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Research paper

Exposure to tricyclic antidepressant nortriptyline affects early-life stages of zebrafish *(Danio rerio)*

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ABSTRACT

Psychiatric drugs are among the leading medications prescribed for humans, with their presence in aquatic environments raising concerns relating to potentially harmful effects on non-target organisms. Nortriptyline (NTP) is a selective serotonin-norepinephrine reuptake inhibitor antidepressant, widely used in clinics and found in environmental water matrices. In this study, we evaluated the toxic effects of NTP on zebrafish (*Danio rerio*) embryos and early larval stages. Developmental and mortality analyses were performed on zebrafish exposed to NTP for 168 h at concentrations ranging from 500 to 46,900 μg/L. Locomotor behaviour and acetylcholinesterase (AChE) activity were evaluated by exposing embryos/larvae to lower NTP concentrations (0.006–500 μg/L). The median lethal NTP concentration after 168 h exposure was 2190 μg/L. Although we did not identify significant developmental changes in the treated groups, lack of equilibrium was already visible in surviving larvae exposed to ≥ 500 μg/L NTP. The behavioural analyses showed that NTP was capable of modifying zebrafish larvae swimming behaviour, even at extremely low (0.006 and 0.088 μg/L) environmentally relevant concentrations. We consistently observed a significant reduction in AChE activity in the animals exposed to 500 μg/L NTP. Our results highlight acute toxic effects of NTP on the early-life stages of zebrafish. Most importantly, exposure to environmentally relevant NTP concentrations may affect zebrafish larvae locomotor behaviour, which in turn could reduce the fitness of the species. More studies involving chronic exposure and sensitive endpoints are warranted to better understand the effect of NTP in a more realistic exposure scenario.

1. Introduction

Pharmaceuticals are widely prescribed around the world and their presence in aquatic environments has gained increased attention in recent years due to concerns about negative effects on non-target organisms (Ross et al., 2012; aus Der Beek et al., 2016). In Brazil, pharmaceutical consumption has increased considerably in recent years, particularly antidepressants (Pivetta et al., 2020). Psychiatric pharmaceuticals have been detected in water bodies in many countries, and are currently classified as emerging pollutants (Calisto and Esteves, 2009; Ojemaye and Petrik, 2019). Dong et al. (2013) proposed a risk-prioritising ranking to identify drugs posing a potential risk to humans and aquatic organisms, in which antidepressants ranked seventh.

A large number of antidepressants have already been identified in multiple water matrices (wastewater, surface water, groundwater, drinking water) (Metcalfe et al., 2010; Lajeunesse et al., 2012; Ma et al., 2018), sludge (Walters et al., 2010; Niemi et al., 2013), and biological tissues of aquatic organisms (Lajeunesse et al., 2011; Arnnok et al., 2017). Antidepressants are known for their specific mode of action and many studies have shown that this type of pharmaceutical may induce side-effects in aquatic organisms, including reduction in aggressiveness,

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disturbance of fertility and development, and inhibition of feeding activity (Henry and Black, 2008; Sánchez-Argüello et al., 2009; Arnnok et al., 2017).

Nortriptyline (NTP) is a tricyclic antidepressant (TCA) used to treat a range of conditions including depression, anxiety disorders, eating disorders and pain syndromes (Haviv et al., 2019). NTP is classified as a serotonin-norepinephrine reuptake inhibitor (SNRI) as it prevents presynaptic reabsorption of serotonin and leads to an increase in postsynaptic receptor activation (Merwar et al., 2020). NTP, together with other TCAs, can be removed in wastewater treatment plants by adsorption onto activated sludge, with a removal efficiency of ~75% (Baker and Kasprzyk-Hordern, 2013; Choi et al., 2018). Since the removal is incomplete, this allows a constant flow of TCAs into the aquatic environment (Aminot et al., 2015; Wu et al., 2015; Choi et al., 2018). The persistence of NTP in aquatic matrices may be due to its limited hydrolysability and biodegradability (Choi et al., 2018). NTP has been detected in water matrices in Brazil and a number of European countries (Greece, Spain and the UK) ranging from 0.04 to 185.8 ng/L (Baker and Kasprzyk-Hordern, 2013; Borova et al., 2014; Ziarrusta et al., 2016; Pivetta et al., 2020).

To date, there is only 1 study that evaluated the potential negative effects of NTP on a non-target aquatic organism (common carp) (Sehonova et al., 2017). The authors evaluated 3 concentrations (10, 100 and 500 μg/L) of 3 TCAs: NTP, amitriptyline and clomipramine. Results suggested that NTP and its mixtures may impair early-life stages of fish, increase mortality, and induce morphological anomalies, histopathological changes and lipid peroxidation (Sehonova et al., 2017). Due to scarce literature, more information about the toxicological effects of NTP exposure is needed, especially in fish.

Danio rerio (zebrafish) is widely used as a model organism in toxicological and environmental health studies (Bambino and Chu, 2017). Interestingly, zebrafish possess all the classic neurotransmitters, including serotonergic and cholinergic systems, making this animal suitable for the study of substances that act on such systems, for instance antidepressants (Panula et al., 2006; Yamamoto et al., 2011). Recently, de Farias et al. (2019) showed that low fluoxetine concentrations impair acetylcholinesterase activity ($\geq$ 0.88 μg/L) and larval zebrafish swimming behaviour ($\geq$ 6 μg/L). Furthermore, bupropion, a norepinephrine-dopamine reuptake inhibitor, was reported to affect zebrafish embryonic development (hatching delay, oedemas and tail deformities) in concentrations > 44 mg/L (Franco et al., 2019).

In this context, the present study aimed to evaluate the toxicity of NTP on zebrafish embryos and larvae using an integrated approach with multiple endpoints (mortality, embryo development, acetylcholinesterase activity and locomotor behaviour), which included environmentally relevant NTP concentrations and sensitive parameters with ecological relevance.

2. Materials and methods

2.1. Chemical and HPLC analysis

Nortriptyline (NTP), Empirical formula: $C_{19}H_{21}N.HCl$, was acquired from C&C Pharmaceuticals (Amapá, Brazil)). NTP sample solutions were maintained under the same environmental conditions as the toxicity tests (SL-24, Solab, Piracicaba, SP, Brazil). To assess NTP stability, sample solutions were analysed daily over a 7-day period by high-performance liquid chromatography (HPLC Prominence, Shimadzu, Kyoto, Japan) following the method reported by Gupta et al. (2010). The HPLC analysis experimental description is provided in the Supplementary Material.

2.2. Test organisms

Zebrafish adults were maintained in tanks with reverse osmosis and activated carbon-filtered water, complemented with salt (Premium Reef Salt, Omega Sea, Sitka, AK, USA), at the Department of Genetics and Morphology, University of Brasília (UnB), Brazil. The animals were raised in the ZebTec housing system (Tecniplast, Buguggiate, Varese, Italy) with a 12:12 h light-dark cycle. The water parameters were strictly maintained: 27.0 ± 1 °C, 650 ± 100 μS/cm, pH 7.0 ± 0.5% and 95% dissolved oxygen saturation. These conditions were maintained for all tests performed.

