POLITÉCNICO DE LEIRIA | ESCOLA SUPERIOR DE TURISMO E TECNOLOGIA DO MAR

The impact of microplastics and copper on sex ratio and oxidative stress: analysis in zebrafish intestine, gonad and brain

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Dissertação para obtenção do Grau de Mestre em Aquacultura

Dissertação realizada sob a orientação da Professora Doutora Sandra Mariza Monteiro e coorientação da Professora Especialista Teresa Maria Coelho Baptista e Doutora Dércia Cabral Santos

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Agradecimentos

Desde cedo, fui confrontado com diversos desafios, tanto de saúde como de vida, que sempre procurei superar, aprendendo com cada experiência. Do mesmo modo, o percurso académico, desde o ensino obrigatório até ao nível superior proporcionou-me ferramentas e conhecimentos essenciais para o meu sucesso no futuro.

Agradeço assim aos meus orientadores pelo tempo e conhecimentos que me dispuseram ao longo de todo este trabalho. Um especial abraço a Professora Teresa por desde a licenciatura ser rígida nas suas correções e estar sempre disponível para nos esclarecer de todas as dúvidas que tenhamos.

Gostaria de expressar um agradecimento e um grande beijinho à Professora Mariza, que, mesmo enfrentando graves problemas de saúde durante a realização desta dissertação, continuou a apoiar-me e a orientar-me com dedicação e generosidade a partir de casa. O seu compromisso mesmo em circunstâncias tão difíceis, foi uma fonte de motivação e apoio para mim, e sou profundamente grato pelo contributo inestimável para este trabalho.

Um grande agradecimento a Professora Maria dos Anjos Pires pelas sugestões e orientações relativas a determinados procedimentos técnicos nos resultados obtidos. Fico-lhe eternamente grato por toda ajuda prestada.

Agradeço à Universidade de Trás-os-Montes e Alto Douro e ao CITAB pelo apoio institucional e pelos recursos que tornaram possível a realização desta dissertação.

Aos meus pais e irmãos, agradeço com todo o amor e carinho, assim como apoio, a motivação, a inspiração e a paciência que sempre me proporcionaram em todo o meu percurso académico e de vida.

Aos meus amigos e colegas de curso pelo suporte emocional. Agradeço em especial ao Henrique, ao Paulo, ao João, ao Afonso, ao Diogo e ao Germano, um gigante obrigado do fundo do coração por me aturarem nos momentos mais difíceis. Um enorme beijinho e abraços para a minha madrinha académica pelo apoio que me deu desde que iniciei esta etapa da vida

Ao meu namorado, que durante toda a realização desta dissertação foi uma âncora de estabilidade emocional e me ofereceu apoio incalculável em diversos pormenores técnicos de bioquímica. Envio-lhe um enorme beijo e, com todo o amor, lhe agradeço por sinceramente tudo.

Assim me despeço da ESTM e desta fase da vida, dando início a uma nova etapa que espero esteja repleta de conquistas, tal como esta foi.

 “Como capitão do meu próprio destino, eu escolho navegar” 

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Resumo

A crescente presença de poluentes emergentes, como microplásticos e metais pesados, representa uma ameaça significativa para os ecossistemas aquáticos e para a aquacultura. Este estudo pretendeu avaliar os efeitos individuais e combinados do cobre (Cu) e dos microplásticos do tipo polietileno (PE) e poliestireno (PS) no desenvolvimento embrionário e na diferenciação sexual do peixe zebra (*Danio rerio*), bem como nas respostas bioquímicas em órgãos-alvo. Ensaios de exposição revelaram que o cobre, em concentrações ambientalmente relevantes, induziu elevada mortalidade embrionária, atrasos no desenvolvimento e alterações na frequência cardíaca. O PE, por si só, promoveu alterações subletais, incluindo um padrão de feminização populacional. A exposição combinada potenciou os efeitos adversos, demonstrando uma interação sinérgica. As análises histológicas confirmaram alterações no processo de diferenciação sexual, enquanto os ensaios bioquímicos evidenciaram respostas específicas por órgão: o cérebro mostrou elevada vulnerabilidade à toxicidade do cobre, as gónadas revelaram limitações nas adaptações antioxidantes e o intestino manifestou modificações pouco evidentes, mas relevantes. Estes resultados sublinham a importância de considerar cenários de exposição múltipla em estudos ecotoxicológicos e destacam o peixe zebra como modelo robusto para estudos sobre os impactos de poluentes emergentes. Conclui-se que a interação entre microplásticos e metais pesados poderá comprometer o sucesso reprodutivo e a sustentabilidade populacional de organismos aquáticos.

