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UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Instituto de Biociências
Câmpus do Litoral Paulista

KAINÃ ROCHA CABRERA FAGUNDES

# MORPHOFUNCTIONAL ASSESSMENT OF SEROTONERGIC SIGNALING IN ZEBRAFISH (*Danio rerio*) EXPOSED TO ENVIRONMENTAL CONCENTRATIONS OF FLUOXETINE

**SÃO VICENTE - SP**

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Tese apresentada à Universidade Estadual Paulista (UNESP), Instituto de Biociências, Campus do Litoral Paulista, para obtenção do título de Doutor em Ciências.

Área de Concentração: Biodiversidade.

Orientadora: Profa. Dra. Renata de Britto Mari

Coorientador(a): Prof. Dr. Denis Modelo de Souza Abessa

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**ATA DA DEFESA PÚBLICA DA TESE DE DOUTORADO DE KAINÃ ROCHA CABRERA FAGUNDES, DISCENTE DO PROGRAMA DE PÓS-GRADUAÇÃO EM BIODIVERSIDADE DE AMBIENTES COSTEIROS, DO INSTITUTO DE BIOCIÊNCIAS - CÂMPUS DO LITORAL PAULISTA.**

Aos 05 dias do mês de setembro do ano de 2025, às 14h, no(a) Salão Nobre do IB/CLP, realizou-se a defesa de TESE DE DOUTORADO de KAINÃ ROCHA CABRERA FAGUNDES, intitulada **Avaliação morfofuncional da sinalização serotoninérgica em zebrafish (*Danio rerio*) exposto a concentrações ambientais de fluoxetina**. A Comissão Examinadora foi constituída pelos seguintes membros: Profa. Dra. RENATA DE BRITTO MARI (Orientador(a) - Participação Presencial) do(a) Departamento de Ciências Biológicas e Ambientais / IB/CLP - UNESP, Dra. JOICE NAIARA BERTAGLIA PEREIRA (Participação Presencial) do(a) Instituto Butantã, Profa. Dra. FLÁVIA DE OLIVEIRA (Participação Presencial) do(a) UNIFESP, Prof. Dr. CARLOS EDUARDO TOLUSSI (Participação Presencial) do(a) Universidade Federal do Ceará, Profa. Dra. KARINA MARTINEZ GAGLIARDO (Participação Presencial) do(a) Universidade Metropolitana de Santos (UNIMES). Após a exposição pelo doutorando e arguição pelos membros da Comissão Examinadora que participaram do ato, de forma presencial e/ou virtual, o discente recebeu o conceito final: Aprovado. Nada mais havendo, foi lavrada a presente ata, que após lida e aprovada, foi assinada pelo(a) Presidente(a) da Comissão Examinadora.

Profa. Dra. RENATA DE BRITTO MARI

*Ser cientista é conseguir fazer de uma gota, uma aquarela*

Fagundes, KRC (2025)

**Dedico essa tese à minha família, à ciência, e à biodiversidade.**

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## Lista de abreviações

**5-HT** – 5-hidroxytryptamin (serotonina)
**AChE** – Achetilcholinesterase
**CECs** – Contaminants of Emerging Concern (Contaminantes de Preocupação Emergente)
**CGs** – Células de Glia (Enteric Glia Cells, dependendo do contexto)
**CNS** – Central Nervous System (Sistema Nervoso Central)
**CPEs** – Contaminantes de Preocupação Emergente
**dpf** – Days Post-Fertilization (dias pós-fertilização)
**ECCs** – Enterochromaffin Cells (Células Enterocromafins)
**EGFP** – Enhanced Green Fluorescent Protein (Proteína Verde Fluorescente Aprimorada)
**ENs** – Enteric Neurons (Neurônios Entéricos)
**ENS** – Enteric Nervous System (Sistema Nervoso Entérico)
**FLX** – Fluoxetina
**GCs** – Goblet Cells (Células Caliciformes)
**HE** – Hematoxilina-Eosina
**HTR1A** – 5-Hydroxytryptamine Receptor 1A (Receptor de Serotonina 1A)
**HTR1B** – 5-Hydroxytryptamine Receptor 1B (Receptor de Serotonina 1B)
**HTR2B** – 5-Hydroxytryptamine Receptor 2B (Receptor de Serotonina 2B)
**HTRs** – Hydroxytryptamine Receptors (Receptores de Serotonina)
**IELs** – Intraepithelial Lymphocytes (Linfócitos Intraepiteliais)
**K factor** – Índice de Condição de Fulton
**MS-222** – Tricaína metanossulfonato (anestésico para peixes)
**NTAOs** – Non-Target Aquatic Organisms (Organismos Aquáticos Não Alvo)
**PAS** – Periodic Acid-Schiff
**RT-qPCR** – Reverse Transcription Quantitative PCR (PCR quantitativo após transcrição reversa)
**SENs** – Serotonergic Enteric Neurons (Neurônios Entéricos Serotoninérgicos)
**SERT** – Serotonin Transporter (Transportador de Serotonina)
**SNE** – Sistema Nervoso Entérico
**SSRI** – Selective Serotonin Reuptake Inhibitor (Inibidor Seletivo da Recaptação de Serotonina)
**tph1/tph2** – Tryptophan Hydroxylase 1/2 (Triptofano Hidroxilase 1 e 2, enzimas de síntese de serotonina)

