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Do Single-Component and Mixtures Selected Organic UV Filters Induce Embryotoxic Effects in Zebrafish (*Danio rerio*)?

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Abstract: UVs are important ingredients in common cosmetic products (e.g., sunscreens, hairsprays, soap). After their use, they can enter the aquatic ecosystem and negatively affect non-target aquatic organisms. The aim of our study was to evaluate acute embryotoxicity of selected organic UVs 2-phenylbenzimidazole-5-sulfonic acid (PBSA), ethylhexyl methoxycinnamate (EHMC), octocrylene (OC), 4-methylbenzylidene camphor (4-MBC) and benzophenone-3 (BP-3). The chemicals were tested both as a single substance and their mixtures. The types of mixtures were chosen as follows: the combination of OC and 4-MBC; the combination of PBSA, EHMC and BP-3 and the combination of all five UV filters. The embryotoxicity was evaluated using a modified method of the Fish Embryo Acute Toxicity Test-OECD guideline 236 and zebrafish (*Danio rerio*) was selected as a suitable fish model organism. The toxic effects were studied by assessing mortality, hatching and the occurrence of malformations at 24, 48, 72 and 96 h post fertilization. The obtained results indicate that especially the mixture of OC and 4-MBC presents a potential risk of embryotoxicity for zebrafish due to a significant increase in mortality, which was 41.7% in the experimental group exposed to 10 µg/L at 96 h post fertilization. Based on our results, the most effected sub-lethal endpoints were hatching and malformation (e.g., edema of pericard, bent spine, yolk edema), but with no statistically significant effect. These results differ within groups with single UVs and with their mixtures, suggesting the interaction of these substances when they are exposed together.

Keywords: personal care products; embryotoxicity; mortality; hatching; sublethal endpoints

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1. Introduction

1.1. Ultraviolet Radiation and Ultraviolet Filters

The exposure to the ultraviolet (UV) radiation has been related to the development of skin cancer. The UV radiation is according to its wavelength and effect divided into three types. UV A radiation (400 to 320 nm) enters skin cells and induces the production of oxidative radicals that can lead to skin aging. UV B radiation (320 to 280 nm) influences the epidermis and DNA cells. UV C radiation is the most dangerous and most absorbed by the ozone layer [1,2]. UV filters (UVs) can minimalize the damage caused by the UV radiation. The UV filters used in personal care products (PCPs) (shampoos, skin lotions, creams, sunscreens, etc.) were developed for the protection of the skin against UV radiation [2,3]. In the Annex VI of EU Cosmetics Products Regulation (EC) No. 1223/2009, 28 UV filters are listed that are allowed in cosmetic products in EU [4]. UVs are of an organic or inorganic origin. Zinc oxide nanoparticles and titanium dioxide belong to inorganic UVs and can reflect the UV radiation. Organic UVs protect skin cells by absorbing the UV rays and their structure contains at least one benzene. Organic UVs belong to the group of aminobenzoates, benzophenones, derivates of camphor and acid cinnamate, salicylates, benzimidazoles, etc. [2].

1.2. Literature Review

Due to a huge consumption of PCPs, UVs have become significant pollutants of the environment [3]. The UVs can enter the aquatic ecosystem by two ways: (a) directly by washing off the sunscreen from the skin, especially during recreational activities or (b) indirectly via the wastewater plants that are not able to eliminate all the micropollutants. Many UVs are lipophilic and due to their physicochemical properties can resist in the aquatic environment. The occurrence of UVs is recorded worldwide, and the UVs are a relevant group of ubiquitously present contaminants of the environment [5,6]. UVs were detected in many biotic (e.g., animal tissues, corals) and abiotic matrices (e.g., surface water, wastewater, beach sand). In surface water, containment of benzophenone-3 (BP-3), octocrylene (OC), homosalate (HMS) and octisalate (OS) was recorded at concentrations of tens to hundreds ng/L [5]. On the base of indirect entering the environment, UVs are detected in municipal wastewater in different countries; in Norway in wastewater effluent between 300 and 8900 ng/L of OC, BP-3 and ethylhexyl methoxycinnamate (EHMC); however, the total concentration of UVs in the sludge was two orders of magnitude higher, between 5 and 51 μg/g, predominantly OC and EHMC [7]. In Portugal, the tested beach sand contained a huge range of UVs (4-methylbenzylidene camphor (4-MBC), EHMC and BP-3) at concentrations of ng/g of the dry weight (DW) [6]. UVs are often detected even in coastal and marine sediments. In the North and Baltic Sea surface sediments, OC was the mainly recorded UV filter with the frequency detection of 79% and it was detected at the concentration of up to 9.7 ng/g DW [8]. In Spain, residues of EHMC, BP-3, OC and 4-MBC were detected in the tap water from metropolitan areas and the city of Barcelona [9].

The aquatic organisms are significant elements for monitoring persistent lipophilic contaminants such as organic UVs. In invertebrates, e.g., corals [5] and mussels [10], various representatives of organic UVs (HMS; OC; EHMC; BP-3) were detected. Moreover, many organic UVs (EHMC; OC; 4-MBC; BP-3) were detected in fish, confirmatory ubiquitous presence of organic UVs in aquatic biota [11,12]. Fent et al. [3] monitored the occurrence of UVs at different trophic levels. EHMC was detected at the concentrations between 22 and 150 ng/g lipids in mussels (*Dreissena polymorpha*), at the concentrations of 337 ng/g lipids in barb (*Barbus barbus*) and within a range of 16 to 701 ng/g lipids in cormorants (*Phalacrocorax* sp.). This increasing concentration of EHMC suggests food-chain accumulation. Many studies are focused on investigating the effect of UVs on non-target organisms (i.e., organism other than the one for which are UVs intend). Recently it was proved that UVs can impair biochemical processes and antioxidant and detoxifying system of the organism. Nataraj et al. [13] proved an increase in lipid peroxidation and a decrease in glutathione-S-transferase (GST) activity in zebrafish (*Danio rerio*) embryo after a 96-h EHMC exposure. Benzophenones (BPs) can induce oxidative stress in liver of carp (*Cyprinus carpio*), especially by decreasing superoxide dismutase (SOD), catalase (CAT) and GST activities. The level of reduced glutathione (GSH) was significantly induced. Induction of oxidative damage and inhibition of CAT activity were observed after 4-MBC exposure in *Solea senegalensis* [14]. All these results point on activating of detoxification process in fish [15].