Zebrafish eggs were obtained by breeding in the iSpawn breeding system (Tecniplast). The day prior to breeding, males and females were sequentially added to the system and separated by a divider, at a ratio of 2 males:1 female. The divider was removed early the next morning and the spawning platform lifted to initiate spawning. The eggs were collected immediately after natural mating, rinsed in water, and checked using a stereomicroscope (Stemi 2000, Carl Zeiss, Jena, Germany). Unfertilised eggs (< 20%), together with those displaying cleavage irregularities or injuries, were discarded.

All experimental procedures were performed in accordance with the International Guiding Principles for Biomedical Research Involving Animals. This study was approved by the University of Brasilia Ethics Committee under Protocol No. 100226/2014.

2.3. Fish embryo toxicity test

The fish embryo toxicity (FET) test was performed based on OECD (Organization for Economic Co-operation and Development) guideline Protocol 236 (OECD, 2013), with adaptations described by de Farias et al. (2019), to provide an overview of NTP toxicity. According to the results of pre-tests, 7 different concentrations (500; 1100; 2300; 4800; 10,300; 22,000; and 46,900 μg/L) were chosen and prepared by successive dilutions of the NTP stock solution. An external control group was also used in accordance with the OECD guidelines. The test was conducted using 60 eggs per treatment, divided into 3 replicates, which were selected and distributed in 24-well microplates. Twenty wells were filled up with 2 mL of the test solution and 4 wells with water (internal plate control, as specified in the OECD guideline).

The test was initiated up to 90 min post fertilisation and lasted for 168 h. Embryos and larvae were kept in a climate chamber (SL-24, Solab, Piracicaba, SP, Brazil) to maintain optimal experimental conditions and were only removed for daily stereomicroscopic analysis. Additionally, solutions were renewed once after 96 h to ensure water quality and the presence of the NTP molecule for the entire exposure period. Before hatching, the following parameters were evaluated: egg coagulation, lack of otolith formation, general delay in development, lack of eye and/or body pigmentation, lack of somite formation, lack of heartbeat, oedemas, lack of detachment of the tail-bud from the yolk sac, lack of yolk sac absorption, and lack of hatching. After hatching, spine malformation, oedema, swim bladder inflation and lack of equilibrium (indicated by living hatching embryos side-lying at the bottom of the microplate well and lack of response to mechanical stimuli) were also examined. All parameters were assessed and quantified as observed or not observed.

2.4. Locomotor behaviour assay

To analyse zebrafish locomotor responses, a wide range of concentrations comprising more realistic environmental scenarios were selected (0.006; 0.088; 1.58; 28.12; 500 μg/L). These concentrations were chosen based on the FET results and previous literature (Baker and Kasprzyk-Hordern, 2013; Borova et al., 2014; Ziarrusta et al., 2016; Pivetta et al., 2020). Zebrafish embryos were distributed in 96-well plates (1 per well) and exposed under similar conditions as those described in Section 2.3 (FET). Locomotion was evaluated for a total of 48 embryos per treatment (3 replicates of 16 embryos), including the control, after 120, 144 and 168 h of NTP exposure. Prior to the behavioural assessment, dead larvae or larvae exhibiting physical abnormalities were discarded and not considered in the analyses.

Larvae movement was evaluated using the ZebraBox (ZEB 478, ViewPoint Life Sciences, Lyon, France) tracking system equipped with a 25-frame-per-second infrared camera over a 20-min period. Temperature stability was maintained at 26 ± 1 °C. Movement was stimulated by applying light:dark intervals as previously described by Irons et al. (2010). Briefly, the test consisted of acclimatising the embryos in the light for 5 min, followed by a 10-min dark period and another 5-min light period. For each replicate, the distance moved and time spent moving at 1-min intervals were recorded for each dark period. The behavioural endpoints measured included: total swimming distance (TSD), total swimming time (TST), and the percentage of slow (SM) and fast (FM) movements. The relative SM (%) is a proportion between slow and total distance moved in each measurement period, while the relative FM (%) is a ratio between fast and total distance moved in each measurement period. A threshold of 5 mm/s was employed to differentiate SM from FM (Franco et al., 2019).

2.5. Biochemical biomarker analysis

To analyse the activity of acetylcholinesterase (AChE), a neuroendocrine biomarker, toxicity tests with zebrafish embryos were performed using the same range of sub-lethal concentrations used in the locomotor behaviour test: 0.006; 0.088; 1.58; 28.12, and 500 μg/L. Tests were performed in 1 L beakers filled with 500 mL of test solution and 250 eggs. After 120, 144 and 168 h of incubation, 10 pools of 15 viable hatched embryos/larvae per concentration were collected into microtubes with 0.5 mL K-phosphate buffer (0.1 M, pH 7.4), frozen in liquid nitrogen and immediately stored at − 80 °C until the day of analysis. Prior to the AChE enzymatic activity measurement, samples were defrosted on ice, homogenised using a sonicator (Ultrasonic Cleaner 2840D, Odontobrás, Ribeirão Preto, SP, Brazil), and centrifuged for 20 min at 10,000 *g* (Mikro 220R, Hettich, Tuttlingen, Germany). The resulting post-mitochondrial supernatant (PMS) was isolated and 40 μL of each sample pipetted into 96-well microplates for enzymatic determination (Jesus et al., 2013).

AChE activity was determined using acetylthiocholine (ASCh) as substrate, measuring the conjugation product between thiocholine (result of the degradation of ASCh) and 5,5-dithiobis-2-nitrobenzoic acid (DTNB) (absorbance increase) at 414 nm, every 20 s, for 5 min, according to the method previously described by Ellman et al. (1961). For enzymatic determination, 40 μL of PMS and 250 μL of the reaction mixture: 75 mM acetylcholine and 10 mM DTNB in K-phosphate buffer (0.1 M, pH 7.2) were used. Acetylcholinesterase activity was expressed as micromoles of substrate hydrolysed per minute per mg of protein (U). Sample protein concentration was quantified using the Bradford Method (at 595 nm) and γ-globulin as the standard (Bradford, 1976). All reactions were performed in quadruplicate and measured spectrophotometrically using a SpectraMax M2 microplate reader (Molecular Devices, Sunnyvale, CA, USA).

2.6. Statistical analysis

Lethal and effective concentrations (LC and EC) were estimated by nonlinear regression analyses (sigmoidal curves). The data were tested for normality and homogeneity of variance using Kolmogorov–Smirnov and Levene's tests, respectively. Significant differences ($p < 0.05$) between the concentrations tested and the control were determined using a one-way ANOVA (with Dunnett's post hoc) for parametric data (lack of equilibrium at 96 and 144 h after NTP exposure and AChE activity), and Kruskal–Wallis test (with Dunn's post hoc) for non-parametric data (locomotor behaviour and lack of equilibrium at 120 and 168 h after NTP exposure). All statistical analyses were carried out using the SigmaPlot 12.5 software (Systat Software, San Jose, CA, USA).

3. Results

3.1. Chemical and HPLC analysis

The HPLC analysis showed that NTP slowly degraded over the course of days (Fig. S1; Tables S1 and S2). After 168 h, a decrease of only ~9% was observed for 25,000 μg/L of NTP solution (Table S2).

