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Developmental, behavioral, and biochemical effects of chronic exposure to sublethal concentrations of organic UV-filter compounds on a freshwater model species

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ABSTRACT

The prevalence of organic/chemical UV-filter compounds in aquatic ecosystems represents a growing environmental issue. The long-term toxicity risks of many UV-filters at environmentally relevant concentrations to aquatic biota are still less studied, especially in the case of invertebrates. This study was designed to evaluate the chronic toxicity of avobenzone (AVO), octocrylene (OCTO), and octinoxate (OCTI), three UV-filters which frequently occur in the aquatic environment, to the water flea (*Daphnia magna*) at an environmentally relevant concentration of 200 ng l^{-1} in a 21-day exposure. Potential alterations in the growth, reproduction, and heart rate were continuously monitored during the treatments. Filtration rate, swimming, and the state of the antioxidant- and metabolic functions were evaluated at the end of exposures. Avobenzone significantly increased the reproductive output, heart rate, and filtration rate, while evoked a significant decrease of swimming behavior, and inhibited the activity of catalase (CAT) and glutathione S-transferase (GST) enzymes. The body size, reproduction, heart rate, and superoxide dismutase (SOD) activity were significantly increased whereas the activity of GST and CAT was significantly reduced by OCTO. OCTI significantly increased reproduction, heart rate, CAT and SOD activity but significantly decreased the swimming behavior. Our results confirmed that chronic exposure to organic UV-filters even at environmentally relevant concentrations affect basic physiological traits and cellular defense pathways in *D. magna*. Highlighting, our observations revealed previously unknown physiological changes (e.g., altered heart rate, filtration rate, SOD activity) caused by the investigated UV-filter compounds. Future research is to be aimed at investigating the mixture effects of these compounds and at the understanding of the potential cellular and molecular mechanisms underlying the changes induced.

1. Introduction

The presence of organic UV-filter compounds in aquatic ecosystems has become a burning issue in environmental sciences. Previous studies have demonstrated that UV-filter compounds enter surface waters both directly (i.e. aquatic recreational activities) and indirectly (i.e. wastewater effluents) (Chatzigianni et al., 2022; de Miranda et al., 2021; O'Malley et al., 2021; Sanchez-Quiles and Tovar-Sanchez, 2015). The concentration range of different organic UV-filters in surface waters is reported from a few ng L^{-1} to a few μg L^{-1} (e.g., avobenzone [AVO]: 1.37–1298 ng l^{-1}; octocrylene [OCTO]: 1–3730 ng l^{-1}) (detailed in **Supplementary Table 1**) (da Silva et al., 2015; Diaz-Cruz et al., 2008; Fenni et al., 2022; Labille et al., 2020; Mitchelmore et al., 2019, 2021; O'Malley et al., 2021).

Previous studies demonstrated that organic UV-filter compounds (e.g., AVO, OCTO, and different benzophenone compounds) primarily occurring in sunscreen cosmetics can cause a wide range of adverse effects (e.g., endocrine disruption) on aquatic organisms and were capable of bioaccumulation and biomagnification, resulting in potential impact on the ecosystem (reviewed by (Chatzigianni et al., 2022; de Miranda

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et al., 2021; Diaz-Cruz and Barcelo, 2009; Kwon and Choi, 2021; Mitchelmore et al., 2021)). From the most widely used organic UV-filter compounds currently approved by the Food and Drug Administration, the bioaccumulation and potential effects of OCTO and AVO have been investigated in the most detail. These studies demonstrated that OCTO exerts different adverse effects (e.g., reproductive changes, elicited oxidative stress, genotoxicity, altered xenobiotic metabolism) on mussels (Falfushynska et al., 2021; Giraldo et al., 2017), crustaceans (Boyd et al., 2021, A. 2023; Park et al., 2017), and fish (Freire Sovierzoski et al., 2023; Gayathri et al., 2023; Yan et al., 2020). AVO, one of the most frequent compound in different sunscreen products, can upon acute and/or chronic exposure cause developmental, behavioral, and biochemical changes in aquatic invertebrates (Bordalo et al., 2022; Boyd et al., 2021; Danovaro et al., 2008; de Paula et al., 2022) and vertebrates (da Silva et al., 2022; Kaiser et al., 2012; Ma et al., 2017). For many currently approved UV-filter compounds (e.g., octinoxate [OCTI]), even basic acute and/or chronic toxicity data to aquatic organisms are still lacking, while knowledge on their potential toxicity risks and modes of action in aquatic invertebrates at environmentally relevant concentrations were less or not examined.

This study was designed to evaluate the chronic effects of AVO, OCTO, and OCTI on the standard ecotoxicology model organism, the water flea (*Daphnia magna*). Neonates were exposed to AVO, OCTO, and OCTI at a concentration of 200 ng l^{-1} for 21 days. At the individual level, growth, reproduction, and heart rate were continuously monitored during the exposure, while feeding performance and swimming behavior were investigated at the end of the treatment. At the biochemical level, alterations in the activity of different biomarker enzymes involved in the xenobiotic metabolism pathways (cytochrome P450 s [ECOD] and glutathione S-transferase [GST]) and antioxidant defense pathways (catalase [CAT] and superoxide dismutase [SOD]) was investigated at the end of the exposure. The relevance of incident alterations induced by the chronic treatments with the selected organic UV-filters in daphnids was further compared based on the Integrated Biomarker Response scores (IBRv2) calculated based on the physiological traits of daphnids recorded by the 21st day of exposure and per treatment. The results of this study were expected to enable a better understanding of the real risks posed by the UV-filters considered to aquatic biota.

2. Materials and methods

2.1. *UV-filter compounds used for chronic exposures and confirmation of compound stability in exposure media*

AVO (CAS No.: 70–356–09–1, Merck, Germany), OCTO (CAS No.: 6197–30–4, Merck, Germany), and OCTI (CAS No.: 5466–77–3, Merck, Germany) were used for the treatments as UV-filtering compounds. From these, 100 µg mL^{-1} standard stock solutions were made in methanol (CAS No.: 64–17–5, VWR, Hungary). 5 µg mL^{-1} working solutions were freshly made in methanol every week from the standard stock solutions and added in the appropriate amount to the water of the experimental glass beakers to reach the 200 ng l^{-1} nominal exposure concentration ($\leq$ 0.01 % final carrier solvent concentration). The choice of methanol as a carrier solvent was based on the results of previous publications that it had no effect on *Daphnia* (David et al., 2012; Dom et al., 2012; Juan-Garcia et al., 2023; R. Svigruha et al., 2023).