Due to their ability to impair hormonal pathways, some of the organic UVs are considered as endocrine disruptors. Blüthgen et al. [16] proved an anti-androgenic effect of BPs in adult male zebrafish (*D. rerio*) by the down-regulation of genes involved in steroidogenesis. 4-MBC caused in zebrafish (*D. rerio*) embryo after 96 h of exposure a downregulation of brain aromatase gene that is involved in normal functioning of the hypothalamus-pituitary-gonadal axis [17]. EHMC showed anti-estrogen and androgen effects on zebrafish (*D. rerio*) in F0 generation after 40 d post fertilization; moreover, this UV filter can accumulate in zebrafish (*D. rerio*) and transfer to the offspring through reproduction and disrupt the nervous and weakened antioxidant capacity of F0 parents and F1 offspring via parental transfer [18]. The neurotoxic potential of EHMC was proved in larval zebrafish by inducing of hypothyroidism [19]. Noteworthy is that recent papers point out the negative influence of EHMC, OC, benzophenone-8 (BP-8) and benzophenone-1

(BP-1) on the marine ecosystem and their responsibility for coral bleaching [20,21]. Based on the recent studies, the State of Hawaii has introduced restrictions on the usage of sunscreens containing EHMC, OC, BP-8 and BP-1 [22].

Some UVs reported an impairment of the embryo development and caused embryotoxicity. Balász et al. [23] proved that the BP-3 decreases the number of hatched embryos (*D. rerio*) and brings about tail and jaw deformation after a 96-h exposure. This delayed hatching and impairment of the embryo development would later lead to the death of the fish. Another representative of benzophenones family, benzophenone-2 (BP-2), causes heart and yolk edema in 5 d after fertilization, reduces the heart rate, erupts blood circulation, enlarges yolk by the accumulation of lipid droplets and disrupts the craniofacial development [24]. The OC affected the transcription of genes related to developmental processes in brain and liver in zebrafish embryo. Moreover, the OC has an impact on hematopoiesis, the formation of blood vessels, blood circulation and fat cell differentiation [25]. The 4-MBC influences the neuronal and muscular development in *D. rerio* embryos after a 3-day exposure. Specifically, the 4-MBC caused abnormal axial curvature in embryos and impaired mobility by disrupting the slow muscle fiber pattern [26]. Quintaneiro et al. [17] studied the effect of the 4-MBC on hatching, heart rate and malformation in zebrafish embryos. The results showed that the 4-MBC delayed the absorption of the yolk sac and pericardial edema and decreased the heart and hatching rate after 48 h of exposure for the highest tested concentration. Torres et al. [27] came to a similar conclusion that the highest tested concentration of 4-MBC decreased the heart and hatching rate. They even observed an abnormal involuntary muscular contraction that could signalize impaired mobility of embryos.

Aquatic organisms are exposed to heterogeneous mixtures of substances, not only to single pollutants [28,29]. These chemicals interact with each other and have potentially additional toxic effect and subsequently could be more environmentally unfriendly when are mixed together [30–32]. Li et al. [33] revealed an adverse effect of a mixture of UVs (BP-3, EHMC, OC) on the development of zebrafish embryos and their impact on the next generation. After 47 days of exposure, the embryo mortality increased and simultaneously the hatching rate in the F1 generation decreased. A mixture of the mentioned UVs did not exhibit any significant effect in the environmentally relevant concentration; however, a negative impact was shown in the next generation even in the environmentally relevant concentration. Worth mentioning is a fact that embryos exposed to UVs in water reported a decreased heart rate in all tested groups.

1.3. Aims of Our Study

The objectives of our study were twofold: (i) to evaluate the effect of UVs as a single-substance and selected organic UVs in mixture on the embryonic development of zebrafish; (ii) due to the lack of literature focused on the interaction in mixtures of UVs we investigate join toxic effect of tested UV filters.

2. Materials and Methods

2.1. Experimental Design and Description of Critical Methods

The embryotoxicity of single UV filters and their mixtures was evaluated using a modified method of the Fish Embryo Acute Toxicity Test (FET)–OECD guideline 236 [34]. FET determines toxicity of chemicals on fish embryonic stages. Organisms were exposed to tested pollutants in increasing concentrations for 96 h. During the test, indicated parameters (coagulation of eggs, lack of somite formation, lack of detachment of the tail bud from the yolk sac, changes in pigmentation, edema of pericard, etc.) were recorded every 24 h. Zebrafish (*D. rerio*) is standard model for fish toxicity research and therefore this fish was selected as a suitable model organism. Fertilized eggs were purchased from a commercial producer (Mendel University in Brno, Czech Republic). At first, eggs were rinsed in water and stereomicroscope (StereoBlue, Euromex) was used for checking of egg quality control and the determination of the development stage as well. For a subsequent use in the

embryonic toxicity test, only fertilized eggs of a maximal 16-cell stage without any obvious irregularities during cleavage were selected.

The fertilized eggs were randomly distributed on 48-well microwell plates, one embryo in each well. Tested eggs were filled up the tested concentrations of solutions with 1 mL. Twenty-four eggs were used for each experimental group (test was performed in duplicate). Simultaneously with the exposure to the tested substances, the control group (only dilution water) and the control group with an appropriate solvent (dilution water with a solvent at concentration 0.01%) were also tested. The test solution and dilution water in the control group were renewed every 24 h by gently draining each chamber and adding a new solution. The individual steps were performed very carefully to avoid potential disturbing of the test embryos. The microwell plates with embryos were stored in the thermostat with parameters set as follows: temperature 26 ± 1 °C and photoperiod 12 h of light and 12 h of darkness. The embryos were monitored at 24, 48, 72 and 96 h post fertilization (hpf). The toxicological impact was represented by the lethal endpoints and development disorders. Mortality, hatching rate and the occurrence of malformations such as changes in pigmentation, body deformation, edema etc. were recorded. The embryo was considered dead if the embryo coagulation (Figure 1), a lack of somite formation, non-detachment of the tail and a lack of heartbeat were detected. All observation was performed according to Nagel [35].

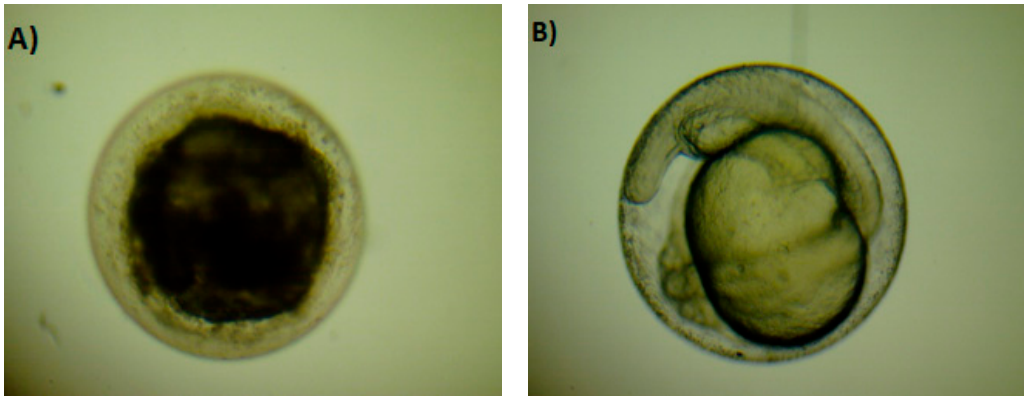

Figure 1. Coagulation of embryo exposed to 10 µg/L of PBSA after 48 h post fertilization (hpf) (**A**). Physiological development of embryo in the control group at 48 hpf (**B**).

