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Chronic Effects of Fluoxetine on *Danio rerio*: A Biochemical and Behavioral Perspective

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Abstract: Fluoxetine is an antidepressant widely used to treat depressive and anxiety states. Due to its mode of action in the central nervous system (selective serotonin reuptake inhibitor (SSRI)), it becomes toxic to non-target organisms, leading to changes that are harmful to their survival. In this work, the effects of fluoxetine on juvenile zebrafish (*Danio rerio*) were evaluated, assessing biochemical (phase II biotransformation—glutathione S-transferase (GST), neurotransmission—acetylcholinesterase (ChE), energy metabolism—lactate dehydrogenase (LDH), and oxidative stress—glutathione peroxidase (GPx)) and behavior endpoints (swimming behavior, social behavior, and thigmotaxis) after 21 days exposure to 0 (control), 0.1, 1 and 10 µg/L. Biochemically, although chronic exposure did not induce significant effects on neurotransmission and energy metabolism, GPx activity was decreased after exposure to 10 µg/L of fluoxetine. At a behavioral level, exploratory and social behavior was not affected. However, changes in the swimming pattern of exposed fish were observed in light and dark periods (decreased locomotor activity). Overall, the data show that juvenile fish chronically exposed to fluoxetine may exhibit behavioral changes, affecting their ability to respond to environmental stressors and the interaction with other fish.

Keywords: pharmaceuticals; selective serotonin reuptake inhibitors; chronic effects; behavior; biochemical biomarkers; environmentally relevance

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1. Introduction

Currently, mental health is a topic of concern, due to the increased stress levels of society. The COVID19 pandemic has increased the pressure on human health, with an increase in the occurrence of depression being reported [1]. Thus, an increase in the consumption of antidepressants is expected and will consequently lead to increased environmental levels and potential effects to biota present in an aquatic environment, the final destination of environmental pollutants. Fluoxetine, known to be a selective serotonin reuptake inhibitor (SSRI), is generally prescribed for the treatment of human depression, anxiety, compulsive behavior, and eating disorders [2–7]. This drug is known to act at the central nervous system, blocking the serotonin transport, leading to its accumulation in the synaptic cleft [4,5,8–13], allowing an attenuation of anxiety and depressive symptoms (anxiolytic effect) [12,14]. Serotonin (5-HT) is a neurotransmitter that has a fundamental role in regulating the development of the brain and spinal cord and, during this development, acts as an important neurotrophic factor in neuronal proliferation, differentiation, axonal growth, migration, and synaptogenesis [15,16]. Additionally, it has the ability to modulate parameters related to behavior such as locomotion, stress, appetite, reproduction, aggressiveness, and social interactions [2,7,13,16–19]. In the freshwater environment, concentrations of fluoxetine have been detected at levels ranging from 0.0004 to 3.645 µg/L in wastewater treatment plants (WWTP) [20–31], 0.0005 to 0.056 µg/L in surface waters and groundwaters [29,31–37] and, for drinking water, the levels vary between 0.0005 and

0.0008 µg/L [38,39]. Previous studies have demonstrated that fluoxetine can be toxic to fish, with exposure resulting in changes at different biological levels, from gene transcription, neurotransmission markers, enzymatic activities (e.g., oxidative stress, metabolism), hormone levels, reproductive processes, and accumulation in various tissues (e.g., brain and liver), resulting in a severe change in the histology of these organs [4–7,10,11,14,40–48]. In addition, this pharmaceutical can cause changes in behavior (e.g., locomotor activity, stress response, feeding, aggression, social and anti-predatory behavior) [4–9,11,15,16,45,49–60].

The main objective of the present study was to evaluate, in juvenile zebrafish, the effects of chronic exposure to sublethal concentrations of fluoxetine at biochemical and behavioral levels. Zebrafish is a model organism widely used in several areas of research such as developmental biology, genetics, neuroscience, ecotoxicology, and behavior, has several advantages such as small size, rapid development, fertilization, and external development, transparent and abundant eggs, and easy maintenance [61,62]. The use of juveniles, that have morphological and behavioral similarities to adults has advantages over the adult stage. As animals have not yet achieved sexual maturation, there is no potential interference of sex-related hormones [63]. Thus, juveniles were exposed for 21 days to 0.1, 1 and 10 µg/L of fluoxetine, and behavior (erratic swimming and thigmotaxis, exploratory swimming (novel tank), and social behavior) were analyzed. Biochemical endpoints were also assessed after 21-days of exposure. The concentrations selected for this study were based on the highest levels detected in Chinese surface waters between 2015 and 2020, 0.101 µg/L in Baiyangdian Lake, Pearl River, Songhua River, Yellow River, Huai River, Hai River, and Liao River [64].

2. Materials and Methods

2.1. Test Chemicals and Test Solutions Preparation

Fluoxetine ($C_{17}H_{18}F_3NO$) was purchased from TCI (Zwijndrecht, Belgium). A stock solution (10 mg/L) of fluoxetine was prepared by dissolution of the compound in culture water. The concentrations used (0.1, 1, and 10 µg/L) were prepared by successive dilutions of the stock in culture water. All other chemicals were analytical grade acquired from Sigma-Aldrich (St. Louis, MI, USA).