First, we studied the stability of the compounds in the exposure media. We added the compounds to the water as a final concentration of 200 ng l^{-1}, hence the concentration of compounds was 100 % at exposure initiation. We kept the tanks with water in the same conditions as the tanks with water and animals during the chronic exposure experiments. After 72 h, measured concentrations of UV-filters in the tanks were of: AVO: 183.3 ± 5.86 ng l^{-1}; OCTO: 166.5 ± 9.16 ng l^{-1}; OCTI: 199.0 ± 4.18 ng l^{-1} (**Supplementary Table 2**). A five-point matrix-matched calibration curve was generated for each compound for the quantitative analysis using water samples containing 10, 25, 50, 100, and 250 ng l^{-1} standards. Detailed sample preparation, solid phase extraction, and HPLC-MS analysis are presented in the **Supplementary information**. We did not detect UV-filter compounds in the water of the control tanks.

2.2. *Experimental animals*

D. magna specimens were obtained from our laboratory-bred culture which has been maintained for more than 7 years at our institute, but its clonal identification remains unknown. The animals are being kept at 21 ± 1 °C (continuously monitored by DL-200T, VoltCraft) on a 16 h:8 h light:dark regime with natural wavelength light and an intensity of 500–700 lx (monitored by MS-200LED, VoltCraft). The animals are being cultured in 500 mL glass beakers containing 450 mL artificial water (31 mg L^{-1} Ca^{2+}, 11.5 mg L^{-1} Mg^{2+}, 6.4 mg L^{-1} Na^{+}; 0.6 mg L^{-1} K^{+}; 1.2 mg L^{-1} Cl^{-}, conductivity 250 µS at 20 °C, hardness 140 mg L^{-1} as $CaCO_3$, alkalinity 114 mg L^{-1} as $CaCO_3$, pH=7.6, and dissolved oxygen 7.4–7.8 mg L^{-1}) consisting of reverse-osmosis water and commercial spring water (Vigano, 1991). The artificial water is renewed twice a week. The specimens are fed three times a week on *Scenedesmus obliquus* (0.5 × 10^{-6} cells mL^{-1}). To avoid using 'first brood neonates' in the chronic exposures, specimens were first randomly selected from the culture and individually placed into beakers. From these beakers, parthenogenetic neonates (< 24 h) from the third brood were randomly selected for the experiments.

2.3. *Chronic treatments*

In *Experiment 1*, to investigate the alterations in developmental, heart rate, and behaviors, the neonates were divided into 4 experimental groups: a) control, b) AVO-exposed, c) OCTO- exposed, and d) OCTI-exposed ($n = 10$ neonates/experimental group). Following the approach of previous studies (Liu et al., 2017; Svigruha et al., 2021, R. 2023; Wang et al., 2016), the neonates were individually exposed to the different UV-filters for 21 days in 100 mL media in 150 mL glass beakers. The control neonates were also kept individually in 100 mL media for the same duration. Each experimental group had three replicates ($n = 30$ total animals/experimental group).

In *Experiment 2*, to investigate the activity of metabolic- and antioxidant enzymes, the neonates were also divided into 4 experimental groups: a) control, b) AVO-exposed, c) OCTO- exposed, and d) OCTI-exposed ($n = 30$ neonates/experimental group). Following the approach of previous studies (Liu et al., 2017; Oliveira et al., 2015; Svigruha et al., 2021, R. 2023; Wang et al., 2016), the neonates were kept together to the different UV-filter compounds for 21 days in 1000 mL media in 1700 mL glass beakers. The control animals were also kept together in 1000 mL media for the same duration. Each experimental group had three replicates ($n = 90$ total animals/experimental group).

Exposure media in the glass beakers during the 21-day exposures was completely changed every third day and the UV-filter compounds were re-added. Based on the recovery results of the preliminary water chemistry experiment, at least 85 % of the 200 ng l^{-1} nominal exposure concentration was continuously maintained for all compounds during the 21-day exposure. The specimens were always fed after the water changes. During the exposures, the maintaining conditions was the same as in the case of the base culture.

2.4. *Investigation of development, reproduction, and heart rate*

The average body size (µm) was determined every second day during the exposures based on the measured length and width parameters of the animals. The reproduction was monitored daily during the treatments and the following standard endpoints were investigated: 1) time of the first egg production, 2) egg number of the first production, 3) maximum egg number (i.e. maximum egg number during the reproductive cycles), 4) total egg number and total number of living offspring (i.e. total neonate number) produced per parent animal surviving at the end of the test. The neonates were always collected and removed after counting the eggs as well as neonates. The heart rate (number of beats ind^{-1} min^{-1}) was evaluated every third day during the exposures by making 1-min video records. All investigations were carried out with a stereomicroscope (LEICA M205C, BioMarker Ltd, Hungary) and evaluated with LAS software (version: 4.12).

2.5. *Evaluation of filtration rate and swimming behavior*

The feeding performance was investigated after the chronic exposure with a modified short-term grazing test performed in a 24-well plate format (Grintzalis et al., 2017; Soares et al., 2009). The assay relies on quantifying the amount of algal cells ingested per unit time by daphnids. Daphnids were individually immersed in 2 mL of artificial water spiked with *R. subcapitata* at a cell density of 1×10^6 cells mL^{-1}. The concentration of algal suspensions at the start- and end of the feeding test (4h) was quantified by means of the change in optical density of the suspensions determined photometrically at 650 nm with a CLARIOstar^{Plus} (BMG LABTECH, Germany) equipment. The initial and final optical densities of algal suspensions were converted to algal cell numbers per mL by means of a calibration curve of serial algal suspension dilutions prepared within the 5×10^4 – 2.5×10^6 cells mL^{-1} density range. Algal cell numbers of calibration dilutions fixed with Lugol were counted using a Burker chamber. The feeding rate was expressed as the number of algal cells consumed per individual per hour [algal cells $ind^{-1\ h\text{-}1}$].

The swimming behavior (swimming velocity, total distance covered, and trajectory) was examined after the 21-day treatments following the method of previous studies (Liu et al., 2022; Rodriguez et al., 2018). The specimens were individually placed in the wells of 6-well culture plates (BioLite™ Microwell Plates, #130,184, Thermo Fisher Scientific, USA) filled with 2 mL of artificial water. The animals were acclimatized for 20 min before the test. The swimming individuals were recorded for 1 min with a German C-mount camera (#VE10320; Loligo Systems). To achieve a more contrasty and shadow-free image, a desktop LED panel with adjustable brightness was used. The records were analyzed with the ToxTrac® v2.98 software which calculated the average speed (mm s^{-1}) and average total distance covered (mm ind^{-1} min^{-1}) and plotted the entire trajectory.