# SUMÁRIO DOS CAPÍTULOS

**General Abstract**

Unconscious consumption and economic forces have intensified the release of contaminants of emerging concern (CECs) into aquatic systems, generating global ecological and health challenges. Among CECs, antidepressants such as fluoxetine (FLX), a selective serotonin reuptake inhibitor (SSRI), are widely prescribed and frequently detected in the environment at concentrations ranging from 0.5 ng $L^{-1}$ levels in surface waters to near 1,000 ng $L^{-1}$ near wastewater effluents. Once released, FLX interacts with conserved serotonergic pathways in non-target aquatic organisms (NTAOs), altering development, behavior, reproduction, and physiology. In zebrafish (*Danio rerio*), FLX exposure has been linked to oxidative stress, neurotoxicity, and gut-liver axis disruption. However, the enteric nervous system (ENS) remains poorly explored as an ecotoxicological target, especially in early life-stages and serotonergic subpopulations. This study aimed to evaluate the morphofunctional effects of environmentally relevant concentrations of FLX on serotonergic signaling and ENS integrity in different zebrafish life-stages. We integrated molecular, morphological, and behavioral analyses to characterize early neurodevelopmental alterations, long-term ENS dynamics, and systemic consequences on gut-liver homeostasis. By establishing the ENS and gut-liver axis as remarkable biomarkers, this research improved the understanding of antidepressant ecotoxicity and highlighted stage-dependent responses in aquatic organisms.

**Keywords:** fluoxetine; zebrafish; serotonergic signaling; enteric nervous system; gut-liver axis; ecotoxicology.

**Resumo Geral**

O consumo inconsciente e as forças econômicas intensificaram a liberação de contaminantes de preocupação emergente (CPEs) em sistemas aquáticos, gerando desafios ecológicos e de saúde globais. Entre os CPEs, antidepressivos como a fluoxetina (FLX), um inibidor seletivo da recaptação da serotonina (ISRS), são amplamente prescritos e frequentemente detectados no ambiente em concentrações que variam de 0,5 ng $L^{-1}$ em águas superficiais a cerca de 1.000 ng $L^{-1}$ perto de efluentes de águas residuais. Uma vez no ambiente, a FLX interage com vias serotoninérgicas conservadas em organismos aquáticos não alvo (OANAs), alterando o desenvolvimento, o comportamento, a reprodução e a fisiologia. No zebrafish (*Danio rerio*), nosso modelo AONAs, a exposição à FLX tem sido associada ao estresse oxidativo, à

neurotoxicidade e à alteração do eixo intestino-fígado. No entanto, o sistema nervoso entérico (SNE) permanece pouco explorado como um alvo ecotoxicológico, especialmente em estágios iniciais da vida e em subpopulações serotoninérgicas. Este estudo teve como objetivo avaliar os efeitos morfofuncionais de concentrações ambientalmente relevantes de FLX na sinalização serotoninérgica e na integridade do SNE em diferentes estágios de vida do zebrafish. Integramos análises moleculares, morfológicas e comportamentais para caracterizar alterações neurodesenvolvimentais inciais, a dinâmica do SNE a longo prazo e as consequências sistêmicas na homeostase intestinal-hepática. Ao estabelecer o SNE e o eixo intestinal-hepático como biomarcadores notáveis, esta pesquisa aprimorou a compreensão da ecotoxicidade antidepressiva e destacou respostas dependentes do estágio em organismos aquáticos.

**Palavras-chave**: fluoxetina; peixe-zebra; sinalização serotoninérgica; sistema nervoso entérico; eixo intestino-fígado; ecotoxicologia.