2.2. Test Organisms

Zebrafish (AB strain) are cultured at the University of Aveiro, Portugal, kept in a ZebTEC (Tecniplast) recirculating system. Culture water is obtained through reverse osmosis and activated carbon filtration of tap water, complemented with salt (Instant Ocean Synthetic Sea Salt, Spectrum Brands), and automatically adjusted for pH (7.5 ± 0.5) and conductivity (conductivity 800 ± 50 mS). Water temperature is maintained at 26 ± 1 °C, and dissolved oxygen is maintained at 95% (7.6 mg/L) saturation or higher. Animals are maintained at 14:10 h light:dark photoperiod. Fish are fed once a day with a commercially available artificial diet (GEMMA Micro 500, Skretting, Tooele, UT, USA). The fish used for this study was approximately 2 months old, having been obtained through crossbreeding and bred to the juvenile stage.

2.3. Animal Exposure

The assay was based on OECD guidelines on Fish Embryo Toxicity Test (OECD 215/230). The concentrations chosen were sublethal (0.1, 1, and 10 µg/L), as they produced a physiological and/or behavioral effect on the exposed organisms, not resulting in their death, which could affect important ecological processes [65]. The juveniles were exposed to small glass aquariums containing 750 mL of test solution. Each concentration contained 12 juveniles, making a total of 48 juveniles. The total exposure duration was 21 days, with 100% renewal of the medium every 3 days. Animals were daily checked for mortality or any signs of stress.

2.4. Behavior Tests

Locomotor activity and thigmotactic behavior were performed after 12 and 21 days of exposure, while the novel tank test and social test were performed after 21 days of exposure. All videos were captured with a model Samsung Zoom Lens 4.9 24.5 mm 1: 3.5 0.9, 27 mm, placed at 28.5 cm distance.

2.4.1. Locomotor and Thigmotactic Behavior

This test was adapted from Correia et al. (2019) [66], assessing swimming behavior, individually, in twelve fish per concentration. Each fish was transferred to a rectangular container (9.4 cm wide and 14.1 cm length) containing 2.5 cm high of the medium. The movement of fish was recorded using the automated video-tracking system Zebrabox (Viewpoint Life Science, Lyon, France), during two periods: 4 min in the dark and 4 min in the light (after 2 initial minutes of acclimatization in dark). The light level was set for 50% intensity, a transparent color background was used and the "detection threshold" value was set to 88. Total swimming distance (TD, mm) and total time (TT, seconds) were recorded. To assess the thigmotactic behavior, two monitoring zones were defined in the recording arena: an inner and an outer zone, allowing the analysis of the tendency to swim near the edges of the container (as a measure of thigmotactic behavior). The angles of the fish trajectory were analyzed using the fish's swimming direction vector and the animal's turn path with 8 classes of angles defined [67]. The assessed classes cover low-amplitude angles (classes 4 and 5) which indicate direct/relax movements; high-amplitude angles (1, 2, 7, and 8) that indicate movements with significant changes in direction, suggesting an erratic swimming behavior (zigzag/stress behavior); and average amplitude angles (classes 3 and 6) indicating average turns.

2.4.2. Novel Tank/Exploratory Swimming Test

This test aimed to evaluate the anxiety level of fish when placed in a new environment [68–70]. To conduct the test, a rectangular aquarium was used (27 cm long and 13.3 cm wide; 12 cm water high) and a camera (model Samsung Zoom Lens 4.9 24.5 mm 1:3.5 0.9, 27 mm) was placed 28.5 cm away from the aquarium, recording the lateral side of it. Twelve fish of each concentration were individually placed in the test aquarium and their behavior was recorded for 5 min [71]. The time spent by each fish in three distinct layers of the aquarium (4 cm height each) was manually analyzed: area 1 (bottom), area 2 (middle), and area 3 (top) as described in a previous study [72].

2.4.3. Social Test

This test was based on the work of Correia et al. (2019) [66], evaluating the tendency of fish to approach a shoal of the same species (a measure of the social behavior of the fish). A zebrafish was placed individually in an aquarium (27 cm long and 13.3 cm wide) adjacent to another (10.5 cm long and 10.5 cm wide), with a shoal of six zebrafish. The individual tested was placed in the middle of the aquarium and the movement was recorded for 5 min (model Samsung Zoom Lens 4.9 24.5 mm 1: 3.5 0.9, 27 mm, placed at 28.5 cm distance). Twelve juveniles of each tested concentration were analyzed, and the time spent by each fish in three different zones of the aquarium (8.8 cm length each) was calculated manually: zone 1—proximity zone (the area closest to the shoal); zone 2—neutral zone close to the shoal (in the middle of the aquarium); and zone 3- neutral zone away from the shoal.

2.5. Biochemical Analysis

After performing the behavioral tests, the fish were euthanized by immersion in an anesthetic solution (ethyl 3-aminobenzoate methanesulfonate salt, 0.06 mg/200 mL) and stored at −80 °C. On the day of analysis, fish were thawed on ice, 1 mL of 0.1 M K-phosphate buffer pH 7.4 was added, and then animals were homogenized by ultrasounds using a KIKA Labortechnik U2005 Control^{TM}. Samples were centrifuged (4 °C, 10,000× g, 15 min), collecting the post mitochondrial supernatant (PMS) used to determine the en-

zymatic activities (acetylcholinesterase (ChE), lactate dehydrogenase (LDH), glutathione S-transferase activity (GST), and glutathione peroxidase activity (GPx)). Enzyme activities were analyzed in 96-well microplates, in triplicate, by spectrophotometric methods (Thermo Scientific Multiskan Spectrum, USA). The ChE activity was determined according to Ellman's method [73] adapted to a microplate [74], at 25 °C and 412 nm. GST activity was determined by the method of Habig et al. (1974) [75] adapted to the microplate. Absorbance was recorded at 340 nm (25 °C). LDH activity was measured according to the methodology described by Vassault (1983) [76], adapted to microplate by Diamantino et al. (2001) [77]. GPx activity was assayed according to the method described by Mohandas et al. (1984) [78] with some modifications for adaptation to microplates. Enzyme activity was quantified at 340 nm (25 °C).

