„ALEXANDRU IOAN CUZA" UNIVERSITY OF IASI

FACULTY OF GEOGRAPHY AND GEOLOGY

DOCTORAL SCHOOL OF GEOSCIENCES

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PHD THESIS ABSTRACT

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Assessment of the Toxicological Effects Induced by the Administration of Chemical Mixtures (Pharmaceutical Compounds and Micro-nanoplastics) in the Model Species *Danio rerio*

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The completion of this doctoral dissertation represents the culmination of a complex academic and personal journey, spanning four years of doctoral training. This achievement would not have been possible without the support, guidance and encouragement of some remarkable people. I would therefore like to express my sincere gratitude to all those who contributed, directly or indirectly, to the completion of this work and to my development both as a researcher and as an individual.

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During my doctoral studies, I had the opportunity to carry out a research internship at the University of Aveiro, Portugal, where a substantial part of the experimental work included in this dissertation was carried out. I am sincerely grateful to my mentors during this period, Professor Isabel Lopes and Dr. Cátia Venâncio, for their exceptional support, invaluable guidance and for providing me with a remarkable scientific and professional environment. Their mentorship contributed significantly to both my scientific training and personal development.

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TABLE OF CONTENT

ABBREVIATIONS LIST

AChE = Acetylcholinesterase

NSAIDs = Non-steroidal anti-inflammatory drugs

ALT = Glutamic-pyruvic transaminase

APX = Ascorbate peroxidase

ASD = Autism spectrum disorder

AST = Transaminase glutamic oxaloacetic

ATCh = Acetylthiocholine

ATR = Attenuated Total Reflectance

ATR-IR = Attenuated Total Reflectance Infrared spectroscopy

BD = Time spent in the right arm

BS = Time spent in the starting arm

BSt = Time spent in the divided left arm

Cd = Cadmium

COX = Cyclooxygenase

dpf = days post fertilization

DT = Total distance traveled

EC = Effective concentration

$EC_{20,\ MIf}$ = Effective concentration inducing 20% malformations

$EC_{50,\ MIf}$ = Effective concentration inducing 50% malformations

FDM = Fused Deposition Modeling

FET = Fish Embryo Toxicity

FTIR = Fourier Transform Infrared Spectroscopy

GPx = Glutation peroxidase

GSH = Glutation

GST = Glutation S-transferase

hpf = hours post fertilization

Ibu = Ibuprofen

Kp = Ketoprofen

LC = Lethal concentration

LD = Linear Dichroism

LDH = Lactat dehydrogenase

LÎ = Confidence limits

MDA = Malondialdehyde

MIC = Minimum inhibitory concentration

MNPL = Micro-nanoplastics

Mp = Meropenem

MPL = Microplastics

n.d. = Not detected

NPL = Nanoplastics

PA = Polyamide

PBT = Persistent, Bioaccumulative and Toxic substances

PE = Polyethylene

PET = Polyethylene terephthalate

PLA= Polylactic acid

PP = Polypropylene

PVC = Polyvinyl chloride

RA = Counterclockwise rotations

ROS = Reactive Oxygen Species

ROUT = Robust Regression and Outlier Removal

RWWTP = Raw Wastewater Treatment Plant

SEM = Scanning electron microscopy

SOD = Superoxide dismutase

TD = Time spent in the decision zone

TI = Time of immobility

TP = Time spent near walls

TrS = Frequency of transitions to the upper half

TS = Time spent in the upper half

TSt = Time spent in the left arm

VÎ = Average swimming speed

VPA = Valproic acid

WWTPs = Wastewater Treatment Plants

KEYWORDS

Zebrafish, pharmaceutical pollutants, micro- and nanoplastics, polylactic acid, anti-inflammatory drugs, antiepileptic drugs, antibiotics.

The doctoral dissertation consists of 211 pages, including 8 pages of Introduction, 17 pages of Current State of Knowledge, 29 pages of Materials and Methods, 93 pages of Results and Discussion and 3 pages of Conclusions. The dissertation contains 73 figures, 2 tables and 256 bibliographical references. In this abstract, the table of contents, the numbering of figures and tables, as well as the list of abbreviations have been kept in the same format as those presented in the doctoral dissertation. The selective bibliography included in this abstract comprises 46 references selected from the total of 256 bibliographical references cited in the doctoral dissertation.

INTRODUCTION

Pollution of the aquatic environment is one of the most significant challenges of our time, driven by the intensification of anthropogenic activities and the widespread use of chemicals in numerous sectors. Among emerging contaminants, pharmaceuticals and plastic debris smaller than 5 mm, namely microplastics and nanoplastics, have attracted considerable attention due to their persistence in aquatic ecosystems and their complex effects on living organisms.

Whether used in human or veterinary medicine, approximately 4,000 pharmaceutical products are manufactured and marketed worldwide for the treatment, prevention, and diagnosis of diseases (SanJuan-Reyes et al., 2017; Alkimin et al., 2020).

Monitoring studies have demonstrated that pharmaceutical compounds cannot be completely removed by conventional wastewater treatment plant (WWTP) processes, resulting in the release of both parent compounds and their transformation products into aquatic environments after use (Diniz et al., 2015; SanJuan-Reyes et al., 2017; Coimbra et al., 2021). These findings have raised concerns about the development of new technologies aimed at eliminating pharmaceutical residues, which are commonly detected at concentrations ranging from ng L^{-1} to µg L^{-1} in surface waters, groundwater and even drinking water supplies (Muambo et al., 2024).

Pharmaceutical pollutants, including antimicrobial agents, antidiabetic drugs, antidepressants, anticonvulsants, analgesics and nonsteroidal anti-inflammatory drugs (NSAIDs), have become a global concern due to their widespread occurrence, persistence in the environment and adverse effects, particularly on aquatic ecosystems (Chelaru et al., 2025) and human health (Muambo et al., 2024; Szarka et al., 2024).

According to previous studies, the simultaneous presence of multiple chemical compounds in the environment may lead to toxicological responses different from those induced by individual contaminants, leading to either increased or reduced toxicity (Gomes et al., 2024; Jijie et al., 2020; Wang et al., 2017; Yang et al., 2021). It has been shown that concomitant exposure to chlorothalonil and prochloraz reduces adverse effects on the metabolism of zebrafish embryos and larvae (Yang et al., 2021). In addition, a study conducted by our research group demonstrated antagonistic effects on the behavior of adult zebrafish and oxidative stress

following short-term exposure to a mixture of cadmium, nickel and deltamethrin (Jijie et al., 2020).

Furthermore, exposure of zebrafish larvae to binary, ternary, quaternary and quinary mixtures of pesticides for 96 h revealed synergistic effects (Wang et al., 2017). Similarly, another study reported more pronounced behavioral changes in *Danio rerio* larvae exposed simultaneously to carbendazim and fipronil compared to those subjected to individual pesticide treatments (Gomes et al., 2024). In addition, co-exposure to cadmium (Cd) and ketoprofen (Kp) for 42 days induced biochemical, behavioral, cognitive and histopathological alterations in adult zebrafish (Madesh et al., 2024a), while parental exposure to Kp in the presence of Cd amplified developmental impairments in the F1 generation, negatively affecting cardiac function and locomotor activity (Madesh et al., 2024b). Synergistic effects have also been reported for ternary mixtures composed of iprodione and pyraclostrobin combined with either pyrimethanil or acetamiprid, while an antagonistic interaction was observed for the quaternary mixture consisting of iprodione, pyrimethanil, pyraclostrobin and acetamiprid (Wang et al., 2018).

In parallel, micro- and nanoplastics (MNPLs), generated by the fragmentation of plastics, have become ubiquitous contaminants in the environment due to the extensive use of thermoplastic polymers, such as polylactic acid (PLA), polypropylene (PP) and low-density polyethylene (LDPE).

These polymers are predominantly used in food packaging, textiles, household products and three-dimensional (3D) printing due to their favorable thermal properties, flexibility and mechanical strength. However, their degradation in aquatic ecosystems generates micro- and nanoscale particles with significant ecotoxicological potential. Their small size and physicochemical characteristics facilitate interactions with chemical pollutants, including pharmaceutical compounds, potentially increasing toxicity and accelerating bioaccumulation processes. Furthermore, these interactions may promote pollutant degradation and subsequent release of hazardous substances into the environment. In this context, investigating both the individual and combined impacts of these contaminants on model organisms, such as *Danio rerio*, is essential for understanding ecotoxicological risks and developing effective pollution mitigation strategies. However, relatively limited information is available regarding the interactions between pharmaceutical compounds and other emerging contaminants, such as MNPLs, which are increasingly detected in aquatic ecosystems.

Microplastics (MPLs) may act as vectors for contaminants, as pharmaceutical compounds can adsorb and desorb from their surfaces. In larger organisms, such as fish, this phenomenon may trigger the so-called “Trojan horse effect”, whereby contaminated particles are ingested or penetrate biological barriers, potentially resulting in increased toxic effects (Atugoda et al., 2021; Hildebrandt et al., 2021; Zhang and Xu, 2022). Although interactions between MNPLs and certain pharmaceutical compounds, such as ibuprofen (Ibu) have been investigated (Barreto et al., 2023), no study has yet addressed the combined effects of ketoprofen (Kp) and PLA-derived MNPLs, despite their potential to produce synergistic, antagonistic or cumulative effects.

Among the types of plastic most frequently identified in environmental samples as fibers and fragments is polypropylene (PP) (Matjašič et al., 2023), probably due to its excellent mechanical characteristics (Hossain et al., 2024) and extensive use in the packaging industry (Sid et al., 2021) and numerous other industrial sectors. Its remarkable properties, including high temperature resistance, chemical stability and durability (Yan et al., 2015; Maddah, 2016), also make PP suitable for the manufacture of products such as funnels, buckets, containers and various laboratory and household implements (Nanthini Devi et al., 2021; Maddah, 2016). To address the existing knowledge gaps, this doctoral research aimed to evaluate how environmentally relevant concentrations of pharmaceutical compounds and MNPLs influence toxicological responses.

In addition, the study took an innovative approach by investigating the individual and combined effects of several classes of contaminants, including pharmaceuticals (anti-inflammatory drugs - ibuprofen [Ibu] and ketoprofen [Kp]; antiepileptic drugs - valproic acid [VPA]; and antibiotics - meropenem [Mp]), as well as MNPL fibers/particles derived from PP and PLA. Both binary and ternary mixtures were evaluated, initially between pharmaceutical compounds and subsequently between pharmaceuticals and MNPLs, allowing comparisons between conventional plastics and PLA-based materials. The experiments were performed using environmentally relevant concentrations, a feature that adds novelty and ecological relevance to the study. Furthermore, exposures were performed in all developmental stages of zebrafish (embryos, larvae, and adults), a species widely recognized as a valuable model organism in toxicological research.

Toxicological effects were evaluated by assessing mortality and malformation rates, total body length and interocular distance in larvae, as well as heart rate and hatching success in embryos. In addition, behavioral analyses were performed following both individual and combined exposures.

Experiments involving adult *Danio rerio* focused mainly on exploratory behavior and social preferences, assessing key parameters of swimming performance. The studies included in this doctoral dissertation are particularly relevant because they address not only the effects of individual pharmaceutical compounds, but also the impact of binary and ternary mixtures, thus reflecting realistic exposure scenarios in aquatic environments where these contaminants commonly coexist. Assessing interactions between pharmaceutical compounds provides critical insight into complex ecotoxicological risks, which may be either amplified or attenuated, depending on the composition of the mixture. Furthermore, investigating the concomitant exposure of aquatic organisms to pharmaceutical compounds and MNPLs is essential, given the increasing prevalence of these emerging contaminants and their potential to disrupt critical physiological processes in early developmental stages.

Therefore, this study contributes to an integrated understanding of synergistic, antagonistic and cumulative effects, highlighting the need for extensive research on multiple mixtures of contaminants and comprehensive risk assessments covering the entire life cycle of exposed organisms.

To achieve the overall aim of this study, the following specific objectives were established:

- **O1.** Determination of the toxic effects of selected pharmaceutical compounds (Ibu, Kp, VPA, and Mp), both individually and in mixtures, on different developmental stages of zebrafish (*Danio rerio*) (embryos, larvae and adults), using concentrations relevant to the environment.
- **O2.** Assessment of the impact of micro- and nanoplastics (MNPLs) derived from PP and PLA, both individually and in combination with the emerging contaminant Kp, on different developmental stages of zebrafish (*Danio rerio*) (embryos, larvae and adults), using concentrations relevant to the environment.

- **O3.** Investigation of the relationships between locomotor parameters and social behavior indicators following exposure of adult zebrafish (*Danio rerio*) to pharmaceutical mixtures and combinations of pharmaceutical compounds and MNPLs.

Due to its interdisciplinary nature, this doctoral dissertation was carried out within a co-tutorship between the Doctoral School of Geosciences and the Doctoral School of Biology of „Alexandru Ioan Cuza" University in Iași, Romania. Part of the experimental work included in this dissertation was carried out within the Center for Environmental and Marine Studies (CESAM) of the University of Aveiro (Portugal) during an Erasmus+ research mobility, as well as within the Department of Biology, Faculty of Sciences, "Vasile Alecsandri" University of Bacău, Romania.

CHAPTER 1. CURRENT STATE OF KNOWLEDGE IN THE FIELD

1.1. General context

Pharmaceuticals and their derivatives are currently a major concern from both a human health and environmental protection perspective (Acs et al., 2022; Moermond et al., 2022). These compounds are frequently detected in aquatic ecosystems; however, their effects remain largely poorly understood, despite increasing research efforts aimed at advancing knowledge in this field.

This situation is mainly due to the excessive global consumption of pharmaceuticals, combined with inadequate wastewater treatment (Ortuzar et al., 2022), as many wastewater treatment plants do not have the technological capacity to completely remove these residues. Even at very low concentrations, pharmaceutical pollutants - often present in the form of nanoparticles that are difficult to detect - may have adverse effects on aquatic populations (Desai et al., 2022).

Discharge of inadequately treated wastewater into surface water bodies, such as rivers and lakes, exposes aquatic ecosystems to active pharmaceutical ingredients, potentially compromising environmental health (Hong et al., 2021) and posing risks to human health (Wilkinson et al., 2022). Furthermore, these waters may subsequently be reintroduced into drinking water supplies, even though conventional treatment processes do not completely remove certain pharmaceutical compounds. Consequently, recent research has focused on the development and implementation of effective strategies for the removal of these contaminants, including biological and chemical approaches, as well as advanced technologies such as ultraviolet (UV) irradiation and activated carbon treatment (Onesios-Barry et al., 2014).

Among the pharmaceutical ingredients most detected in wastewater are antiepileptic, antidiabetic, anti-inflammatory and antibiotic compounds. Numerous studies have reported the occurrence of phenytoin, an antiepileptic drug, in wastewater treatment plant effluents, hospital wastewater, surface water and even drinking water supplies (Cardoso-Vera et al., 2022). Strategies aimed at reducing water contamination by pharmaceutical pollutants must be sustainable. This requires the development and implementation of an adequate infrastructure for

wastewater collection and treatment, along with the adoption of advanced technologies capable of completely removing active pharmaceutical substances.

An immediate solution could involve the responsible use of medicines through controlled prescriptions and the implementation of stricter dosage regulations. In this regard, Moermond et al. (2022) proposed an environmentally oriented "green" approach, based on the identification and implementation of key criteria for environmental protection. This strategy aims to reduce the impact of pharmaceutical residues on aquatic organisms and key trophic levels, minimize environmental exposure by reducing emissions or improving (bio)degradability in the environment, avoid the use of PBT substances (persistent, bioaccumulative and toxic compounds) and mitigate ecotoxicological risks. In this context, Moermond et al. (2022) developed a graphical representation of the proposed framework, which has been adapted and extended in the present thesis to facilitate a clearer understanding of the phenomenon (**Fig. I.1.**).