2.6. *Investigation of metabolic- and antioxidant enzymes activities*

The enzyme activity assays were made according to our previous publications (Acs et al., 2023; R. Svigruha et al., 2023). A schematic overview of the workflow is presented in **Supplementary Figure 1**. All measurements were performed with the CLARIOstar^{Plus} equipment.

To investigate the activity of GST, CAT, and SOD, 10 individuals were randomly selected from each replicate of each experimental group and pooled per replicate (i.e. $n = 3$ pooled samples/experimental group). The samples were homogenized in 500 μL phosphate buffer saline (0.5 M; pH = 7.4) with a TissueLyser LT device (QIAGEN, Germany) at 50 Hz for 3 min. Following centrifugation (10,000 g for 15 min at 4 °C), the resulted supernatants were aliquoted according to the assay requirements set in the protocols of the assay kits for GST (GST Assay Kit, #CS0410, Merck, Germany), CAT (Catalase Assay Kit, #A22180, Invitrogen, USA), and SOD (SOD Assay Kit, #19,160, Merck, Germany). For the in vivo ECOD activity measurement, 10 individuals were randomly selected from each replicate of each experimental group and individually placed in the wells of 96-well-round bottom clear plates. The ECOD assay is based on the in vivo EROD activity method used in fish embryos with modifications (Gaaied et al., 2019; Le Bihanic et al., 2013; Schiano Di Lombo et al., 2021), which was further optimized for daphnids by our research group (Acs et al., 2023). The detailed methodology and calculations for all measurements are presented in the **Supplementary information**. All enzyme activities were normalized to the total protein content (#B6916, Bradford Assay Kit, Merck, Germany).

2.7. *Integrated biomarker response (IBRv2)*

Changes caused by UV-filter compound exposures in the selected physiological traits of daphnids were integrated by means of the IBRv2 approach (Catteau et al., 2022; Sanchez et al., 2013). This method sums up the deviations from reference conditions of the biomarkers considered, where the reference values are the average biomarker levels observed in control groups. First, the mean of each biomarker per treatment condition were calculated ($\overline{X}_{i,j}$) (where i = biomarker labelling, j = the treatment condition). Mean $\overline{X}_{i,j}$ values were further converted into induction/inhibition indices ($Y_{i,j}$) relative to the reference biomarker baselines of control daphnids according to eq. (1):

$$Y_{i,j} = \log\left(\frac{\overline{X}_{i,j}}{X0_{i,j}}\right) \tag{1}$$

Next, deviation indices from the reference baselines were calculated for each biomarker by standardizing the $Y_{i,j}$ data relative to their standard deviation (Eq. 2):

$$A_{i,j} = \frac{Y_{i,j}}{\sigma_{Y_{i,j}}} \tag{2}$$

In eq. (2) $\sigma_{Y_{i,j}}$ represents the standard deviation of the induction/inhibition indices $Y_{i,j}$ determined for a particular biomarker within treatment groups of the same exposure condition. The validity of this standardizing approach was demonstrated in (Catteau et al., 2022).

Finally, the IBRv2 was calculated by summing up the absolute values of the deviation indices of all biomarkers per treatment condition. The absolute values of deviation indices determined for each physiological endpoint ($A_{i,j}$) were plotted in star charts to better outline deviations of physiological traits caused by exposures from the reference states.

2.8. *Statistical analysis*

Statistical analysis was performed using OriginPro 2018 software (OriginLab Corp., Northampton, Massachusetts, USA) and R v4.2.0 (R Core Team, 2022) programming environment. The normality of the datasets was investigated using the Shapiro-Wilk test and the homogeneity of variances between groups was investigated using Levene's statistic.

In the case of body size and heart rate, two-way repeated-measures ANOVA was performed to study the effect of time, treatment, and time x treatment interaction. This analysis was followed by ANOVA and Scheffe's post hoc test or Kruskal-Wallis test with Dunn's post hoc test to identify significant differences between control and treatment groups within a given time point (observation day). The time of the first egg production data, the maximum egg number per individual data, the total egg number data, the total neonate number data, and the swimming behavior data were analyzed using Kruskal-Wallis test with Dunn's post hoc test. The egg number in the first production data, the filtration rate data, and the different enzymatic activity data were analyzed using one-way ANOVA with Scheffe's post hoc test.

3. Results

3.1. *Mortality and growth*

No significant mortality was observed during the 21-day exposures (**Supplementary Table 3**). Fig. 1**A** presents the average body size of the specimens during the different exposures. Two-way repeated-measures ANOVA revealed significant effects of time (observation days) [F(6, 228) = 371.758, $P \leq 0.001$] and treatment [F(3, 228) = 7.261, $P \leq 0.001$], but not significant time x treatment interaction [F(18, 228) = 1.144, $P > 0.05$]. Further analysis with ANOVA and Scheffe's post hoc test