2.2. Overview of Tested Chemicals

The selected eggs of zebrafish were exposed to a broad range of concentrations of various organic UV filters 2-phenylbenzimidazole-5-sulfonic acid (PBSA), EHMC, OC, 4-MBC, BP-3, which can be commonly detected in surface water worldwide [5,8]. All standards were obtained from Sigma-Aldrich (Czech Republic) with chemical purity ≥99%. Because of pure solubility of the individual substances, suitable solvents were used for preparing the stock solutions (dimethyl sulfoxide for the solution of PBSA, EHMC and BP-3; ethanol for the solution of 4-MBC and OC). The total concentration of the solvents was always 0.01% (10 µL in 100 mL). The stock solution was prepared daily in dilution water in dark glass. The dilution water (pH = 7.8) was prepared according to ISO 7346 [36]. The tested concentrations of the individual UV filters were as follows: PBSA—0.1, 1, 10, 100, 500, 1000 and 2000 µg/L; EHMC—0.1, 1, 10, 50, 100, 500, 1000 and 2000 µg/L; OC—0.1, 1, 10, 50, 100 and 250 µg/L; 4-MBC—0.1, 1, 10, 50, 100 and 250 µg/L; BP-3—0.1, 1, 10, 50, 100, 500, 1000 and 2000 µg/L. In addition to the individual substances, various mixtures of these UV filters were tested. The types of mixtures were chosen as follows: the combination of OC and 4-MBC; the combination of PBSA, EHMC and BP-3 and the combination of all five UV filters. The used concentrations of these individual UV filters in mixtures were 0.1; 10 and 100 µg/L. The lowest concentrations used in our embryonic toxicity test corresponded to the environmentally relevant concentrations of these substances in surface

water. Higher concentrations were chosen as a multiple of the lowest one to determine the potential concentration-dependent relationship. A restriction in the choice of the used concentrations was also the limited solubility of the selected UV filters.

2.3. Statistical Evaluation

The statistical analysis was carried out using statistical software Unistat for Excel 6.5. (Czech Republic). The chi-square (χ^2) test of independence was used to determine it there is a significant difference between monitored variables (mortality, hatching rate and occurrence of malformations). Pearson's chi-squared test (contingecy table—k × m) and Yates correction (contingency table—2 × 2) were used to determine whether is a statistically significant difference between frequencies of monitored variables. The statistically significant difference was considered when $p < 0.05$ (*) and $p < 0.01$ (**). All data are expressed in percentage and the data of the hatching rate and the occurrence of malformations are always based on the surviving embryos at a given time. Due to a non-significant difference in all monitored indices between the control group and the control groups with a solvent, for next evaluation only the control group was used.

3. Results

3.1. Mortality

The cumulative mortality of embryos after the exposure to single substances of UVs and their mixtures was recorded at 24, 48, 72 and 96 hpf. Results including the statistical evaluation are shown in Table 1 and in Supplementary Materials S1 and S2.

Table 1. Cumulative mortality (%) of zebrafish embryos exposed to various mixtures of UV filters during 96 h. The bold font and asterisk indicate a significant difference ($p < 0.05$) between the control and experimental groups at the same time of exposure. *—no mortality was observed in both control group and control groups with solvents.

Test Substance	Group	24 hpf	48 hpf	72 hpf	96 hpf
-	control *	0	0	0	0
OC + 4-MBC	0.1 µg/L	12.5	20.8	20.8	20.8
	10 µg/L	20.8	**29.2 ***	**33.3 ***	**41.7 ***
	100 µg/L	0	0	4.2	4.2
PBSA + BP-3 + EHMC	0.1 µg/L	4.2	4.2	4.2	4.2
	10 µg/L	0	0	0	0
	100 µg/L	0	0	0	0
PBSA + EHMC + OC + 4-MBC + BP-3	0.1 µg/L	4.2	4.2	4.2	4.2
	10 µg/L	0	0	0	0
	100 µg/L	8.3	8.3	8.3	8.3

Abbreviations: BP-3—benzophenone-3; EHMC—ethylhexyl methoxycinnamate; hpf—hours post fertilization; 4-MBC—4-methylbenzylidene camphor; OC—octocrylene; PBSA—2-phenylbenzimidazole-5-sulfonic acid.

No mortality was observed in both control group and control groups with solvents. Similarly, no mortality was recorded in the embryonic toxicity test with BP-3 (data not shown). Rare mortality was observed in other embryonic toxicity tests with single substances of organic UV filters. The highest mortality was found in the embryonic toxicity test with 4-MBC in the experimental group exposed to 100 µg/L (12.5%). But this difference was tested as non-significant compared to the control group. On the other hand, more frequent mortality was found in the embryonic toxicity tests with mixtures of organic UV filters, especially in the toxicity test with the combination of OC and 4-MBC in the experimental group exposed to 10 µg/L. The statistical analysis revealed significant differences between the control and this experimental group at 48, 72 and 96 hpf as well.

3.2. Hatching

The results of the hatching rate (expressed in %) in the individual embryonic toxicity tests are highlighted in Figures 2–7 and in Supplementary Materials S1 and S2.

In almost all experimental groups, hatching was recorded at 72 hpf. Earlier hatching at 48 hpf was observed only in few experimental groups exposed to individual substances of selected UV filters (4-MBC—50 µg/L; PBSA—10 µg/L and EHMC—2000 µg/L), but no significant differences ($p > 0.05$) were found compared to the control group and experimental groups at this observation time. The first hatching in the control groups was recorded at 72 hpf, but all control embryos were hatched at 96 hpf. Similarly, most embryos in the groups exposed to single substances or mixtures of UV filters finished their hatching at 96 hpf. No significant differences ($p > 0.05$) in the hatching rate were found between the control and experimental groups at 96 hpf.

