT	3	76.7 \pm 13.3	76.7 \pm 15.3	80.0 \pm 8.7
ET	3	86.7 \pm 5.8	82.2 \pm 3.6	96.3 \pm 3.6
DHHB	3	77.8 \pm 18.9	76.7 \pm 18.6	96.6 \pm 3.1

For each UV filter tested at the concentration of 10 $\mu\text{g g}^{-1}$, no significant mortality was observed compared to the solvent control (*t*-test, $p > 0.05$). Hatching success decreased after exposure to OC ($p < 0.05$). This means that OC induced late hatching, which could impact fish development under environmental conditions. On the contrary, no effect on hatching success was observed for ES, HS, DBT, ET, and DHHB under our exposure conditions.

Cardiotoxicity

No significant change in heart beat was observed for the OC ($p = 0.2472$), HS ($p = 0.8892$), DBT ($p = 0.2345$), ET ($p = 0.3792$) or DHHB ($p = 0.0363$) treatments compared to the solvent control.

These results are in the range of cardiac frequency measured in previous studies [39–44]. On the contrary, ES significantly increased the cardiac frequency by 19.7% compared to the solvent control (Figure 1, $p < 0.0001$). This result are in the range of the studies of Lucas et al. [42,43] who observed a 10.2% heart beat increase after exposure to the UV filter BEMT and 12.5% heart beat increase after parental exposure to polycyclic aromatic hydrocarbons. The increase was not lethal and may be due to only a stress response; this requires further investigation. In fact, pollutants are known to increase stress hormone level such as cortisol which could impair cardiac frequency [44,45]. In addition, antioxidant defenses or detoxification processes could be triggered by exposure to UV filters to prevent oxidative damage [46]. These additional energy-consuming activities could induce an increase in heart rate to meet the increased oxygen demand to produce more energy. This could in turn increase SMR and impair aerobic metabolic scope. To complete these results, it will be interesting to look at cardiac morphological deformities and any other ventricular cardiac dysfunctions such as measurement of the stroke volume, in order to observe all the cardiac function.

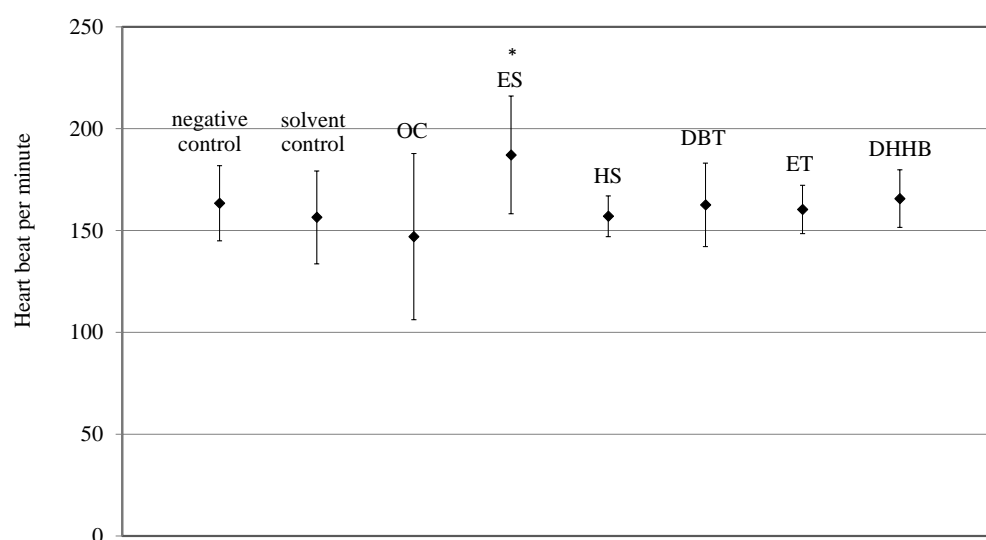


Figure 1. Cardiac frequency of 5 dpf larvae zebrafish (*Danio rerio*) exposed to one of the following treatments: controls, exposure to OC, ES, HS, DBT, ET or DHHB. Mean values \pm standard deviation are plotted; * indicates a significant difference from the solvent control experiment (t -test, $p < 0.05$).

Aerobic Metabolic Scope

All the results regarding aerobic metabolism are synthesized in Figure 2. SMR, AMR or AMS measured in 5 dpf embryo-larvae were not significantly different from the solvent control for OC ($p_{SMR} = 0.2034$, $p_{AMR} = 0.2858$, $p_{AMS} = 0.4514$), ES ($p_{SMR} = 0.8161$, $p_{AMR} = 0.1335$, $p_{AMS} = 0.1787$), HS ($p_{SMR} = 0.3923$, $p_{AMR} = 0.7012$, $p_{AMS} = 0.8401$), DBT ($p_{SMR} = 0.4365$, $p_{AMR} = 0.9130$, $p_{AMS} = 0.8555$), DHHB ($p_{SMR} = 0.4081$, $p_{AMR} = 0.8107$, $p_{AMS} = 0.9271$) and ET ($p_{SMR} = 0.6172$, $p_{AMR} = 0.8899$, $p_{AMS} = 0.9971$). Therefore, comparison with other studies is rather limited since the UV filters tested are not the same in both studies. These results are similar to those reported by Lucas et al. for other UV filters: benzophenone-3 (BP3), butyl methoxydibenzoylmethane (BM), bis-ethylhexyloxyphenol methoxyphenyl triazine (BEMT), and methylene bis-benzotriazolyl tetramethylbutylphenol (MBBT). At the concentration tested UV filters do not seem to impact the metabolic regulation. The lack of effect suggested that zebrafish larvae had overall capacity to sustain oxygen supply for oxygen-consuming functions in these conditions of exposure [47].