## General Introduction

The world lives for consumption, and consumption kills the world. The Oxford English defines consumption in: The process of using resources to satisfy human wants or needs. The more we need, the more we use. So, what are the consequences of using without conscience? Are our needs above the environmental balance?

The Industrial Revolution introduced new technologies and possibilities; however, destructive mode of production is still above the conscious consumption (Falasca-Zamponi, 2011). As society, the needs are from humans, but the desires are made by corporations. Conrad (2007) recognize in his book *The Medicalization of Society* that pharmaceutical companies have become so important that they have displaced physicians as a main driver of the medicalization process.

As consequence, tons of pharmaceuticals and other products are eliminated in the environment daily (Brusseau & Artiola, 2019). Most of them are considered contaminants of emerging concern (CECs), i.e., chemicals or materials 'naturally occurring, manufactured or whose toxicity or persistence are likely to significantly alter the metabolism of a living being' (Sauvé & Desrosiers, 2014). These CECs have increased significantly in aquatic systems (Loos et al., 2013).

CECs comprise a variety of contaminants that have only recently appeared in water, or that are of recent concern because they have been detected at concentrations significantly higher than expected, and/or their risk to human and environmental health may not be fully understood (Gros et al., 2008; Starling et al., 2019). CECs include natural and artificial chemical substances and their by-products, comprising pharmaceuticals, personal care products (PPCPs), flame retardants (FRs), pesticides, artificial sweeteners (ASWs), nanoparticles, microplastics and their transformation products, but also antibiotic resistant bacteria (ARB), antibiotic resistant genes (ARG) and, more recently, the SARS-CoV-2 virus (Broccoli et al., 2021; Gros et al., 2010; Jelic et al., 2011; Keerthanan et al., 2021; Köck-Schulmeyer et al., 2021; Pastorino, Nocita, et al., 2021; Pastorino, Pizzul, et al., 2021; Praveena et al., 2019; Primel et al., 2017; Sicuro et al., 2020; Xu et al., 2021).

Antidepressants are considered CECs and they are a group of drugs to treat psychiatric disorders (Metz et al., 2023), which can be classified as tricyclic antidepressants (TCA), serotonin reuptake inhibitors (SSRIs), serotonin-norepinephrine reuptake inhibitors (SNRIs), and monoamine oxidase inhibitors (MAOIs) according to their mechanisms of action (Fitzgerald

& Watson, 2019). Antidepressants have become among the most prescribed medications worldwide (Diaz-Camal et al., 2022; Pazzagli et al., 2022).

The pharmacological mechanisms of antidepressants contribute not only to their therapeutic effects but also to their environmental risks. The primary mechanisms of action of TCAs, SSRIs, and SNRIs. TCAs act by inhibiting the reuptake of both serotonin and norepinephrine, leading to increased neurotransmitter levels in the synaptic cleft (Gillman, 2007). SSRIs selectively block serotonin reuptake, while SNRIs target both serotonin and norepinephrine reuptake, though with a higher selectivity ratio for norepinephrine (Brunello, 2002; McDonald, 2017). As consequence, these mechanisms can interfere with neurotransmitter systems in aquatic species, leading to altered behavior, reproduction, and development (Liu et al., 2025).

Fluoxetine (FLX) is the first and represents the main SSRI antidepressant class. FLX is a racemic mixture of R- and S-fluoxetine hydrochloride, and the chronic treatment of SSRIs elicits a series of neurobiological changes relevant for antidepressant activity. In target organisms, the chronic modulation of FLX, as SSRI, is reducing/blocking the serotonin (5-HT) transporter (SERT) but not affecting its mRNA levels (Fuller et al., 1974; Rudnick, 2006; Wong et al., 1974). FLX is prescribed for depression, obsessive-compulsive disorder, bulimia nervosa and panic disorder, increasing serotonergic activity in the Central Nervous System (CNS) (P. De Silva, 2014; Myers, 2007).

FLX can be introduced into the environment as a result of various anthropogenic sources, the main one being the irregular domestic disposal of expired drugs, incomplete absorption followed by physiological elimination, and the direct disposal of partially treated pharmaceutical effluents (Glaser et al., 2020). Additionally, part of FLX that is not metabolized, is eliminated, and enters the environment via the faecal and urinary excretion (J. Zhao et al., 2024).