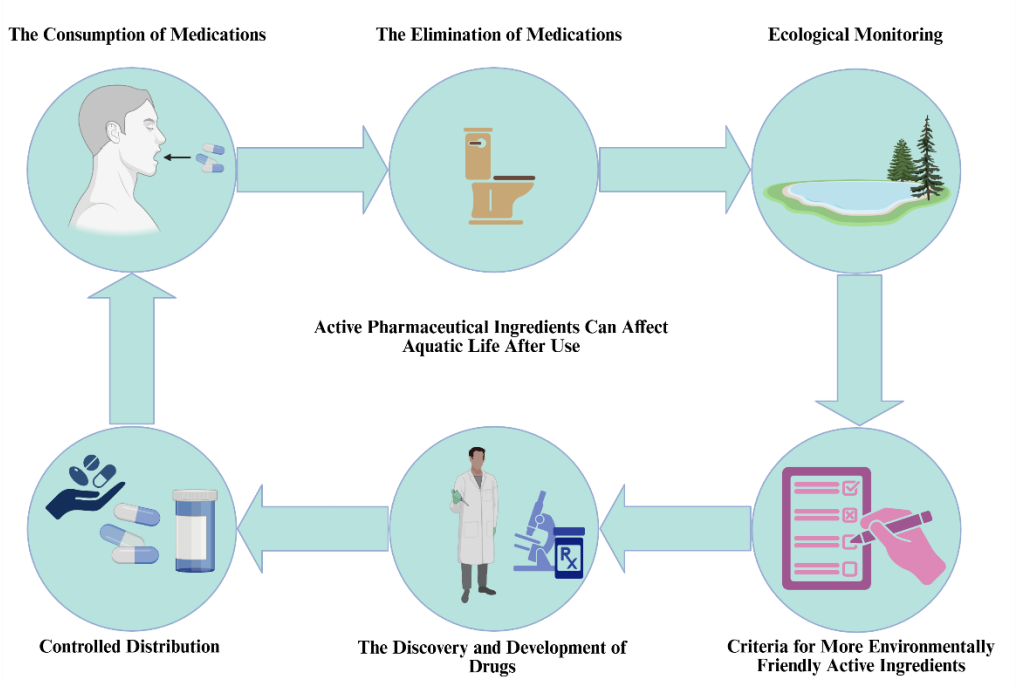

Fig. I.1. Graphical illustration of the life cycle of pharmaceutical products under the implementation of ecological sustainability criteria; created using BioRender (adapted from Moermond et al., 2022).

1.2. Pharmaceutical substances

An active pharmaceutical compound is defined as a substance used in the development of a medicinal product which, when incorporated during the manufacturing process, becomes its active ingredient. This compound is designed to exert pharmacological, immunological or metabolic action with the aim of restoring, correcting or modifying physiological functions or contributing to the establishment of a medical diagnosis.

Pharmaceutical pollution is currently a major global environmental concern (Wohler et al., 2020), driven by the presence of both active pharmaceutical ingredients and inert substances, such as polymeric materials used in packaging. These materials have attracted considerable scientific interest (Desai et al., 2022) due to their potential to exert adverse effects on both the environment and human health (Millarhouse et al., 2020; Wilkinson et al., 2022). Such contaminants may be harmful even at very low concentrations (Desai et al., 2022). Consequently, recent research has focused on developing and implementing effective strategies for eliminating pharmaceutical residues from the environment (Onesios-Barry et al., 2014).

The occurrence of these substances in the environment is mainly determined by consumers through the use and disposal of medicines. Only a small fraction of the administered pharmaceuticals is metabolized, while the rest is excreted, to a limited extent through sweat, but predominantly through urine and feces. As a result, these compounds enter wastewater streams and are subsequently released into the environment.

As Ortúzar et al. (2022) have highlighted, advanced biological treatment technologies - including modified conventional activated sludge systems, aerobic granular sludge systems, moving bed biofilm reactors, membrane bioreactors and Anammox processes - play a central role in improving the efficiency of wastewater treatment. Their schematic representation provides a useful framework for understanding the operating principles of these systems and how by-products, such as residual sludge, may contribute to the transfer and dissemination of pharmaceutical compounds in the environment, as illustrated in **Figure I.2.**

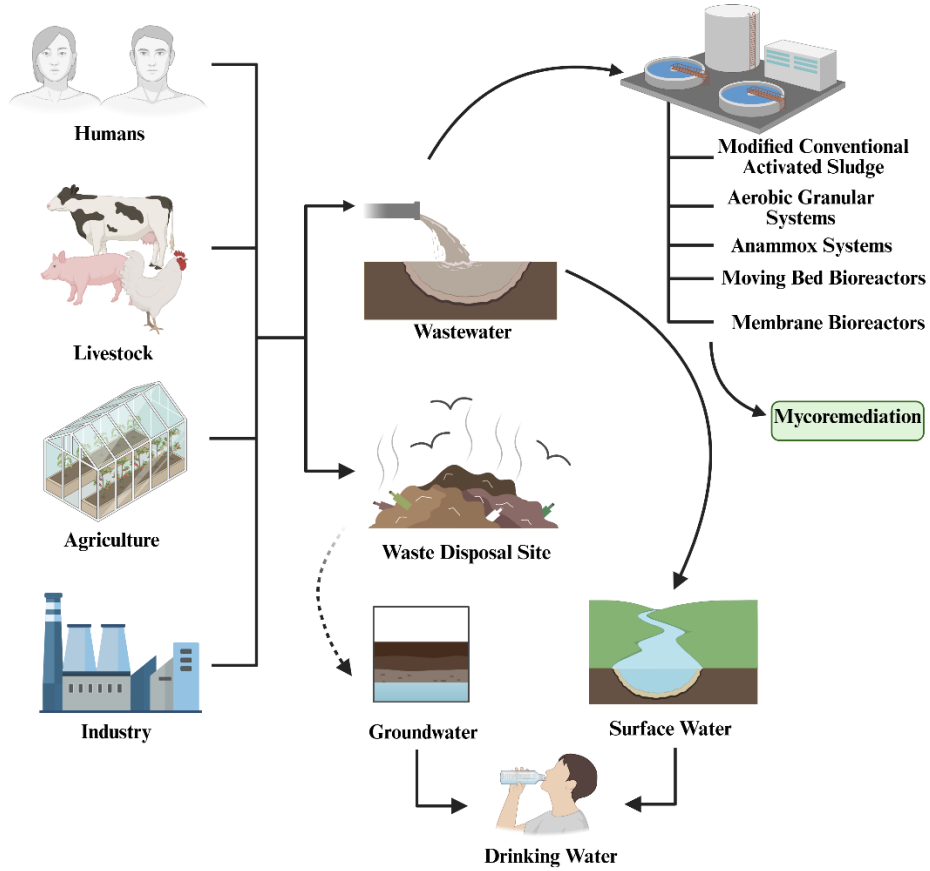

Fig. I.2. Main sources of pharmaceutical pollutants in the environment. Illustration created using BioRender, adapted from Ortúzar et al. (2022) and Chelaru et al. (2026b).

1.3. Micro-nanoplastics

Micro- and nanoplastics (MNPLs) are plastic particles ≤ 5 mm in size, originating from primary sources, such as microbeads and synthetic fibers, and from secondary sources resulting from the degradation of larger plastic debris, including polyethylene terephthalate (PET) bottles and plastic bags (Andrady, 2011; Song et al., 2024).

The rapid growth of plastic production and the continued reliance on fossil fuels for plastic manufacturing have contributed to numerous environmental challenges and adverse health effects. As a result, plastic pollution has become a growing threat to natural ecosystems, human health and the global climate (Karali et al., 2024). As illustrated in **Figure I.3.**, micro- and nanoplastics originate from a wide range of primary and secondary sources associated with human activities across multiple sectors.

Fig. I.3. Main sources of microplastic contamination in the environment. Illustration created using BioRender and adapted from Kataria et al. (2024).

Micro- and nanoplastics (MNPLs) act as vectors for a wide range of pollutants, including pharmaceutical compounds, heavy metals and persistent organic pollutants. Once ingested by aquatic organisms, these contaminant-laden particles may induce oxidative stress, cellular toxicity and metabolic dysfunction (Atugoda et al., 2021; Klavins et al., 2022; Narwal et al., 2024; Savuca et al., 2024; Abdollahi et al., 2025). Due to their large specific surface area and hydrophobic properties, MNPLs exhibit a high sorption capacity for persistent organic contaminants. Furthermore, desorption of these substances in aquatic environments may facilitate their transfer to exposed organisms, thus increasing ecotoxicological risks. To provide a clearer understanding of this phenomenon, a graphical representation was developed, as shown in **Figure I.6.**

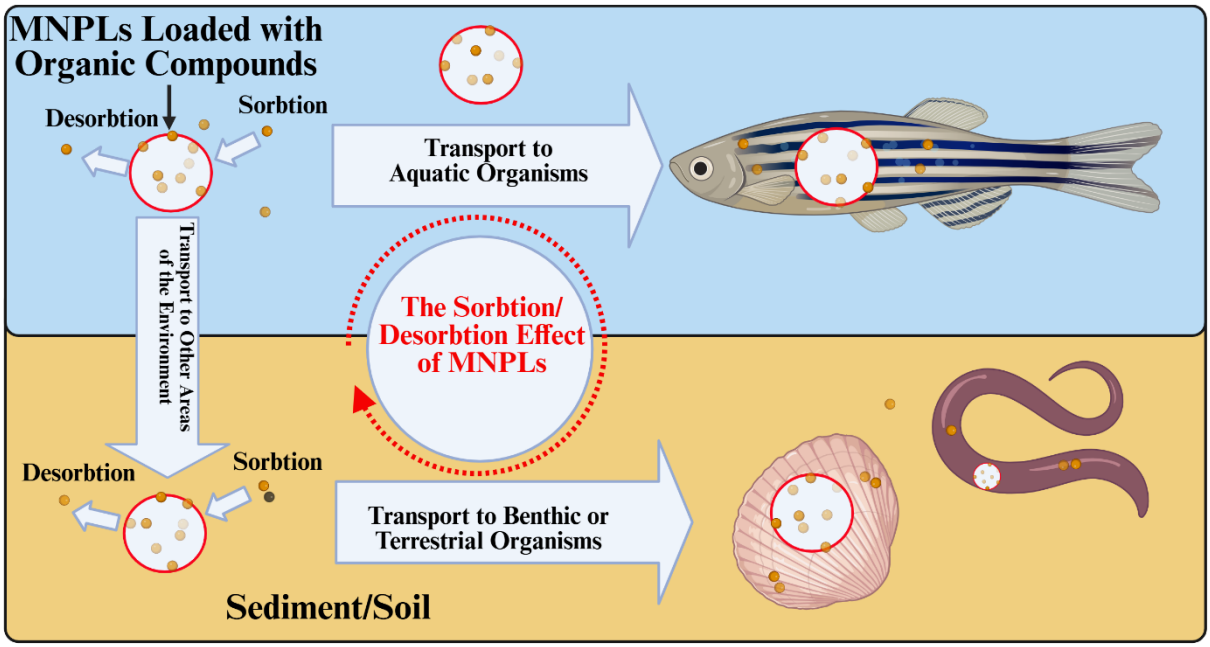

Fig. I.6. The "Trojan Horse" phenomenon in aquatic environments. Created using BioRender (adapted from Zhang and Xu, 2020).

Given the complexity of the sources, mechanisms and impacts of microplastics and nanoplastics (MNPLs) on both the environment and human health, an integrated approach is needed to comprehensively illustrate these interactions and their interconnected effects.

CHAPTER 2. MATERIALS AND METHODS

2.1. Zebrafish (Danio rerio) – Animal model used

In the present study, the toxicity of four pharmaceutical compounds was evaluated in zebrafish (*Danio rerio*) at different developmental stages (embryos, larvae and adults) by assessing oxidative stress biomarkers, behavioral endpoints and acetylcholinesterase (AChE) activity. The effects of ibuprofen (Ibu), ketoprofen (Kp), meropenem (Mp) and valproic acid (VPA) were investigated both individually and in binary and ternary mixtures over a 96-hour exposure period. In addition, a distinct investigation focused on the combined effects of pharmaceutical compounds and polypropylene (PP) and polylactic acid (PLA) micro- and nanoplastics (MNPLs), using experimental models spanning all developmental stages of zebrafish.

Zebrafish (*Danio rerio*) is a sensitive and widely used model organism for assessing neurotoxicity induced by hazardous compounds, due to its low maintenance costs, high fecundity, diverse behavioral repertoire, and significant functional, molecular, and genetic similarities to humans (Zhang et al., 2024; Zhao et al., 2024).

Therefore, toxicological assessments were performed using both embryos and larvae, providing a comprehensive perspective on how organisms at early developmental stages respond to exposure to various external stressors, including chemical and physical agents. These developmental stages allow for direct investigation of fundamental biological processes under controlled conditions. While embryos allow for the assessment of developmental toxicity during early ontogeny, larval stages provide insight into how contaminants interfere with already functional tissues and physiological systems. In addition, the absence of the chorion in larvae may increase their sensitivity to environmental contaminants compared to embryos. By integrating these two critical stages of ontogenetic development, the experimental approach provides a more robust framework for interpreting toxicological responses and a more accurate understanding of the potential impact of environmental contaminants on aquatic species.

2.2. Ethical Approval

All procedures related to the acclimation, reproduction and experimental handling of zebrafish (*Danio rerio*) were performed in accordance with current Romanian legislation regulating the use of animals in research and were approved by the Ethics Committee of the Faculty of Biology, Alexandru Ioan Cuza University of Iași, Romania (approvals no. 343/09.02.2023, 1273/19.05.2023, 96/18.06.2024 and 5526/03.12.2025).

2.3. Exposure to pharmaceutical compounds

2.3.1. Test Substances

Ibuprofen (Ibu) oral solution (400 mg 100 mL^{-1}), ketoprofen (Kp) solution (50 mg mL^{-1}), valproic acid (VPA) tablets (500 mg), and meropenem (Mp) powder vials (500 mg) were obtained through the institution from local licensed pharmacies. Stock solutions of Ibu (100 mg L^{-1}), Kp (100 mg L^{-1}), VPA (100 mg L^{-1}), and Mp (100 mg L^{-1}) were prepared de novo by dissolving the corresponding compounds in distilled water.

Adult zebrafish (*Danio rerio*) were used to assess oxidative stress biomarkers and acetylcholinesterase (AChE) activity following exposure to the tested pharmaceutical compounds. After exposure, reagents purchased from Merck (Darmstadt, Germany) were used for total protein quantification, oxidative stress analyses, and AChE activity determination.

2.3.2. Single and combined exposure to ibuprofen and valproic acid

- Exposure of *Danio rerio* embryos at 5 hpf (hours post-fertilization)

Exposure began at 5 hpf and ended at 96 hpf, with organisms being assessed at 24-hour intervals, and key developmental endpoints were monitored by stereomicroscopy. Throughout the experiment, organisms were maintained under controlled conditions, at a temperature of 28.0 ± 0.5 °C and a light/dark photoperiod of 14:10 h. To ensure the stability of the experimental conditions, the culture medium was renewed daily throughout the exposure period. Heart rate was evaluated at 48 hpf, while locomotor behavior under light and dark conditions was assessed at 96 hpf. Subsequently, larvae were photographed using a DinoEye microscope camera to

perform biometric measurements, including total body length, interocular distance, and lens diameter.

- Exposure of *Danio rerio* larvae starting at 72 hpf (hours post-fertilization)

Exposure began at 72 hpf and ended at 168 hpf, with organisms being assessed at 24-hour intervals to monitor key developmental endpoints using stereomicroscopy. Throughout the experiment, organisms were maintained under controlled conditions at a temperature of 28.0 ± 0.5 °C and a light/dark photoperiod of 14:10 h. To ensure the stability of the experimental conditions, the culture medium was renewed every 24 hours during the exposure period. At the end of the exposure, larval behavior was assessed under light and dark conditions, followed by photography using a DinoEye microscope camera for biometric analyses, including total body length, interocular distance, and ocular lens diameter.

