

Fluoxetine can make marine organisms unhappy: a study on the sub-lethal effects on marine invertebrates

PAULY, G.F.E.*; VALLIM, A. L.; MERGUIZO, R.A.C.; DANIEL, G.; RAMAGLIA, A. C.; CORTEZ, F. S.; MIRALDO, M. C.; ROJAS, E. G.; CRUZ, A.C.F. & ABESSA, D. M. S.

Instituto de Biociências, Campus do Litoral Paulista, Universidade Estadual Paulista, São Vicente-SP, Brasil

Received April 05, 2019; Accept September 10, 2021

Abstract

The environmental effects caused by selective serotonin reuptake inhibitor drugs have been investigated for marine organisms and coastal ecosystems but are scarce in neotropical organisms. This investigation aimed to evaluate the sublethal effects of fluoxetine on the embryonic development of the sea urchin *Echinometra lucunter* and the survival and swimming behavior of the brine shrimp *Artemia* sp. The organisms were exposed to four different concentrations of fluoxetine (30, 300, 3000 and 30000 ng L⁻¹) and to a negative control (filtered seawater), following the respective standard testing protocols. We verified a significant reduction of the embryos development to pluteus larvae, starting from 3000 ng L⁻¹ (54.0±10.9% normal larvae), in comparison with the controls (83.5±3.1%). The non-observed effect concentration (NOEC) was estimated at 300 ng L⁻¹, and the lowest observed effect concentration (LOEC) was 3000 ng L⁻¹. In the behavior tests with *Artemia* sp, no significant adverse effects were reported for mobility, swimming speed and inactivity time. These results show that Fluoxetine can interfere on the development of species like the sea urchin *E. lucunter*, but short term exposure did not affected the swimming behavior of the brine shrimp *Artemia* sp. Fluoxetine presents thus a potential to affect marine biota and disrupt the equilibrium of the coastal ecosystems.

Key words: Ecotoxicology, marine assessment, sublethal effects, fluoxetine, *Artemia* sp., *Echinometra lucunter*.

INTRODUCTION

The presence of pharmaceuticals and personal care products (PPCPs) in the aquatic ecosystems is of main concern and recently has received attention from scientists, legislators and environmental managers (Kolpin *et al.*, 2002; Sumpter, 2003; Vystavna *et al.*, 2012; giebultowicz & Nałęcz-Jawecki, 2014). Despite many urban regions and large cities are located on the coastal zone (Martínez *et al.*, 2007), constituting major sources of PPCPs, the adjacent marine and estuarine environments have been little studied for this problem (Gaw *et al.*, 2014). In the developed countries, where wastewater management is well established, the main sources of PPCPs to coastal waters include wastewater treatment plants (WWTPs) (Daughton, 2007), followed by ships, irregular release of

domestic sewage, agricultural and livestock residues and aquaculture effluents (Gaw *et al.*, 2014). In countries and regions where wastewaters are not properly collected or treated, the input of sewage to the sea includes diffuse sources, urban drainage and/or its intentional discharge into the sea. Moreover, many coastal cities discharge sewage into the sea throughout sewage outfalls after a pre-conditioning process, which does not remove the contaminants from the effluent.

Once in the aquatic environment, the PPCPs and their metabolites may be found in the water column, sediments, suspended solids and accumulated in the soft tissues of the aquatic biota, such as fish and invertebrates (Ramirez *et al.*, 2009; Birsch *et al.*, 2015; Biel-Maeso *et al.*, 2018). Because PPCPs are bioactive compounds synthesized with therapeutically purposes, and because many metabolic

*Corresponding author: Guacira Eufrazio; e-mail: guaeufrazio@gmail.com

pathways are conserved in the most of living organisms, PPCPs may influence non target organisms, affecting their reproduction, growth, sexual differentiation, immune system and behavior (Meguid *et al.*, 2000; Brooks *et al.*, 2006).