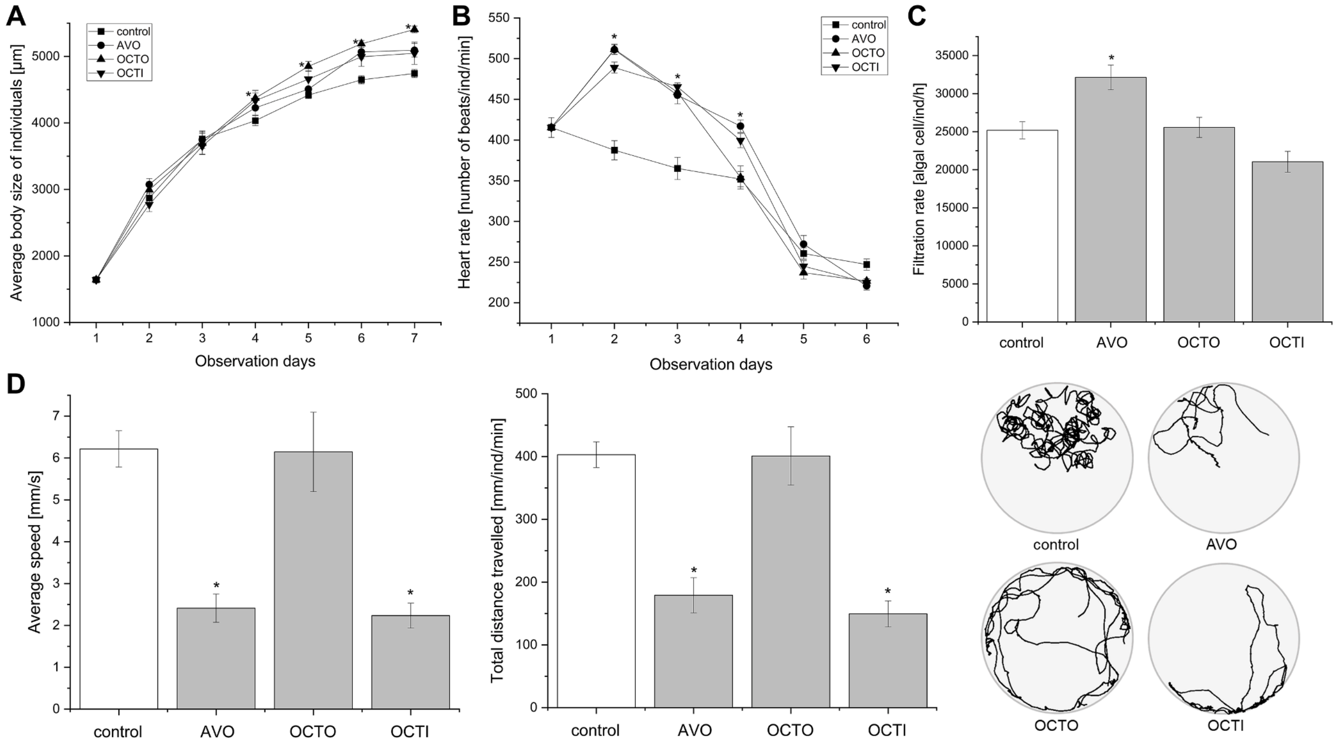

Fig. 1. Changes in the body size (**A**), heart rate (**B**), filtration rate (**C**), and swimming activity (**D**), of *D. magna* specimens in the control, avobenzone- (AVO), octocrylene- (OCTO), and octinoxate (OCTI)-treated groups. Each data point (**A, B**) and bar (**C, D**) represents mean ± SEM ($n = 10$ animals/replicate/group). The white column corresponds the control group and the grey columns indicate the treated groups. Significant differences to the control group are marked by asterisks ($*P < 0.05$).

(observation day 5) or Kruskal-Wallis and Dunn's post hoc test (observation day 4, 6, 7) showed that the body size in the OCTO-exposed group was significantly increased ($P \leq 0.05$) from the 4th observation day to the end of the experiment. On the last observation day, the body size of the individuals in the OCTO-exposed group (5405.46 ± 49.76, $P \leq 0.001$) was 13 percent larger than that of the control group (4743.71 ± 61.26).

3.2. Effects of UV-filter exposure on reproduction

Table 1 shows the findings of the exposures on different reproductive endpoints. No significant difference was observed in the number of days until the first egg production or in the number of eggs in the first production between the different experimental groups. However, the maximum egg number laid per animal ($\chi^2 = 21.90$, $P \leq 0.001$) significantly changed during the 21-day exposures: in comparison with the control group (7.9 ± 0.69), the maximum egg number significantly increased in the AVO-treated (13.4 ± 1.08, $P \leq 0.01$), OCTO-treated (16.5 ± 2.60, $P \leq 0.001$), and OCTI-treated (13.4 ± 1.50, $P \leq 0.01$) groups. In the case of the total egg number [F(3, 35) = 12.61, $P \leq 0.001$] and total neonate number ($\chi^2 = 12.64$, $P \leq 0.01$) produced per individual, only OCTO had a significant effect. Animals in the OCTO-treated group produced a significantly higher number of eggs (54.7 ± 4.04, $P \leq 0.001$) and neonates (40.3 ± 4.93, $P \leq 0.01$) compared to the control group (23.6 ± 2.35 and 15.7 ± 1.57, respectively).

Table 1
Changes in the reproductive performance of *D. magna* during the 21-day exposures to avobenzone (AVO), octocrylene (OCTO), and octinoxate (OCTI), respectively. The reproduction was monitored every day. Values represent mean ± SEM for all endpoints (i.e. the given values are the mean of the values of individuals surviving at the end of the test). Significant results, in comparison with the control, are indicated by asterisks (ANOVA or Kruskal-Vallis test, $**P \leq 0.01$ and $***P \leq 0.001$).

Treatments	First egg production (days)	Egg number in the first production	Maximum egg number	Total egg number	Total neonate number
Control	8.3 ± 0.76	6.3 ± 0.63	7.9 ± 0.69	23.6 ± 2.35	15.7 ± 1.57
AVO	8.7 ± 0.70	7.9 ± 0.95	**13.4 ± 1.08****	37.2 ± 3.77	23.0 ± 3.95
OCTO	8.2 ± 0.80	6.6 ± 0.99	**16.5 ± 2.60*****	**54.7 ± 4.04*****	**40.3 ± 4.93****
OCTI	8.8 ± 0.35	7.8 ± 1.19	13.4 ± 1.50**	37.1 ± 3.93	20.0 ± 1.90

3.3. Behavioral and physiological responses

Fig. 1**B** presents the effects of UV-filter compounds on the heart rate of *D. magna*. Two-way repeated-measures ANOVA revealed significant effects of time (observation days) [F(5, 194) = 556.99, $P \leq 0.001$], treatment [F(3, 194) = 31.75, $P \leq 0.001$], and time x treatment interaction [F(15, 194) = 14.38 $P \leq 0.001$]. Further analysis revealed that on the observation day 2 [F(3, 39) = 53.93, $P \leq 0.001$), the heart rate significantly increased in the AVO-treated (511.1 ± 18.59, $P \leq 0.001$), OCTO-treated (511.6 ± 19.76, $P \leq 0.001$), and OCTI-treated (489.1 ± 21.41, $P \leq 0.001$) groups compared to the control group (387.5 ± 11.83). On the observation day 3 ($\chi^2 = 20.69$, $P \leq 0.001$), the heart rate was also significantly higher in all treated groups (AVO: 455.1 ± 10.7, $P \leq 0.01$; OCTO: 458.8 ± 6.43, $P \leq 0.01$; OCTI: 465.4 ± 4.87, $P \leq 0.001$) than the control (365.1 ± 13.62). On the 4th observation day ($\chi^2 = 19.79$, $P \leq 0.001$), in comparison with the control group (352 ± 9.32), specimens in the AVO-treated group (417.1 ± 7.61, $P \leq 0.001$) and OCTI-treated group (399.5 ± 8.75, $P \leq 0.05$) showed a significantly increased heart rate, while OCTO did not induce significant change. From the 5th observation day, no significant difference could be detected between the experimental groups.