- Exposure of adult *Danio rerio* (8 months old)

Adult zebrafish (*Danio rerio*), wild strain AB, 8 months old, with an average body mass of 0.74 ± 0.1 g and an average standard length of 40 ± 2 mm, were obtained from an authorized and certified breeder in Iași, Romania, in accordance with national animal welfare regulations. The fish were initially acclimated to laboratory conditions for 14 days in a 92 L aquarium and subsequently transferred to 10 L experimental aquaria, where they were maintained for an additional 3 days to allow for further adaptation. The exposure period lasted 96 hours, after which individuals were assessed separately, in a randomized order, according to the exposure conditions.

During the acclimation and experimental periods, fish were maintained in aquaria with continuous aeration, daily renewed dechlorinated water and carefully controlled water quality parameters (temperature: 26 ± 1 °C, pH: 7.5, dissolved oxygen: 7.2 ± 0.1 mg L^{-1}, ammonia concentration: < 0.004 ppm and conductivity: 500 µS), under a 14:10 h light/dark photoperiod. Fish were fed twice a day with a standard commercial dry diet (TetraMin™ flakes), at a ration corresponding to approximately 2% of their body mass per day.

To ensure the stability of the experimental conditions, the exposure environment was renewed every 24 h throughout the exposure period. Behavioral assessments were performed individually in a dedicated testing room, between 09:00 and 17:00, using the novel tank test and the social preference test, following previously established protocols (Jijie et al., 2020; Paduraru et al., 2024).

2.3.3. Single and combined exposure to meropenem, ketoprofen and valproic acid

- Exposure of *Danio rerio* embryos at 5 hpf (hours post-fertilization)

Exposure began at 5 hpf and ended at 96 hpf, with each experimental condition including n = 10 organisms. Embryos were exposed individually, with one organism per well in 24-well plates, and the E3 exposure medium was renewed daily. Throughout the experiment, organisms were maintained under controlled conditions in a Mikrotest climate chamber at 28.0 ± 0.5 °C, under a 14:10 h light/dark photoperiod. To ensure the stability of the experimental conditions, the culture medium was replaced every 24 h throughout the exposure period.

Assessments were performed at 24-hour intervals, with key developmental endpoints monitored by stereomicroscopy. Cardiac activity was assessed at 48 hpf, while hatching rate was determined at 72 hpf. At 96 hpf, larval behavior was assessed under light and dark conditions, followed by photography using a DinoEye microscope camera for biometric analyses, including total body length, interocular distance, and ocular lens diameter.

- Exposure of *Danio rerio* larvae starting at 72 hpf (hours post-fertilization)

Exposure began at 72 hpf and ended at 168 hpf, with each experimental condition including n = 10 organisms. Larvae were exposed individually, one organism per well in 24-well plates, and the E3 exposure medium was renewed daily.

Throughout the experiment, organisms were maintained under controlled conditions in a Mikrotest climate chamber at 28.0 ± 0.5 °C, under a 14:10 h light/dark photoperiod. To ensure stability of the experimental conditions, the culture medium was replaced every 24 h throughout the exposure period.

Assessments were performed at 24-hour intervals, with key developmental endpoints monitored by stereomicroscopy. Cardiac activity and heart rate were assessed at the beginning of the exposure period (72 hpf), while hatching rate was previously determined prior to larval exposure. At 168 hpf, larval behavior was assessed under light and dark conditions, followed by photography using a DinoEye microscope camera for biometric analyses, including total body length, interocular distance, and ocular lens diameter.

- Exposure of adult *Danio rerio* (6 months old).

Adult zebrafish (*Danio rerio*), AB strain, 8 months old, with an average body mass of 0.74 ± 0.1 g and an average standard length of 40 ± 2 mm, obtained from an authorized and certified breeder in Iași, Romania, in accordance with national animal welfare regulations, were

used. The fish were initially acclimated to laboratory conditions for 14 days in a 92 L aquarium, after which they were transferred to 10 L experimental aquariums and maintained for an additional 3 days for adaptation. The exposure lasted for 96 h, after which the individuals were assessed individually, in a randomized order, depending on the exposure condition.

During the acclimation and experiment, the fish were maintained in aquaria with continuous oxygenation, dechlorinated water renewed daily and constantly controlled quality parameters (temperature 26 ± 1 °C, pH 7.5, dissolved oxygen 7.2 ± 0.1 mg L^{-1}, ammonia concentration < 0.004 ppm and conductivity 500 µS), under a 14:10 h light/dark cycle and a standard diet, the fish being fed twice a day with dry commercial food (TetraMin™ flakes), in an amount equivalent to approximately 2% of body mass per day.

To ensure the stability of the experimental conditions, the environment was changed daily throughout the exposure period. Behavioral assessments were performed individually, in a dedicated room, between 09:00 and 17:00, using the novel aquarium test and the social preference test, according to previously described work protocols (Jijie et al., 2020; Paduraru et al., 2024).

2.4. Combined exposure to pharmaceutical compounds and micro-nanoplastics

2.4.1. Substances and materials used

Ketoprofen (Kp) in liquid form (50 mg mL^{-1}), identical to that used in previous experiments, was used as a pharmaceutical compound to evaluate the effects of nonsteroidal anti-inflammatory drugs (NSAIDs) on aquatic organisms. Stock solutions were prepared by dilution in distilled water, followed by homogenization by shaking.

The micro- and nanoplastics (MNPLs) used in this study consisted of polypropylene (PP) and polylactic acid (PLA), selected due to their widespread use in packaging, textiles and biodegradable materials. PLA particles were used as micro- and nanoplastics, and their structural characterization was performed by Fourier transform infrared spectroscopy (FTIR) using a Bruker µ-FTIR Lumos II spectrometer (Bruker, Germany) equipped with an ATR module (Ge crystal). Spectral analyses were performed using reference libraries, including the BPAD Bruker Polymer ATR Library, the KIMW ATR-IR Polymer Library and the BIBL ATR-FTIR-Library Complete, in the spectral range 4000–680 cm^{-1}, at a resolution of 4 cm^{-1} and an

accumulation of 64 scans. Instrument control and IR spectrum acquisition were carried out using the OPUS software package.

The morphology of microplastics was analyzed by scanning electron microscopy (SEM) using a HITACHI S-3400 N system (Hitachi Science Systems, Tokyo, Japan) and by optical microscopy using a conventional stereomicroscope (Celestron), to confirm their chemical composition, particle size, and surface morphology.

For PP, two particle sizes were used, adapted to the developmental stage of the exposed organisms. In the case of *Danio rerio* embryos and larvae, micro- and nanoscale particles were used to reproduce realistic ingestion and contact scenarios in early developmental stages. For adult fish, exposure was performed using PP microplastics in the form of fibers smaller than 1 mm. Both types of MNPL were characterized by FTIR spectroscopy and SEM to confirm the identity of the material (PLA and PP) and to assess their surface properties.

2.4.2. Single and combined exposure to ketoprofen and polypropylene micro-nanoplastics

- Exposure of *Danio rerio* embryos at 5 hpf (hours post-fertilization)

Exposure began at 5 hpf and ended at 120 hpf, with organisms being assessed at 24-hour intervals and key developmental endpoints being monitored by stereomicroscopy. Throughout the experiment, organisms were maintained under controlled conditions in a sealed growth chamber at 28.0 ± 0.5 °C, under a 14:10 h light/dark photoperiod. To ensure stability of the experimental conditions, the culture medium was renewed every 48 h during the exposure period.

Heart rate was monitored at 48 hpf, while hatching success was evaluated at 72 hpf. At 120 hpf, larval behavior was assessed under light and dark conditions, followed by photography using a DinoEye microscope camera for biometric analyses, including total body length, interocular distance, and ocular lens diameter.

- Exposure of *Danio rerio* larvae starting at 72 hpf (hours post-fertilization)

Larval exposure began at 72 hpf and ended at 192 hpf (cycle per leg), with organisms assessed at 24-hour intervals and key developmental endpoints monitored by stereomicroscopy. Throughout the experiment, organisms were maintained under controlled conditions in a sealed growth chamber at 28.0 ± 0.5 °C, under a 14:10 h light/dark photoperiod. To ensure stability of

the experimental conditions, the culture medium was renewed every 48 h during the exposure period.

Behavioral assessments were performed under both light and dark conditions. At the end of the experiment, larvae were photographed using a DinoEye microscope camera for biometric analyses, including total body length, interocular distance, and ocular lens diameter.

- Exposure of adult *Danio rerio* (8 months).

Adult zebrafish (*Danio rerio*), AB strain, 8 months old, with an average body mass of 0.73 ± 0.1 g and an average length of 39 ± 3 mm, were used. The fish were purchased from a local authorized supplier in Iași, Romania, and acclimated to laboratory conditions for 14 days in a 92 L aquarium (60 × 30 × 45 cm; L × w × h) prior to the experiment. Specimens of both sexes were housed together at an approximate 1:1 ratio, and white paper dividers were placed between the holding aquariums to reduce visual interactions and external stressors.

Subsequently, the fish were exposed for 96 h to Kp, MNPL of PP and the binary mixture (Kp and PP). Behavioral testing was performed in a dedicated room, between 09:00–17:00, with specimens being assessed individually, with one fish from each group being tested daily, in a randomized order depending on the exposure condition. During the experiment, the fish were fed twice a day with dry commercial food (TetraMin™ flakes) in an amount equivalent to approximately 2% of their body weight per day, and environmental conditions were maintained constant, according to previously described protocols (Jijie et al., 2020; Paduraru et al., 2024). To ensure the stability of the experimental conditions and a controlled exposure, the water in all experimental conditions was completely renewed at 24 h intervals, during the exposure period.

2.4.3. Single and combined exposure to ketoprofen and polylactic acid micro-nanoplastics

- Exposure of *Danio rerio* embryos at 5 hpf (hours post-fertilization)

Exposure began at 5 hpf and ended at 120 hpf, with organisms being scored at 24-hour intervals and key developmental endpoints monitored by stereomicroscopy. Throughout the experiment, organisms were maintained under controlled conditions in a sealed growth chamber at 28.0 ± 0.5 °C, under a 14:10 h light/dark photoperiod. To ensure stability of the experimental conditions, the culture medium was renewed every 48 h during the exposure period.

Cardiac activity was monitored at 48 hpf, while hatching success was evaluated at 72 hpf. At 120 hpf, larval behavior was assessed under both light and dark conditions, followed by

photography using a DinoEye microscope camera for biometric analyses, including total body length, interocular distance, and ocular lens diameter.

- Exposure of *Danio rerio* larvae starting at 72 hpf (hours post-fertilization)

Larval exposure began at 72 hpf and ended at 192 hpf, with organisms being assessed at 24-hour intervals and key developmental endpoints being monitored by stereomicroscopy. Throughout the experiment, organisms were maintained under controlled conditions in a sealed growth chamber at 28.0 ± 0.5 °C, under a 14:10 h light/dark photoperiod. To ensure stability of the experimental conditions, the culture medium was renewed every 48 h during the exposure period.

Behavioral assessments were performed under both light and dark conditions. At the end of the experiment (192 hpf), larvae were photographed using a DinoEye microscope camera for biometric analyses, including total body length, interocular distance, and ocular lens diameter.

- Exposure of adult *Danio rerio* (8 months old)

Adult zebrafish (*Danio rerio*), AB strain, 8 months old, with an average body mass of 0.72 ± 0.1 g and an average body length of 40 ± 3 mm, were obtained from a local authorized supplier in Iași, Romania. Before the experiment, the fish were acclimated to laboratory conditions for 14 days in a 92 L aquarium (60 × 30 × 45 cm; L × W × H). Both sexes were kept together in a ratio of approximately 1:1, and white paper partitions were placed between the test aquariums to minimize visual interactions and external stressors. Subsequently, the fish were exposed for 96 hours to ketoprofen (Kp), polylactic acid (PLA), and their mixture. Behavioral assessments were conducted in a dedicated testing room between 09:00 and 17:00, with each individual being assessed separately. One fish from each experimental group was tested daily, in a randomized order, depending on the exposure conditions.

During the experiment, fish were fed twice a day with a commercial dry food (TetraMin™ flakes). Environmental conditions were maintained constant according to previously described protocols (Jijie et al., 2020; Paduraru et al., 2024). To ensure the stability of the experimental conditions and controlled exposure, the exposure medium was renewed every 24 h during the experimental period.

2.5. Tests performed

- **Light/dark test**

The present thesis investigated the exposure of early developmental stages (embryos and larvae) to pharmaceutical compounds and micro-nanoplastics (MNPL), which required conditioning adult zebrafish for reproduction and subsequent collection of embryos.

Following exposure to the test substances and compounds, a light/dark locomotor test was performed on *Danio rerio* larvae. In this assay, larvae were initially allowed to acclimate to ambient light conditions for 2 min. Subsequently, abrupt alternation between light and dark phases was used to elicit a stress response analogous to anxiety-like behavior. Behavioral changes induced by these transitions were recorded and analyzed as indicators of potential neurobehavioral effects associated with the experimental treatments.

- **Novel Tank Test**

To evaluate exploratory behavior, a rectangular aquarium (30 cm × 20 cm × 17 cm; length × width × height) filled with 6 L of dechlorinated tap water was used. Two video cameras were positioned orthogonally to simultaneously record top and frontal views of fish movements. In addition, the side walls and the front face of the aquarium were divided into two distinct zones. The exploratory behavior of each adult zebrafish was recorded and subsequently analyzed using EthoVision XT software, version 16 (Noldus Information Technology, Wageningen, The Netherlands).

- **Social Preference Test**

The experiment was conducted in a T-maze (70 cm × 50 cm × 10 cm; length × width × height), divided into five zones: the left arm, the decision zone, the right arm, the start arm, and the conspecific zone, separated by a transparent plastic partition. The maze was filled with 5 L of water, and three zebrafish were placed in the conspecific zone located at the end of the left arm. For each experimental fish, the time spent in each zone and the frequency of entries into the decision zone, as well as the left, right, and start arms, were recorded and analyzed using the same tracking software used in the Novel Tank test, namely EthoVision XT version 16 (Noldus Information Technology, Wageningen, The Netherlands).

2.6. Assessment of oxidative stress and acetylcholinesterase activity

After completion of behavioral assessments, adult zebrafish were euthanized by hypothermic shock (2–4 °C) in a beaker containing ice-cold water, in accordance with the recommendations of the American Veterinary Medical Association (AVMA). After confirming the absence of vital signs, each specimen was homogenized using a 7-ml KIMBLE Dounce tissue grinder (Sigma-Aldrich, St. Louis, MO, USA) in ice-cold 0.1 M phosphate-buffered saline (PBS; pH 7.4) at a ratio of 10% (w/v). The homogenates were subsequently centrifuged at 5,500 rpm for 15 min at 4 °C, and the resulting supernatant was collected for biochemical analyses. Total protein content was determined using the Bradford method.

Acetylcholinesterase (AChE) activity was determined according to the method described by Boiangiu et al. (2021), with minor modifications. For each independent sample, two fish brains were homogenized in ten volumes of extraction buffer and centrifuged at 14,000 rpm for 10 minutes at 4 °C. Subsequently, 50 µL of supernatant was mixed with 450 µL of sodium phosphate buffer (0.25 M, pH 7.4), 50 µL of 5,5′-dithiobis-(2-nitrobenzoic) acid (DTNB, 1 mM) and 25 µL of acetylthiocholine iodide (ATCh, 5 mM). After incubation for 10 minutes at 37 °C, the reaction was terminated by adding 900 µL of acetone. Absorbance was measured at 412 nm using a Specord 210 Plus spectrophotometer (Analytik Jena, Germany). AChE activity was expressed in nanomoles of ATCh hydrolyzed per minute per milligram of protein.

2.7. Statistical analysis

The normality of the data was assessed using the Shapiro-Wilk test implemented in GraphPad Prism version 10.4.1 (GraphPad Software, San Diego, CA, USA). Significant outliers were identified using the ROUT method to ensure that the data sets were suitable for further analyses. Results were expressed as mean ± standard deviation (SD), and the threshold for statistical significance was set at $p < 0.05$.

For embryos and larvae, the effects of treatment under light and dark conditions were initially evaluated using one-way analysis of variance (one-way ANOVA), followed by Dunnett's post hoc test to identify differences between treatment groups and the control group within each lighting condition separately, using a 95% confidence interval. Subsequently,

differences between light and dark phases were assessed using two-way analysis of variance (two-way ANOVA), followed by the Šidák post-hoc test, also using a 95% confidence interval.