In the 1940's decade, Hanfliger and Schindler firstly synthesized the tricyclic antidepressants (TCAs) (Kwon & Armbrust, 2006), which were used as medicines in the treatment of psychiatric disorders, by acting as serotonin reuptake inhibitors (SRRI) (Sánchez-Argüello *et al.*, 2009) and metabolism inhibitors (Mayberg *et al.*, 2000). Fluoxetine (FLX) is a TCA widely used for the treatment of both adults and children. This compound is the active principle of the commercial drug Prozac®, and produce a norfluoxetine, a metabolite that reduce important enzyme activities *in vivo*, as cytochrome P450 (Goodnick & Goldstein, 1998). FLX is the most studied TCA, with results indicating acute and chronic toxicity to aquatic organisms (Brooks *et al.*, 2003; Fent *et al.*, 2006; Weinberger & Kapler, 2014; Luis *et al.*, 2016; Cortez *et al.*, 2019).

Acute effects of FLX were already observed in algae ((Brooks *et al.*, 2003; Johnson *et al.*, 2007), gastropods (Fong & Molnar, 2013), bivalves (Chen *et al.*, 2015; Cortez *et al.*, 2019) and fish (Brooks *et al.*, 2003; Stanley *et al.*, 2007). In terms of chronic toxicity, increased reproduction was observed in *Daphnia magna* (Flaherty & Dodson, 2005), while *Ceriodaphnia dubia* exhibited reduced reproductive rates (Brooks *et al.*, 2003). Still, little information is available regarding chronic toxicity of FLX (Ansai *et al.*, 2016), with a no-observed effect concentration (NOEC) of 0.47 µg L⁻¹ (Nentwig, 2007) and a low-observed effect concentration (LOEC) of 447 µg L⁻¹ (Henry *et al.*, 2004). At relevant environmental concentrations, this compound is known to cause behavioral effects in aquatic organisms, as documented for crabs (Peters *et al.*, 2017), amphipods (Bossus *et al.*, 2014), and fish (Barry, 2013), and caused biochemical and cytogenotoxic effects in mussels (Cortez *et al.*, 2019) and fish (Duarte *et al.*, 2019), and oxidative stress and reduction of population density in the marine rotifer *Brachionus koreanus* (Byeon *et al.*, 2020). Most studies were conducted with freshwater organisms (Mennigen *et al.*, 2010; Schultz, *et al.* 2011; Dzieweczynski & Hebert, 2012; Kohlert, *et al.*, 2012; Gaw *et al.*, 2014; Lamichhane *et al.*, 2014; Weinberger & Kapler, 2014; Silva *et al.*, 2015; Kalichak *et al.*, 2016); however, the few studies made with marine organisms suggest a higher sensitivity to FLX (Sverdrup *et al.*, 2002).

Considering that fluoxetine, like as most of contaminants, ends up in the marine environment, the assessment of its toxic effects on the biota is a critical issue, in order to allow the determination of its toxic thresholds, subsidize ecological risk assessments for this compound (Sverdrup *et al.*, 2002) and especially the estimation of maximum acceptable levels in the environment. In this sense, toxicity tests consist of reliable tools to identify and determine the effects of substances on aquatic organisms. Protocols assessing the effects of pollutants on the embryo-larval of invertebrates, such as bivalves and echinoderms, are sensitive and widely accepted and used

worldwide (Ghirardini *et al.*, 2001). Similarly, assessing behavioral changes in organisms allow understanding the chances of survival and reproductive success (Anufrieva & Shadrin, 2014).

This investigation aimed to evaluate the sublethal effects of the fluoxetine to marine organisms, considering the swimming behavior of *Artemia* sp. and the embryonic development of the sea-urchin *Echinometra lucunter*.

MATERIAL AND METHODS

Test substance - Fluoxetine

The pharmaceutical Fluoxetine (IUPAC name N-methyl-3-phenyl-3-[4-(trifluoromethyl)phenoxy]propan-1-amine) (CAS Number 56296-78-7; molecular weight 345.79; purity ≥ 98%; which molecular formula is C₁₇H₁₈F₃NO) as well as all other reagents used in this study were purchased from Sigma Aldrich (Steinheim, Germany). FLX is a selective serotonin reuptake inhibitor and consists of an effective substance to treat the symptoms of human depression (O'Shea, 1991). The compound is stable at normal conditions, with a fusion temperature ranging about 158.4 – 158.9 °C. Its solubility in water is 14 mg mL⁻¹ at 25°C and its pKa is 4.6 (Risley & Bopp, 1990).