The results of the exposures on the filtration rate are presented in

Fig. 1**C**. The feeding rate of the *Daphnia* specimens [F(3, 39) = 11.19, $P \leq 0.001$) was significantly higher in the AVO-treated group (32,142.85 ± 1620.61, $P \leq 0.05$) than the control (25,187.96 ± 1127.81). Neither the animals of the OCTO-treated group nor the OCTI-treated group showed significant changes.

In the case of swimming behavior (Fig. 1**D**), both AVO (2.41 ± 0.33, $P \leq 0.05$) and OCTI (2.23 ± 0.29, $P \leq 0.05$) caused a significant decrease in the average speed ($\chi^2 = 21.90$, $P \leq 0.001$) in comparison with the control group (6.21 ± 0.43). Accordingly, compared to the control group (403.00 ± 20.44), the total distance covered ($\chi^2 = 21.48$, $P \leq 0.001$) also decreased significantly in the AVO-treated group (179.06 ± 28.09) and OCTI-treated group (149.41 ± 20.71). However, the OCTO-treated group showed no significant changes.

3.4. *Changes of metabolic- and antioxidant enzymes activities*

Fig. 2 presents the alterations induced by UV-filter compounds on various enzymatic activity. The chronic exposures to UV-filters caused no significant change in ECOD activity (Fig. 2**A**). In the case of GST activity [F(3, 11) = 9.85, $P \leq 0.01$), in contrast to the control group (0.36 ± 0.01), both AVO (0.21 ± 0.02, $P \leq 0.05$) and OCTO (0.24 ± 0.01, $P \leq 0.05$) caused a significant decrease (Fig. 2**B**). CAT activity [F(3, 11) = 141.39, $P \leq 0.001$) was significantly reduced in the AVO-exposed group (2.81 ± 0.05, $P \leq 0.05$) and OCTO-exposed group (1.18 ± 0.03, $P < 0.05$), while a significant increase was found in the OCTI-exposed group (5.74 ± 0.25, $P \leq 0.05$) in comparison with the control (4.83 ± 0.22) (Fig. 2**C**). Animals in OCTO-exposed group (41.81 ± 3.07, $P \leq 0.05$) and OCTI-exposed group (65.07 ± 3.59, $P \leq 0.05$) showed significantly higher SOD activity [F(3, 11) = 37.07, $P \leq 0.001$) in comparison with the control (26.63 ± 0.48) (Fig. 2**D**).

3.5. *Integrated biomarker response (IBRv2) evaluation*

The relevance of differences in the bioactive potential of AVO, OCTO and OCTI towards *Daphnia* was evaluated based on the IBRv2 scores determined by summing up the absolute deviation indices of the following parameters: growth, main reproduction parameters (total egg number, total neonate number), heart rate, swimming distance, swimming speed, and enzymatic activity endpoints (ECOD, GST, SOD, CAT) recorded by the 21st day of exposure (Fig. 3). The IBRv2 scores determined per UV-filter treatment revealed minor differences in the bioactive potential of compounds the environmental tested (200 ng l^{-1}), resulting in the following values: AVO – 11.31, OCTO – 13.65, OCTI – 12.36. Compound specific alteration features were observable for OCTO