3.2. Fish embryo toxicity test

The cumulative percentage of zebrafish embryo/larvae mortality after 168 h exposure to 7 NTP concentrations (from 500 to - 46,900 μg/L) is displayed in Fig. 1. The 2 highest NTP concentrations (22,000 and 46,900 μg/L) were completely lethal to the embryos after 48 h exposure. All embryos at these concentrations died before hatching (Fig. 1). For the 2300, 4800 and 10,300 μg/L NTP concentrations, mortality was progressive, increasing with exposure time (Fig. 1). At 4800 μg/L NTP, almost all embryos hatched after 72 h, but no organism survived after 144 h. Likewise, for 10,300 μg/L NTP, the hatching rate was ~50% after 72 h, however, the mortality rate reached 100% after 120 h exposure. On the other hand, the lower NTP concentrations (500 and 1100 μg/L) and the control presented similar profiles, with very low death rates after 168 h (Fig. 1). As expected, the LC_{50} calculations (the exposure concentration resulting in 50% mortality) indicated that embryos/larvae became more sensitive to NTP as the exposure time increased; thus we observed the lowest LC_{50} (2190 μg/L) after 168 h (Table 1).

Changes in hatching and developmental formation (such as tail formation, yolk sac absorption, spine malformation, among others, as described in material and methods) showed no significant difference ($p > 0.05$) between the groups treated with NTP compared with the control (data not shown). Fig. 2A and B shows normal embryo and larvae with no apparent morphological malformations, respectively. The only noteworthy sub-lethal parameter was the lack of equilibrium (Fig. 2C and D and Fig. 3). Larvae with lack of equilibrium presented both non-inflated (Fig. 2C) and inflated swim bladders (Fig. 2D).

After 96 h of NTP exposure, equilibrium alterations were already observed in 1100, 2300 and 4800 μg/L NTP samples when compared to the control ($p < 0.001$) (Fig. 3). At the lowest concentration (500 μg/L), larvae with lack of equilibrium were observed after 144 h exposure in comparison to the control ($p < 0.001$). Although not statistically significant, increased equilibrium alterations were also identified after 120 h exposure to all 4 of the lower concentrations tested (Fig. 3). Similarly, after 168 h, there was an increase in the larvae with altered equilibrium at NTP concentrations: 500, 1100 and 2300 μg/L, however, the data was only significant for 1100 μg/L ($p < 0.05$) when compared to the control group. The EC_{50} of the lack of equilibrium was calculated (Table 1). The EC_{50} for the lack of equilibrium was 1247 μg/L after 96 h, 24.4 times lower than the LC_{50} for the same exposure time.

3.3. Locomotor behaviour analysis

The ZebraBox results indicated that NTP exposure induced changes in zebrafish larvae swimming activity in dark periods (Fig. 4 and Fig. 5). Regarding TSD, larvae exposed to 500 μg/L NTP showed a significantly lower total distance moved than the control group ($p < 0.05$) after 120, 144 and 168 h (Fig. 4A–C). A significant decrease in TSD was also observed in organisms exposed to 28.12 μg/L NTP after 144 and 168 h ($p < 0.05$), when compared to the control (Fig. 4B and C). In contrast, a slight, but significant increase in TSD was observed in animals exposed to 0.088 μg/L NTP after 144 h in comparison to the control group ($p < 0.05$) (Fig. 4B). TST was also analysed and a significant reduction was observed in comparison to the control for larvae exposed to 28.12 μg/L NTP after 144 and 168 h ($p < 0.05$), and 500 μg/L NTP after 120 and 144 h ($p < 0.05$) (Fig. 4D–F). Interestingly, we also detected a significant TST decrease in animals exposed to the lowest NTP concentration (0.006 μg/L) after 144 h in comparison to the control group

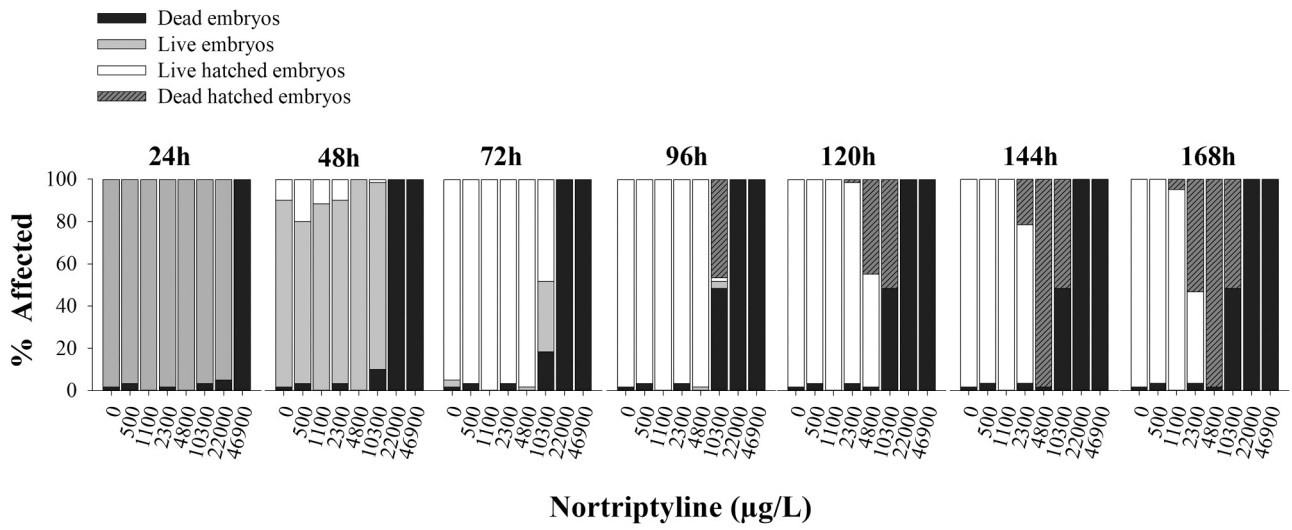

Fig. 1. Overview of nortriptyline (NTP) effects on zebrafish early-life stages during 168 h exposure. The proportion of dead, alive and hatched organisms is represented by the different coloured bars.

Table 1
Effects of nortriptyline (NTP) on zebrafish embryos development after 168 h of exposure. EC_{50} and LC_{50} values were calculated using nonlinear regression analyses (standard errors in brackets). Values are presented in µg/L.

Time (h)	L(E)C_{50}	95% confidence interval
Mortality		
24	30,369 (0[a])	n.d
48	13,853 (1.9)	9800–17800
72	13,141 (1.6)	9600–16600
96	7970 (0[a])	n.d
120	4920 (0.08)	4700–5100
144	3000 (0.1)	2200–3000
168	2190 (0.03)	2100–2200
Lack of equilibrium		
96	1247 (81.3)	1070–1424
120	552 (0[a])	n.d
144	432.2 (13.8)	400–463
168	390.3 (0[a])	n.d

[a] parameter not measured - values are indicative only; n.d. endpoint not determined.

($p < 0.05$) (Fig. 4E).