In this investigation, nominal concentrations of FLX were prepared by the direct dilution of FLX in seawater previously filtered through a 45µm pore acetate membrane. Four test concentrations were used (30, 300, 3000, 30000 ng L⁻¹), plus a negative control which consisted of filtered seawater in which FLX was virtually absent (0 ng L⁻¹). The test concentrations were selected based on previous information regarding the environmental levels of FLX and its toxic levels to other species (Mesquita *et al.*, 2011; Franzellitti *et al.*, 2015).

Embryonic development toxicity test - Echinometra lucunter

In this bioassay, we evaluated the effects of FLX on the development of embryos of the sea urchin *Echinometra lucunter* after exposing the fertilized eggs to the different chemical concentrations. The tests were conducted following the standard protocol described by ABNT (2012).

Adult individuals of *E. lucunter* (Linnaeus, 1758) were collected at Palmas island, Guarujá - Brazil (24°00'29.47"S - 46°19'30.34"W) and transferred to the laboratory, where spawning was stimulated by osmotic induction (injection of 2.5 mL KCl 0,5M in their coelomic cavities). The ovules were released in a glass beaker containing filtered seawater in an acetate membrane of 0.45 µm. Prior to *in-vitro* fertilization, the eggs were examined under microscope for their morphology and viability. The sperm was stored dry in a little beaker kept on ice, until their activation by addition of filtered seawater.

The fertilization was made by adding a 2-ml aliquot of sperm solution into the ovules solution, followed of a gentle agitation for 2h, in order to allow fecundation. Then,

sub-samples were examined on microscope to confirm the formation of the fertilization membrane; a viable test should present a minimum of 80% fertilized eggs. Next, about 500 eggs were introduced in each replicate, which consisted of glass tube tests with 10 ml of test-solutions (i.e. the FLX concentrations or the control). Four replicates were used for each concentration. After about 36 hours (time necessary to the embryos reach the pluteus stage), the test was finished, by the addition of 0.5 mL tamponed formaldehyde (10%) in each replicate. Further, the first 100 embryos of each replicate were examined on microscope, in order to determine the normal development rates and the presence of abnormalities, such as delays, morphological alterations, or absence of development.

Behavioral toxicity test - *Artemia* sp.

In the bioassay for identifying behavior effects in adults of brine shrimps (*Artemia* sp.), 30 healthy males were selected by random from a stock population acquired from a shop. These animals were introduced into glass beakers containing 200 mL of FLX concentrations (or the control) and exposed for 48 hours, in a static experiment. The physical chemical parameters were controlled during the experiment, as follows: photoperiod of 16h:8h (clear-dark), salinity 35 ± 1 , and temperature of 25 ± 2 °C.

At the end of the experiment, 12 animals from each treatment were randomly separated and individually placed in Petri dishes (with mean diameters of 95.7 ± 4.5 mm) containing a water layer enough to allow only the horizontal swimming (about 1-2 mm deep). Then, 6-minutes films were made with each animal, from which the first 5 minutes corresponded to the acclimation period and were discarded from the analyses. In the last minute of each film, the swimming behavior of the animals was evaluated for the following endpoints: mean swimming speed, total distance covered, and inactivity time (Venkateswara Rao *et al.*, 2007). A computer aided tracking video system was employed (Ethovision XT; Noldus Information Technology, Wageningen, Netherlands) to automatize the readings of the respective behaviors.

Statistical Analyses

The results of the test with sea urchin embryos were firstly used to calculate the inhibition concentration of 50% development (IC_{50}), by using the Trimmed Spearman-Kärber method (Hamilton *et al.*, 1977). The data were checked for

normality and variance homogeneity by the Shapiro-Wilk's and Levene's tests, respectively. Because the embryonic development data were not normal (Shapiro-Wilks $p=0.011$) and heterocedastic (Levene $p=0.0007$), the results were analyzed by the Kruskal-Wallis test, followed by the Dunn's multiple comparison, in order to calculate the respective NOEC and LOEC.