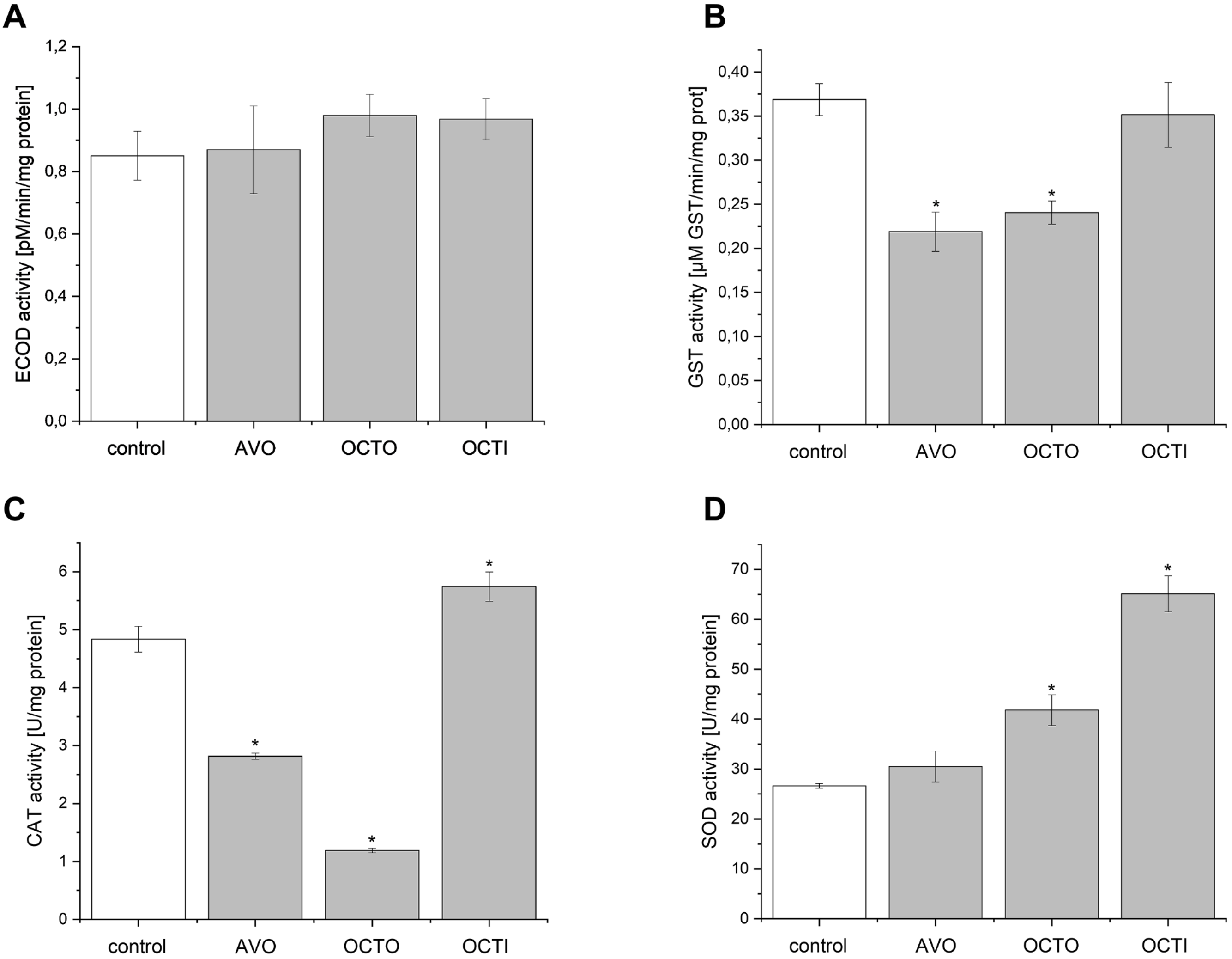

Fig. 2. Changes in the activity of ethoxycoumarin O-deethylase (ECOD) (**A**), glutathione S-transferase (GST) (**B**), catalase (CAT) (**C**), and superoxide dismutase (SOD) (**D**) after the 21-day exposures to avobenzone (AVO), octocrylene (OCTO), and octinoxate (OCTI). Each bar represents mean ± SEM (**A**: $n = 10$ individual animals/replicate/group; **B-D**: $n = 10$ pooled animals/replicate/group). The white column corresponds the control group and the grey columns indicate the treated groups. Significant differences to the control group are highlighted by asterisks (*$P < 0.05$). The ECOD and GST measurements are kinetic assays, while the CAT and SOD measurements are non-kinetic assays.

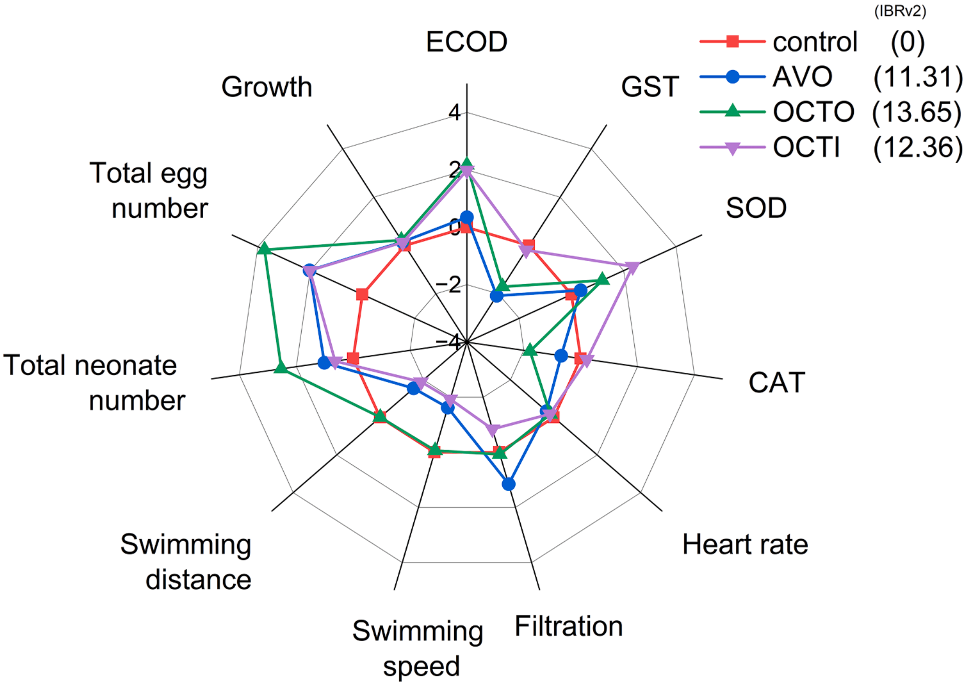

Fig. 3. Integrated biomarker response scores (IBRv2) and star plot representing deviation indices of physiological traits (ethoxycoumarin O-deethylase activity (ECOD), glutathione S-transferase (GST), superoxide dismutase (SOD), catalase (CAT), heart rate, filtration activity, swimming speed, swimming distance, total neonate number, total egg number and growth) computed for daphnids exposed to UV-filters per treatment.

with significantly higher growth and reproductive output of daphnids as well as significantly increased CAT activity. Depressed swimming behavior parameters and GST and CAT inhibition were the major contributors to the IBRv2 scores for AVO, while for OCTI the most important alterations were manifested in significantly depressed swimming behavior and induced antioxidant enzymes (CAT, SOD).

4. Discussion

Previous in vitro and in vivo studies revealed many adverse effects of both inorganic and organic UV-filters on algae, corals, mollusks, arthropods, echinoderms, and vertebrates (Chatzigianni et al., 2022). However, only a few studies examined the acute and chronic effects of AVO, OCTO, and OCTI individually and/or in mixture on *D. magna* (Boyd et al., 2021, A. 2023, 2024; de Paula et al., 2022; Park et al., 2017). These earlier studies mainly focused on the developmental, growth, and reproductive alterations, but did not investigate other behavioral and biochemical changes, including swimming, heart rate, ECOD activity (**Supplementary Table 4**). In the present study, we have performed more detailed assessment at the behavioral and biochemical levels to gain additional information on the toxic mode of action of these organic UV-filter compounds in aquatic ecosystems. Importantly, compared to previous studies which even used tens or hundreds of μg l^{-1} exposure concentrations (such levels do not occur in the environment), another novelty of the present study is the usage of an environmentally-relevant 200 ng l^{-1} concentration based on the previous environmental analytical studies (**Supplementary Table 1**).

Studies investigating the general toxicity of AVO, OCTO and OCTI to *Daphnia* demonstrated acute (48 h EC) toxicity thresholds within 0.03 – 3.18 mg l^{-1} concentration ranges (Boyd et al., 2021; Park et al., 2017), while complete mortality following 7 days exposure of daphnids to 150 μg l^{-1} AVO and 7.5 μg l^{-1} OCTO were reported by (Boyd et al., 2021). Chronic toxicity investigations of OCTI in *Daphnia* revealed insignificant dose dependent mortality within the 0.03 – 75 μg l^{-1} dose range (Lambert et al., 2021). Our results comply with the toxicity data reported in these studies as none of the UV-filters tested caused any significant lethality at the 200 ng l^{-1} dose applied during 21 days of exposure.