2.6. Statistical Analysis

SigmaPlot V.14.0 for Windows was used for statistical analyses. The normality (Shapiro-Wilk test) and homogeneity of variances (Brown-Forsythe test) were tested. For the analysis of locomotor activity and thigmotactic behavior, a Two-Way ANOVA was used to detect differences in total swimming distance (concentration vs. condition as factors) and for the distance traveled in the outer zone (concentration vs. condition as factors). One-Way ANOVA (Holm–Sidak method) was used to analyze the data from the novel tank test, social test, and biochemical responses (Dunnett's Method). When there was no normality of data, Kruskal–Wallis One Way Analysis was used. The level of significance for all statistical analyzes was set to 0.05.

3. Results

3.1. Locomotor Activity and Thigmotactic Behavior

After 12 days of exposure to fluoxetine, no differences across treatments were observed in terms of total distance moved during light and dark periods (Figure 1A). On the other hand, differences in the time spent swimming was observed between light and dark conditions for control, 0.1 and 1 µg/L of fluoxetine, with fish swimming for longer periods during the light period (Figure 1B). Furthermore, fluoxetine significantly increased fish swimming time during the dark period at concentrations 1 and 10 µg/L (Two-Way ANOVA, 1 µg/L: $p = 0.002$; 10 µg/L: $p = 0.003$) (Figure 1B). In terms of thigmotactic behavior, no significant differences were observed between dark and light conditions in the total distance traveled in the outer zone (Figure 1C). When considering the time spent in the outer zone, the control group presented significant differences in response to dark and light conditions (Two-Way ANOVA, Control: $p = 0.003$), spending more time in the outer zone during the light period. However, this trend was not observed in fish exposed to fluoxetine, where no differences were observed during dark and light periods (Figure 1D). After 21 days exposure, fish exposed to all fluoxetine concentrations showed significant decreases in the swimming activity during the light period, when compared to control (Two-Way ANOVA, 0.1 µg/L: $p = 0.022$; 1 µg/L: $p = 0.010$; 10 µg/L: $p = 0.041$) (Figure 2A). In terms of total time spent swimming, fish spent more time swimming during the light period (as observed at 12 days exposure), a pattern that was not altered by fluoxetine exposure (Figure 2B). Both parameters analyzed for fish thigmotactic behavior (total distance and time spent in the outer zone) remained similar across dark and light conditions as well as across fluoxetine treatments (Figure 2C,D).

The measurement of the angles during the fish path, after 12 days exposure, showed that the frequency of class 1 angles (high amplitude angles) was much higher during the light, where all treatments, including the control, showed > 2-fold more frequency than that during the dark period (Figure 3A). Contrary to class 1 angles, class 4 angles (low amplitude angles) were significantly more frequent during the dark period (Figure 3B). However, no effect was observed for class 1 and class 4 angle frequency. After 21 days of exposure, profiles of turn angle frequencies, across dark and light periods were similar to those observed at day 12, and not affected by fluoxetine exposure (Figure 3C,D). In the

remaining classes of angles, no effects of fluoxetine exposure were observed after 12 and 21 days of exposure (data not shown).