For adult *Danio rerio*, statistical analyses were performed exclusively for the light phase. Differences between experimental and control groups were assessed using one-way ANOVA followed by Dunnett's post hoc test. In experiments requiring comparisons between all treatment groups, Tukey's post hoc test was applied using a 95% confidence interval. To investigate the relationships between behavioral and biochemical parameters, Pearson correlation analysis was performed with a 95% confidence interval, allowing the assessment of associations between the measured variables.

Determination of lethal and sublethal concentrations for embryos and larvae was performed using Probit analysis. Results of acute toxicity tests were expressed as LC_{50} and EC_{50} values, together with the corresponding 95% confidence limits. Parameters used for these calculations included mortality, hatching success and observed developmental abnormalities.

The minimum sample size required to detect a medium effect size was estimated using G*Power version 3.1.9.7 (“Heinrich Heine” University Düsseldorf, Germany), with a significance level set at $\alpha = 0.05$ and a statistical power of 80%.

Pairwise associations between behavioral variables were examined using the Pearson correlation coefficient (r). Correlations were calculated between each locomotor parameter and both social preference and biochemical parameters. All variables were first assessed for normality and linearity to confirm the adequacy of the Pearson correlation analysis. The sample size (n = 20/n = 40) for each correlation corresponded to the total number of fish with complete data available for the two variables being compared. Correlation coefficients and associated p-values were calculated using the entire dataset without aggregating results across treatments, thus allowing assessment of continuous relationships independent of treatment group membership. Statistical significance was assessed at $\alpha = 0.05$.

CHAPTER 3. RESULTS AND DISCUSSION

3.1. Experimental study no. 1: Individual and Combined Effects of Ibuprofen and Valproic Acid on Zebrafish Development and Behavior

The aim of this study was to evaluate the effects of exposing zebrafish to environmentally relevant concentrations of ibuprofen (Ibu; 20 µg L^{-1}), valproic acid (VPA; 3 µg L^{-1}) and their binary mixture.

The exposure was performed at three developmental stages - embryonic (5 hpf), larval (72 hpf) and adult (8 months) - each exposure lasting 96 hours, in order to capture stage-specific responses during zebrafish ontogeny.

3.1.1. Exposure of *Danio rerio* embryos at 5 hpf (hours post-fertilization)

- Heart rate and biometric parameters assessed

Exposure of zebrafish embryos to environmentally relevant concentrations of Ibu, VPA, and their mixture did not result in statistically significant changes in heart rate, total body length, or interocular distance compared to the control group ($p > 0.05$; Fig. III.1a–c).

Although no significant changes in ocular lens diameter were observed following exposure to Ibu or VPA alone ($p > 0.05$), combined exposure to Ibu and VPA resulted in a significant decrease in lens diameter ($p = 0.022$) compared to the control group (Fig. III.1d).

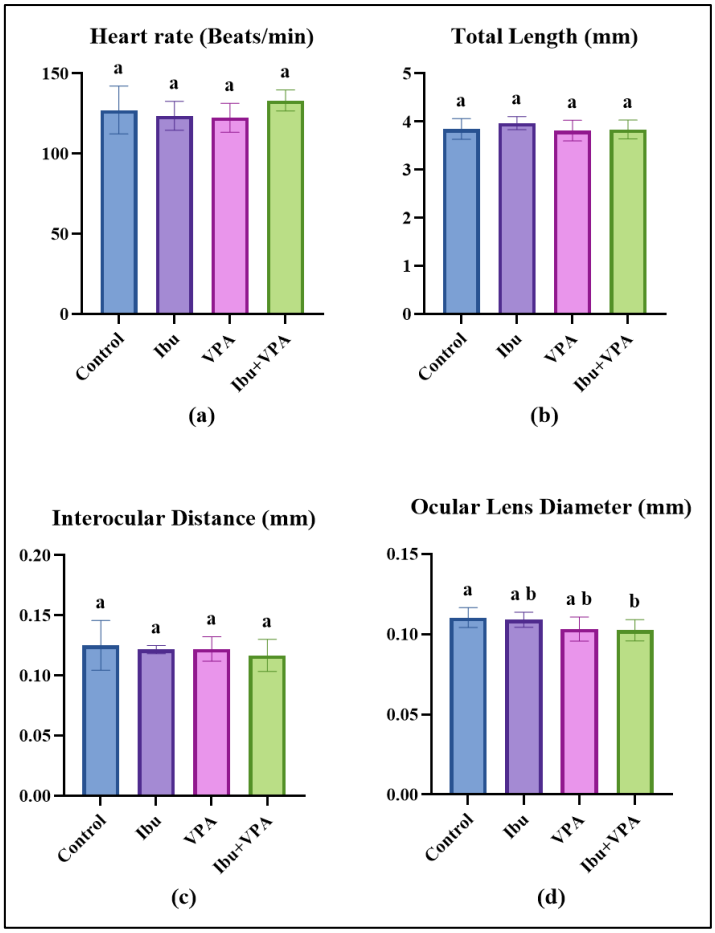

Fig. III.1. Heart rate and biometric parameters measured at the embryonic stage (5 hpf) in *Danio rerio*, exposed for 96 hours to individual pharmaceuticals and mixtures (Ibu = 20 µg L^{-1}; VPA = 3 µg L^{-1}). The monitored parameters included: (a) heart rate (beats/min), (b) total length (mm), (c) interocular distance (mm), (d) ocular lens diameter (mm). Data are presented as mean ± SD (n = 10 animals per group) and were analyzed using one-way ANOVA, followed by Tukey's test. Common letters indicate groups that do not differ significantly from each other.

3.1.2. Exposure of *Danio rerio* larvae at 72 hpf (hours post-fertilization)

- Biometric measurements taken in the larval stage

In the case of simple and mixed exposure of larvae (72 hpf) to pharmaceutical substances, no statistically significant differences were observed for the monitored parameters ($p > 0.05$) - Fig. III.3.

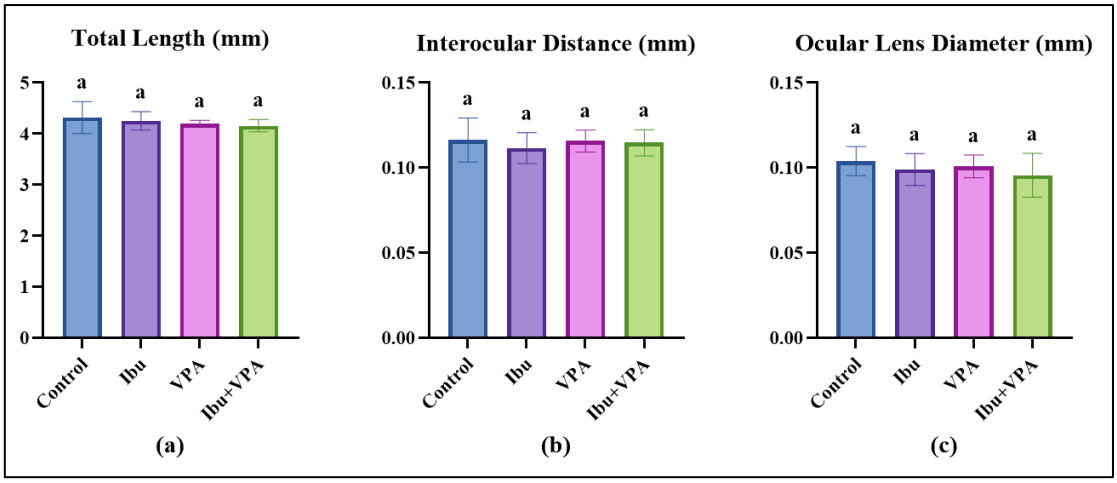

Fig. III.3. Biometric measurements performed on larval stage (72 hpf) of *Danio rerio* exposed for 96 hours to individual pharmaceuticals and mixtures (Ibu = 20 µg L^{-1}; VPA = 3 µg L^{-1}). Parameters monitored included: (a) total length (mm), (b) interocular distance (mm), (c) ocular lens diameter (mm). Data are presented as mean ± SD (n = 10 animals per group) and were analyzed using one-way ANOVA followed by Tukey's test. Common letters indicate groups that are not statistically significantly different from each other.

3.1.3. Exposure of adult *Danio rerio* (8 months old)

- Novel Tank Test

In the new aquarium test, from a lateral perspective, the group treated with VPA traveled a significantly greater total distance than the control group ($p = 0.025$) - Fig. III.6. (a). In the case of immobility time, a significant increase was observed for the group treated with Ibu ($p = 0.009$) compared to the group treated with Ibu+VPA - Fig. III.6. (b). The group treated with Ibu+VPA spent significantly less time in the upper half of the aquarium compared to all other treated groups, including the control group ($p < 0.001$) - Fig. III.6. (c).

In addition, the group treated with Ibu+VPA spent significantly more time in the lower half compared to all other groups ($p < 0.001$) - Fig. III.6. (d). Regarding clockwise rotations, a significant increase was observed in the group treated with Ibu+VPA compared to the control group ($p = 0.017$) and the group treated with Ibu ($p = 0.032$) - Fig. III.6. (e). No statistically significant differences were observed in the case of counterclockwise rotations ($p > 0.005$) - Fig. III.6. (f).

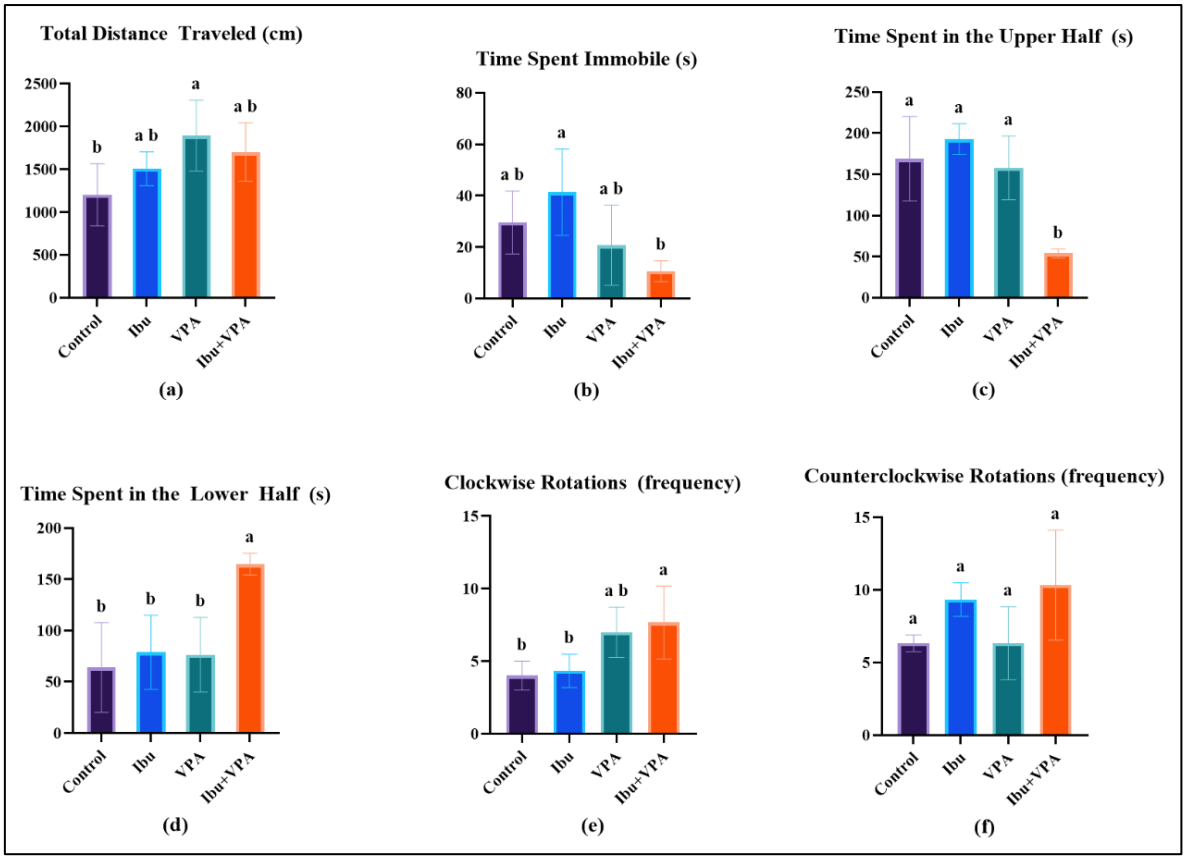

Fig. III.6. Effects of exposure to ibuprofen (Ibu) and valproic acid (VPA), individually and in combination (Ibu = 20 µg L^{-1}; VPA = 3 µg L^{-1}) on the locomotion of adult zebrafish. Behavioral parameters measured included: (a) total distance traveled (cm), (b) time spent immobile (s), (c) time spent in the upper half, (d) time spent in the lower half (s), (e) clockwise rotations (frequency), (f) counterclockwise rotations (frequency). Data are presented as mean ± SD (n = 5 animals per group, with a sex ratio males/females of 3:2) and were analyzed using one-way ANOVA followed by the Tukey's test. Common letters indicate groups that are not statistically significantly different from each other.

3.1.4. Discussion of the study

The study revealed that exposure to environmentally relevant concentrations of ibuprofen (Ibu) and valproic acid (VPA) induces stage-dependent behavioral effects in *Danio rerio*, while combined exposure frequently produces non-additive, predominantly antagonistic responses. In embryos and larvae, no significant changes in the main morphological parameters (heart rate, total length, interocular distance) were observed, suggesting the absence of any obvious general toxicity. However, VPA and the Ibu+VPA mixture reduced the diameter of the ocular lens in embryos, indicating a possible subtle impairment of visual function. In parallel,

behavioral changes were more sensitive than morphological indicators, with changes in distance traveled, activity time, and light and dark-dependent behavior being observed.

In early stages of development, VPA caused changes in locomotor activity and stress-related behavioral responses, while Ibu reduced activity in light conditions and induced compensatory responses in the dark. The increased preference for the edge of the wells, especially in the dark, suggests the emergence of early anxiogenic responses. In contrast, the combined exposure did not generate evident cumulative effects on locomotor activity, which supports the existence of antagonistic interactions between the two compounds. These results indicate that the effects of pharmaceutical contaminants are manifested mainly at the functional and neurobehavioral level, before the appearance of obvious morphological changes.

In adult fish, exposure to Ibu and Ibu+VPA altered exploratory behavior, and VPA significantly affected social preference, leading to reduced interactions with conspecifics and suggesting persistent neurobehavioral alterations. Overall, the results demonstrate that exposure to environmentally relevant concentrations of Ibu and VPA may influence zebrafish behavior throughout ontogeny, from embryos to adults, while the effects of the mixture are difficult to predict due to antagonistic interactions between the compounds. These observations highlight the importance of assessing pharmaceutical contaminants throughout the entire developmental cycle and including mixture effects in ecotoxicological assessments.

3.2. Experimental study no. 2: Individual and combined effects of meropenem, valproic acid and ketoprofen on zebrafish development and behavior

The aim of the study was to evaluate the effects of environmentally relevant concentrations of meropenem (Mp), valproic acid (VPA), ketoprofen (Kp) and their mixtures on zebrafish (*Danio rerio*) at the embryonic, larval, and adult stages. In embryos and larvae, developmental, toxicity, and locomotor behavior parameters were analyzed under light and dark conditions, including mortality, malformations, biometric measurements and swimming activity. In adults, after 96 hours of exposure, exploratory, anxiety, and social behaviors were assessed by the novel tank test and the social preference test. In addition, oxidative stress markers (SOD, MDA, GPx) and acetylcholinesterase (AChE) activity were determined, and the relationships between behavioral and biochemical responses were investigated by Pearson correlation analysis.