Earlier hatching was recorded especially in the experimental groups exposed to various mixtures of UV filters at 72 hpf. Significant differences compared to the control group were found in the experimental groups exposed to the mixture of OC and 4-MBC at the concentration of 100 µg/L ($p < 0.05$), further to the mixture of PBSA, BP-3 and EHMC at the concentrations of 10 and 100 µg/L (both at $p < 0.05$) and to the mixture of all five UV filters at the concentrations of 0.1 ($p < 0.01$) and 10 µg/L ($p < 0.05$). Accelerated hatching was revealed also in the groups treated by a single substance of organic UV filters. A significant difference in the hatching rate was noted after the exposure to OC at the concentration of 1 µg/L. On the other hand, hatching retardation was found in embryos exposed to selected concentrations of 4-MBC and BP-3 as single test substances. Highly significant delay in hatching ($p < 0.01$) was observed especially in the highest concentrations of the test substances (4-MBC—10, 50, 100 and 250 µg/L; BP-3—2000 µg/L). No significant changes in the hatching rate ($p > 0.05$) were observed in the embryonic toxicity tests with PBSA and EHMC as single substances at 72 hpf.

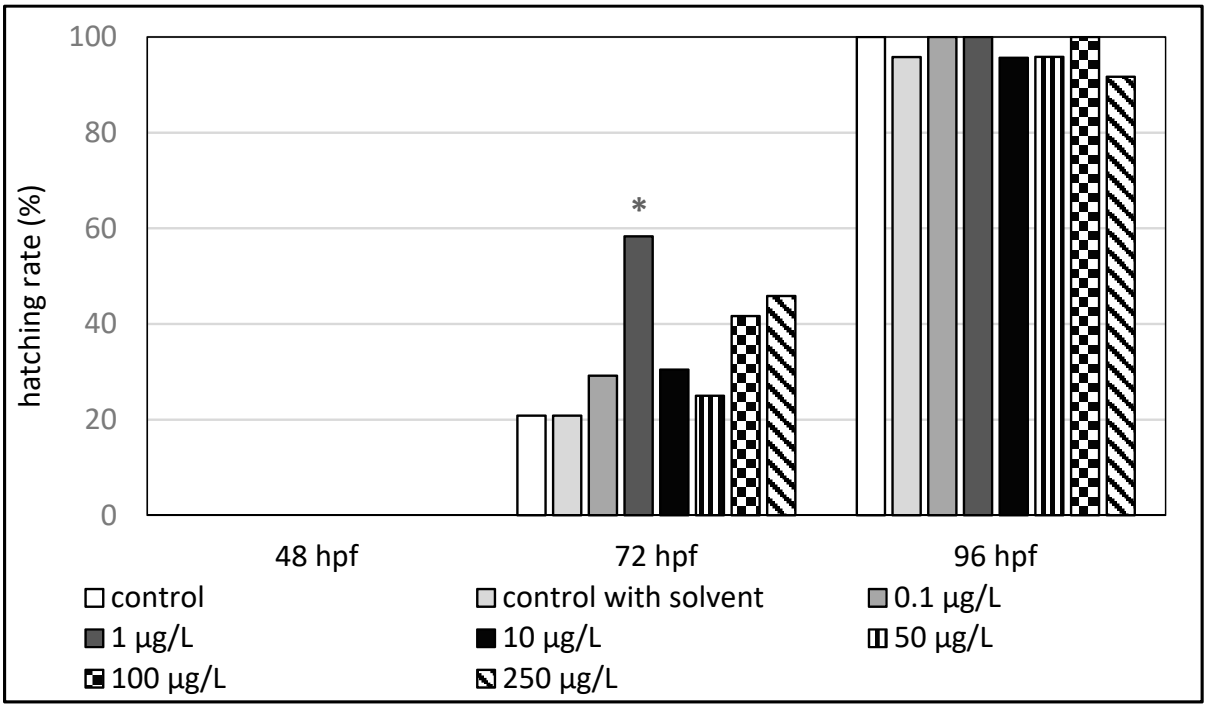

Figure 2. Hatching rate (%) of zebrafish embryos exposed to octocrylene (hpf—hours post fertilization). The asterisk indicates a significant difference (* $p < 0.05$) between the control and experimental groups at the same time of exposure.

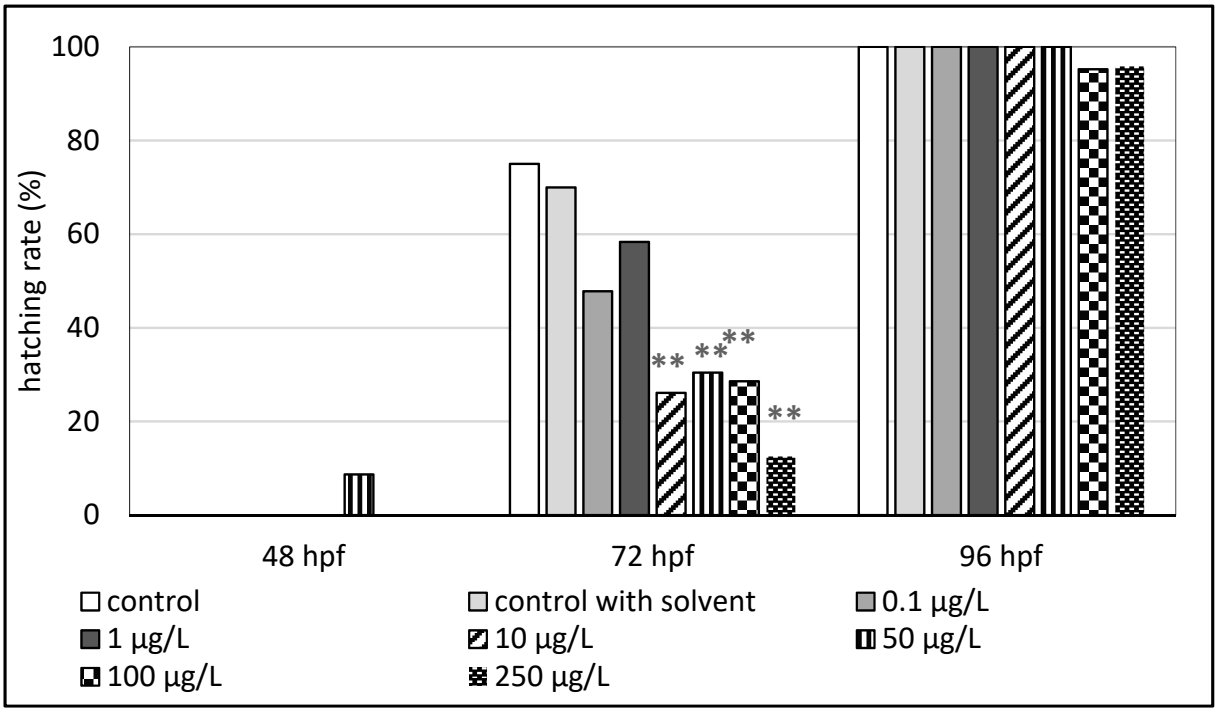

Figure 3. Hatching rate (%) of zebrafish embryos exposed to 4-methylbenzylidene camphor (hpf—hours post fertilization). The asterisk indicates a significant difference (** $p < 0.01$) between the control and experimental groups at the same time of exposure.