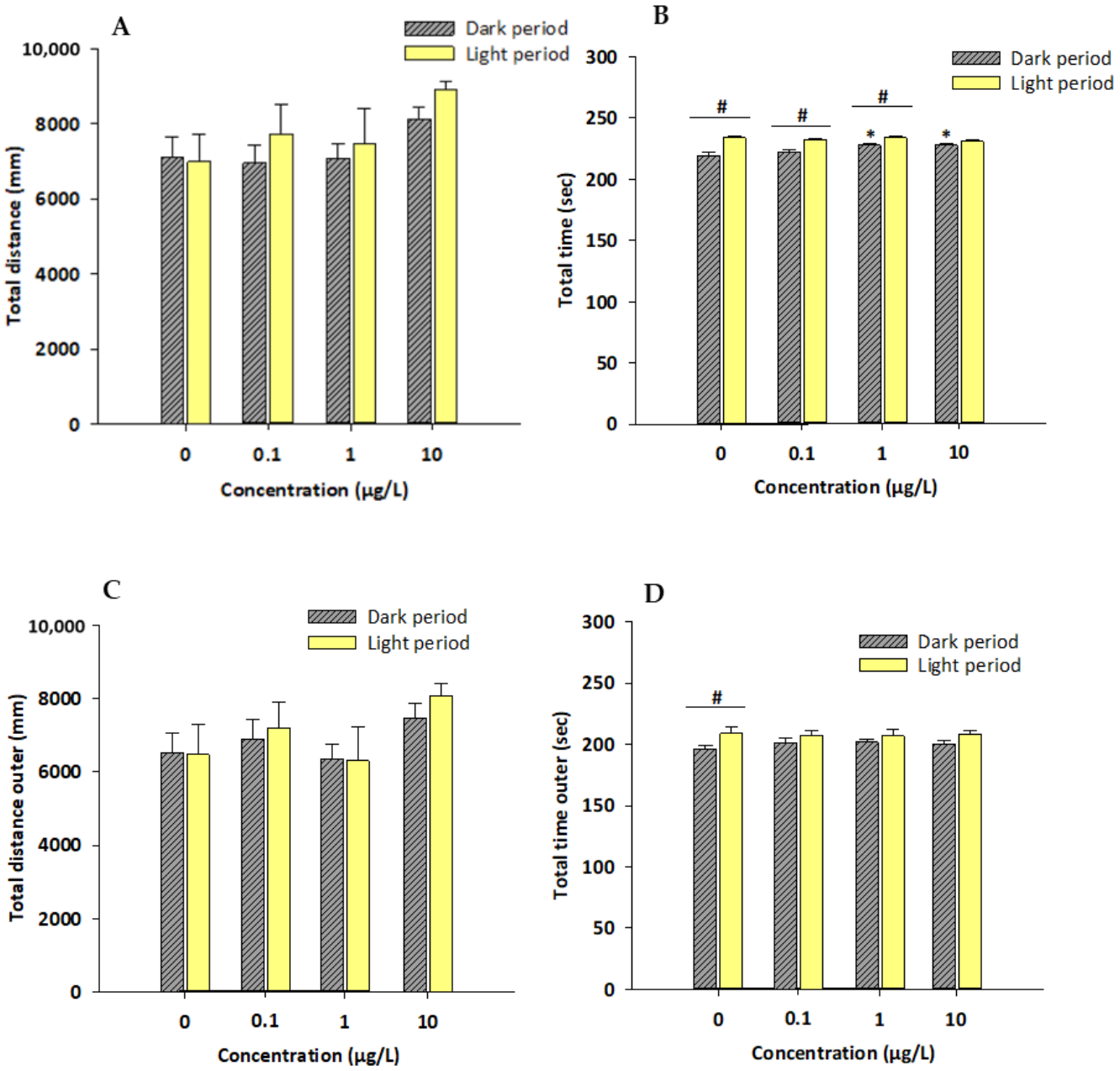

Figure 1. Effects of fluoxetine concentrations on locomotor activity and thigmotactic behavior of juvenile zebrafish, in response to dark and light cycles, after 12 days exposure. (**A**)—Total swimming distance traveled by the juvenile zebrafish; (**B**)—Total time spent swimming; (**C**)—Total distance traveled in the outer zone; (**D**)—Total time spent swimming in the outer zone. Results are expressed as mean values ± standard error (Supplementary Material, Table S1). Yellow bars represent the light period and grey bars represent the dark period. Asterisks (*) indicate differences towards the control and the symbol "#" indicates differences between dark and light periods in their respective concentrations (Two-way ANOVA, Holm–Sidak method, $p < 0.05$).