Chronic exposure of daphnids to organic UV-filters was proven to alter the growth of daphnids causing in general decreases in body size above the 0.5 μg l^{-1} doses. Transient decreases in the body size of daphnids exposed over five generations to AVO (6.59 μg l^{-1}) and OCTO (~ 0.6 μg l^{-1}) were demonstrated by (A. Boyd et al., 2023). Moreover, in a later 21 days exposure study to the organic UV-filters octisalate and homosalate (10 – 100 μg l^{-1}) significant reductions within 10 % in the body length of daphnids were recorded (Boyd et al., 2024). Chronic exposure to OCTI at 75 μg l^{-1} was shown to cause a significantly increased body size in *Daphnia*, while lower concentrations (30 and 700 ng l^{-1}) were not effective (Lambert et al., 2021). In the present study, we found that exposure of daphnids to 200 ng l^{-1} of each UV-filter tested initiated an increase in the body length of daphnids from the 4th observation day onward, which became statistically significant for the daphnids subjected to OCTO treatment. Increased growth in daphnids were observed upon incubation to elevated temperature (Im et al., 2019) or following exposures to endocrine-disrupting chemicals such as acetaminophen (Lari et al., 2017). Elevated temperature is known to increase the somatic growth rate of ectotherms by amplifying their metabolism (Somero et al., 2017). Increased growth upon exposure to endocrine disruptors may evolve either because the chemical has the potential to enhance the activity of growth-promoting hormones as ecdysone, or may inhibit hormones that block growth, as farnesoate (deFur, 2004). In our case, the potential for OCTO to exert endocrine disrupting effects on daphnids upon chronic exposure may be presumed. However, this presumption needs further research for justification. As reduced lipid stores were evidenced for daphnids with increased growth rates that was initiated by low chemical stress (Moro et al., 2024), it suggests that even in our case a poorer viability of OCTO treated daphnids may not be excluded.

The effects of AVO, OCTO, and OCTI on the reproductive performance of *D. magna*, including time to maturity, number of broods, brood size, total offspring number, and reproductive effort, have previously been investigated in detail (**Supplementary Table 4**). Similar to previous studies (Boyd et al., 2021, A. 2023; de Paula et al., 2022), our results showed no change in the time required to reach the first egg production or in the egg number of the first egg production. However, the maximum egg number per animal was significantly increased in all treated groups. According to a previous study, after three weeks exposure of < 24 h old neonates to AVO (20 μg L^{-1}), significantly increased the number of neonates produced per individual, but OCTO (0.5 μg L^{-1}) had no effect on this parameter (Boyd et al., 2021). Moreover, higher concentrations of AVO (25 μg l^{-1}) or OCTO (3 μg l^{-1}) caused some multigenerational effects manifested only in transient and minor decreases in the total reproductive effort (A. Boyd et al., 2023). Our assessments showed that OCTO at 200 ng l^{-1} concentration significantly increased the total egg number and total neonate number produced per individual. A previous study investigated the reproductive effects on the F1 generation born to parents chronically exposed to 75 μg/L OCTI for 21 days and found no significant changes in the total number of offspring or time to maturity (Lambert et al., 2021). Similarly, OCTI exposure in the present study did not affect these endpoints at a lower exposure concentration. Of note, the baseline egg number of the control group in the present work was in line with previous studies (Boyd et al., 2021; Liu et al., 2017; Luna et al., 2015; Svigruha et al., 2021, R. 2023), but the significant changes (i.e. an increase in the maximum egg number or total egg number) were detectable during the treatments. Increased reproductive rate upon environmental- or chemical stress are increasingly reported for many invertebrates and this phenomenon is considered as a “spawn and die” response to unfavorable conditions (Blewett et al., 2017; Boyd et al., 2021; Schmitt et al., 2008). An increased number of neonates within a timeframe may facilitate a phenotypic adaptation, a phenomenon specifically observed for species with relatively short lifespan (Chatterjee et al., 2019). Nevertheless, evidence exists that upon low chemical stress, similar maternal resource investment to increased reproductive output may result in less resources per offspring, which imply that newborns are smaller in size and possess less lipid reserves (Campos et al., 2016; Moro et al., 2024).

In contrast to the reproductive performance, potential alterations induced by AVO, OCTO, and OCTI in behavioral activities are much less investigated (**Supplementary Table 4**). Lambert and her co-workers did not find changes in the swimming speed of F1 generation (24 h old) born to parents exposed to OCTI for 21 days (Lambert et al., 2021). However, as far as we know, the direct effects of AVO, OCTO, and OCTI on the heart rate, filtration rate, and swimming behavior of *D. magna* have not been investigated until now. Our results revealed that the heart rate transiently increased in all treated groups (between the second and fourth observation days), but it reached the control level to the end of the exposure. Few studies demonstrated for various contaminants to cause increased heart beating frequency in daphnids. Chronic exposure of daphnids to environmental concentrations of butylone (a chiral synthetic cathinone used in wastewater treatment) led to an increased heart rate in developing daphnids (Carvalho et al., 2024). Similarly, exposure to azoxystrobin resulted in an increased heart beating frequency (Lari et al., 2024). It is suggested that a common feature of chemicals showing sympathomimetic effects is to elicit increased blood pressure and heart rate in organisms (Dolan et al., 2018). In general, chemicals that reduce cellular respiration cause an increased heart rate in organisms to compensate the reduced metabolic output (Lari et al., 2017; Smirnov, 2013). Only AVO caused a significant alteration (increase) in filtration rate, yet no correlation was found between the filtration rate and body size, suggesting that available overall energy budget of daphnids may have varied across treatments. Average swimming speed was found to significantly decrease after exposure to AVO and OCTI resulting in a significantly decreased total swimming distance. The exact cellular and molecular mechanisms underlying these physiological changes remain to be explored by future studies.