Palavras-chave: Polietileno, Poliestireno, Metais Pesados, *Danio rerio*, Ecotoxicologia.

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Abstract

The increasing prevalence of emerging pollutants, such as microplastics and heavy metals, poses a major threat to aquatic ecosystems. This study aimed to investigate the individual and combined effects of copper (Cu), polyethylene (PE) and polystyrene (PS) microplastics on embryonic development and sexual differentiation in zebrafish (*Danio rerio*), as well as biochemical responses in target organs. Exposure trials revealed that copper, at environmentally relevant concentrations, caused high embryonic mortality, developmental delays, and altered heart rates. PE exposure alone induced sublethal effects, including a feminisation trend in sex differentiation. Co-exposure amplified these adverse outcomes, confirming a synergistic interaction. Histological analyses revealed disruptions in sexual differentiation, while biochemical assays demonstrated organ-specific responses. The brain appeared highly vulnerable to copper neurotoxicity, the gonads showed limited antioxidant adaptation, and the intestine displayed subtle but significant changes. These findings highlight the ecological relevance of multi-contaminant exposure scenarios and reinforce zebrafish as a powerful model for toxicological studies. Overall, the interaction between microplastics and heavy metals may compromise reproductive success and population sustainability in aquatic organisms.

Keywords: Polyethylene, Polystyrene, Heavy Metals, *Danio rerio*, Ecotoxicology.

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List of Acronyms

CAT	Catalase
CDNB	1-chloro-2,4-dinitrobenzene
Cu	Copper
Dpf	Days post-fertilization
DCFH-DA	2',7'-dichlorofluorescein diacetate
DFC	Dichloroflurescein
DNPH	2,4-dinitrophenylhydrazine
E3	Embryo medium 3
GPx	Glutathione Peroxidase
GR	Glutathione Reductase
GSH	Reduced Glutathione
GSSG	Oxidized Glutathione
GST	Glutathione S-Transferase
Hpf	Hours post-fertilization
HPG	Hypothalamic-pituitary-gonadal
LDH	Lactate Dehydrogenase
MDA	Malondialdehyde
Mix	Mixture
MPs	Microplastics
MS-222	Tricaine Methanesulfonate
NPs	Nanoplastics
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NEM	N-ethylmaleimide
NBT	Nitroblue Tetrazolium
OPT	O-phthalaldehyde
OSI	Oxidative Stress Index
PBSA	2-phenylbenzimidazole-5-sulphonic acid
PE	Polyethylene

PET	Polyethylene Terephthalate
PP	Polypropylene
PS	Polystyrene
PVC	Polyvinylchloride
RAS	Recirculating Aquaculture System
ROS	Reactive Oxygen Species
SD	Standard Deviation
SOD	Superoxide Dismutase
TBARS	*(Thiobarbituric Acid Reactive Substances)* detects Lipid Peroxidation products
U	Units

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Introduction

1. Aquaculture and Fisheries

In recent years, we have witnessed a rapid increase in the global population, which has had an adverse impact on fish populations and various non-commercial wildlife species, (FAO, 2024). The pressure on aquatic ecosystems arises from efforts to satisfy the dietary requirements of a growing population, alongside the manufacturing and utilization of disposable products or materials that do not decompose, leading to increased pollution in water bodies, (WWF, 2024).

According to FAO's annual report, aquaculture has the potential to play a significant role in the world economy, accounting for 10 to 12% of the global population's livelihoods, (FAO, 2022). This importance stems from the gradual yet exponential growth that the sector has undergone since 1970, managing in 2022 to surpass the fisheries sector, representing 51% of all world production of aquatic animals (FAO, 2024), how can be observed on Figure 1.