3.2.1. Exposure of *Danio rerio* embryos at 5 hpf (hours post-fertilization)

- Heart rate and measured biometric parameters

Embryo exposure to anti-inflammatory (Kp), antibiotics (Mp), and antiepileptics (VPA) substances, by simple administration or in binary and ternary mixtures, was carried out for 96 hours, aiming to observe key parameters for the early stages of zebrafish, parameters presented in Fig. III.10.: heart rate (a), total length (b), interocular distance (c) and diameter of the ocular lens (d).

No statistically significant differences were observed in terms of heart rate between the control group and the other groups treated with pharmaceutical products ($p > 0.05$) - Fig. III.10. (a). However, in the case of embryos exposed to Mp, a significant increase was observed compared to the binary mixtures with Mp+VPA and VPA+Kp ($p < 0.05$) and to the ternary treatment group ($p < 0.01$) - Fig. III.10. (a).

Regarding the total length of the organisms at the end of the exposure period (96 h), no statistically significant differences were observed ($p > 0.05$) - Fig. III.10. (b). However, for the interocular distance, statistically significant differences were observed between the control group and the other groups treated with pharmaceutical substances, consisting of a significant decrease between the control group vs. Mp and Mp+VPA ($p < 0.05$) and the control group vs. VPA+Kp and Mp+Kp ($p < 0.01$) and between the control group vs. VPA, Kp, and VPA+Mp+Kp ($p < 0.001$) - Fig. III.10. (c).

For a more detailed evaluation, the diameter of the ocular lens was analyzed, where a statistically significant decrease was observed compared to the control group, more precisely control vs. VPA, control vs. Kp, control vs. Mp+VPA, control vs. VPA+Kp ($p < 0.01$). No statistically significant differences were recorded for the comparisons between the control group vs. the Mp-exposed group, control vs. the VPA+Mp+Kp-exposed group, and control vs. the VPA+Mp+Kp-exposed group ($p > 0.05$) - Fig. III.10. (d).

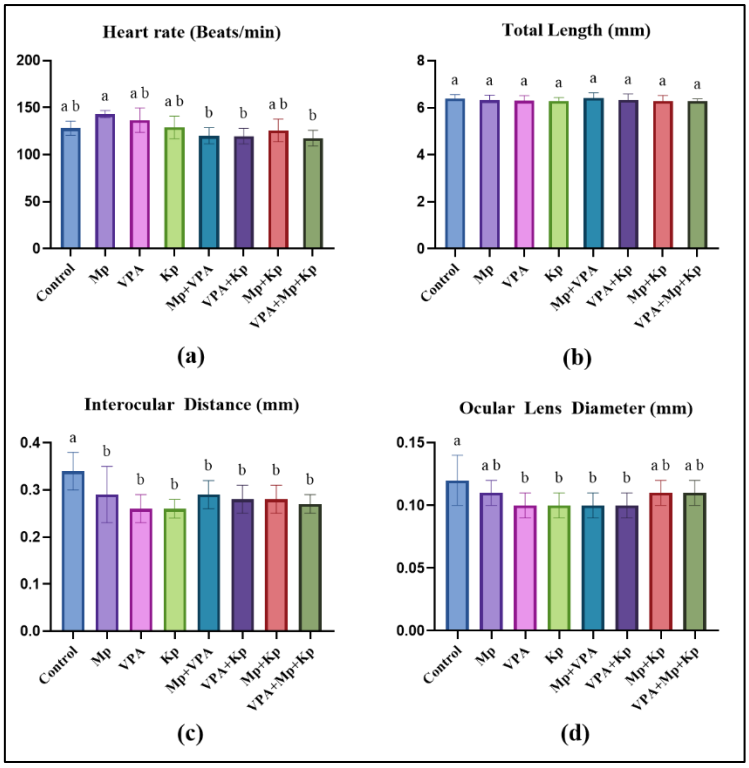

Fig. III.10. Heart rate and biometric parameters measured at the embryonic stage (5 hpf) in *Danio rerio*, exposed for 96 hours to individual pharmaceuticals and mixtures (Mp = 1 µg L^{-1}; VPA = 3 µg L^{-1}; Kp = 5 µg L^{-1}). The monitored parameters included: (a) heart rate (beats/min), (b) total length (mm), (c) interocular distance (mm), (d) ocular lens diameter (mm). Data are presented as mean ± SD (n = 10 animals per group) and were analyzed using one-way ANOVA followed by Tukey's test. Common letters indicate groups that are not statistically significantly different from each other.

3.2.2. Exposure of *Danio rerio* larvae at 72 hpf (hours post-fertilization)

- Biometric measurements taken in the larval stage

The exposure of *Danio rerio* larvae (72 hpf) to anti-inflammatory (Kp), antibiotics (Mp), and antiepileptics (VPA) substances, administered individually or in binary and ternary mixtures, was carried out for 96 hours. This developmental stage is of interest because the organisms are no longer protected by the chorion, being in direct contact with the pharmaceutical pollutants studied. Thus, the key parameters for this stage were monitored, as shown in Fig. III.12., more specifically: total length (a), interocular distance (b), and ocular lens diameter (c).

Regarding the total length of the larvae at the end of the 96-hour exposure to the pharmaceuticals administered individually or in mixture, compared to the control group, no

statistically significant changes were observed ($p > 0.05$) - Fig. III.12. (a). On the other hand, the fish in the Mp-treated group showed a statistically significant decrease compared to the fish in the groups exposed to Kp, Mp+VPA, VPA+Kp, Mp+Kp ($p < 0.01$) - Fig. III.12. (a).

Regarding the interocular distance of the larvae treated with pharmaceutical substances, it was found that, compared to the organisms in the control group, those in the Mp+VPA and Mp+Kp groups did not show statistically relevant differences - Fig. III.12. (b). However, the groups treated with Mp and VPA showed a statistically significant decrease compared to the control group ($p < 0.01$) and, in addition, a more pronounced difference was observed compared to the group treated with Kp ($p < 0.001$) - Fig. III.12. (b).

The Kp-treated group showed the greatest decrease among all treatments, showing statistical significance compared to the Mp+Kp, VPA+Mp+Kp ($p < 0.05$), and Mp+VPA ($p < 0.01$) exposed groups - Fig. III.12. (b). Regarding the diameter of the ocular lens, the Mp+Kp-treated group recorded a statistically significant increase compared to the control group ($p < 0.05$) - Fig. III.12. (c). The Mp+Kp-exposed group ($p < 0.05$) showed a statistically significant increase compared to the Mp-treated group. In addition, the Kp-exposed groups, namely Mp+Kp ($p < 0.001$) and VPA+Mp+Kp ($p < 0.05$), showed a statistically significant increase compared to the VPA-exposed group. The binary mixture of Mp+VPA caused a decrease in the diameter of the ocular lens compared to the group exposed to Mp+Kp ($p < 0.01$) - Fig. III.12. (c).

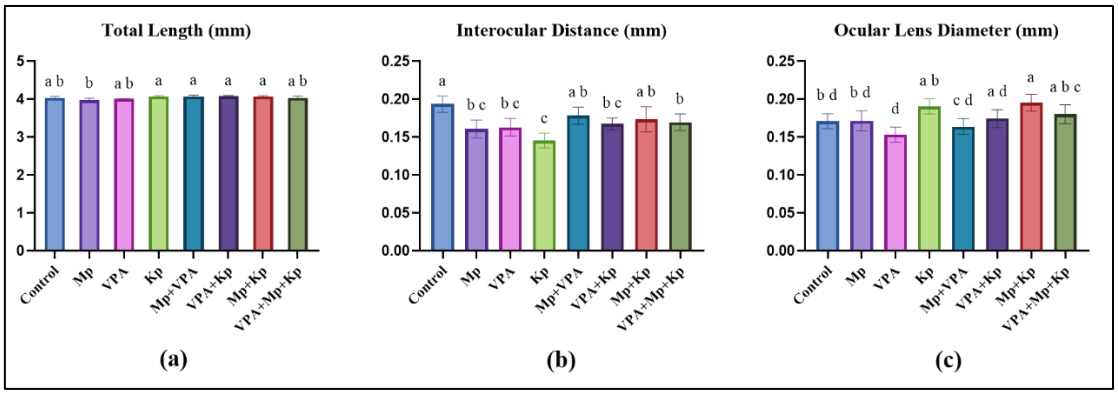

Fig. III.12. Biometric measurements were performed on larval stage (72 hpf) of *Danio rerio*, exposed for 96 hours to individual and mixed pharmaceutical products (Mp = 1 µg L^{-1}; VPA = 3 µg L^{-1}; Kp = 5 µg L^{-1}). The monitored parameters included: (a) total length (mm), (b) interocular distance (mm), (c) ocular lens diameter (mm). Data are presented as mean ± SD (n = 10 animals per group) and were analyzed using one-way ANOVA followed by Tukey's test. Common letters indicate groups that do not differ significantly from each other.

3.2.3. Exposure of adult *Danio rerio* (6 months old)

- Novel Tank Test

Zebrafish exposed to VPA and Mp + Kp showed a considerable increase in the distance traveled ($p < 0.001$) compared to the control group during the 5 minutes of video recording, performed with a camera placed in front of the test aquarium - Fig. III.15. (a). Furthermore, the VPA + Kp group showed a significant decrease in average swimming speed (8.17 ± 1.96 cm s^{-1}), while the VPA (14.13 ± 1.19 cm s^{-1}) and Mp + Kp (14.53 ± 1.31 cm s^{-1}) groups had the highest average speeds compared to the control group (1.05 ± 1.21 cm s^{-1}) - Fig. III.15. (b). As can be seen in Fig. III.15. (c)., fish treated with Mp and VPA + Kp for 4 days showed a significant increase ($p < 0.001$) in immobility time, approximately 1.6-fold. Unlike the control group and the group exposed to Mp, which explored the entire aquarium, fish exposed to the other individual or combined pharmaceutical treatments mainly explored the bottom of the aquarium (geotaxis), suggesting a behavior associated with anxiety - Fig. III.15. (d).

Although the zebrafish treated with VPA and Kp, both individually and in combination with Mp, frequently entered the upper half of the tank, the time spent in the upper part of the aquarium was significantly reduced compared to the control group.

The frequency of transitions to the upper part of the tank was significantly lower ($p < 0.001$) in the VPA + Kp (3.7-fold) and VPA + Mp + Kp (5.8-fold) groups compared to the control group - Fig. III.15. (e). In addition, acute exposure to VPA ($p = 0.001$ vs. control) or Kp ($p < 0.001$ vs. control), as well as to the Kp + Mp mixture ($p < 0.001$ vs. control), demonstrated a significant increase in counterclockwise rotations - Fig. III.15. (f).

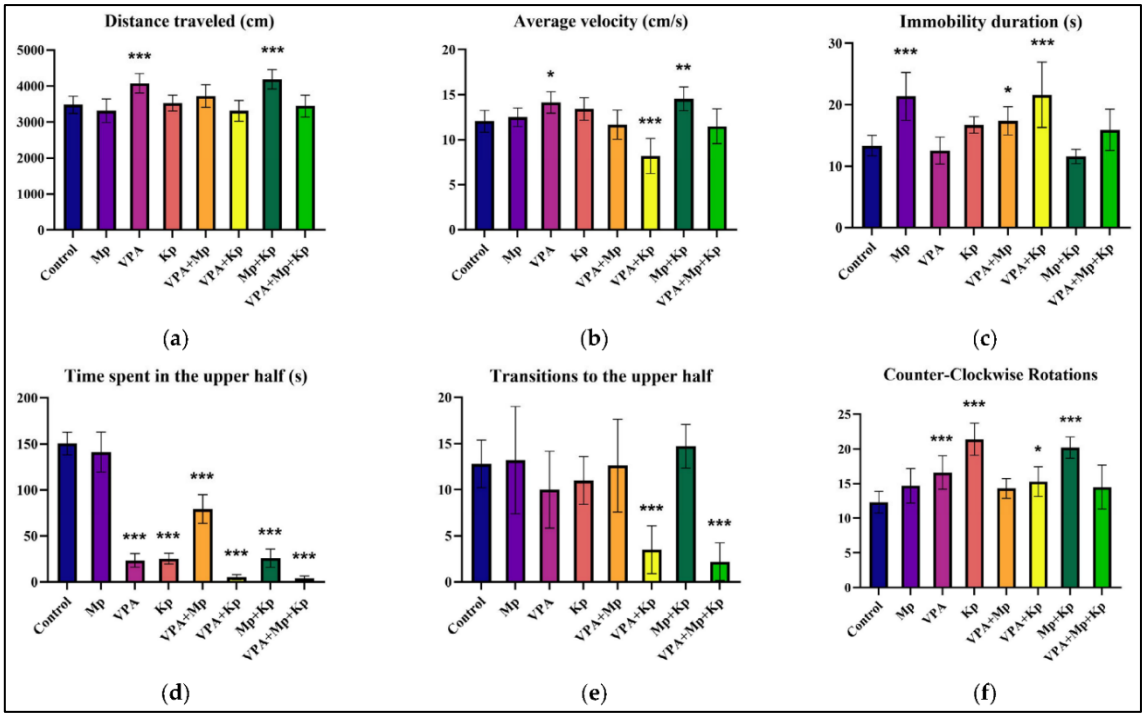

Fig. III.15. Behavioral changes in adult zebrafish exposed to individual pharmaceuticals and mixtures (Mp = 1 µg L^{-1}; VPA = 3 µg L^{-1}; Kp = 5 µg L^{-1}) for 96 hours. Behavioral parameters measured included: (a) distance traveled (cm), (b) average velocity (cm s^{-1}), (c) immobility duration (s), (d) time spent in the upper half (s), (e) transitions to the upper half, and (f) counterclockwise rotations. Data are presented as mean ± SD (n = 10 animals per group, with a male/female sex ratio of 1:1) and were analyzed using one-way ANOVA followed by Dunnett's test. Statistically significant differences are indicated as follows: * $p < 0.05$; ** $p < 0.01$; and *** $p < 0.001$ (Chelaru et al., 2025).

3.2.4. Discussions of the study

The study evaluated the effects of valproate (VPA), ketoprofen (Kp), meropenem (Mp), and their combinations on zebrafish (*Danio rerio*), integrating behavioral, biochemical, and neurotoxic responses. The results revealed significant changes in locomotor and anxiety-related behavior.

In the novel tank test, VPA and the Mp+Kp combination increased locomotor activity, while Mp and the ternary mixture (VPA+Mp+Kp) reduced the distance traveled. These effects are supported by observations in the embryonic and larval stages, where changes in distance traveled, active time, and preference for peripheral areas indicated the early onset of neurobehavioral disorders. In addition, most treatments increased thigmotaxis and anxiety-related behaviors, suggesting an impairment of exploratory capacity and stress response.

In adults, the most obvious effects were observed on social behavior. Fish exposed to the combinations VPA+Kp and VPA+Mp+Kp showed a reduced preference for conspecifics, choosing more frequently the area devoid of social stimuli, indicating sociability deficiencies. These results are consistent with the changes observed in studies on VPA exposure and suggest that neurobehavioral effects may persist throughout development. At the same time, the increase in counterclockwise rotations and the preference for peripheral areas of the aquarium support the existence of anxiogenic responses and changes in information processing at the neuronal level.

Biochemical analyses have confirmed that exposure to drugs and their mixtures induces oxidative stress and neurotoxicity. Most treatments resulted in increased MDA levels and changes in antioxidant enzyme activities (SOD and GPx), indicating a disruption of the oxidative balance.

In addition, acetylcholinesterase (AChE) activity, an important marker of nerve function, increased in the VPA-treated group but was inhibited in some VPA-containing mixtures, suggesting complex interactions between compounds and different neurotoxic mechanisms. Overall, the results demonstrate that pharmaceutical mixtures can produce behavioral and biochemical effects that differ from those generated by individual substances, highlighting the need to evaluate contaminants under mixed exposure conditions, more similar to those found in the natural environment.

3.3. Experimental study no. 3: Individual and combined effects of ketoprofen and polypropylene micro-nanoplastics on zebrafish development and behavior

The aim of the study was to evaluate the individual and combined effects of ketoprofen (Kp) and polypropylene particles (PP) on zebrafish (*Danio rerio*) at the embryonic, larval, and adult stages. In embryos and larvae, developmental and toxicity parameters were analyzed, including mortality, malformations, hatching rate, heart rate, morphometric parameters, and locomotor behavior under light and dark conditions.