Larvae behaviour, in terms of slow and fast movements, also changed after NTP exposure (Fig. 5). We observed a reduction in SM and an increase in FM in the animals exposed to the lowest NTP concentration (0.006 µg/L) after 120, 144 and 168 h, when compared to the control ($p < 0.05$) (Fig. 5A–F). A significant reduction in SM (Fig. 5A) and an increase in FM (Fig. 5D) were also detected at 0.088 µg/L, in comparison to the control ($p < 0.05$), but only after 120 h exposure. At 500 µg/L NTP, an increase in SM (Fig. 5C) and a reduction of FM (Fig. 5F), in comparison with the control ($p < 0.05$), were observed uniquely after 168 h exposure. Interestingly, we observed a significant reduction in SM at 28.12 µg/L, (Fig. 5A) and an increase in FM (Fig. 5D), compared to the control ($p < 0.05$) after 120 h, and the opposite pattern (increase in SM and reduction in FM) after 144 and 168 h of exposure (Fig. 5B and C and Fig. 5E and F).

3.4. Biochemical biomarker analysis

NTP inhibited AChE enzymatic activity in the exposed larvae. The inhibition of the enzyme activity was more consistent at 500 µg/L when compared to the control ($p < 0.001$), occurring after 120, 144 and 168 h (Fig. 6A–C). Reduced AChE activity was observed at lower concentrations, but only after 168 h NTP exposure, specifically at 0.088 and 1.58 µg/L in comparison to the control group ($p < 0.001$) (Fig. 6C).

4. Discussion

The continuous release of antidepressants into water systems affects the health of aquatic organisms (Arnnok et al., 2017). Nevertheless, information about the toxicity of these compounds in non-target organisms remains scarce. In this regard, our study investigated NTP toxicity in zebrafish early-life stages using a multilevel approach considering environmentally relevant concentrations, together with ecologically important parameters such as locomotor behaviour.

Exposure to NTP had a significant impact on the survival of zebrafish embryos and larvae. Mortality was dependent on concentration and time, with LC_{50} values of 30,369 and 2190 µg/L for 24 and 168 h exposure, respectively. Our results corroborate those obtained by Yang

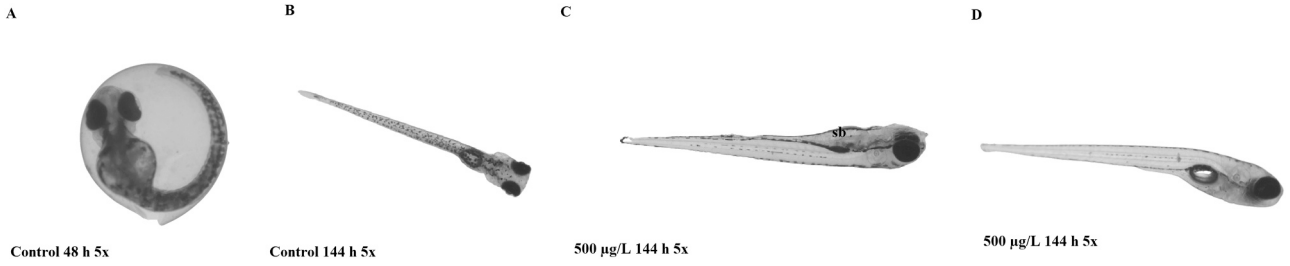

Fig. 2. Representative images of a zebrafish embryo and larvae obtained from the fish embryo toxicity (FET) test. (A) Normal embryo, (B) Normal larvae, and (C-D) larvae with lack of equilibrium. sd: non-inflated swim bladder. The concentration of nortriptyline (NTP) is shown in each picture, together with the exposure time and magnification used.

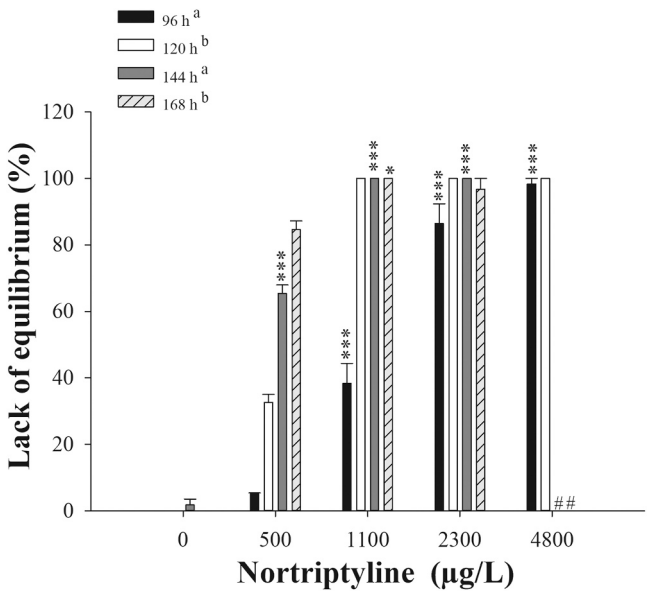

Fig. 3. Effects of nortriptyline (NTP) on the lack of equilibrium of zebrafish larvae from 96 h to 168 h exposure. Values represented per mean ± the standard error of the mean (SEM). *($p < 0.05$) or ***($p < 0.001$): indicate significant differences in relation to the respective control groups (larvae not exposed to NTP for equal time). [a]: one-way ANOVA (Dunnett's post-hoc). [b]: Kruskal–Wallis (Dunn's post-hoc). #: lack of equilibrium not evaluated due to the mortality of all exposed organisms. Statistical analyses were performed considering only the surviving larvae for each time and concentration.

et al. (2014), who investigated the toxicity of another TCA in zebrafish - amitriptyline - and determined an LC_{50} of 27,200 µg/L (24 h). Interestingly, the metabolism of amitriptyline produces NTP. Although *Danio rerio* is an important model organism for studying the toxicity of psychiatric drugs (Yang et al., 2014; de Farias et al., 2019; Franco et al., 2019), there are currently no studies that evaluated NTP-related mortality, or other sublethal parameters, in this species. In the literature, there is only one study that examined NTP toxicity in fish (Sehonova et al., 2017). The authors showed that 500 µg/L NTP increased mortality in early-life stages of common carp (*Cyprinus carpio*), with 100% mortality reported after 22 days of exposure (Sehonova et al., 2017). The high mortality of animals exposed to the highest NTP concentrations may be the result of heart failure (Sehonova et al., 2017). Cardiac toxicity is one of the main side effects caused by TCAs (Stewart et al., 2013; Wang et al., 2018).

In the FET test, equilibrium alterations were observed in larvae exposed to the lower NTP concentrations (500–4800 µg/L) after 96, 120, 144 and 168 h. After 168 h, the EC_{50} for the lack of equilibrium was 390 µg/L, a concentration 5.6 times lower than the LC_{50} for the same exposure period, indicating its sensitivity to predict lethal outcomes. In *Cyprinus carpio*, NTP exposure for 120 h at 500 µg/L also caused changes in swimming: the fish was no longer able to swim, floating apathetically at the bottom of the tank (Sehonova et al., 2017). Lack of equilibrium was also reported in zebrafish larvae exposed to other antidepressants, such as fluoxetine and bupropion (de Farias et al., 2019; Franco et al., 2019). Although there is a relationship between equilibrium and swim bladder inflation (Lindsey et al., 2010), we identified larvae with lack of equilibrium presenting both inflated and non-inflated swim bladders, suggesting that other factors such as neurological disorders may contribute to this phenotype. In the environment, alterations in larvae swimming behaviour can compromise feeding and the ability to avoid predators, directly impacting survival (Painter et al., 2009).