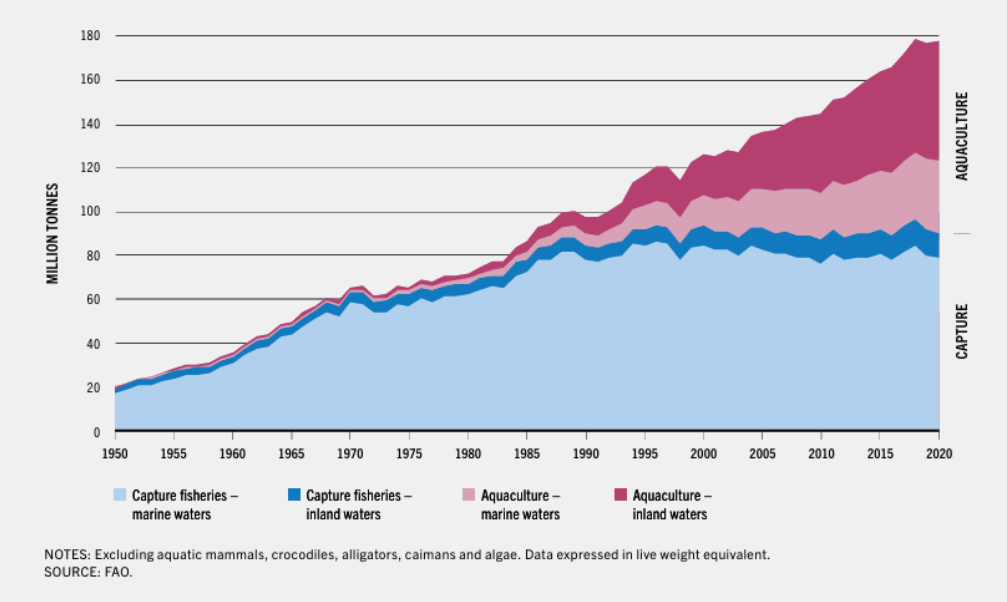

Figure 1 - World Capture Fisheries and Aquaculture Production, (FAO, 2024).

To meet the market demand, aquaculture has increasingly focused on optimization, aiming to achieve higher product density without expanding production space, (Xu & Zhang, 2013).

However, both aquaculture and fisheries are affected by pollution in aquatic environments, to which they may also contribute through the materials used in

their activities. In aquaculture, contamination by certain pollutants, specifically microplastics and heavy metals, can derive from external sources, such as rivers, seas, land and the atmosphere, or from internal sources including the materials used in pipes, water filtration equipment, fishing gear or feed (Wu et al., 2023).

2. Plastics

One of the most widely used raw materials in today's world is plastic, which can be found in all kinds of products, including bottles, pipes, food containers and even construction materials.

Plastics are polymeric materials that can be moulded or shaped, typically through the application of heat and pressure. This plasticity is often combined with other distinctive properties like low density, low electrical conductivity, transparency and toughness. These characteristics have enabled the development of a wide family of polymers, such as polystyrene (PS), polyethylene (PE), polyethylene terephthalate (PET), polyvinylchloride (PVC) and polypropylene (PP), among others, (Rodriguez, 2024).

The greatest concern, however, is not only the pollution of the environment, especially the aquatic ecosystems, as exemplified by floating "plastic islands", but also the degradation of the macroplastics in microplastics (MPs) and nanoplastics (NPs), (Ziani et al., 2023).

Microplastics are solid particles or polymeric matrices with regular or irregular shapes (Frias & Nash, 2018) that, in general, don't exceed 5 mm in size, (Arthur et al., 2009). These particles can be classified into two categories: primary MPs that are intentionally produced in this size range, and secondary MPs, which result from the fragmentation or wear of plastic-containing products widely distributed in the environment, (GESAMP, 2015).

There are various pathways through which MPs enter the aquaculture environment, (Chen et al., 2021; Liu et al., 2019), and many studies have demonstrated that their accumulation in aquaculture systems represents a significant problem, (Bordós et al., 2018; Ma et al., 2020; Wang et al., 2020). This production sector can be mainly affected by these contaminants in two ways: (i) through the external environment or (ii) internally, through the ageing of equipment, feed and packaging materials. Figure 2 summarizes the potential pathways of contamination.