In adults, after 96 hours of exposure, behaviors associated with locomotion, anxiety, and social interaction were evaluated through the novel tank test and the social preference test.

3.3.1. Exposure of *Danio rerio* embryos at 5 hpf (hours post-fertilization)

- Physiological and morphological parameters measured in the embryonic stage

Following the FET test (mixtures of Kp with PP) performed on the embryonic stage of the *Danio rerio* species (Fig. III.20.), the percentage of mortality and morphological abnormalities upon exposure to $EC_{20,\ MIf}$ amounted to 50%, and at $EC_{50,\ MIf}$ a total of 60% - Fig. III.20. (a).

For heart rate and hatching rate, no statistically significant differences were identified - Fig. III.20. (b), (c).

For the interocular distance of the embryos at 120 h of exposure, it is noteworthy that both levels of Kp induced a significant decrease in this parameter (mean interocular distance of 0.116 ± 0.004 mm and 0.123 ± 0.009 mm for the $EC_{20,\ MIf}$ and $EC_{50,\ MIf}$ groups, respectively), compared to the organisms in the control group (mean interocular distance of 0.141 ± 0.015 mm) ($p < 0.05$) - Fig. III.20. (d).

For exposure to mixtures with PP, the exposed organisms presented a significantly lower interocular distance than that of the control group ($p < 0.05$) - Fig. III.20. (d), except for the groups exposed to $EC_{20,\ MIf}$ + 0.1 µg L^{-1} PP and $EC_{50,\ MIf}$ + 10 µg L^{-1} PP.

Regarding total length, statistically significant differences were identified only for the group exposed to $EC_{50,\ MIf}$, where the mean total length was 4.157 ± 0.274 mm, compared to the control group, where the mean total length was 4.392 ± 0.123 mm - Fig. III.20. (e).

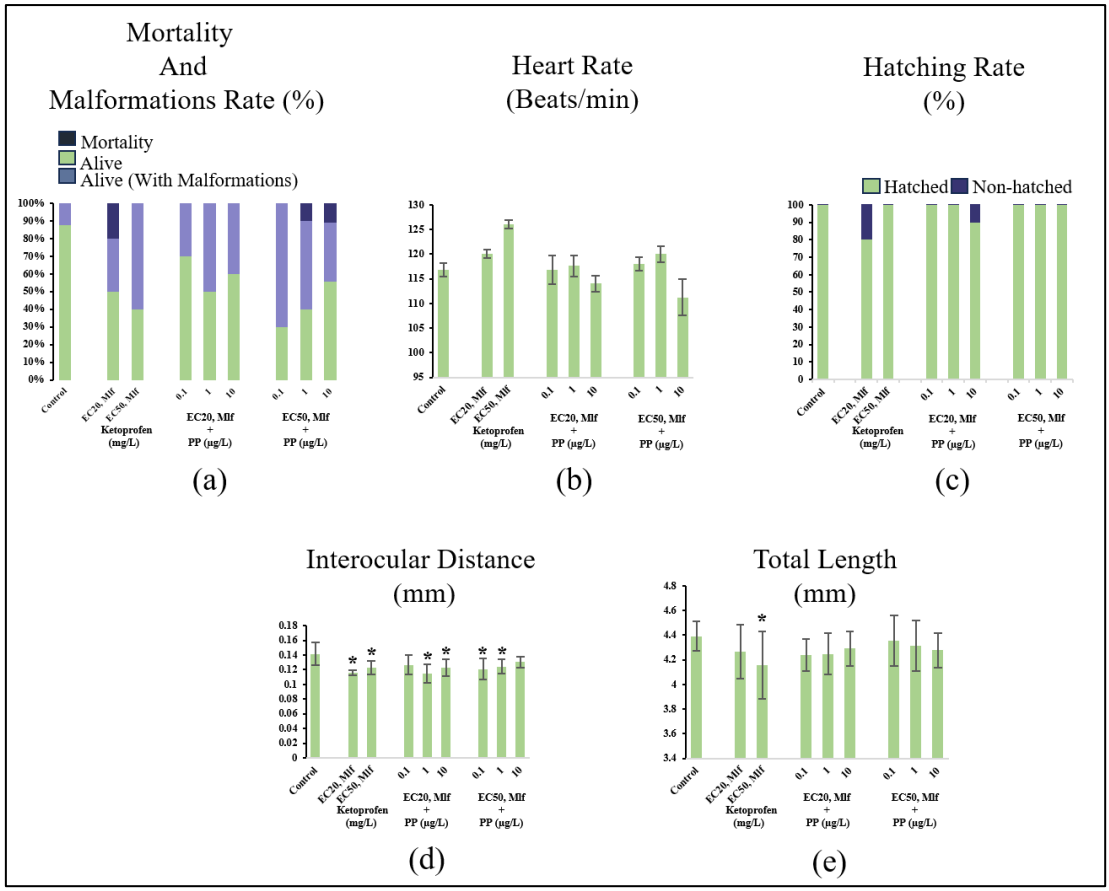

Fig. III.20. Physiological and morphological parameters measured at the embryonic stage (5 hpf) in *Danio rerio*, exposed for 120 h to mixtures of ketoprofen (Kp) at concentrations inducing 20 and 50% malformations ($EC_{20,\ Mlf}$ and $EC_{50,\ Mlf}$, expressed in mg L^{-1}) and polypropylene micro-nanoplastics (PP) at concentrations of 0.1; 1 and 10 µg L^{-1}. The monitored parameters included: (a) mortality and malformations rate (%), (b) heart rate (beats/min), (c) hatching rate (%), (d) interocular distance (mm), (e) total length (mm). Data are presented as mean ± SD (n = 10 animals per group) and were analyzed using one-way ANOVA followed by Dunnett's test. Statistically significant differences are indicated as follows: * $p < 0.05$.

3.3.2. Exposure of *Danio rerio* larvae at 72 hpf (hours post-fertilization)

- Physiological and morphological parameters measured in the larval stage

Regarding the exposure of larvae to Kp, for $EC_{20,\ Mlf}$ and $EC_{50,\ Mlf}$, mortality and morphological abnormalities together accounted for a 50% effect in both cases, while up to 40% were observed for the groups treated with $EC_{20,\ Mlf}$ + 1 and 10 µg L^{-1} PP, as well as up to 40% for the groups treated with $EC_{50,\ Mlf}$ + 1 µg L^{-1} PP - Fig. III.24. (a).

No statistically significant differences were identified for the two biometric landmarks, namely the interocular distance and total length ($p > 0.05$) - Fig. III.24. (b), (c).

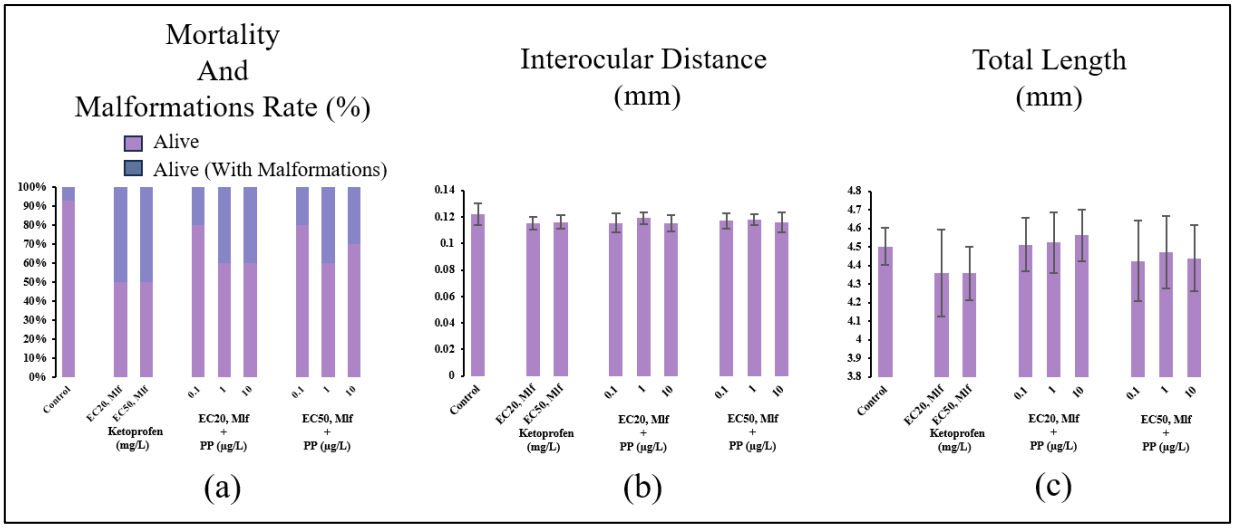

Fig. III.24. Physiological and morphological parameters measured in the larval stage (72 hpf) of *Danio rerio*, exposed for 120 h to ketoprofen (Kp) and polypropylene micro-nanoplastics (PP) at concentrations of 0.1; 1 and 10 µg L^{-1}. The parameters monitored included: (a) mortality and malformations rate, (b) interocular distance, (c) total length. Data are presented as mean ± SD (n = 10 animals per group) and were analyzed using one-way ANOVA followed by Dunnett's test.

3.3.3. Exposure of adult *Danio rerio* (8 months old)

- Novel Tank Test

The results of the Novel Tank test, after 96 hours of exposure to Kp, PP, and their mixture, showed no statistically significant differences in terms of total distance traveled ($p > 0.05$) - Fig. III.28. (a). Regarding the immobility time, a significant increase was observed in the Kp+PP treated group compared to the control group ($p < 0.001$) - Fig. III.28. (b). Statistically significant differences were reported in in terms of time spent in the upper half of the aquarium, where the control group explored significantly more than the PP treated group ($p < 0.001$), Kp+PP ($p = 0.02$), and with no statistical significance compared to the Kp treated group ($p = 0.061$) - Fig. III.28. (c). In addition, another parameter of interest was the time spent in the lower area of the aquarium, where the control group spent significantly less time compared to Kp ($p = 0.020$), PP ($p < 0.001$), and Kp+PP ($p < 0.001$) - Fig. III.28. (d).

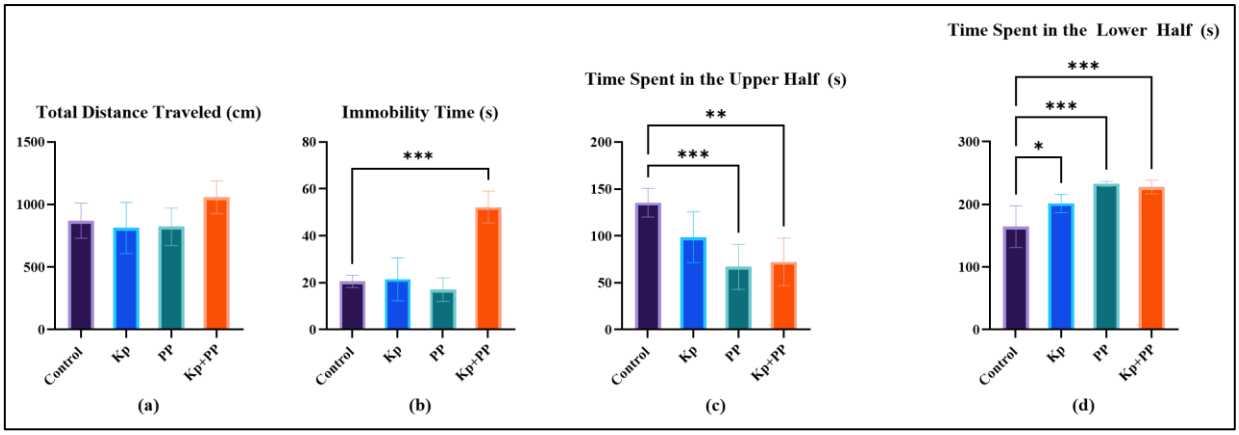

Fig. III.28. Effects of exposure to ketoprofen (Kp) and polypropylene (PP), individually and in mixture (Kp = 5 µg L^{-1}; PP = 2 mg L^{-1}) on the locomotion of adult zebrafish. The measured behavioral parameters included: (a) total distance traveled (cm), (b) immobility time (s), (c) time spent in the upper half (s), and (d) time spent in the lower half (s) of the aquarium. Data are presented as mean ± SD (n = 10 animals per group, with a male/female sex ratio of 1:1) and were analyzed using one-way ANOVA, followed by Dunnett's test. Statistically significant differences are indicated as follows: * $p < 0.05$; ** $p < 0.01$; and *** $p < 0.001$.

3.3.4. Discussion of the study

The study demonstrated that exposure to ketoprofen (Kp), polypropylene (PP), and their mixtures produces stage-dependent effects in *Danio rerio*, with the most pronounced effects observed in embryonic and larval stages. Kp induced a dose-dependent increase in malformations, and mixtures with PP exacerbated the toxic effects, including delayed hatching, increased mortality and changes in certain morphological parameters, such as interocular distance. Although heart rate and total length were not significantly affected, the results indicate a high sensitivity of early stages to simultaneous exposure to pharmaceutical contaminants and micro-nanoplastics.

At the behavioral level, Kp+PP mixtures generated more severe effects than individual exposures, both in embryos and larvae, as well as in adults. In the early stages, significant changes were observed in locomotor activity, distance traveled, speed of movement, immobility, and preference for the edges of the wells, suggesting avoidance and stress responses. In adults, the same trend was manifested by increased immobility time, reduced exploration of the upper area of the aquarium, and a preference for the lower area, behaviors characteristic of anxiety. Exposure to Kp and PP also led to increased clockwise rotations, indicating possible alterations in sensory processing and motor control.

The results obtained suggest that effects induced in early stages may persist and influence adult behavior. Fish exposed to Kp, PP, and the Kp+PP mixture showed a reduction in social preference and a tendency to avoid conspecifics, indicating impaired social interactions. Overall, the study highlights that the association between ketoprofen and micro-nanoplastics amplifies toxic and behavioral effects compared to individual exposures, underscoring the increased vulnerability of embryos and larvae and the importance of assessing the combined effects of emerging contaminants throughout the entire developmental process.

3.4. *Experimental study no. 4: Individual and combined effects of ketoprofen and polylactic acid micro-nanoplastics on zebrafish development and behavior*

The study investigated the combined effects of ketoprofen (Kp) and PLA micro-nanoplastics on zebrafish (*Danio rerio*) at the embryonic, larval, and adult stages. For embryos and larvae, mortality, malformations, morphological development and locomotor behavior were assessed, while for adults, exploratory, anxious and social behaviors were analyzed after a 96-hour exposure. The behavioral parameters monitored included locomotor activity, immobility, swimming speed, rotations, and preference for certain areas of the test environment.

3.4.1. Exposure of *Danio rerio* embryos at 5 hpf (hours post-fertilization)

- Physiological and morphological parameters measured in the embryonic stage

In the FET test, performed at the embryonic stage of *Danio rerio* (Fig. III.33.), the mortality rate and the malformation rate were analyzed together, being considered as the total effect.

These accounted for approximately 50% of the response at the $EC_{20,\ Mlf}$ concentration and reached up to 80% in the case of exposure to $EC_{50,\ Mlf}$ - Fig. III.33. (a).

For exposures to mixtures with $EC_{20,\ Mlf}$, and PLA, the effects observed were approximately similar to those recorded in individual exposures. At the higher concentration of $EC_{50,\ Mlf}$ together with PLA, the impact was even more pronounced, with minimum values of 70% and maximum values reaching 80%, while no effects were observed in the control group - Fig. III.33. (a).

In addition, the heart rate was statistically significantly lower for the groups exposed to $EC_{50, Mlf}$ and $EC_{50, Mlf}$ + 10 µg L^{-1} PLA, compared to the control group - Fig. III.33. (b).