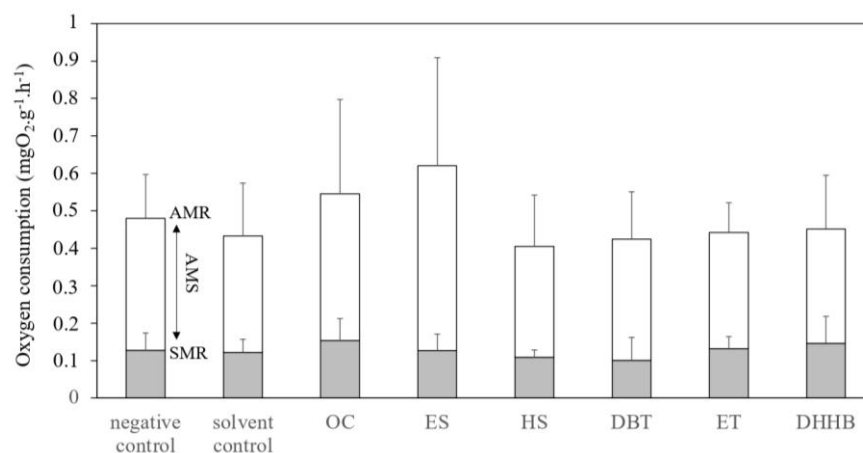


Figure 2. Active metabolic rate (AMR), standard metabolic rate (SMR), and aerobic metabolic scope (AMS) of 5 dpf *Danio rerio* larvae in controls or after exposure to one of the six UV filters tested: OC, ES, HS, DBT, ET, DHHB. The results are expressed as the mean values \pm standard deviation. No significant difference was observed between the solvent control and UV filters regardless of the metabolic rate considered (t -test, $p < 0.05$).

This is only the second work on the impact of UV filters on fish metabolism [35]. Even if the metabolic rates of ES were not significantly different from those of the control, the high AMR may be related to the tachycardia observed in larvae. In fact, higher AMR results from increase of energy demand. To produce more energy, organism increased oxygen consumption which extend cardiac frequency increases to regulate oxygen transport.

4. Conclusions

The current study showed that OC significantly delayed hatching and that ES significantly increased the heartbeat rate of embryo-larvae after sediment exposure. Our findings are consistent with numerous studies that report negative effects, especially for OC, on aquatic organisms (review by Lozano et al.). Since very high sediment concentrations of OC have been reported, it can be inferred that OC could significantly impact the local reproductive success of fish laying eggs on sediment. HS, DBT, DEBT, and ET did not induce any significant effect under our exposure conditions regardless of the physiological endpoint studied. The test concentrations were higher than those encountered in a natural environment. In our experimental conditions and using standardized artificial sediment, UV filters toxicity towards zebrafish embryo-larvae was low. However, xenobiotics bioavailability and toxicity may be affected by the nature and relative concentration of organic matter as well as many other parameters such as ionic strength and salinity. It could be interesting to test different sediment composition in future experiments.

This work is the first report on the effect of DHHB, DBT and ET on fish development and larval metabolism. Some countries are beginning to address the environmental impact of sunscreen products, but more ecotoxicity data are urgently needed. In particular, a clearer definition of the products being marketed as “reef-safe” is necessary and should follow-up the advances of science in this matter. Considering the regular release and persistence of UV filters in the aquatic environment, long-term exposure studies should also be conducted. As UV filters are usually applied in mixtures, additional work should assess their combined effect on the environment.

Author Contributions: All authors contributed to the study’s conception and design. Material preparation, data collection and analysis were performed by J.L. and V.L. was responsible for fish rearing. A.M.S.R. and D.S. helped with chemical protocols and analysis. P.L. supervised the project. The first draft of the manuscript was written by J.L., and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Data Availability Statement: The datasets used and analyzed during the current study are available from the corresponding author upon reasonable request.

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Conflicts of Interest: The work reported in this article was financed in the context of the Pierre Fabre Laboratories' Conscious Care Approach and the Skin Protect Ocean Respect commitment of the brand Eau Thermale Avène. It was neither supervised nor audited by Pierre Fabre Laboratories. The interpretation and views expressed in this manuscript are not those of the company. The authors have no relevant financial or nonfinancial interest to disclose.

Ethical Approval/Consent to Participate: This study was conducted under the agreement of the Animal Care Committee N° A66-01-601.

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