To better understand the toxic effects of NTP on zebrafish, we performed locomotor behavioural assays using a range of concentrations, including environmentally relevant levels. We focused specifically on dark periods, since it is known that zebrafish larvae present more

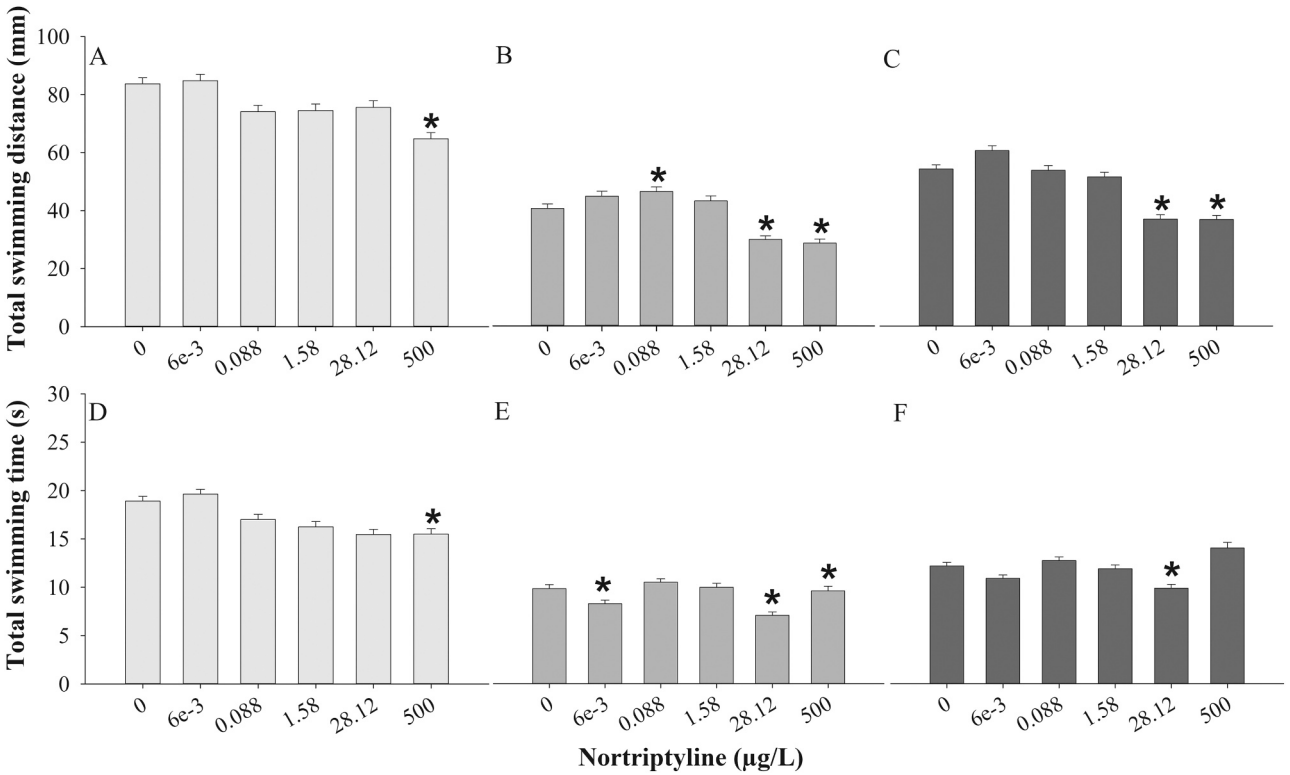

Fig. 4. Total swimming distance (TSD) and total swimming time (TST) of zebrafish larvae after exposure to nortriptyline (NTP) in the dark. The results are presented after 120 h (A and D), 144 h (B and E) and 168 h (C and F) of NTP exposure. Values represented per mean ± the standard error of the mean (SEM). *: indicates significant differences in relation to the control group ($p < 0.05$) by Kruskal–Wallis and Dunn's post-hoc test.

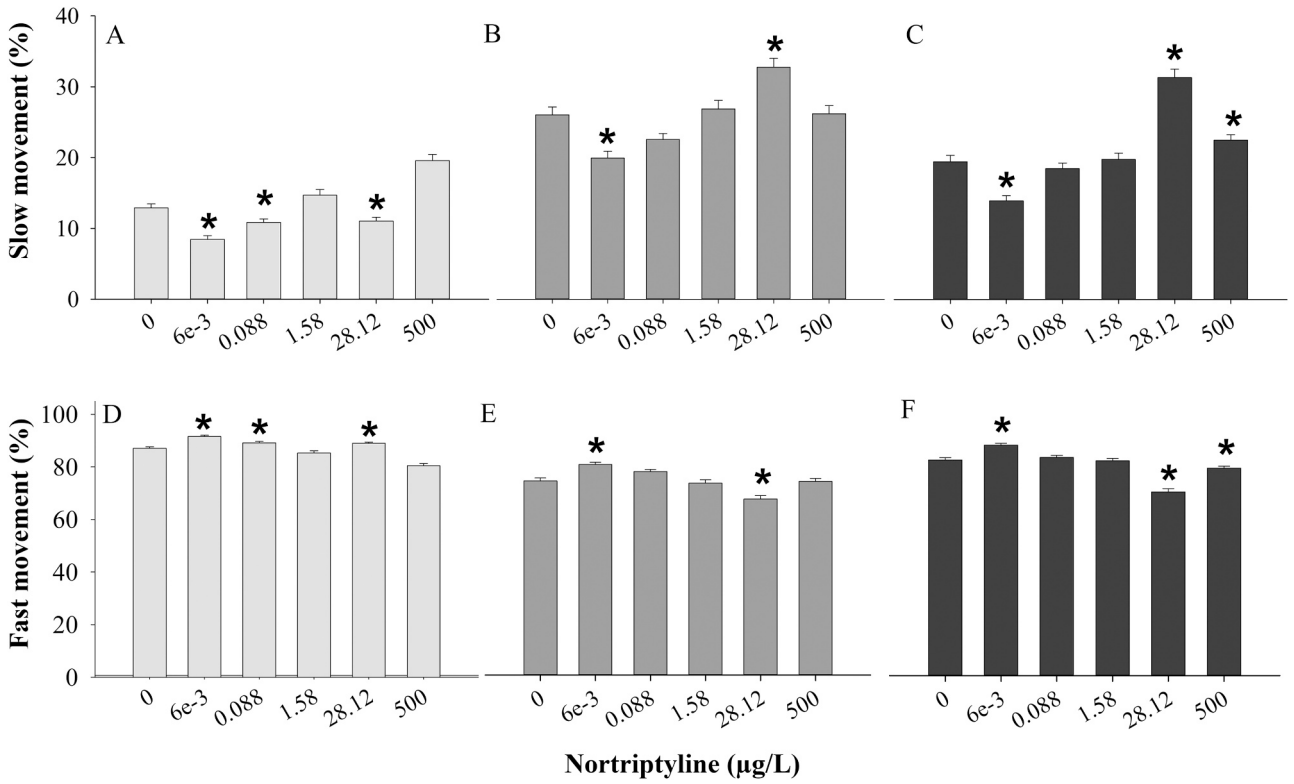

Fig. 5. Percentage of slow distance moved (SM) and percentage of fast distance moved (FM) of zebrafish larvae after exposure to nortriptyline (NTP) in the dark. The results are presented after 120 h (A and D), 144 h (B and E) and 168 h (C and F) of NTP exposure. Values represented per mean ± the standard error of the mean (SEM). *: indicates significant differences in relation to the control group ($p < 0.05$) by Kruskal–Wallis and Dunn's post-hoc test.