Embryo hatching was delayed by 10% at $EC_{20, Mlf}$ and up to 40% at $EC_{50, Mlf}$, compared to the control group - Fig. III.33. (c). The groups exposed to the mixture of $EC_{20, Mlf}$ + 1 µg L^{-1} PLA showed a hatching rate of only 50%, while the mixture of $EC_{20, Mlf}$ + 10 µg L^{-1} PLA showed a hatching rate of 40%. The mixtures of $EC_{50, Mlf}$, and 0.1; 1, respectively 10 µg L^{-1} PLA showed a hatching rate of 100% - Fig. III.33. (c).

At the end of the exposure period, both levels of Kp induced a significant decrease in interocular distance, with mean values of 0.127 ± 0.009 mm and 0.128 ± 0.007 mm for the groups exposed to $EC_{20, Mlf}$, and $EC_{50, Mlf}$, respectively, compared to the organisms in the control group (0.145 ± 0.009 mm) ($p < 0.05$) - Fig. III.33. (d).

In addition, exposure to mixtures of PLA and Kp resulted in significantly lower values of interocular distance compared to those recorded in the control group ($p < 0.05$) - Fig. III.33. (d), the values corresponding to $EC_{50, Mlf}$ + PLA being generally lower than those observed for $EC_{20, Mlf}$ + PLA.

For total length, statistically significant differences were obtained only for the groups exposed to $EC_{50, Mlf}$, where the mean total length was 4.238 ± 0.153 mm, compared to the control group, where the mean total length was 4.375 ± 0.205 mm - Fig. III.33. (e).

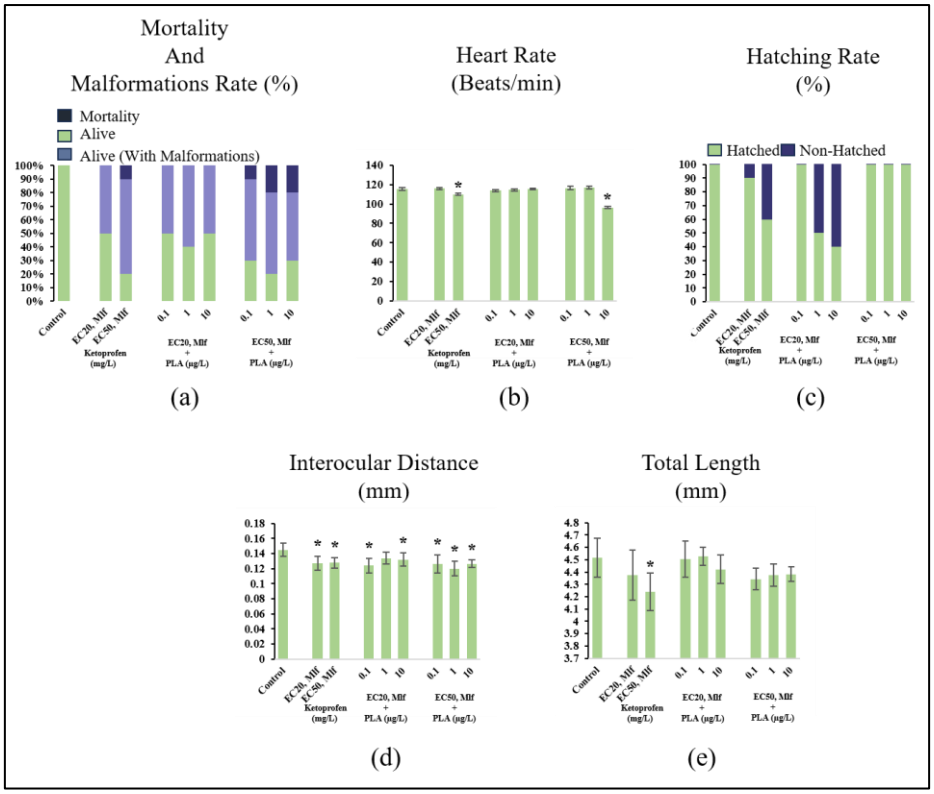

Fig. III.33. Physiological and morphological parameters measured at the embryonic stage (5 hpf) in *Danio rerio* exposed to mixtures of ketoprofen (Kp) at concentrations inducing 20 and 50% malformations ($EC_{20,\ Mlf}$ and $EC_{50,\ Mlf}$, expressed in mg L^{-1}) and polylactic acid micro-nanoplastics (PLA) at concentrations of 0.1; 1; 10 µg L^{-1}. The monitored parameters included: (a) mortality and malformations rate (%), (b) heart rate (beats/min), (c) hatching rate (%), (d) interocular distance (mm), (e) total length (mm). Data are presented as mean ± SD (n = 10 animals per group) and were analyzed using one-way ANOVA followed by Dunnett's test. Statistically significant differences are indicated as follows: * $p < 0.05$.

3.4.2. Exposure of *Danio rerio* larvae at 72 hpf (hours post-fertilization)

- Physiological and morphological parameters measured in the larval stage

For larvae exposed to individual concentrations of Kp, mortality and malformations together accounted for approximately 30% for the group exposed to $EC_{20,\ Mlf}$ and reached up to 70% for the group exposed to $EC_{50,\ Mlf}$ - Fig. III.37. (a).

Regarding the mixtures of Kp and PLA micro-nanoplastics, the effects were more pronounced. For the group exposed to $EC_{20,\ Mlf}$ + PLA, the lowest effect was 20%, while the maximum value reached 50%. For the group treated with $EC_{50,\ Mlf}$ + PLA, the effect ranged between 70% and 80%, indicating a statistically significant increase in the mortality and malformation rate compared to individual exposures - Fig. III.37. (a). No statistically significant

changes were observed for the interocular distance and total length of the larvae after exposure ($p > 0.05$) - Fig. III.37. (b), (c).

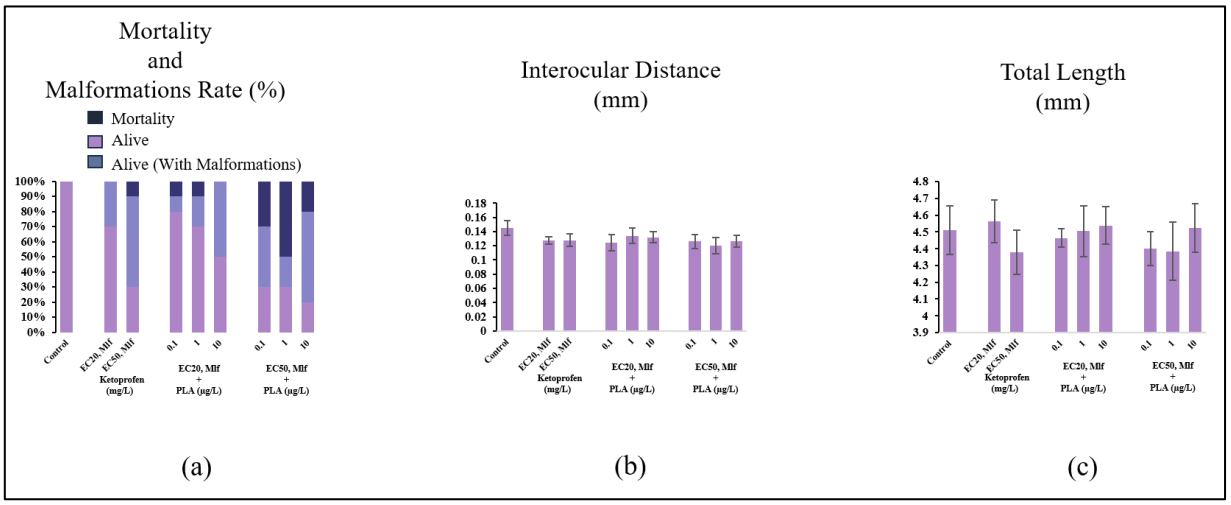

Fig. III.37. Physiological and morphological parameters measured in the larval stage (72 hpf) of *Danio rerio* exposed for 120 h to ketoprofen (Kp) and polylactic acid (PLA) micro-nanoplastics at concentrations of 0.1; 1 and 10 µg L^{-1}. The monitored parameters included: (a) mortality and malformation rate, (b) interocular distance, (c) total length. Data are presented as mean ± SD (n = 10 animals per group) and were analyzed using one-way ANOVA followed by Dunnett's test.

3.4.3. Exposure of adult *Danio rerio* (8 months old)

- Novel Tank Test

Regarding the total distance traveled, no statistically significant differences were observed ($p > 0.05$) - Fig. III.41. (a), but swimming speed was significantly lower in the PLA-treated group ($p = 0.002$) compared to the control group - Fig. III.41. (b).

No statistically significant differences were observed for the exposed groups compared to the control group in terms of total activity time ($p > 0.05$) - Fig. III.41. (c).

Regarding immobility time, a significant increase was observed among all treated groups ($p < 0.001$), compared to the control group - Fig. III.41. (d).

To compare the exploratory behavior of the fish in the control group with that of the treated groups, the aquarium was divided into two halves and the time spent in each half was measured. In the lower half, no statistically significant differences were observed between the treated groups and the control group ($p > 0.05$) - Fig. III.41. (f).

A statistically significant decrease in the time spent in the upper half was observed in the Kp+PLA treated group ($p = 0.027$), compared to the control group - Fig. III.41. (e). No

statistically significant differences were observed between the other treated groups and the control group ($p > 0.05$).

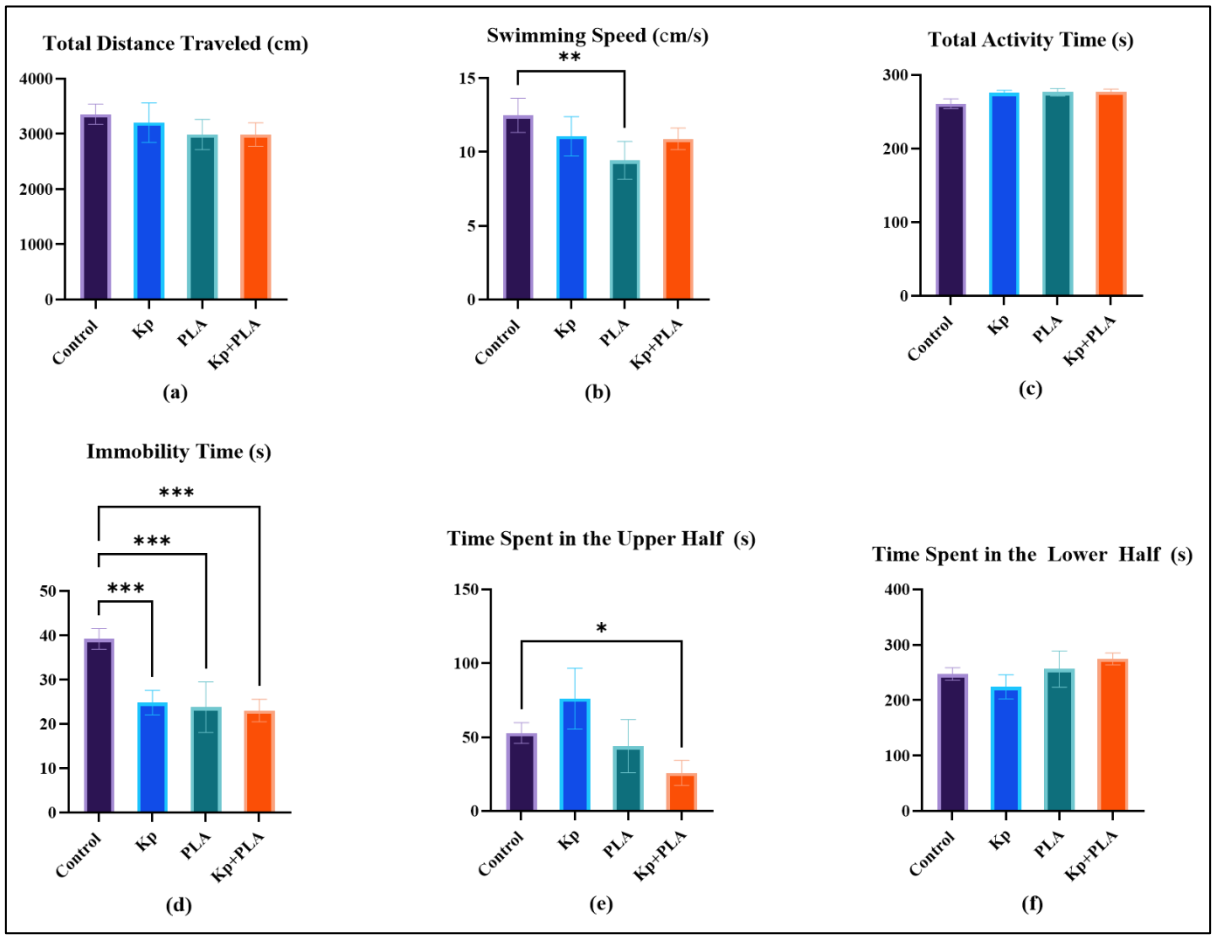

Fig. III.41. Effects of exposure to ketoprofen (Kp) and polylactic acid (PLA), individually and in mixture (Kp = 10 µg L^{-1}; PLA = 10 µg L^{-1}) for 96 hours. The measured behavioral parameters included: (a) total distance traveled (cm), (b) swimming speed (cm s^{-1}), (c) total activity time (s), (d) immobility time (s), (e) time spent in the upper half (s), and (f) time spent in the lower half (s). Data are presented as mean ± SD (n = 10 animals per group, with a male/female sex ratio of 1:1) and were analyzed using one-way ANOVA followed by Dunnett's test. Statistically significant differences are indicated as follows: * $p < 0.05$; ** $p < 0.01$; and *** $p < 0.001$.

3.4.4. Discussion of the study

The study revealed that PLA micro-nanoplastics (MNPL) administered individually did not produce significant effects on the frequency of mortality or malformation compared to the control group. In contrast, their association with ketoprofen (Kp) caused obvious behavioral changes at all developmental stages investigated. Both embryos and larvae exposed to the Kp+PLA mixture showed a marked increase in clockwise and counterclockwise rotations,

especially in dark conditions, suggesting alteration of normal movement patterns and the emergence of stress-related responses even at early stages of development.

These effects persisted into adulthood, where exposure to the Kp+PLA mixture resulted in a significant increase in counterclockwise rotations, a behavior recognized as an indicator of stress, anxiety, and neurological dysfunctions in *Danio rerio*. The concordance between the responses observed in embryos, larvae, and adults suggests that exposure to the mixture may induce persistent neurobehavioral disorders throughout ontogeny, highlighting a potential toxicity-enhancing effect through the interaction between the pharmaceutical contaminant and plastic particles.

An important result of the study is that PLA, although considered a biodegradable material and an environmentally friendly alternative to conventional plastics, was not shown to be devoid of biological effects. Exposure of embryos and larvae to PLA MNPLs, especially in combination with Kp, generated sublethal and lethal effects more pronounced than those previously reported for some conventional plastics. These results underscore the need to reevaluate the ecotoxicological impact of bioplastics and demonstrate that the status of a material as "eco-friendly" does not guarantee the absence of risks to aquatic organisms, especially in early stages of development.

CONCLUSIONS

This study demonstrates that the ecotoxicological impact of emerging contaminants in aquatic environments is conditioned by interactions between substances, both in the case of mixtures of pharmaceutical compounds and in their combinations with micro-nanoplastics (MNPL). Analysis of the results obtained on the *Danio rerio* experimental model indicates that biological responses differ depending on the developmental stage and the type of exposure and cannot be predicted based on the individual effects of the substances. Thus, exposure to mixtures results in toxic effects significantly different from exposure to individual contaminants.

In conclusion, the results of this study support the need for modern and integrated approaches in the assessment of ecotoxicological risks. The integration of behavioral and neurofunctional parameters provides a more sensitive and realistic perspective on the effects of emerging contaminants. Furthermore, the data obtained demonstrate that biodegradable plastics cannot be automatically considered safe and that the interaction between chemical pollutants and MNPL may generate biological effects with potential impact on the survival and functioning of aquatic organisms.