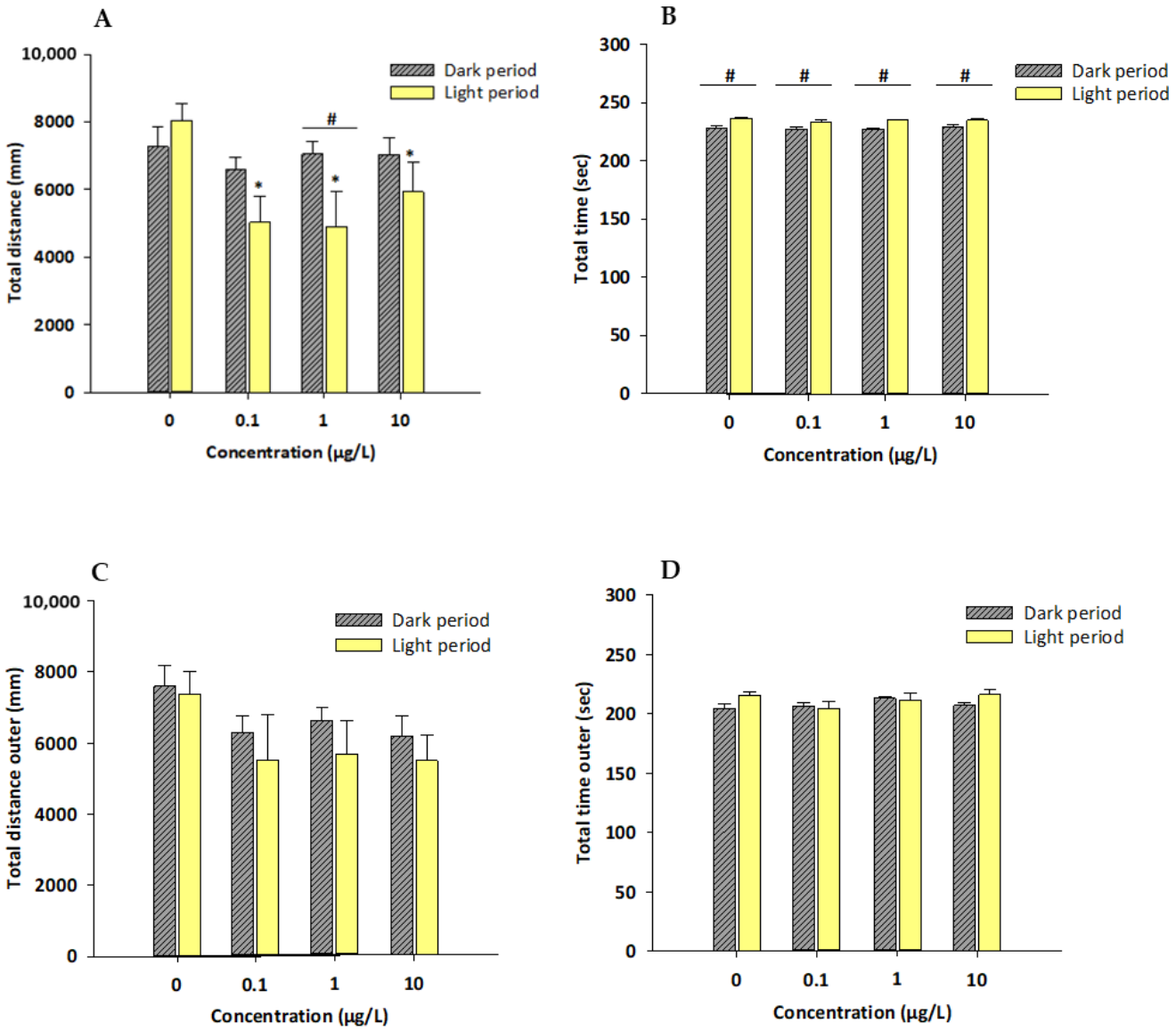

Figure 2. Effects of fluoxetine concentrations on locomotor activity and thigmotactic behavior of zebrafish, in response to dark and light cycles, after 21 days exposure. (**A**)—Total swimming distance traveled by the juvenile zebrafish; (**B**)—Total time spent swimming; (**C**)—Total swimming distance traveled in the outer zone; (**D**)—Total time spent swimming in the outer zone. Results are expressed as mean values ± standard error (Supplementary Material, Table S2). Yellow bars represent the light period and grey bars represent the dark period. Asterisks (*) indicate differences towards the control and the symbol "#" indicates that the dark and light periods are different from each other in their respective concentrations (Two-way ANOVA, Holm–Sidak method, $p < 0.05$).