The behavioral endpoints covered distance and mean swimming speed presented normal distribution and homogeneous variances (Shapiro-Wilks $p=0.548$ and Levene $p=0.333$; and Shapiro-Wilks $p=0.979$ and Levene $p=0.333$, respectively), thus they were analyzed by one-way analysis of variance (ANOVA) followed by the Tukey's test. For the inactivity time, the data were not normal nor homocedastic ($p<0.05$), thus they were analyzed by the Kruskal-Wallis test followed by the Dunn's multiple comparison.

RESULTS

Embryonic development toxicity test - *Echinometra lucunter*

The physical-chemical parameters of the test-solutions are presented in the Table 1, and they remained within the acceptable ranges, according to the test protocol for the species (ABNT, 2012).

The control group presented embryonic development above 80% (Figure 1), that is the minimum acceptable, according to ABNT (2012). Significant differences were detected among treatments (Kruskal-Wallis; $H_{(4,N=20)}=13.912$, $p=0.007$). Embryos of *E. lucunter* exposed to $30,000 \text{ ng L}^{-1}$ exhibited reduced embryonic development rates (Dunn $p=0.025$). The inhibition concentration to 50% organisms after 36h (IC_{50} - 36h) was calculated as $25,000.9 (12,884.9 - 48,745.6) \text{ ng L}^{-1}$. The LOEC was estimated as $30,000 \text{ ng L}^{-1}$ while the NOEC was 3000 ng L^{-1} .

Behavioral toxicity test - *Artemia* sp.

The physical-chemical parameters of the test-solutions are presented in the Table 2, and they remained within the acceptable ranges, according to the test protocol for the species (ABNT, 2012).

In the test evaluating effects on the swimming behavior of *Artemia* sp., there were no significant differences between

Table 1. Physical-chemical parameters measured at the end of the embryonic experiment with the sea-urchin *Echinometra lucunter*.

Fluoxetine concentrations (ng L^{-1})	Total ammonia (mg L^{-1})	Unionized ammonia (ug L^{-1})	Temperature (°C)	pH	Salinity	Dissolved oxygen (mg L^{-1})
0	14.13	0.25	25 ± 2	7.59	35	6.60
30	15.57	0.30	25 ± 2	7.63	35	5.40
300	25.42	0.46	25 ± 2	7.60	35	5.20
3000	22.36	0.41	25 ± 2	7.61	35	5.30
30000	17.55	0.29	25 ± 2	7.57	35	4.90

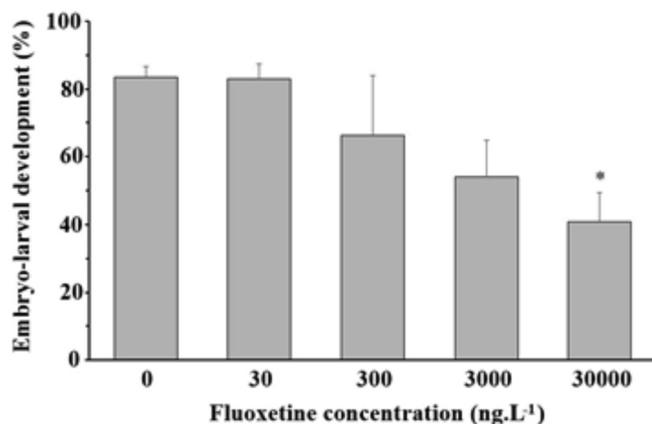


Fig. 1. Percent of normal embryo-larval development of *Echinometra lucunter* at different concentrations of fluoxetine. * = statistical difference with the control. The error bars show the respective standard deviations.

total covered distances in all concentrations tested (Figure 2A – ANOVA; $F_{4,45}=1,842$, $p=0.137$). Regarding the mean swimming speed, no significant differences were detected as well (Figure 2B – ANOVA; $F_{4,45}=1,838$, $p=0.137$), as well for the inactivity time (Figure 2C – Kruskal-Wallis; $H(4,n=50)=4.041$, $p=0.399$).