SELECTIVE BIBLIOGRAPHY

1. Abdollahi, S., Raissi, H., & Farzad, F. (2025). The role of microplastics as vectors of antibiotic contaminants via a molecular simulation approach. *Scientific Reports*, *15*(1), 27007. https://doi.org/10.1038/s41598-025-12799-6.
2. Ács, A., Liang, X., Bock, I., Griffitts, J., Ivánovics, B., Vásárhelyi, E., Ferincz, Á., Pirger, Z., Urbányi, B., & Csenki, Z. (2022). Chronic Effects of Carbamazepine, Progesterone and Their Mixtures at Environmentally Relevant Concentrations on Biochemical Markers of Zebrafish (*Danio rerio*). *Antioxidants*, *11*(9), 1776. https://doi.org/10.3390/antiox11091776.
3. Alkimin, G. D., Soares, A. M. V. M., Barata, C., & Nunes, B. (2020). Evaluation of ketoprofen toxicity in two freshwater species: Effects on biochemical, physiological and population endpoints. *Environmental Pollution*, *265*(Pt B), 114993. https://doi.org/10.1016/j.envpol.2020.114993.
4. Andrady, A. L. (2011). Microplastics in the marine environment. *Marine Pollution Bulletin*, *62*(8), 1596–1605. https://doi.org/10.1016/j.marpolbul.2011.05.030.
5. Atugoda, T., Vithanage, M., Wijesekara, H., Bolan, N., Sarmah, A. K., Bank, M. S., You, S., & Ok, Y. S. (2021). Interactions between microplastics, pharmaceuticals and personal care products: Implications for vector transport. *Environment International*, *149*, 106367. https://doi.org/10.1016/j.envint.2020.106367.
6. Barreto, M., Lopes, I., & Oliveira, M. (2023). Micro(nano)plastics: A review on their interactions with pharmaceuticals and pesticides. *TrAC Trends in Analytical Chemistry*, *169*, 117307. https://doi.org/10.1016/j.trac.2023.117307.
7. Cardoso-Vera, J. D., Elizalde-Velázquez, G. A., Islas-Flores, H., Mejía-García, A., Ortega-Olvera, J. M., & Gómez-Oliván, L. M. (2021). A review of antiepileptic drugs: Part 1 occurrence, fate in aquatic environments and removal during different treatment technologies. *Science of The Total Environment*, *768*, 145487. https://doi.org/10.1016/j.scitotenv.2021.145487.
8. Chelaru, I.-A., Ciausu, R.-A., Savuca, A., Ureche, C., Nicoara, M. N., Ciobica, A. S., Andronic, G.-A., Ambrosie, R.-M., & Ureche, D. (2025). Effects of environmentally relevant ibuprofen and valproic acid exposure on zebrafish behavior. *Biomolecules and Biomedicine*. https://doi.org/10.17305/bb.2026.14030.
9. Chelaru, I.-A., Strungaru-Jijie, R., Nicoara, M., Mirila, D., Ciobica, A., & Ureche, D. (2025). Single and Combined Effects of Meropenem, Valproic Acid, and Ketoprofen on Adult Zebrafish Behavior, Oxidative Stress, and Acetylcholinesterase Activity. *Pharmaceuticals*, *18*(8), 1096. https://doi.org/10.3390/ph18081096.
10. Coimbra, R. N., Escapa, C., & Otero, M. (2021). Removal of Pharmaceuticals from Water: Conventional and Alternative Treatments. *Water*, *13*(4), 487. https://doi.org/10.3390/w13040487.
11. Desai, M., Njoku, A., & Nimo-Sefah, L. (2022). Comparing Environmental Policies to Reduce Pharmaceutical Pollution and Address Disparities. *International Journal of Environmental Research and Public Health*, *19*(14), 8292. https://doi.org/10.3390/ijerph19148292.
12. Diniz, M. S., Salgado, R., Pereira, V. J., Carvalho, G., Oehmen, A., Reis, M. a. M., & Noronha, J. P. (2015). Ecotoxicity of ketoprofen, diclofenac, atenolol and their photolysis

byproducts in zebrafish (*Danio rerio*). *The Science of the Total Environment*, *505*, 282–289. https://doi.org/10.1016/j.scitotenv.2014.09.103.

13. Gomes, S. da S., da Silva, J. F., Padilha, R. M. O., de Vasconcelos, J. V. A., Negreiros Neto, L. G. de, Marrs, J. A., & Cadena, P. G. (2024). Behavioral Effects of the Mixture and the Single Compounds Carbendazim, Fipronil, and Sulfentrazone on Zebrafish (*Danio rerio*) Larvae. *Biomedicines*, *12*(6), 1176. https://doi.org/10.3390/biomedicines12061176.
14. Hildebrandt, L., Nack, F. L., Zimmermann, T., & Pröfrock, D. (2021). Microplastics as a Trojan horse for trace metals. *Journal of Hazardous Materials Letters*, *2*, 100035. https://doi.org/10.1016/j.hazl.2021.100035.
15. Hong, X., Zhao, G., Zhou, Y., Chen, R., Li, J., & Zha, J. (2021). Risks to aquatic environments posed by 14 pharmaceuticals as illustrated by their effects on zebrafish behaviour. *The Science of the Total Environment*, *771*, 145450. https://doi.org/10.1016/j.scitotenv.2021.145450.
16. Hossain, Md. T., Shahid, Md. A., Mahmud, N., Habib, A., Rana, Md. M., Khan, S. A., & Hossain, Md. D. (2024). Research and application of polypropylene: A review. *Discover Nano*, *19*(1), 2. https://doi.org/10.1186/s11671-023-03952-z.
17. Jijie, R., Paduraru, E., Simionov, I.-A., Faggio, C., Ciobica, A., & Nicoara, M. (2023). Effects of Single and Combined Ciprofloxacin and Lead Treatments on Zebrafish Behavior, Oxidative Stress, and Elements Content. *International Journal of Molecular Sciences*, *24*(5). https://doi.org/10.3390/ijms24054952.
18. Jijie, R., Solcan, G., Nicoara, M., Micu, D., & Strungaru, S.-A. (2020). Antagonistic effects in zebrafish (*Danio rerio*) behavior and oxidative stress induced by toxic metals and deltamethrin acute exposure. *The Science of the Total Environment*, *698*, 134299. https://doi.org/10.1016/j.scitotenv.2019.134299
19. Karali, N., Khanna, N., & Shah, N. (n.d.). *Climate Impact of Primary Plastic Production*.
20. Kataria, N., Yadav, S., Garg, V. K., Rene, E. R., Jiang, J.-J., Rose, P. K., Kumar, M., & Khoo, K. S. (2024). Occurrence, transport, and toxicity of microplastics in tropical food chains: Perspectives view and way forward. *Environmental Geochemistry and Health*, *46*(3), 98. https://doi.org/10.1007/s10653-024-01862-2.
21. Klavins, M., Klavins, L., Stabnikova, O., Stabnikov, V., Marynin, A., Ansone-Bertina, L., Mezulis, M., & Vaseashta, A. (2022). Interaction between Microplastics and Pharmaceuticals Depending on the Composition of Aquatic Environment. *Microplastics*, *1*(3), 520–535. https://doi.org/10.3390/microplastics1030037.
22. Maddah, H. (2016). *Polypropylene as a Promising Plastic: A Review*. https://www.semanticscholar.org/paper/Polypropylene-as-a-Promising-Plastic%3A-A-Review-Maddah/682060d2941b3eb5421aab0ac49a28ead79ece58.
23. Madesh, S., Sudhakaran, G., Meenatchi, R., Manikandan, K., Dhayanithi, N. B., Almutairi, M. H., Almutairi, B. O., Guru, A., & Arockiaraj, J. (2024)a. Neurobehavioral and bioaccumulative toxicity in adult in-vivo zebrafish model due to prolonged cadmium exposure in the presence of ketoprofen. *Journal of Biochemical and Molecular Toxicology*, *38*(11), e70005. https://doi.org/10.1002/jbt.70005.
24. Madesh, S., Sudhakaran, G., Murugan, R., Almutairi, M. H., Almutairi, B. O., Kathiravan, M. K., & Arockiaraj, J. (2024)b. Parental (F0) exposure to Cadmium and Ketoprofen induces developmental deformities in offspring (F1): A transgenerational toxicity assessment in zebrafish model. *The Science of the Total Environment*, *950*, 175319. https://doi.org/10.1016/j.scitotenv.2024.175319.

25. Matjašič, T., Mori, N., Hostnik, I., Bajt, O., & Kovač Viršek, M. (2023). Microplastic pollution in small rivers along rural-urban gradients: Variations across catchments and between water column and sediments. *The Science of the Total Environment*, *858*(Pt 3), 160043. https://doi.org/10.1016/j.scitotenv.2022.160043.
26. Moermond, C. T. A., Puhlmann, N., Brown, A. R., Owen, S. F., Ryan, J., Snape, J., Venhuis, B. J., & Kümmerer, K. (2022). GREENER Pharmaceuticals for More Sustainable Healthcare. *Environmental Science & Technology Letters*, *9*(9), 699–705. https://doi.org/10.1021/acs.estlett.2c00446.
27. Muambo, K. E., Kim, M.-G., Kim, D.-H., Park, S., & Oh, J.-E. (2024). Pharmaceuticals in raw and treated water from drinking water treatment plants nationwide: Insights into their sources and exposure risk assessment. *Water Research X*, *24*, 100256. https://doi.org/10.1016/j.wroa.2024.100256.
28. Nanthini Devi, K., Raju, P., Santhanam, P., Dinesh Kumar, S., Krishnaveni, N., Roopavathy, J., & Perumal, P. (2021). Biodegradation of low-density polyethylene and polypropylene by microbes isolated from Vaigai River, Madurai, India. *Archives of Microbiology*, *203*(10), 6253–6265. https://doi.org/10.1007/s00203-021-02592-0.
29. Narwal, N., Kakakhel, M., Katyal, D., Yadav, S., Rose, P., Rene, E., Rakib, M. R., Khoo, K. S., & Kataria, N. (2024). Interactions Between Microplastic and Heavy Metals in the Aquatic Environment: Implications for Toxicity and Mitigation Strategies. *Water, Air, & Soil Pollution*, *235*. https://doi.org/10.1007/s11270-024-07343-7.
30. Onesios-Barry, K. M., Berry, D., Proescher, J. B., Sivakumar, I. K. A., & Bouwer, E. J. (2014). Removal of pharmaceuticals and personal care products during water recycling: Microbial community structure and effects of substrate concentration. *Applied and Environmental Microbiology*, *80*(8), 2440–2450. https://doi.org/10.1128/AEM.03693-13.
31. Ortúzar, M., Esterhuizen, M., Olicón-Hernández, D. R., González-López, J., & Aranda, E. (2022). Pharmaceutical Pollution in Aquatic Environments: A Concise Review of Environmental Impacts and Bioremediation Systems. *Frontiers in Microbiology*, *13*, 869332. https://doi.org/10.3389/fmicb.2022.869332.
32. Paduraru, E., Jijie, R., Simionov, I.-A., Gavrilescu, C.-M., Ilie, T., Iacob, D., Lupitu, A., Moisa, C., Muresan, C., Copolovici, L., Copolovici, D. M., Mihalache, G., Lipsa, F. D., Solcan, G., Danelet, G.-A., Nicoara, M., Ciobica, A., & Solcan, C. (2024). Honey Enriched with Additives Alleviates Behavioral, Oxidative Stress, and Brain Alterations Induced by Heavy Metals and Imidacloprid in Zebrafish. *International Journal of Molecular Sciences*, *25*(21). https://doi.org/10.3390/ijms252111730.
33. SanJuan-Reyes, N., Gómez-Oliván, L., Islas-Flores, H., Martínez, M., García Medina, S., Dublán-García, O., & Hernández-Navarro, M. (2017). Occurrence of Pharmaceuticals in the Environment. In *Handbook of Environmental Chemistry*. https://doi.org/10.1007/698_2017_142.
34. Savuca, A., Chelaru, I.-A., Balmus, I.-M., Curpan, A.-S., Nicoara, M. N., & Ciobica, A. S. (2024). Toxicological Response of Zebrafish Exposed to Cocktails of Polymeric Materials and Valproic Acid. *Sustainability*, *16*(5), 1–14.
35. Sid, S., Mor, R. S., Kishore, A., & Sharanagat, V. S. (2021). Bio-sourced polymers as alternatives to conventional food packaging materials: A review. *Trends in Food Science & Technology*, *115*, 87–104. https://doi.org/10.1016/j.tifs.2021.06.026.
36. Song, J., Wang, C., & Li, G. (2024). Defining Primary and Secondary Microplastics: A Connotation Analysis. *ACS ES&T Water*, *4*(6), 2330–2332. https://doi.org/10.1021/acsestwater.4c00316.

37. Szarka, A., Vnuková, L., Keršňáková, Z., Viktoryová, N., & Hrouzková, S. (2024). Contamination with Pharmaceuticals in Aquatic Environment: Focus on Analytical Methodologies. *Applied Sciences*, *14*(19). https://doi.org/10.3390/app14198645.
38. Wang, Y., Wu, S., Chen, J., Zhang, C., Xu, Z., Li, G., Cai, L., Shen, W., & Wang, Q. (2018). Single and joint toxicity assessment of four currently used pesticides to zebrafish (*Danio rerio*) using traditional and molecular endpoints. *Chemosphere*, *192*, 14–23. https://doi.org/10.1016/j.chemosphere.2017.10.129.
39. Wang, Y., Yang, G., Dai, D., Xu, Z., Cai, L., Wang, Q., & Yu, Y. (2017). Individual and mixture effects of five agricultural pesticides on zebrafish (*Danio rerio*) larvae. *Environmental Science and Pollution Research International*, *24*(5), 4528–4536. https://doi.org/10.1007/s11356-016-8205-9.
40. Wilkinson, J. L., Boxall, A. B. A., Kolpin, D. W., Leung, K. M. Y., Lai, R. W. S., Galbán-Malagón, C., Adell, A. D., Mondon, J., Metian, M., Marchant, R. A., Bouzas-Monroy, A., Cuni-Sanchez, A., Coors, A., Carriquiriborde, P., Rojo, M., Gordon, C., Cara, M., Moermond, M., Luarte, T., … Teta, C. (2022). Pharmaceutical pollution of the world's rivers. *Proceedings of the National Academy of Sciences of the United States of America*, *119*(8), e2113947119. https://doi.org/10.1073/pnas.2113947119.
41. Wöhler, L., Niebaum, G., Krol, M., & Hoekstra, A. Y. (2020). The grey water footprint of human and veterinary pharmaceuticals. *Water Research X*, *7*, 100044. https://doi.org/10.1016/j.wroa.2020.100044.
42. Yan, G., Bo, P., Chunyu, Z., & Xuequan, Z. (2015). Morphology of Polypropylene in-Reactor Alloys. *Huaxue Jinzhan*, *27*(12), 1815–1821.
43. Yang, G., Wang, Y., Li, J., Wang, D., Bao, Z., Wang, Q., & Jin, Y. (2021). Health risks of chlorothalonil, carbendazim, prochloraz, their binary and ternary mixtures on embryonic and larval zebrafish based on metabolomics analysis. *Journal of Hazardous Materials*, *404*(Pt B), 124240. https://doi.org/10.1016/j.jhazmat.2020.124240.
44. Zhang, H., Zhu, C., Zhao, J., Zheng, R., Xing, J., Li, Z., Zhang, Y., & Xu, Q. (2024). The enhanced hepatotoxicity of isobavachalcone in depigmented zebrafish due to calcium signaling dysregulation and lipid metabolism disorder. *Journal of Applied Toxicology: JAT*, *44*(6), 919–932. https://doi.org/10.1002/jat.4593.
45. Zhang, M., & Xu, L. (2020). Transport of micro- and nanoplastics in the environment: Trojan-Horse effect for organic contaminants. *Critical Reviews in Environmental Science and Technology*, *52*, 1–37. https://doi.org/10.1080/10643389.2020.1845531.
46. Zhao, W., Chen, Y., Hu, N., Long, D., & Cao, Y. (2024). The uses of zebrafish (*Danio rerio*) as an in vivo model for toxicological studies: A review based on bibliometrics. *Ecotoxicology and Environmental Safety*, *272*, 116023. https://doi.org/10.1016/j.ecoenv.2024.116023.