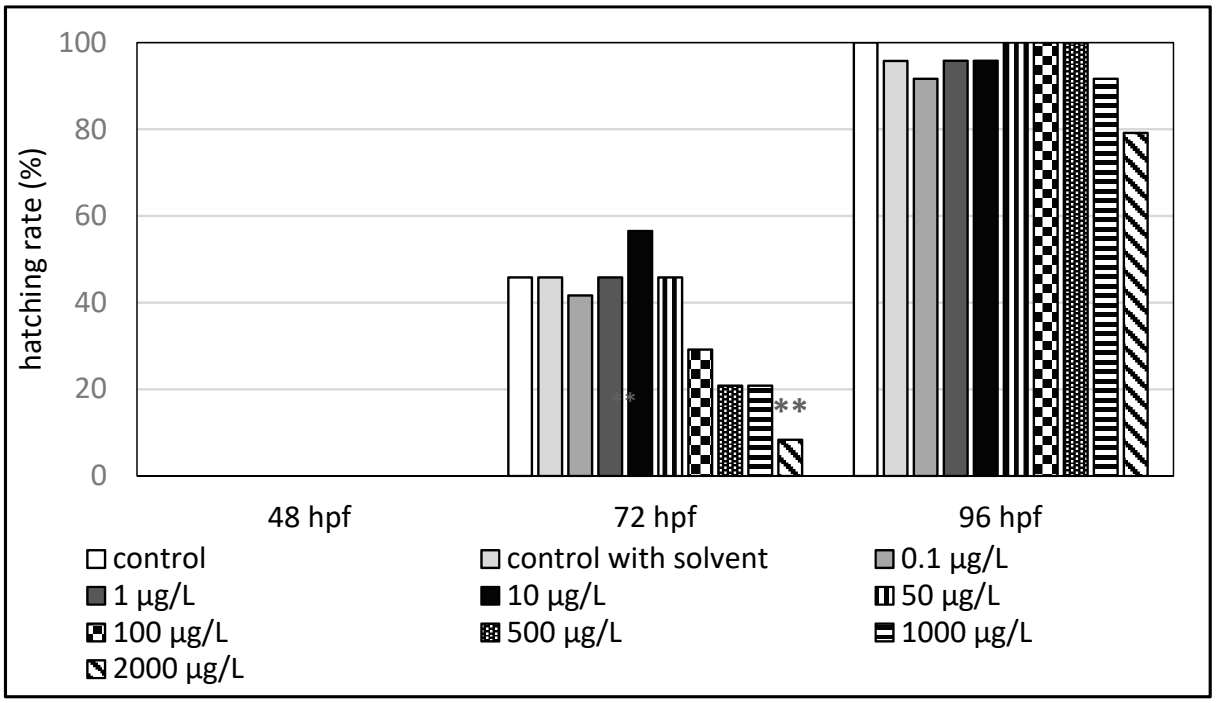

Figure 4. Hatching rate (%) of zebrafish embryos exposed to benzophenone-3 (hpf—hours post fertilization). The asterisk indicates a significant difference (** $p < 0.01$) between the control and experimental groups at the same time of exposure.

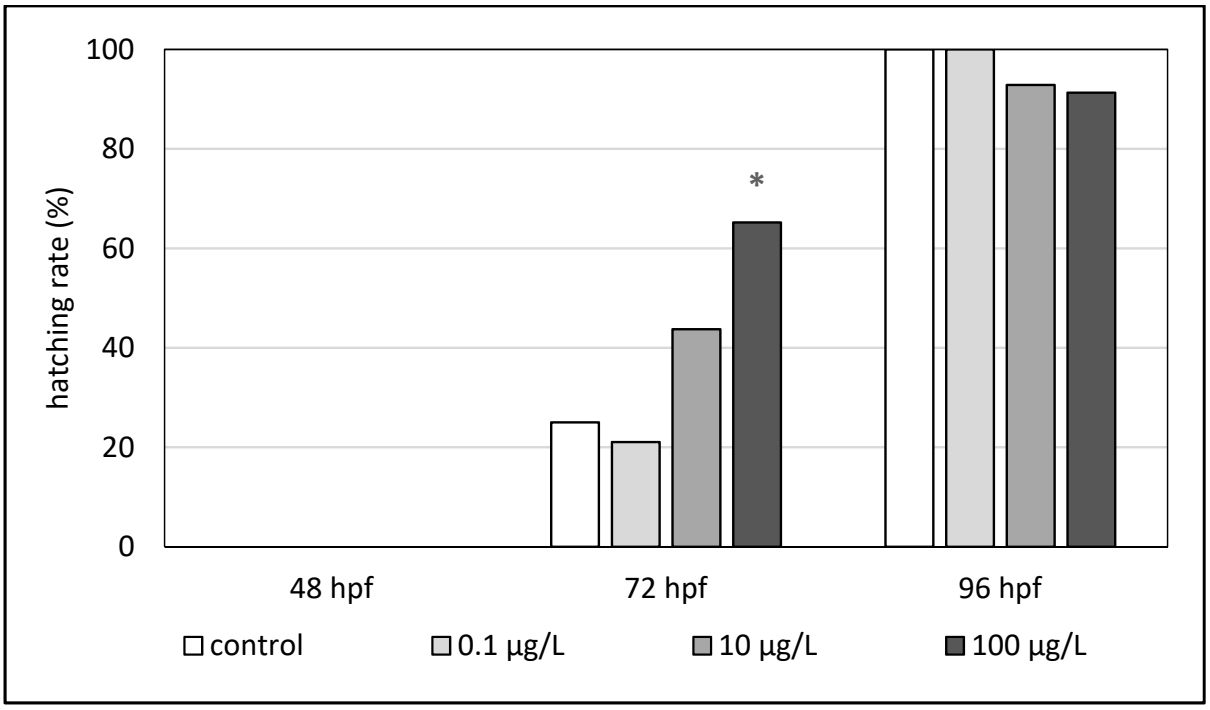

Figure 5. Hatching rate (%) of zebrafish embryos exposed to the combination of octocrylene and 4-methylbenzylidene camphor (hpf—hours post fertilization). The asterisk indicates a significant difference (* $p < 0.05$) between the control and experimental groups at the same time of exposure.