The CECs, once in environment, affects non-target aquatic organisms (NTAOs) and they may have similar biological functions and receptors (Arnold et al., 2014). FLX modulates a series of biological markers in diverse NTAOs, reviewed by Correia et al. (2023), from invertebrates to vertebrates, at molecular, morphological and behavior levels (Correia et al., 2023; Cortez et al., 2019; Pauly et al., 2021). Environmentally relevant concentrations of FLX in aquatic systems range from as low as 0.4 ng $L^{-1}$ in open waters to as high as 930 ng $L^{-1}$ near wastewater treatment plants (Benotti & Brownawell, 2009; Cortez et al., 2019; de Souza et al., 2021; González Alonso et al., 2010; Kolpin et al., 2002; Lajeunesse et al., 2008; MacLeod et al., 2007; Metcalfe et al., 2003, 2010; Schultz et al., 2010; L. J. G. Silva et al., 2015;

Snyder, 2008; Styrishave et al., 2011; Vasskog et al., 2008).

In aquatic systems, FLX disrupts key biological processes in fish, affecting development, behavior, reproduction, neurochemistry, and physiology. In early life stages of *Danio rerio* (zebrafish), FLX altered embryo hatching, heart rate, larval size, oxidative balance, gene expression, and neurotransmitter levels (Correia et al., 2023), as well as larval enteric neurogenesis (Fagundes et al., 2025), leading to behavioral impairments and teratogenic effects (Orozco-Hernández, Gómez-Oliván, Elizalde-Velázquez, Heredia-García, et al., 2022).

In juveniles and adults of *D. rerio*, FLX induced anxiety-like and sedative behaviors (Orozco-Hernández et al., 2022b), altered energy metabolism (Pinto et al., 2024), and impaired reproduction (Venkatachalam et al., 2023). In *Cichlasoma dimerus*, FLX reduced feeding (Dorelle et al., 2020) and acted as a mild endocrine disruptor (Dorelle et al., 2017), while in multiple freshwater species, some effects persisted or were concentration-dependent (Hubená et al., 2020). Most of these results are linked to oxidative stress (Orozco-Hernández et al., 2023; Orozco-Hernández et al., 2022a; Pinto et al., 2024), and neurotoxicity (Orozco-Hernández et al., 2022b).

As described above, most studies focus on acute toxicity or isolated biomarkers and long-term effects, particularly about the integration between the intestine, liver, and enteric nervous system (ENS) are barely known. The ENS is considered a new and outstanding ecotoxicological biomarker and our group has been published articles in different fish species that can define the ENS as a key morphofunctional biomarker (Fagundes et al., 2025; Gonçalves et al., 2020; Marinsek et al., 2018, 2022).

The ENS is an extensive intrinsic nervous system in the gut and it is autonomous from CNS and controls a sort of functions in the intestine (Furness, 2012). The ENS is composed of neurons aggregated as ganglia or diffused and nerve fibers that supply effector tissues, including the muscle of the gut wall, the epithelial lining, intrinsic blood vessels and gastroenteropancreatic endocrine cells (Furness, 2012). The serotoninergic enteric neurons represent 2–3 % of 5-HT synthesized in the ENS (Terry & Margolis, 2016).

The teleostean ENS, such as zebrafish, is anatomically and functionally closely resembled the one in mammals, with some differences and simpler, mostly containing myenteric neurons, and they are arranged as neuronal pairs or single neurons, instead of clustered in ganglia (Lickwar et al., 2017; Marinsek et al., 2018; Wallace et al., 2005; Z. Wang et al., 2010).

The 5-HT is synthesized by tryptophan hydroxylase (TPH) in the peripheric cells, as isoform TPH1, such as enterochromaffin cells (ECCs) (Côté et al., 2003; Walther & Bader, 2003);

pancreatic β cells (Kim & Ho, 2010; Paulmann et al., 2009); adipocytes (Stunes et al., 2011) and osteoclasts (Chabbi-Achengli et al., 2012). And the TPH2 occurs and acts in neurons of the CNS and in the ENS (Côté et al., 2003; Walther & Bader, 2003). The gut contains over 95 % of the body's 5-HT and the concentration of 5-HT magnitude in the intestine is higher than in the brain (Gershon, 1999a). 5-HT is a growth factor which stimulates the development of late-arising enteric neurons in the primitive ENS (Fiorica-Howells et al., 2000; X. Zhou & Galligan, 1999).

The 5-HT receptors (HTRs) are found throughout the body in various tissues including brain, spinal cord, heart, blood, and gut (Gresch, 2013). There are extensive types of HTRs, and HTR1A is the major 5-HT inhibitory receptor, which is found in a variety of tissues in vertebrates, including brain and ENS (Kirchgessner et al., 1996; Norton et al., 2008). Among the serotonin receptors, HTR1A is the most sensitive to FLX under therapeutic use (Pei et al., 2016).