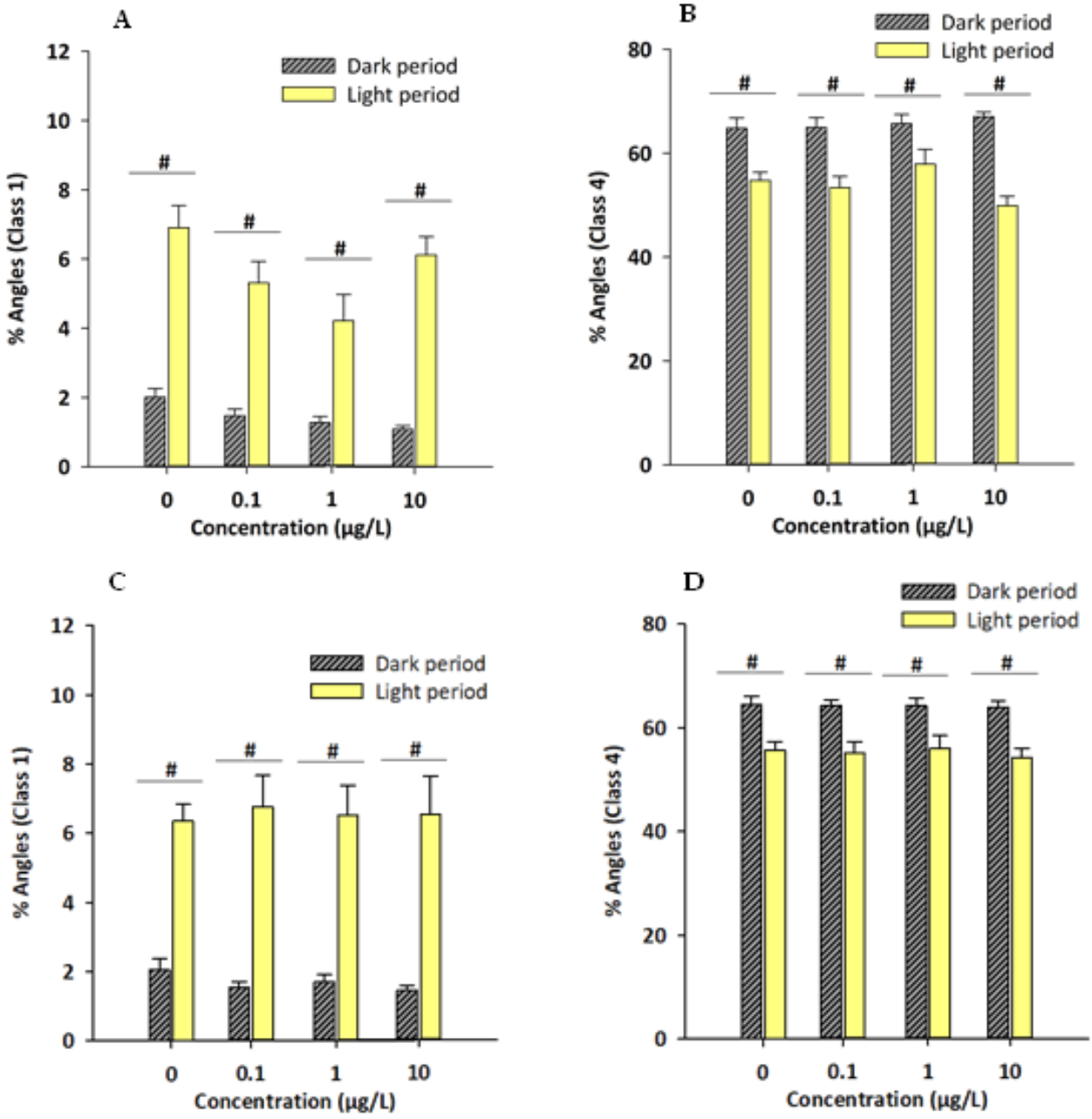

Figure 3. Percentage of frequency of class 1 and 4 angles (mean values ± standard error (Supplementary Material, Table S3)) after 12 (**A**,**B**) and 21 days (**C**,**D**). Yellow bars represent the light period and grey bars represent the dark period. Asterisks (*) indicate differences towards the control and the symbol "#" indicates that the dark and light stimuli are different from each other in their respective concentrations (Two-way ANOVA, Holm–Sidak method, $p < 0.05$).

3.2. Novel Tank/Exploratory Swimming Test

In the novel tank test, the time fish spent swimming in the three areas was not affected by fluoxetine exposure (Figure 4) (One-Way ANOVA, Bottom: $p = 0.145$; Middle: $p = 0.246$; Top: $p = 0.398$). This test was performed only after 21 days of exposure to minimize the stress induced by human handling.

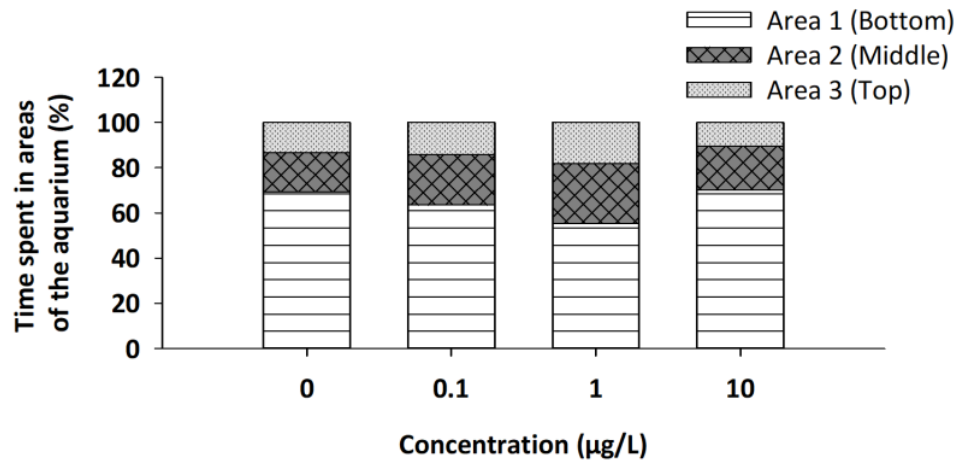

Figure 4. Effects of fluoxetine concentrations on zebrafish exploratory behavior (swimming behavior at bottom, middle, and top layers of the tank), after 21-days exposure. Results are expressed as a percentage of time spent in tank layers (Supplementary Material, Table S4).

3.3. Social Test

Fish exposed to fluoxetine and control fish seem to spend more time in the area near the shoal (area 1) (Figure 5), with no significant changes due to fluoxetine exposure (One-Way ANOVA, Zone 1: $p = 0.632$; Zone 2: $p = 0.654$; Zone 3: $p = 0.832$). This test was also performed only after 21 days of exposure to minimize the stress induced by human handling.

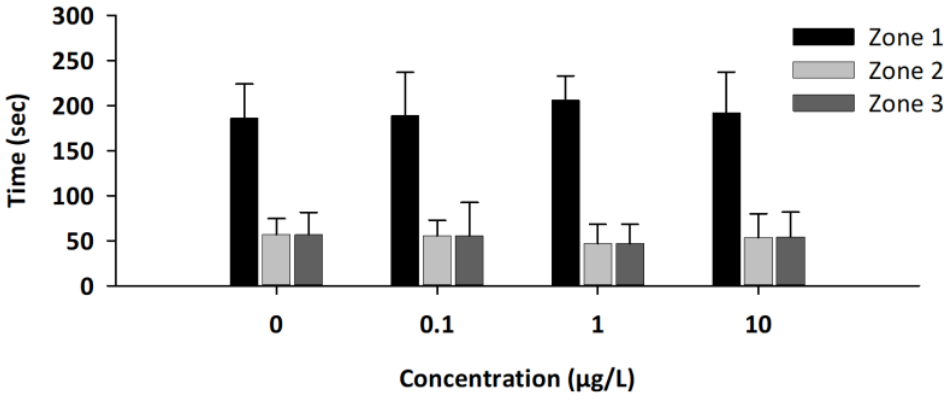

Figure 5. Effects of fluoxetine concentrations on the social behavior of zebrafish, after 21-days exposure, discriminated between zone 1—Proximity Zone, zone 2—Neutral zone near the shoal, and zone 3—Neutral zone away from the shoal. Results are expressed as mean values ± standard error (Supplementary Material, Table S5).

3.4. Biochemical Analysis

The 21-days exposure to fluoxetine slightly decreased GST activity, at the highest concentration, although non-significantly (One-Way ANOVA, $p = 0.101$) (Figure 6A). A similar response was observed for GPx activity at 1 and 10 µg/L, with significant differences, only observed for the latter concentration (One-Way ANOVA, 10 µg/L: $p = 0.015$) (Figure 6B). No effects were observed for ChE and LDH activities (Figure 6C,D).