DISCUSSION

This study evaluated some biological effects of FLX on marine invertebrates, namely the embryonic development

of the sea-urchin *E. lucunter* and the swimming behavior of the crustacean *Artemia* sp. Fluoxetine is ranked within the top 20 most threatening pharmaceuticals to the environment, due to its ability to disrupt the endocrine systems of non-target organisms and the risks associated to its release in the environment (Kumar & Xagorarakis, 2010).

On the other hand, no differences in the swimming behavior of *Artemia* sp. were detected (Figure 2) independently of the FLX concentration. According to the literature, other organisms exhibited alterations in their behavior after the exposure to FLX. The estuarine crab *Carcinus maenas* presented alteration of its locomotion when exposed to 120 $\mu\text{g L}^{-1}$ of FLX (Mesquita *et al.*, 2011); the time required to cover long distances was significantly increased in comparison to the control. De Lange *et al.* (2006) observed that for the freshwater amphipod *Gammarus pulex* exposed to 10 and 100 ng L^{-1} FLX the locomotion time was 65% smaller than that exhibited by the control animals. Other studies reported cytotoxic effects of FLX in marine invertebrates, after exposure at 30 ng L^{-1} (Franzellitti *et al.*, 2014). Moreover, studies reported chronic toxicity in adult vertebrates and invertebrates. Weinberg II & Klaper (2014) observed a more aggressive behavior and negative effects in the defense and construction of nests in the fathead minnow *Pimephales promelas* exposed at fluoxetine concentrations $<1 \mu\text{g L}^{-1}$, while Stanley *et al.* (2007) observed feeding disturbances in the same species. Ding *et al.* (2017) reported reduction of the enzymatic activity of the acetylcholinesterase (AChE) in *D. magna* exposed to concentrations up to 5 $\mu\text{g L}^{-1}$ FLX. In studies conducted with

Table 2. Physical-chemical parameters measured at the beginning of the two days exposure to different concentrations of fluoxetine and before the 1 minute shoot of adult males of *Artemia* sp.

Fluoxetine concentrations (ng L^{-1})	Total ammonia (mg L^{-1})	Unionized ammonia ($\mu\text{g L}^{-1}$)	Temperature ($^{\circ}\text{C}$)	pH	Salinity	Dissolved oxygen (mg L^{-1})
0	<LD	<LD	25 ± 2	7.63	35	4.70
30	<LD	<LD	25 ± 2	7.59	34	4.80
300	<LD	<LD	25 ± 2	8.01	35	4.90
3000	<LD	<LD	25 ± 2	7.77	35	5.00
30000	0,57	6,03	25 ± 2	8.17	35	4.50

LD: detection limit

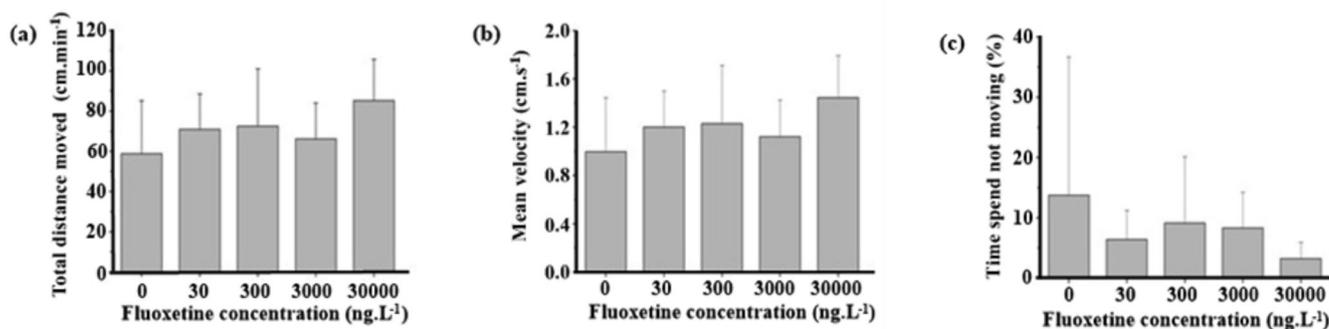


Fig. 2a. Distance traveled by adult individuals of *Artemia* sp. in 1 minute after two days of exposure to different concentrations of fluoxetine; Fig. 2b. Average speed of adult individuals of *Artemia* sp. in 1 minute after two days of exposure to different concentrations of fluoxetine; Fig. 2c. Downtime of adult individuals of *Artemia* sp. in 1 minute after two days of exposure to different concentrations of fluoxetine. Error bars show the respective standard deviations.