HTR1A is found in brain in two distinct populations, autoreceptors and heteroreceptors (Piszczek et al., 2015). HTR1A autoreceptors are expressed on serotoninergic neurons in the raphe nuclei and inhibit the firing rate of serotoninergic neurons (Blier & de Montigny, 1994; Evrard et al., 1999; Pompeiano et al., 1992). Heteroreceptors are expressed widely in brain and in the body (Casanovas et al., 1999; Pompeiano et al., 1992). One of the roles of 5HTR1A, when activated by pharmacological compounds, is to reduce anxiety (File et al., 1996).

In target organisms, FLX is also known to promote neuroprotection and post-embryonic neurogenesis in dorsal hippocampus (Khodanovich et al., 2018; Lim et al., 2009; Y. Zhao et al., 2020), primarily due to the increased availability of 5-HT in mammals by inhibiting SERT. However, environmental concentrations of FLX inhibit acetylcholinesterase (AChE) activity in zebrafish larvae (de Farias et al., 2019). In contrast, in adult zebrafish, exposure to environmentally relevant concentrations of FLX for 21 days did not affect AChE activity in either the head or body (Pinto et al., 2024).

The effects of environmentally relevant concentrations of FLX on the ENS of NTAOs remain poorly defined, despite its importance as an ecotoxicological biomarker and key regulator of gut-brain communication. Since most serotonin is synthesized in the gut, FLX-induced disruptions may affect gastrointestinal, immune, and behavioral functions. While studies often focus on larvae, it is also essential to investigate adults, as they allow evaluation of long-term systemic consequences, including alterations in the gut-liver axis, serotonergic signaling and ENS integrity. Assessing both life stages in zebrafish, our NTAO model, provides a

comprehensive understanding of FLX impacts, supporting their use as a model for pharmacology and ecotoxicology.

**Aim:** To evaluate the morphofunctional effects of environmentally relevant concentrations of FLX on serotonergic signaling and ENS in zebrafish (*Danio rerio*) as NTAO model in different life-stages.

*Specific aims:*

1. Characterize the effects of environmentally relevant concentrations of FLX on serotonergic signaling components in zebrafish larvae.
2. Assess the impact of fluoxetine on enteric neurogenesis and serotonergic enteric neurons (SENs) during early development.
3. Investigate behavioral alterations in larvae related to serotonergic disruption (e.g., locomotion, light-dark preference).
4. Evaluate the long-term effects of environmentally relevant concentrations of FLX on ENS and SENs dynamics of adult zebrafish.
5. Investigate the effects of environmentally relevant concentrations of FLX exposure on the gut-liver axis, including intestinal barrier integrity, inflammatory responses, and hepatic condition.
6. Integrate molecular, morphofunctional, and behavioral data to establish the ENS and gut-liver axis as reliable biomarkers of fluoxetine ecotoxicity.

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The Chapter II was written as article format and it is planned to be published in the journal Science of The Total Environment.

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## GENERAL CONCLUSIONS

The study " demonstrated that exposure to environmentally relevant concentrations of FLX, an Emerging Contaminant of Concern (CEC), significantly and adversely modulates serotonergic signaling in the zebrafish across different life stages.

In larvae, FLX acted as an early-life disruptor, altering the expression of serotonin transporters and receptors and impairing enteric neurogenesis, which was manifested in notable changes in behavioral responses. These findings underscore the high vulnerability of aquatic organisms during their early development. In contrast, chronic exposure in adults revealed profound systemic consequences, where the drug affected the Enteric Nervous System (ENS) and the crucial gut-liver axis. This impact resulted in complex morphological, immunological, and metabolic alterations, reinforcing the idea that pharmaceutical pollution has a reach extending beyond the primary neurochemical targets.

These findings provide a strong foundation for establishing the ENS, the serotonergic pathways, and the gut-liver axis as sensitive and essential biomarkers in assessing FLX ecotoxicity. Our integrated approach, which combined molecular, morphofunctional, and behavioral analyses across distinct life stages, allowed for a comprehensive perspective on how antidepressants influence NTAOs.

In synthesis, this work highlights the critical importance and the urgent need for understanding the impact of neuroactive contaminants on aquatic ecosystems. By validating the zebrafish as a robust model in pharmaceutical ecotoxicology, we emphasize the pressing requirement for more stringent regulatory attention. It is imperative that regulatory bodies mitigate the environmental and ecological risks posed by CECs, ensuring the health and sustainability of aquatic ecosystems in the face of increasing contamination by pharmaceuticals such as FLX.