Many environmental pollutants are known to alter xenobiotic metabolism and antioxidant defense pathways (Oliveira et al., 2015), hence enzymatic biomarkers are generally applied to assess the biochemical responses to different pollutants (Liu et al., 2017; Oliveira et al., 2015; R. Svigruha et al., 2023; Wang et al., 2016). Cytochrome P450-mediated reactions in phase I (such as demethylation) and phase II conjugation enzymes (such as GST) are responsible for the biotransformation of xenobiotics (Sokolova et al., 2012). Enzymes of antioxidant defense system, such as CAT and SOD, have a key role in the elimination of reactive oxygen species (ROS) (Kurutas, 2016). Many organic UV-filters including AVO and OCTO have been shown to affect the activity of these biomarker enzymes in vertebrates (Ma et al., 2017; Meng et al., 2021). Although a previous study suggested that UV-filters may also interfere with the activity of enzymes involved in the xenobiotic metabolism and antioxidant defense pathways in *D. magna* (A. Boyd et al., 2023), this has not yet been thoroughly investigated. Only one study investigated the possible effects of acute AVO (4.4 μg l^{-1}), OCTO (4.4 μg l^{-1}) and OCTI (0.2 μg l^{-1}) treatment on GST and CAT activity in the F0 and F1 generations and demonstrated a significant increase in CAT activity due to the OCTI exposure but no change was found in GST activity (de Paula et al., 2022). Our investigations revealed no alteration in the activity of ECOD after chronic AVO, OCTO, and OCTI exposure at 200 ng L^{-1} concentration, suggesting that phase I enzymes may not be involved in the metabolization of these compounds in *D. magna*. However, the GST activity were significantly decreased by AVO and OCTO, suggesting that these compounds may exert an inhibitory effect at the phase II metabolization pathways. Exposure to AVO at our applied concentration did not alter SOD activity, but OCTO and OCTI showed a significant induction of this anti-ROS enzyme. CAT activity was also increased by OCTI, but AVO and OCTO significantly inhibited the activity of this enzyme. Our findings indicate that AVO, OCTO, and OCTI have a strong effect on both phase II metabolization pathways and antioxidant defense system. The inhibitory effect detected in case of CAT and GST activity, and increased heart rate after chronic exposure to AVO and OCTO are suggesting a similar mode of action if these compounds, which may differ from OCTI effect pathway. OCTO and OCTI showed an inducing effect on SOD activity, indicating the induction of the antioxidant system. SOD represent the first defense line of antioxidant activity against ROS, producing H_2O_2 as a byproduct, and CAT in peroxisomes and glutathione-peroxidases in mitochondria, among other second-line enzymes and non-enzymatic scavengers eliminate the nascent H_2O_2 (Faria et al., 2009). The inhibition of CAT activity may be attributed to excess ROS if SOD, or other first line antioxidants are unable to eliminate the superoxide radicals which in turn are able to ©nhibit CAT or GPxSe, and proteins inhibiting other antioxidant enzymatic activities (Halliwell and Gutteridge, 2015) worsen deleterious ROS effects, resulting in damaging other pathways and general energetic shortage (Li et al., 2010; Viarengo et al., 1995).

The mode of action of organic UV-filter compounds is largely unknown in aquatic invertebrates. In vertebrates, UV-filters have been shown to bind to different nuclear steroid receptors and to have multiple hormonal activities, hence they are considered as endocrine disrupting chemicals (e.g., thyroid- or estrogen-like disruption) (Diaz-Cruz and Barcelo, 2009; Ka and Ji, 2022; Kwon and Choi, 2021; Yan et al., 2020; Zhang et al., 2016). Given that there is no gene for nuclear sex steroid, nuclear glucocorticoid, and nuclear thyroid receptors in arthropods including *D. magna* (Baker, 2019; Markov et al., 2017; Morthorst et al., 2023), the perception of UV-filters and the exact cellular and molecular underpinnings are unknown. It can be assumed that the possible mode of action may occur via endocrine-related nuclear receptors. For example, the results of previous studies indicated the potential of UV-filters for endocrine disruption of the ecdysone pathway (Lambert et al., 2021; Yang et al., 2021); however, there have also been contradictions regarding this question (A. Boyd et al., 2023). Speculatively, if organic UV-filters can interact with the nuclear ecdysone receptor (nEcR) or with the nEcR-Retinoid X receptor complex, they could alter the expression of some key genes, such as vitellogenin (Gouveia et al., 2018), which could, for example, explain the altered reproduction observed in the present study. These compounds might also be able to bind to membrane receptor (e.g., membrane EcR) causing endocrine disruption via rapid cellular changes. Future research is to be aimed at investigating the interaction between organic UV-filters and well-known endocrine-related receptors of *Daphnia* to clarify the exact mechanisms. Another explanation, supported by a recent study (A. Boyd et al., 2023), can be that there is no receptor involved at all but the absorption and metabolization of UV-filters involve re-allocation of resources and/or enhanced energy usage which can be manifested in measurable changes at the behavioral and biochemical level, as also seen in this study.

Integration of deviation indices of physiological traits from baseline levels in exposed daphnids evidenced closely similar adverse effects of chronic exposure to environmental concentrations of AVO, OCTO and OCTI nevertheless, it outlined also some specific adverse features as impaired growth and reproduction for OCTO, significantly reduced locomotion for AVO and OCTI and apparent inhibition of the Phase II conjugation pathway for AVO and OCTO.

5. Conclusions

The widespread prevalence of organic UV-filter compounds in aquatic ecosystems has generated a worldwide concern as they can induce several alterations in aquatic invertebrates and vertebrates. Our experiments confirmed that organic UV-filters were able to exert deleterious effects in *D. magna* even at an environmentally relevant (200 ng L^{-1}) concentration. Our results showed physiological alterations in *D. magna* by AVO, OCTO, and OCTI at both of the individual and biochemical levels. Our investigations also revealed behavioral changes that had not been investigated previously (e.g., swimming activity, filtration rate). Our observations suggest that although low environmental concentrations (ng l^{-1} to a few μg l^{-1}) of UV-filters are not lethal to aquatic organisms, but their presence may result in decrease in energetic stress and fitness, and changes in aquatic ecosystems. Since these compounds mainly occur in mixtures in the surface waters, further studies should aim to investigate the mixture effects of organic UV-filters to better mimic the environmental situation. Moreover, in most environments, the residues of UV-filters are exposed to UV-rays. As pointed out by a previous study (Kim et al., 2023), the structural changes caused by UV-rays can also affect toxicity of these compounds. Hence, follow-up studies should also consider this important point. Finally, future research is to be aimed at investigating the interaction between organic UV-filters and well-known endocrine-related nuclear or membrane receptors of *Daphnia* to reveal the cellular and molecular mechanisms underlying changes induced, for example, in reproduction.

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CRediT authorship contribution statement

Zoltán Németh: Writing – review & editing, Visualization, Methodology, Investigation, Funding acquisition, Data curation. **Réka Svigruha:** Writing – review & editing, Visualization, Methodology, Investigation, Data curation. **András Ács:** Writing – review & editing, Investigation. **Anna Farkas:** Writing – review & editing, Methodology, Investigation, Data curation. **Kálmán Tapolczai:** Writing – review & editing, Data curation. **Károly Elekes:** Writing – review & editing. **István Fodor:** Writing – original draft, Visualization, Methodology, Data curation, Conceptualization. **Zsolt Pirger:** Writing – review & editing, Supervision, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

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Data availability

Data will be made available on request.

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