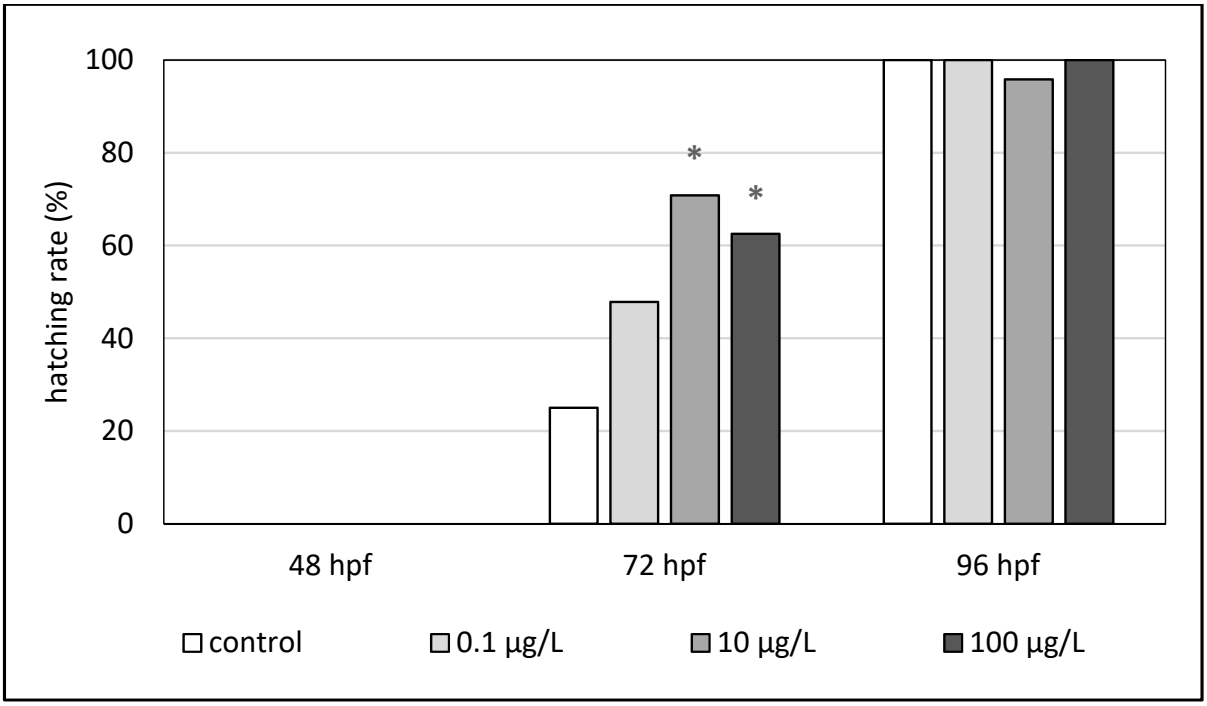

Figure 6. Hatching rate (%) of zebrafish embryos exposed to the combination of 2-phenylbenzimidazole-5-sulfonic acid, benzophenone-3 and ethylhexyl methoxycinnamate (hpf—hours post fertilization). The asterisk indicates a significant difference (* $p < 0.05$) between the control and experimental groups at the same time of exposure.

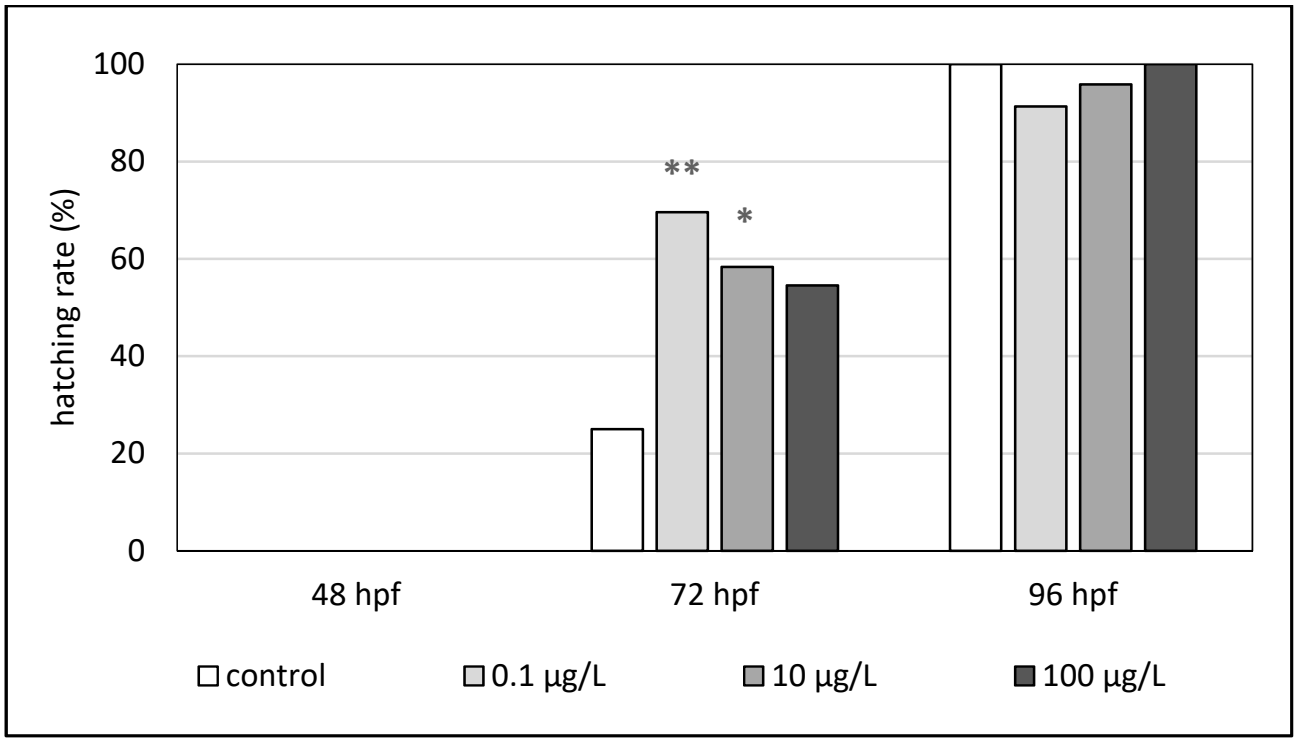

Figure 7. Hatching rate (%) of zebrafish embryos exposed to the combination of all five organic UV filters (2-phenylbenzimidazole-5-sulfonic acid, ethylhexyl methoxycinnamate, octocrylene, 4-methylbenzylidene camphor, benzophenone-3) (hpf—hours post fertilization). The asterisk indicates a significant difference (* $p < 0.05$, ** $p < 0.01$) between the control and experimental groups at the same time of exposure.