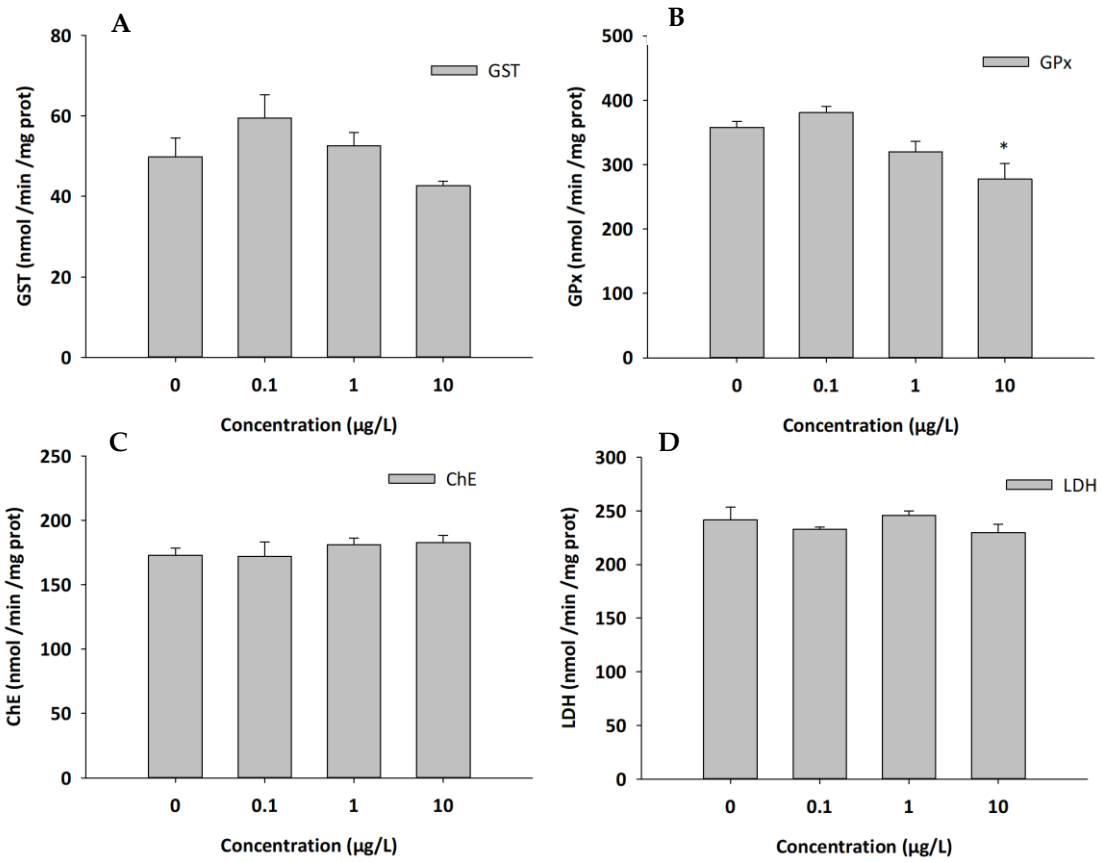

Figure 6. Effects of fluoxetine concentrations on zebrafish enzymatic activities: (**A**)—Glutathione S-transferase (GST) activity; (**B**)—Glutathione peroxidase (GPx) activity; (**C**)—Cholinesterase (ChE) activity; (**D**)—Lactate Dehydrogenase (LDH) activity. Results are expressed as mean values ± standard error (Supplementary Material, Table S6). Asterisks (*) indicate differences towards the control (One Way ANOVA, Dunnett's Method, $p < 0.05$).

4. Discussion

The present study evaluated different behavior endpoints associated with anxiety/stress response (locomotion, thigmotaxis, and exploratory behavior), sociability (social test), and biochemical changes associated with neurotransmission, biotransformation, and oxidative stress, after chronic exposure of juvenile zebrafish to the SSRI antidepressant fluoxetine. In general, the data showed that, in the low µg/L range, fluoxetine can cause mild biological alterations in the tested endpoints affecting fish locomotor activity which, in the long term, may compromise population fitness. The concentrations tested in this study are considered environmentally relevant, and fluoxetine levels similar to those tested in the present study, and even lower, have already been detected in the environment. For example, in Baiyangdian Lake, Pearl River, Songhua River, Yellow River, Huai River, Hai River, and Liao River, fluoxetine levels up to 0.101 µg/L were detected [79,80].

Alternating light and dark changes are often used to assess the stress response in zebrafish larvae, where, larvae respond to the shift from light to dark with a burst of activity [81,82]. However, this assessment is scarcely used in juvenile fish. In the present study, after 12 days of exposure to fluoxetine, zebrafish juveniles altered swimming time during the dark period at the highest concentrations. Curiously, the total distance traveled during this period was unaffected by fluoxetine, which indicates a decrease in swimming velocity and, therefore, a lower effect on the stress reaction to this condition. After 21 days of exposure, the change in locomotor activity during the light period became more evident. In a study carried out by Zindler et al. (2020) [60], fluoxetine reduced the swimming distance of embryos in the dark and changed the maximum speed during light periods, after 96 h

exposure. Thigmotaxis is seen as the tendency of an animal placed in a new environment, to remain close to the walls and avoid the center of the aquarium. In fish, the persistence of this activity in the outer zone can be considered a measure of anxiety [69,83,84]. Fluoxetine did not affect this behavior following 21 days of exposure.