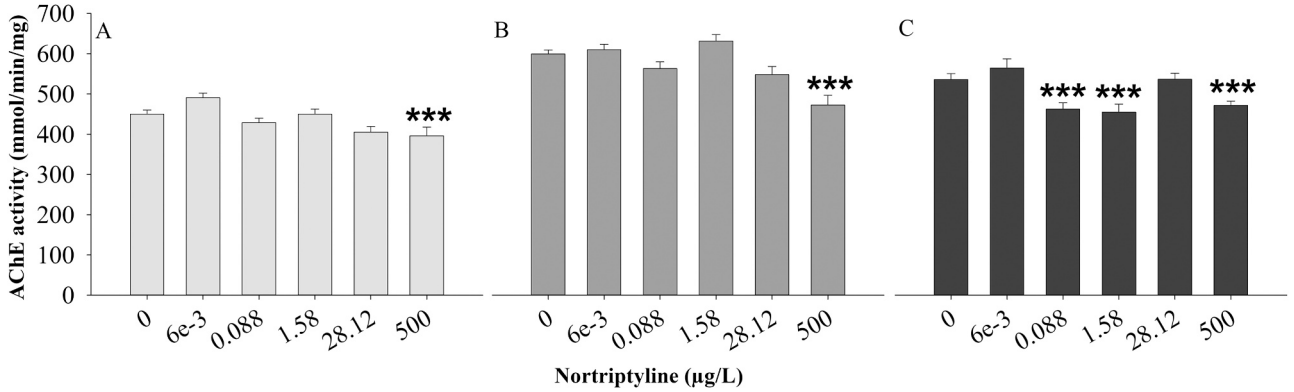

Fig. 6. Acetylcholinesterase (AChE) activity in zebrafish larvae after exposure to nortriptyline (NTP) for 120 h (A), 144 h (B) and 168 h (C). Values represented per mean ± the standard error of the mean (SEM). ***: indicates significant differences in relation to the control group ($p < 0.001$) by one-way ANOVA and Dunnett's post-hoc test.

activity in dark conditions. For all of the locomotor behaviour endpoints analysed (TSD, TST, SM and FM), significant differences were observed in treated groups compared with control after 120, 144 and 168 h of NTP exposure, in at least 1 of the concentrations evaluated. Other studies that evaluated different SNRIs also observed behavioural alterations in fish (Painter et al., 2009; Meshalkina et al., 2018). Exposure to environmentally relevant concentrations of venlafaxine (0.5 µg/L) negatively affected the predator avoidance behaviour of *Pimephales promelas* larvae (Painter et al., 2009). Similarly, adult *D. rerio* exposed to 50 µg/L amitriptyline for 2 weeks reduced both the swimming distance and average speed (Meshalkina et al., 2018).

Neurological dysfunction induced by toxicant exposure is likely to result in behavioural changes (Tierney, 2011). NTP increases the presence of serotonin and norepinephrine in the synaptic clefts (Merwar et al., 2020), with both neurotransmitters implicated in zebrafish behaviour (Herculano and Maximino, 2014; Singh et al., 2015). In fact, serotonin, among other neurotransmitters, has been linked to the regulation of arousal, stress, anxiety, and aggressive behaviour (Herculano and Maximino, 2014); while norepinephrine has also been associated with the modulation of arousal and wakefulness in *D. rerio* (Singh et al., 2015). Considering that zebrafish possess a well-developed and evolutionarily conserved serotonergic and noradrenaline system (Maximino and Herculano, 2010; Yamamoto et al., 2011; Stewart et al., 2013), it is plausible to assume that NTP caused the negative effects observed in this study, especially the lack of equilibrium and locomotor alterations.

We identified contrary effects of NTP on SM/FM parameters in zebrafish locomotor activity. The organisms exposed to environmentally

relevant NTP concentrations (0.006 and 0.088 μg/L) (Baker and Kasprzyk-Hordern, 2013; Borova et al., 2014; Ziarrusta et al., 2016) presented a reduction in SM and an increase in FM in comparison to the control, mainly after 120 h. Conversely, we consistently observed a significant increase in SM and a reduction in FM in animals exposed to 28.12 and 500 μg/L NTP, when compared to the control. Biphasic effects of psychiatric drugs on zebrafish locomotor behaviour were also observed in previous studies involving fluoxetine and valproate (Cowden et al., 2012; de Farias et al., 2019). At extremely low concentrations (0.006 and 0.088 μg/L), NTP appears to increase arousal, offering a possible explanation for the increased FM. Although only significant for the 0.088 μg/L NTP concentration after 144 h; in general, we also observed a slight increase of TSD in the organisms exposed to the lowest concentrations, which can also be related to the increase in arousal (Herculano and Maximino, 2014).

For the 28.12 and 500 μg/L NTP concentrations, the consistent reduction in TSD and FM, together with the increase in SM, mainly after 168 h of exposure, may be linked to hyperserotonemia. In humans, serotonin syndrome is caused by excessive serotonergic activity, resulting in disorientation, muscle rigidity and arrhythmia, among other symptoms (Gillman, 2006; Bartlett, 2017). Continued use of serotonergic antidepressants can lead to the appearance of this phenomenon (Bartlett, 2017). Zebrafish constitutes a potential model to study phenotypes relating to hyperserotonemia (Stewart et al., 2013). In fact, hypolocomotion is one of the main behaviours observed in the animals exposed to serotonergic drugs (Stewart et al., 2013).

In the biochemical test, we detected a significant reduction in AChE activity in the animals exposed to 500 μg/L NTP, in comparison to the control, after 120, 144 and 168 h. The 28.12 μg/L concentration, albeit not significant, also inhibited the AChE enzyme after 120 and 144-h exposures. Very low NTP concentrations do not seem to affect AChE activity consistently, although we also found a significant decrease in enzymatic activity at 0.088 and 1.58 μg/L after 168 h, when compared to the control. Previously, a reduction in AChE activity caused by NTP was reported in the electrical tissue of *Electrophorus electricus* (electric eel) (Nunes-Tavares et al., 2002). The inhibition of AChE activity has been related to behavioural changes in fish (Scott and Sloman, 2004). Acetylcholine accumulation may impair muscle cells, resulting in a reduction of locomotor activity (Tierney, 2011). In addition to its main enzymatic function, AChE has an important morphogenic role in axonal growth (Bigbee et al., 1999). We consistently observed locomotor alterations and AChE inhibition in animals exposed to 500 μg/L NTP. Our results suggest that at higher NTP concentrations (≥ 500 μg/L), the AChE inhibition may contribute to behavioural disruption in zebrafish larvae.