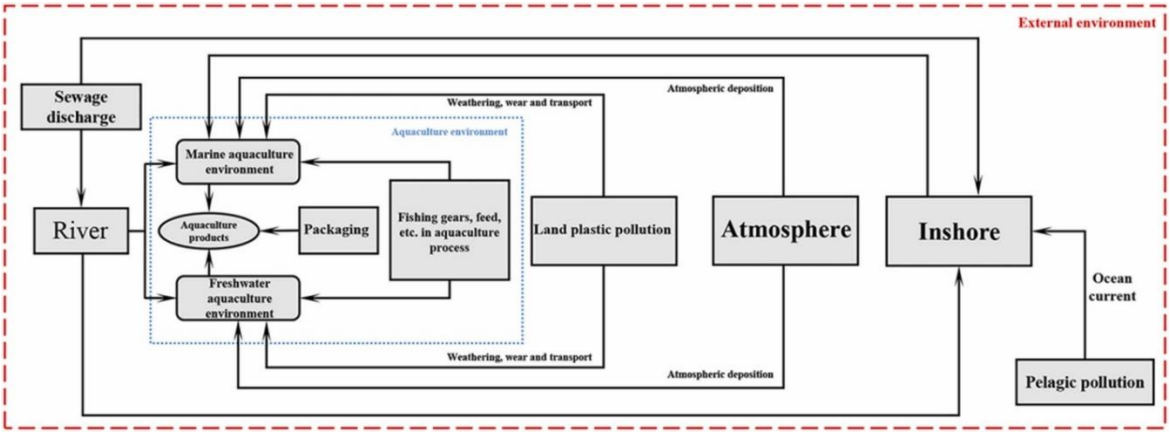

Figure 2 - Ways of microplastics introduced into aquaculture environments and aquaculture products, (Wu et al., 2023).

Polyethylene (PE) is the second most used commercial polymer (El-Sherif et al., 2022) and one of the major sources of environmental toxins, with the potential to harm wildlife, (Rochman et al., 2013). PE is not biodegradable and persists for a long time in water and soil, as well as clogging drains and sewage lines in and near cities, (El-Sherif et al., 2022). The widespread use of low-density PE and PVC raises significant environmental concerns and impacts to the terrestrial and aquatic ecosystems, as shown by the obstructions in the intestines of fish, birds and marine mammals, (Barnes et al., 2009).

Some of the impacts of MPs, particularly PE, have been highlighted by Matias et al. (2023), who showed that *Dicentrarchus labrax* exhibited changes in behaviour and growth, with the MPs potentially linked to neurotoxicity. In the same study, the fish also presented tissue damage, oxidative stress, and accumulation of MPs in edible tissues. Another study focusing on PE, reported that organs such as gills, liver and brain from juvenile *Cyprinus carpio* were damaged, and this effect was amplified when combined with 4-nonylphenol, a common chemical used as a stabilizer and antioxidant in the production of PE, (Ammar et al., 2022; Hahladakis et al., 2017). Additional cases illustrating the effects of MPs in different species are summarized in Table I.

Table I: Some examples of studies done with microplastics.

Contaminant	Species	Type of Impact	Reference
Microfibers and Nanoplastics	*Symphysodon aequifasciatus*	Growth Performance; Swimming and Predatory Behavior; Neurobehavioral Toxicity; Neurotransmitter Levels; Gut Microbiota Dysbiosis; Molecular Pathways	(Huang et al., 2021)

Microplastics (PVC and PET)	*Dicentrarchus labrax*	Oxidative stress and cellular damage	(Espinosa et al., 2019)
Microplastics (PS)	*Danio rerio*	Oxidative stress and a decrease in heart rate	(Pitt et al., 2017)
Microplastics	*Clarias gariepinus*	Tissue damage in the gills	(Karami et al., 2016)
Triclosan and Microplastics	*Perna canaliculus*	Increase oxidative stress markers like SOD activity and lipid peroxidation	(Webb et al., 2020)
Microplastics	*Tegillarca granosa*	Increase in immunotoxicity of sertraline	(Shi et al., 2020)
Microplastics (PS) and inorganic substances	*Thunnus orientalis*	High juvenile mortality	(Okada et al., 2014)
Amino-modified polystyrene nanoparticles	*Artemia franciscana*	Impaired growth, neurotoxicity, oxidative stress, and genetic alterations	(Mosconi et al., 2023)