Regarding the measurement of angles during the fish's path, the high amplitude angles (class 1) showed no effects of fluoxetine exposure, after 12 and 21 days of exposure. High amplitude angles are indicators of erratic swimming behavior (stress behavior) and the decrease in its frequency manifests the anxiolytic effect of fluoxetine on the stress behavior of exposed juveniles.

The novel tank test aims to assess the exploratory behavior in zebrafish, focusing on measures of "vertical" behavior patterns. This test explores the tendency of a fish to dive and stay at the bottom of a new environment (geotaxis), before exploring the upper areas. This test is used extensively to assess the effects of a wide variety of compounds, including pharmacological chemicals [69,70]. Drugs with antidepressant and anxiolytic properties have the ability to modify this behavior, consequently leading the fish to explore the new space sooner and spend less time in the bottom [71,85,86]. In the present study, there was no evidence of effects over fish geotaxis behavior. Similar to this study, Marcon et al. (2016) [87] also failed to observe any changes in fish behavior to a novel environment following 7 days of exposure to 10 µg/L of fluoxetine. On the other hand, other studies, using embryos and adult zebrafish, and adult *Oryzias latipes* (Medaka), have reported effects of fluoxetine over their locomotion parameters in concentrations ranging from 0.01 µg/L to 10,000 µg/L, and exposure periods ranging from 3 min to 30 days. These studies indicate that fluoxetine decreases stress levels and increases fish exploratory behavior, with longer swimming periods at the top of the tank. In addition, there is also a decrease in freezing, lower latency to enter the top area, and an increase in the number of entries into the top area [5,9,14–16,49,51,56–58,88]. All these parameters indicate that fluoxetine reduces anxiety and stress levels in the fish brain system, resulting in more relaxed behavior.

The zebrafish is a social fish, living in shoals in its natural habitat [51,86,89–91]. This social interaction minimizes the risk of predation and if an organism is isolated, it can trigger anxiety-like behavior [92], reducing serotonin levels in the nervous system [93]. The change in social behavior can impact the reproduction and survival of the species. In this study, chronic fluoxetine exposure did not alter fish social behavior. However, other studies, for example, Giacomini et al. (2016) [51] found that a 15-min exposure to 50 µg/L fluoxetine decreased social interaction in adult zebrafish, with animals spending less time near the shoal.

Biochemical biomarkers are considered an alteration in a biological response, being sensitive measures of molecular, biochemical, and cellular interactions, capable of signaling the effects of exposure to environmental chemical compounds and often used as indicators of exposure and effects in ecotoxicology studies [94–98]. Previous studies have observed the effects of fluoxetine on the antioxidant defenses of fish [43,44]. In this work, there was a reduction in antioxidant responses (GPx) only at the highest concentration. In the literature, there are reports of a decrease/inhibition of GST activity. In juveniles of *Argyrosomus regius*, a 15 days exposure to 0.3 and 3 µg/L fluoxetine resulted in GST activity inhibition [43]. *Pseudorasbora parva* juveniles exposed for 42 days to 200 µg/L fluoxetine also displayed GST inhibition in the liver and gills [44]. The reduction in the activity of biotransformation enzymes could be related to the negative regulation of genes involved in the detoxification pathways, as observed by Cunha et al. (2016) [40]. The effects of fluoxetine on antioxidant responses and biotransformation processes have been widely reported, with increased or decreased activity of enzymes belonging to these groups (CAT, SOD, GSH/GSSH, LPO/MDA, GST, and EROD activity). The different fluoxetine concentrations tested, exposure lengths, species, and life stages tested may be related to the variability of responses [40–44,46]. However, all these studies evidence and support the potential of fluoxetine to promote long-term biochemical effects.

5. Conclusions

In this work, the effects of low concentrations of fluoxetine in juvenile zebrafish were evaluated, at the behavioral and biochemical levels. The main effects included the alteration of the locomotor activity in the light period. The low impact at the biochemical level suggests that chronic exposure to high concentrations of fluoxetine could interfere with the antioxidant pathway. These results are important and clearly demonstrate the potential of fluoxetine to alter normal behavioral patterns in the juvenile stages of fish, and therefore, this scenario needs to be considered in environmental risk assessments.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/app12042256/s1. Table S1: Mean values for locomotor activity and thigmotactic behavior, after 12 days exposure, in all concentrations tested; Table S2: Mean values for locomotor activity and thigmotactic behavior, after 21 days exposure, in all concentrations tested; Table S3: Mean values for path angles (class 1 and 4), after 12- and 21-days exposure; Table S4: Percentage of time spent in tank layers (area 1 (bottom), area 2 (middle) and area 3 (top)); Table S5: Mean values for social test in zone 1 (proximity zone), zone 2 (neutral zone near the shoal) and zone 3 (neutral zone away from the shoal); Table S6: Mean values for biochemical analysis (glutathione S-transferase activity (GST), glutathione peroxidase activity (GPx), acetylcholinesterase (ChE) and lactate dehydrogenase (LDH) activity).

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Institutional Review Board Statement: All experimental procedures followed International Guiding Principles for Biomedical Research Involving Animals (EU 2010/63) and were previously approved by the ethics committee and the responsible national legal authority "Direção Geral de Alimentação e Veterinária".

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Conflicts of Interest: The authors declare no conflict of interest.

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