3.3. The Occurrence of Malformations

No malformations were observed in the control groups during the embryonic toxicity test. Only a rare malformation (4.2%) in the control group and control group with a solvent were observed in the embryonic toxicity test with the 4-MBC during the whole monitoring period. No malformations were also found in embryos treated by single substances of UVs such as BP-3, EHMC and OC. At 72 and 96 hpf, rare occurrence of edema of pericard (Figure 8) was found in the embryonic toxicity test with 4-MBC at the concentrations of 10 and 250 µg/L (4.2% and 8.3%, respectively) but with a non-statistically significant difference ($p > 0.05$) compared to the control group. Similarly, only rare ($p > 0.05$) occurrence of malformations was noticed in the experimental group exposed to 10 µg/L of PBSA at 48, 72 and 96 hpf.

Surprisingly, no malformation was observed in embryos exposed to the mixture of all five organic UV filters during the whole monitoring (Supplementary Materials—Table S3). Only rare occurrence of malformations was found in embryos exposed to the mixture of PBSA, BP-3 and EHMC at the lowest test concentration at 48 hpf (4.2%) and 72 hpf (4.3%). In contrast, numerous malformations such as total deformation, curvature of the spine, yolk sac edema, edema of pericard, bent spine, undeveloped tail, but still non-significant difference ($p > 0.05$) compared to the control group, were recorded in the embryonic toxicity test with the mixture of OC and 4-MBC (Figure 8).

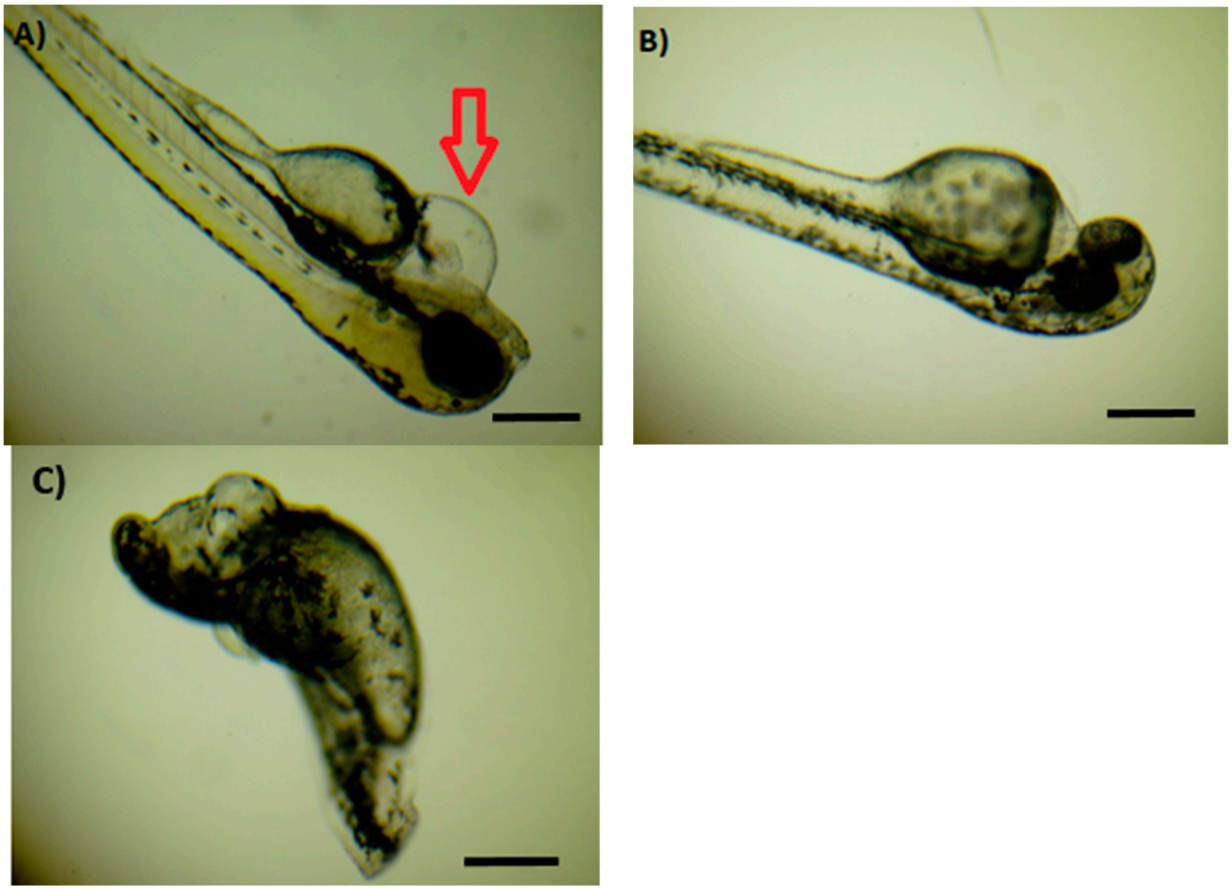

Figure 8. Pericard edema of zebrafish larvae in the group exposed to 250 µg/L of 4-methylbenzylidene camphor at 72 h post fertilization (hpf) (**A**). Zebrafish larvae in the control group at 72 hpf (**B**). Total body deformation of zebrafish larvae in group exposed to 10 µg/L of 4-methylbenzylidene camphor and octocrylene at 72 hpf (**C**). Scale bar = 0.4 mm.

4. Discussion

Due to the extensive use of PCPs (e.g., sunscreens, lotions, shampoos, decorative cosmetics) with UVs, these substances have become a significant micro-pollutant of our environment and extensive scientific research has been performed to investigate their toxicological effect on non-target organisms (e.g., fish, corals, mussels) [37]. Aquatic organisms are exposed to various of micropollutants from their early life stage to their adulthood [38–44]. It has been revealed that UVs are able to bioaccumulate in the organism and biomagnificate through the food chain [3,43,45]. To our best knowledge, recent papers have focused mainly on the determination of embryotoxicity of single substances. Based on the data of the occurrence in aquatic ecosystem [5–9], we aimed to extend our research to the mixtures of UVs which are naturally present together in the aquatic environment and observe how these substances interact.

Our experiment was based on the examination of single UVs and their mixtures after 96 h of exposure in zebrafish embryos. The toxic effects were evaluated by the lethal endpoint and sub-lethal endpoints (i.e., the hatching rate, the formation of somites, morphological development, spontaneous movement and the occurrence of edema).