3. Heavy Metals

Heavy metals are well known for their environmental toxicity. To be classified as a heavy metal, an element generally needs to have a relatively high density (> 5 g/cm³) and be toxic to living organisms and ecosystems, even at low concentrations, (Helmenstine, 2024). In aquaculture, which involves the controlled production of aquatic organisms such as fish, crustaceans, molluscs and aquatic plants, in freshwater, brackish or marine environments, under controlled conditions (Emenike et al., 2021), heavy metal contamination is a major concern due to its negative impacts on both cultured animals and human consumers. Currently, aquaculture accounts for nearly 50% of all fish intended for direct human consumption and 43% of the total seafood supply, (Emenike et al., 2021). This underscores the importance of understanding and monitoring contamination sources to prevent incidents like the one that occurred in Minamata, Japan. Metals such as mercury, lead, cadmium and copper can persist in the environment and food chain for long periods of time.

Copper, in particular, is an essential nutrient, but can become toxic at elevated concentrations. Its bioavailability depends on multiple factors such as the water hardness and pH, (Erickson et al., 1996). Copper enters aquatic systems from both natural sources and anthropogenic sources. Natural sources include the erosion of copper-rich rocks and soils, as well as volcanic activity, (Aquatic Life Criteria - Copper | US EPA, 2024). Human activities, such as the use of copper-based pesticides and fungicides, (Comber et al., 2022) and urban runoff, also contribute to copper contamination in rivers and groundwater. Industrial activities,

particularly metal manufacturing and mining, release copper through wastewater discharges, with mining activities being a major contributor, (Aquatic Life Criteria - Copper | US EPA, 2024). All these sources can negatively impact aquatic ecosystems and aquaculture operations.

Copper is known to damage various tissues, in both aquatic organisms and humans. In fish, it can affect gill structure, impairing, respiratory efficiency, and induce hypoxia. For example, Rainbow Trout (*Oncorhynchus mykiss)*, are highly sensitive to copper, with acute toxicity thresholds as low as 5–30 µg/L, leading to reduced oxygen uptake even at low concentrations, (Dethloff & Bailey, 1998). Tilapia, *Oreochromis niloticus*, can tolerate higher copper levels compared to the trout, but can still experience gills damage and liver swelling, with acute toxicity observed between 5 and 40 mg/L, (Alkobaby & Wahed, 2017). Additional studies on copper toxicity in fish are summarized in Table II.

Table II: Some examples of studies done with heavy metals.

Contaminant	**Species**	**Type of Impact**	**Reference**
Heavy metals (Ni,Cu,Zn,Pb,Cr,Cd, As)	*Scylla olivacea*	Oxidative stress, metabolic disruptions, changes in protein expression and potential DNA damage	(Razali et al., 2024)
Acetamiprid and Cd	*Danio rerio*	Synergistic acute toxicity, inhibited growth, developmental malformations, disrupted glucose, lipid, and amino acid metabolism, oxidative stress, and altered gene expressions related to metabolism	(Hu et al., 2023)
Heavy metal (Hg)	*Gibbula umbilicalis*	Inhibition of cholinesterase activity, impaired behaviour and potential effects on energy allocation	(Cabecinhas et al., 2014)
Heavy metal (Cd,Cr,Cu,Zn)	*Odontesthes bonariensis*	Reduced sperm quality decreased fertilization and hatching rates, and lower embryo and larval survival, with severe effects in higher concentrations.	(Gárriz & Miranda, 2020)

4. Mixture of Microplastics and Heavy Metals

It is essential to understand the combined negative impacts of MPs and heavy metals such as copper. Expanding knowledge on their interactions with *in vivo,* both in larvae and adult zebrafish, is important given the rising levels of these contaminants in aquatic environments. Some studies, such as that of Santos et al. (2021), have reported a potential connection between MPs and copper, reinforcing the need for further research on the subject. In their study, a vector-like effect was observed, whereby MPs facilitated metal toxicity, leading to reduced survival rates and impaired growth in larvae. Additional studies addressing these combined effects are summarized in Table III.

Table III: Some examples of studies done with mixtures of microplastics and heavy metals.