5. Conclusions

To our knowledge, this is the first study that investigated the possible adverse effects of NTP on the early-life stages of zebrafish using a multilevel assessment methodology. The FET test showed that NTP concentrations ≥ 4800 μg/L caused 100% mortality after 144 h exposure (168 h, LC_{50}: 2190 μg/L). Although we did not observe significant developmental changes, a lack of equilibrium in the surviving larvae was evident in ≥ 500 μg/L NTP concentrations. The locomotor assay revealed that very low NTP concentrations (0.006 and 0.088 μg/L) are capable of impairing *D. rerio* swimming behaviour. Furthermore, the biochemical analysis suggested that in concentrations ≥ 500 μg/L, the decrease in AChE activity may contribute to disturbing zebrafish larvae locomotor behaviour. Collectively, our results indicate that short periods of NTP exposure are toxic to zebrafish embryos/larvae. Concerningly, we observed behavioural toxicity in environmentally relevant NTP concentrations, suggesting that very low concentrations of the drug can negatively influence the survival rates of non-target organisms in the environment. Further studies using chronic exposure and more specific endpoints are necessary to better understand the toxic effect of NTP in more realistic exposure scenarios.

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CRediT authorship contribution statement

Ana C. Oliveira: Formal analysis, Investigation, Writing - original draft, Visualization. **Maria L. Fascineli**: Formal analysis, Investigation, Writing - review & editing. **Thayres S. Andrade**: Formal analysis, Writing - review & editing. **Diego Sousa-Moura**: Formal analysis, Investigation. **Inês Domingues**: Resources, Writing - review & editing, Funding acquisition. **Níchollas S. Camargo**: Formal analysis, Investigation. **Rhaul Oliveira**: Conceptualization, Methodology, Writing - review & editing. **Cesar K. Grisolia** Conceptualization, Methodology, Resources, Writing - review & editing, Supervision, Project administration, Funding acquisition. **Rolando A.R. Villacis** Conceptualization, Methodology, Validation, Data curation, Writing - review & editing, Visualization, Supervision.

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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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2020.111868.