For organic UVs, high NOEC is determined since many of them (BP-3, EHMC and OC) did not report any embryotoxicity for zebrafish in the environmentally relevant concentrations [2]. In our experiment, statistically significant mortality was not recorded in any tested group with the single UVs exposure. This result is surprisingly positive regarding to the results of Jang et al. [30] experiment where the BP-3 treated group exhibited higher mortality. Li et al. [33] recorded 100% mortality of zebrafish embryos after the exposure of OC at 700 µg/L. To compare that, in our experiment the highest tested concentration

was 250 μg/L of OC with no recorded mortality. The only recorded statistically significant mortality was for the mixture of OC and 4-MBC at the concentration of 10 μg/L after 96 hpf. This result would present a potential embryotoxicity risk of OC and 4-MBC for zebrafish.

Based on our results, we recorded the disruption of the hatching process more often than mortality. The only individually tested UV filter that caused significantly accelerated hatching was OC at the concentration of 1 μg/L. This is contrary to the research carried out by Blüthgen et al. [25], where OC did not show any toxicity effect on the hatching rate in zebrafish embryos for any tested concentration, which were even higher than we used in our experiment.

The 4-MBC and BP-3 were the only single test substances that affected the hatching process by hatching retardation. Balász et al. [23] came to a similar conclusion when the BP-3 exposure caused a decrease in the hatched embryos after 96 hpf. Delayed hatching was also recorded after benzophenone exposure in zebrafish embryos [46]. Focusing only on the 4-MBC means that when we tested the 4-MBC alone, the hatching of zebrafish embryos was retarded. However, when we tested the 4-MBC with OC in a mixture, earlier hatching was recorded. This outcome suggests an interaction of the 4-MBC and OC when they are present together, apparently with a dominant effect of OC. The interaction of UVs in a mixture was recently noticed by Li et al. [33] when they recorded an interrelation between the BP-3, EHMC and OC for zebrafish at an environmental level resulting in reduced toxic effects on the embryonic development and suggesting their antagonistic effect.

Surprisingly, the earlier hatching was recorded in the testing group treated by the mixture of all five UVs even in the environmentally relevant concentration. To conclude, all these results suggest a dominant effect of OC, considering the hatching rate. The earlier hatching recorded in the mixture of PBSA, BP-3 and EHMC would lead to the synergism or antagonism of these substances, and they need further investigation and consideration to understand the whole process and specify the accurate toxicological assessment.

If we consider the results of the observed malformation in our experiment, it is necessary to mention, that no statistically significant impairment of the development was observed, especially for each single tested UV filter. On the contrary, recent papers revealed the disruption of the development after the BPs exposure [23,24,47]. After 96 hpf, pericardial and yolk sac edema, deformed jaw, dilated gut or an impairment of craniofacial development were observed. Nataraj et al. [13] have tested the adverse effect of EHMC and its photoproduct on the development of zebrafish embryo. After the 96 hpf exposure, lesions of the muscle fibers and yolk sac, along with an increasing heart rate and hatching delay were revealed. On the other hand, in our experiment, only rare malformations were recorded in the mixture of PBSA, BP-3 and EHMC. Jang et al. (2016) [30] have tested a mixture of EHMC and BP-3 together in *Daphnia magna*. They assume that the EHMC and BP exhibit a synergistic effect and may cause a combined toxic effect. Based on our results and considering the result of testing every single UV filter, it seems that the mixture of PBSA, BP-3 and EHMC could have some potentiated effect, but this presumption need further investigation.

Our result of single 4-MBC with no significant effect on the development differs from recent papers. Quintaneiro et al. [17] observed after 4-MBC in zebrafish embryo developmental malformation including notochord curvature, delayed absorption of the yolk sac and pericardial edema, and Torres et al. [27] recorded an increase in the abnormal involuntary muscular contraction after the highest tested concentration of 4-MBC. However, if we focus on other tested mixtures of UVs (e.g., OC and 4-MBC; all five UVs together), a certain trend of interaction of these substances may be considered. After the OC and 4-MBC exposure, numerous morphological abnormalities were recorded (e.g., yolk edema, edema of pericard, bent spine, undeveloped tail), but when testing all five UVs together, the impairment of the development was not so common. These results might be pointing at a combined toxic effect of UVs when they are introduced together. This hypothesis needs to be further investigated in detail to confirm our prediction.

5. Conclusions

To conclude, during our experiment the organic UVs tested as a single substance did not cause the death of embryos in the early life stage and did not even an impair the development of zebrafish. Although many deformations (e.g., edema of pericard, bent tail or undeveloped tail) and the decrease in hatching after UVs exposure would not be lethal for the embryo, this handicap could lead to the death of the adult fish lately. On the other hand, it is important to realize that aquatic organisms are exposed to these substances in mixtures. Considering results of hatching rate and mortality after UVs mixture exposure, we suppose that UVs could have an additional toxic effect, especially mixture of OC and 4-MBC with probably dominant effect of OC. In contrast, the mixture of all five UVs did not cause such extensive changes in hatching rate or mortality. These results could lead to antagonism between tested UVs. To better understand the mixture toxicity, further research of various combination of UVs mixtures is required.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/w13162203/s1, Table S1: Cumulative mortality (%) of zebrafish embryos exposed to octocrylene (OC) and 4-methylbenzylidene camphor (4-MBC) as single substances (hpf—hours post fertilization) during 96 h. No significant differences ($p > 0.05$) were observed between the control and experimental groups at the same time of exposure. *—no mortality was observed in both control group and control groups with a solvent., Table S2: Cumulative mortality (%) of zebrafish embryos exposed to 2-phenylbenzimidazole-5-sulfonic acid (PBSA) and ethylhexyl methoxycinnamate (EHMC) as single substances (hpf—hours post fertilization) during 96 h. No significant differences ($p > 0.05$) were observed between the control and experimental groups at the same time of exposure. *—no mortality was observed in both control group and control groups with a solvent. Table S3: The occurrence of malformations (%) in zebrafish embryos exposed to the mixture of octocrylene and 4-methylbenzylidene camphor (hpf—hours post fertilization). No significant differences ($p > 0.05$) were observed between the control and experimental groups at the same time of exposure. *—no malformations were observed in both control group and control groups with solvents. Figure S1: Hatching rate (%) of zebrafish embryos exposed to 2-phenylbenzimidazole-5-sulfonic acid (hpf—hours post fertilization). No significant differences were observed between the control and experimental groups at the same time of exposure. Figure S2: Hatching rate (%) of zebrafish embryos exposed to ethylhexyl methoxycinnamate (hpf—hours post fertilization). No significant differences were observed between the control and experimental groups at the same time of exposure.

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