Contaminant	Species	Type of Impact	Reference
Heavy metals (Hg,Cd,Pb,Cu,Zn) and PCBs	*Capitella teleta*	Neurotoxicity, oxidative stress and impaired growth and feeding	(Gomes et al., 2014)
POPs (PCBs, and PCDEs) and heavy metals (Hg,Cd)	*Prionace glauca*	DNA damage, lipid peroxidation, oxidative stress potential disruption to ecological functions	(Alves et al., 2016)
Microplastics and heavy metals (Hg)	*Dicentrarchus labrax*	Neurotoxicity, oxidative stress, altered energy metabolism and increased mercury bioaccumulation in tissues	(Barboza et al., 2017)
Microplastics and heavy metal (Cu)	*Danio rerio*	Increased mortality, growth inhibition, neurotoxicity, oxidative stress, lipid peroxidation, and disruption of metabolic and antioxidant systems	(Santos et al., 2021)
Nanoplastics and heavy metals (Cu)	*Danio rerio*	Developmental toxicity, intestinal inflammation, oxidative stress, mitochondrial dysfunction and gut microbiota dysbiosis	(Rong et al., 2024)

5. Zebrafish as a scientific model

Danio rerio, widely known as the zebrafish, is an indigenous freshwater fish of South Asia, and has been broadly distributed across parts of India, Bangladesh, Nepal, Myanmar and Pakistan, although can also be found in Sri Lanka and rivers draining into the Arabian Sea, (Spence et al., 2007). It belongs to the family Cyprinidae and gained importance as a model organism in scientific research due

to its unique characteristics, as shown by Ribas & Piferrer, (2013). According to the same author, a suitable fish model for aquaculture research is one that fulfils the follow four conditions: Basic biological features possessed by the most important cultured species; Exhibits similar physiological responses to cultured fish; Short life cycle and easy and inexpensive to breed; Many resources that facilitate research in most areas.

Additionally, the transparency of zebrafish embryos allows observing developmental processes in real-time, (Veldman & Lin, 2008). The life cycle of *Danio rerio* (Figure 3), is divided into four main stages: fertilization and embryonic development, larval, juvenile and adult.

The embryonic development was first described by Kimmel et al. (1995), who defined seven distinct periods within the first 72 hours post-fertilization (hpf). After hatching, the larval stage extends approximately 30 days post-fertilization (dpf). During this phase, zebrafish exhibit a rapid growth rate, organ maturation and the development of the sensory systems, (Çalışkan & Alturfan, 2021; Kimmel et al., 1995).

The juvenile stage follows, beginning around 30 dpf and continuing until roughly 90 dpf. At this stage, the fins, scales and pigmentation patterns are fully developed, sexual differentiation occurs, and gonadal maturation begins. The adult stage starts after ~90 dpf, when the fish are fully developed and reproductively mature, (Parichy et al., 2009). Under laboratory conditions, zebrafish typically live 3-4 years, whereas in the wild their lifespan is about one year, (Gerhard et al., 2002).

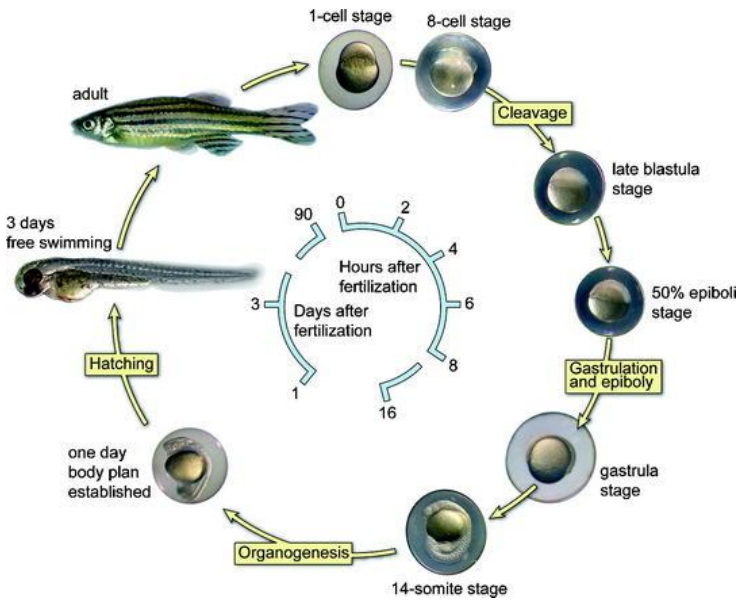

Figure 3: Life cycle of Danio rerio, (Themes, 2016).