Danio rerio, Chai *et al.* (2021) observed impairment of cardiac tissue and arrhythmia in organisms exposed to 100 ng L⁻¹ of FLX, while Zindler *et al.* (2020) reported behavioral changes related with stress, at environmentally relevant concentrations (2.9 nM), and an additive effect when FLX was mixed with its metabolite norfluoxetine.

Comparing the responses of the two species tested, embryos of *E. lucunter* were more sensitive than *Artemia* sp. Previous studies reported that adults of *Artemia* sp. were resistant to environmental changes and chemical substances (Allender *et al.*, 2012; Peixoto *et al.*, 2019). Thus, such tolerance could explain the absence of significant effects of FLX on the behavior of *Artemia* sp. in the present study. Minguez *et al.* (2014) showed that nauplii of *Artemia salina* were more resistant than organisms from other trophic levels, when acute effects were compared.

The results of the present investigation also indicated that toxicity occurred at concentrations higher than those reported in the environment. Fluoxetine concentrations in superficial coastal waters are one order of magnitude lower than the observed toxicity. The mean concentration of FLX in effluents of the WWTP of Seixal (Portugal) was 946 ng L⁻¹ (Salgado *et al.*, 2011), while Birch *et al.* (2015) found a maximum concentration of 36 ng L⁻¹ in surface waters from an estuary of Sydney, Australia. Other studies conducted in the San Francisco Bay (USA), Mediterranean Sea (Israel) and Pacific Ocean (USA) reported respective maximum and mean concentrations of FLX as 90 ng L⁻¹ and 66 ng L⁻¹ (Nödler *et al.*, 2014). Jiang *et al.* (2014) reported concentrations of FLX below the detection limit in coastal waters from Taiwan. In Brazil, Cortez *et al.* (2019) reported concentrations up to 0.58 ng L⁻¹ in coastal waters from the Santos Bay, in the São Paulo State. In the same study, the authors reported enzymatic alterations in soft tissues of the mussel *Perna perna* exposed to concentrations ranging between 30 and 300 ng L⁻¹ FLX.

Despite the toxic concentrations of FLX obtained in our study are above the environmental concentrations previously reported for coastal surface waters around the world (Jiang *et al.*, 2014; Nödler *et al.*, 2014; Birch *et al.*, 2015), this fact not necessarily indicates lack of environmental risks associated to this compound, because only two species were tested, and tests consisted of short-term exposures. Moreover, the environmental concentrations of FLX in Brazil are unknown, despite the discharge of untreated sewage in most regions. Thus more ecotoxicological studies are required, considering different trophic levels, and especially long-term exposure, in order to provide robust information to determine potential environmental risks associated to the FLX.

Moreover, like other pharmaceuticals, FLX tends to occur in the environment associated with other compounds, especially those present in sewage, such as household products, PPCPs, metals, oil and its derivatives, ammonia and other chemicals (Birch *et al.*, 2015; Ding *et al.*, 2017). Thus, the combination of FLX with other chemicals, in complex mixtures, may produce environmental risks, even if the concentrations are low. In this sense, further studies considering the combined effects of fluoxetine and other chemicals are also required.

ACKNOWLEDGEMENTS

The authors thank the staff and logistic support from the Center of Investigation on Aquatic Pollution and Ecotoxicology (NEPEA), the Laboratory of Sustainable Aquaculture – FINEP, and the Ecotoxicology Laboratory “Professor Caetano Bellibon” from UNISANTA. DMSA thanks CNPq by the PQ fellowship, while ALV, GFE, EGR, MCM, RACM, and ACR thank CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) by the support. ACFC and GD thank FAPESP – Fundação de Amparo à Pesquisa de São Paulo (grants # 2016/02029-4, and # 2016/04768-9, respectively). FSC thanks CNPq by the financial support (grants #481358/2012-9 and #481553/2012-6).

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