References

Aminot, Y., Litrico, X., Chambolle, M., Arnaud, C., Pardon, P., Budzindki, H., 2015. Development and application of a multi-residue method for the determination of 53 pharmaceuticals in water, sediment, and suspended solids using liquid chromatography-tandemmass spectrometry. Anal. Bioanal. Chem. 407, 8585–8604.
Arnnok, P., Singh, R.R., Burakham, R., Pérez-Fuentetaja, A., Aga, D.S., 2017. Selective uptake and bioaccumulation of antidepressants in fish from effluent-impacted Niagara river. Environ. Sci. Technol. 51, 10652–10662.
aus der Beek, T., Weber, F.A., Bergmann, A., Hickmann, S., Ebert, I., Hein, A., Küster, A., 2016. Pharmaceuticals in the environment–global occurrences and perspectives. Environ. Toxicol. Chem. 35, 823–835.
Baker, D.R., Kasprzyk-Hordern, B., 2013. Spatial and temporal occurrence of pharmaceuticals and illicit drugs in the aqueous environment and during wastewater treatment: new developments. Sci. Total. Environ. 454–455, 442–456.
Bambino, K., Chu, J., 2017. Zebrafish in toxicology and environmental health. Curr. Top. Dev. Biol. 124, 331–367.
Bartlett, D., 2017. Drug-induced serotonin syndrome. Crit. Care Nurse 37, 49–54.
Bigbee, J.W., Sharma, K.V., Gupta, J.J., Dupree, J.L., 1999. Morphogenic role for acetylcholinesterase in axonal outgrowth during neural development. Environ. Health Perspect. 107 (Suppl. 1), 81–87.
Borova, V.L., Maragou, N.C., Gago-Ferrero, P., Pistos, C., Thomaidis, N.S., 2014. Highly sensitive determination of 68 psychoactive pharmaceuticals, illicit drugs, and relatedhuman metabolites in wastewater by liquid chromatography-tandem mass spectrometry. Anal. Bioanal. Chem. 406, 4273–4285.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of micro- gram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.
Calisto, V., Esteves, V.I., 2009. Psychiatric pharmaceuticals in the environment. Chemosphere 77, 1257–1274.
Choi, J.W., Zhao, Y., Bediako, J.K., Cho, C.W., Yun, Y.S., 2018. Estimating environmental fate of tricyclic antidepressants in wastewater treatment plant. Sci. Total Environ. 634, 52–58.
Cowden, J., Padnos, B., Hunter, D., MacPhail, R., Jensen, K., Padilla, S., 2012. Developmental exposure to valproate and ethanol alters locomotor activity and retino-tectal projection area in zebrafish embryos. Reprod. Toxicol. 33, 165–173.
Dong, Z., Senn, D.B., Moran, R.E., Shine, J.P., 2013. Prioritizing environmental risk of prescription pharmaceuticals. Regul. Toxicol. Pharmacol. 65, 60–67.
Ellman, G.L., Courtney, K.D., Andres Jr., V., Feather-Stone, R.M., 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem. Pharmacol. 7, 88–95.
de Farias, N.O., Oliveira, R., Sousa-Moura, D., de Oliveira, R.C.S., Rodrigues, M.A.C., Andrade, T.S., Domingues, I., Camargo, N.S., Muehlmann, L.A., Grisolia, C.K., 2019. Exposure to low concentration of fluoxetine affects development, behaviour and acetylcholinesterase activity of zebrafish embryos. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 215, 1–8.
Franco, M.B., Andrade, T.S., Sousa-Moura, D., Lopes da Silva, M., Machado, F.I.B., Camargo, N.S., Domingues, I., Oliveira, R., Grisolia, C.K., 2019. Exposure to dilute concentrations of bupropion affects zebrafish early life stages. Chemosphere 222, 175–183.
Gillman, P.K., 2006. A review of serotonin toxicity data: implications for the mechanisms of antidepressant drugaction. Biol. Psychiatry 59, 1046–1051.
Gupta, M., Jain, A., Verma, K.K., 2010. Determination of amoxapine and nortriptyline in blood plasma and serum by salt-assisted liquid-liquid microextraction and high-performance liquid chromatography. J. Sep. Sci. 33, 3774–3780.
Haviv, Y., Zini, A., Sharav, Y., Almoznino, G., Benoliel, R., 2019. Nortriptyline compared to amitriptyline for the treatment of persistent masticatory myofascial pain. J. Oral Fac. Pain Headache 33, 7–13.
Henry, T.B., Black, M.C., 2008. Acute and chronic toxicity of fluoxetine (selective serotonin reuptake inhibitor) in western mosquito fish. Arch. Environ. Contam. Toxicol. 54, 325–330.
Herculano, A.M., Maximino, C., 2014. Serotonergic modulation of zebrafish behavior: towards a paradox. Prog. Neuropsychopharmacol. Biol. Psychiatry 55, 50–66.
Irons, T.D., MacPhail, R.C., Hunter, D.L., Padilla, S., 2010. Acute neuroactive drug exposures alter locomotor activity in larval zebrafish. Neurotoxicol. Teratol. 32, 84–90.
Jesus, F.T., Oliveira, R., Silva, A., Catarino, A.L., Soares, A.M., Nogueira, A.J., Domingues, I., 2013. Lethal and sub lethal effects of the biocide chlorhexidine on aquatic organisms. Ecotoxicology 22, 1348–1358.
Lajeunesse, A., Gagnon, C., Gagné, F., Louis, S., Cejka, P., Sauvé, S., 2011. Distribution of antidepressants and their metabolites in brook trout exposed to municipal wastewaters before and after ozone treatment–evidence of biological effects. Chemosphere 83, 564–571.
Lajeunesse, A., Smyth, S.A., Barclay, K., Sauvé, S., Gagnon, C., 2012. Distribution of antidepressant residues in wastewater and biosolids following different treatment processes by municipal wastewater treatment plants in Canada. Water Res. 46, 5600–5612.
Lindsey, B.W., Smith, F.M., Croll, R.P., 2010. From inflation to flotation: contribution of the swimbladder to whole-body density and swimming depth during development of the zebrafish (Danio rerio). Zebrafish 7, 85–96.
Ma, L.D., Li, J., Li, J.J., Liu, M., Yan, D.Z., Shi, W.Y., Xu, G., 2018. Occurrence and source analysis of selected antidepressants and their metabolites in municipal wastewater and receiving surface water. Environ. Sci. Process. Impacts 20, 1020–1029.
Maximino, C., Herculano, A.M., 2010. A review of monoaminergic neuropsychopharmacology in zebrafish. Zebrafish 7, 359–378.
Merwar, G., Gibbons, J.R., Hosseini, A.S., Saadabadi, A., 2020. Nortriptyline. StatPearls [Internet]. StatPearls Publishing, Treasure Island, FL.
Meshalkina, D.A., Kysil, E.V., Antonova, K.A., Demin, K.A., Kolesnikova, T.O., Khatsko, S.L., Gainetdinov, R.R., Alekseeva, P.A., Kalueff, A.V., 2018. The effects of chronic amitriptyline on zebrafish behavior and monoamine neurochemistry. Neurochem. Res. 43, 1191–1199.
Metcalfe, C.D., Chu, S., Judt, C., Li, H., Oakes, K.D., Servos, M.R., Andrews, D.M., 2010. Antidepressants and their metabolites in municipal wastewater, and downstream exposure in an urban watershed. Environ. Toxicol. Chem. 29, 79–89.
Niemi, L.M., Stencel, K.A., Murphy, M.J., Schultz, M.M., 2013. Quantitative determination of antidepressants and their select degradates by liquid chromatography/electrospray ionization tandem mass spectrometry in biosolids destined for land application. Anal. Chem. 85, 7279–7286.
Nunes-Tavares, N., Nery da Matta, A., Batista e Silva, C.M., Araújo, G.M., Louro, S.R., Hassón-Voloch, A., 2002. Inhibition of acetylcholinesterase from Electrophorus electricus (L.) by tricyclic antidepressants. Int. J. Biochem. Cell Biol. 34, 1071–1079.
OECD, 2013. Test no. 236: fish embryo acute toxicity (FET) test. OECD Guidelines for the Testing of Chemicals, Section 2. OECD, Paris.
Ojemaye, C.Y., Petrik, L., 2019. Occurrences, levels and risk assessment studies of emerging pollutants (pharmaceuticals, perfluoroalkyl and endocrine disrupting compounds) in fish samples from Kalk Bay harbour, South Africa. Environ. Pollut. 252, 562–572.
Painter, M.M., Buerkley, M.A., Julius, M.L., Vajda, A.M., Norris, D.O., Barber, L.B., Furlong, E.T., Schultz, M.M., Schoenfuss, H.L., 2009. Antidepressants at environmentally relevant concentrations affect predator avoidance behavior of larval fathead minnows (Pimephales promelas). Environ. Toxicol. Chem. 28, 2677–2684.
Panula, P., Sallinen, V., Sundvik, M., Kolehmainen, J., Torkko, V., Tiittula, A., Moshnyakov, M., Podlasz, P., 2006. Modulatory neurotransmitter systems and behavior: towards zebrafish models of neurodegenerative diseases. Zebrafish 3, 235–247.
Pivetta, R.C., Rodrigues-Silva, C., Ribeiro, A.R., Rath, S., 2020. Tracking the occurrence of psychotropic pharmaceuticals in Brazilian wastewater treatment plants and surface water, with assessment of environmental risks. Sci. Total Environ. 727, 138661.
Ross, V., Gunnarsson, L., Fick, J., Larsson, D.G., Rudén, C., 2012. Prioritising pharmaceuticals for environmental risk assessment: Towards adequate and feasible first-tier selection. Sci. Total Environ. 1 (421–422), 102–110.
Sánchez-Argüello, P., Fernández, C., Tarazona, J.V., 2009. Assessing the effects of fluoxetine on Physa acuta (Gastropoda, Pulmonata) and Chironomusriparius (Insecta, Diptera) using a two-species water-sediment test. Sci. Total Environ. 407, 1937–1946.
Scott, G.R., Sloman, K.A., 2004. The effects of environmental pollutants on complex fish behaviour: integrating behavioural and physiological indicators of toxicity. Aquat. Toxicol. 68, 369–392.
Sehonova, P., Plhalova, L., Blahova, J., Doubkova, V., Marsalek, P., Prokes, M., Tichy, F., Skladana, M., Fiorino, E., Mikula, P., Vecerek, V., Faggio, C., Svobodova, Z., 2017. Effects of selected tricyclic antidepressants on early-life stages of common carp (Cyprinus carpio). Chemosphere 185, 1072–1080.
Singh, C., Oikonomou, G., Prober, D.A., 2015. Norepinephrine is required to promote wakefulness and for hypocretin-induced arousal in zebrafish. Elife 4, e07000.
Stewart, A.M., Cachat, J., Gaikwad, S., Robinson, K.S., Gebhardt, M., Kalueff, A.V., 2013. Perspectives on experimental models of serotonin syndrome in zebrafish. Neurochem. Int. 62, 893–902.
Tierney, K.B., 2011. Behavioural assessments of neurotoxic effects and neurodegeneration in zebrafish. Biochim. Biophys. Acta 1812, 381–389.
Walters, E., McClellan, K., Halden, R.U., 2010. Occurrence and loss over three years of 72 pharmaceuticals and personal care products from biosolids-soil mixtures in outdoor mesocosms. Water Res. 44, 6011–6020.
Wang, S.M., Han, C., Bahk, W.M., Soo-Jung, L., Patkar, A.A., Masand, P.S., Pae, C.U., 2018. Addressing the side effects of contemporary antidepressant drugs: a comprehensive review. Chonnam Med. J. 54, 101–112.
Wu, M., Xiang, J., Que, C., Chen, F., Xu, G., 2015. Occurrence and fate of psychiatric pharmaceuticals in the urban water system of Shanghai, China. Chemosphere 138, 486–493.
Yamamoto, K., Ruuskanen, J.O., Wullimann, M.F., Vernier, P., 2011. Differential expression of dopaminergic cell markers in the adult zebrafish forebrain. J. Comp. Neurol. 519, 576–598.
Yang, M., Qiu, W., Chen, J., Zhan, J., Pan, C., Lei, X., Wu, M., 2014. Growth inhibition and coordinated physiological regulation of zebrafish (Danio rerio) embryosupon sublethal exposure to antidepressant amitriptyline. Aquat. Toxicol. 151, 68–76.
Ziarrusta, H., Mijangos, L., Prieto, A., Etxebarria, N., Zuloaga, O., Olivares, M., 2016. Determination of tricyclic antidepressants in biota tissue and environmental waters by liquidchromatography-tandem mass spectrometry. Anal. Bioanal. Chem. 408, 1205–1216.