Because of these well-defined developmental stages, together with their short generation time, *D. rerio* has become a widely used model organism in diverse areas of research, for example, biomedicine, drug development, aquaculture and behavioural studies. Its popularity is largely due to its high genetic similarity to humans (approximately 70%) (Howe et al., 2013); the transparency of the embryos and its rapid development, (Tavares & Lopes, 2013).

This versatility has led to the development of internationally recognized assays, such as the Fish Embryo Acute Toxicity (FET) test, standardised by the OECD (2013). Furthermore, zebrafish have been successfully used in disease modelling, as shown by Frantz and Ceol (2020), who employed the species to study novel treatments for melanoma, the most aggressive form of skin cancer.

This study aims to better understand the effects of MPs and copper on sex ration and oxidative stress induction in key organs of zebrafish (*Danio rerio*), namely the gonads, brain and intestine. Through histological analysis of the gonadal sexual differentiation, and a statistical evaluation of oxidative stress biomarkers in adult specimens, this work seeks to clarify whether exposure to these contaminants can alter the sex ratio, affect reproductive processes and and disrupt metabolic defence mechanisms. Understanding these effects is particularly relevant for aquaculture, where co-exposure to MPs and copper may compromise fish health, reproduction, and product quality, ultimately affecting both sustainability and food safety.

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Material and Methods

1. Ethics Statement

All procedures were conducted in agreement with the European Directive on the protection of animals used for scientific purposes (2010/63/EU) and its transposition to the Portuguese law (Decreto-lei 113/2013), ensuring minimal animal stress and discomfort.

2. Management of breeding animals

Zebrafish (*Danio rerio*) adults were maintained according to procedures at the Ecotoxicology Lab – University of Trás-dos-Montes and Alto Douro, Vila Real. Briefly, the animals were kept at a temperature between 26 ± 2°C, under a 14:10 h light–dark cycle in a RAS system supplied with dechlorinated, aerated, charcoal-filtered, and UV-sterilized tap water. Water parameters were routinely monitored and adjusted to remain within the optimal range for the species (pH 7.7 ± 0.2; hardness 3–4 dGH; dissolved oxygen 6 ± 1 mg/L; conductivity 376 µS/cm; ammonium 0.08 ± 0.06 mg/L; nitrite 0.01 ± 0.01 mg/L). The animals were fed once a day with a commercial diet with a size 400-600µm (SPAROS, Portugal).

For experimental purposes, fertilized eggs were obtained by inducing natural spawning of adult zebrafish at the beginning of the light cycle, using groups of two males and one female. Collected eggs were gently rinsed with embryo water, disinfected with bleach, and washed again, following established procedures:

1. Preparation of the buffer solution by mixing 4x4.16µL of Embryo medium 3 (E3), 50x concentrated solution into 1L of deionized water.
2. Preparation of the disinfection solution composed of 5% of Choramine T in 100 mL of E3 buffer.
3. The eggs were immersed in the disinfection solution for 90 seconds, followed by another two rounds of 90 seconds in the buffer solution.

After this procedure, the eggs were transferred to 50ml goblets.

3. Exposure solutions (Copper and Microplastics)

A stock solution of 1 g Cu/L was prepared by dissolving copper (II) sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$, Merck, Darmstadt, Germany) in distilled water and stored at 4 °C for subsequent dilution and preparation of the exposure solutions (15 µg/L, Cu 1; and 30 µg/L, Cu 2). Virgin polyethylene and polystyrene beads, white in color, spherical in shape, and ranging in size between 2 and 4 mm, were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). To mimic the size of microplastics commonly found in aquatic environments and to fit the zebrafish

mouth opening, the beads were ground and sieved until obtaining particles of ≤ 100 µm. The concentration used in this experiment for both types of microplastics was 1 mg/L, corresponding approximately to 2.79×10^5 particles/L for PE-MPs and 3.09×10^5 particles/L for PS-MPs (calculated based on the density of each polymer, 0.95 g/mL and 1.05 g/mL, respectively, and the formula $3\pi r^3\delta/4$) (Santos et al., 2020).

4. Exposure trial draw

For this experiment, six treatments were established, each with three replicates (n = 100 embryos per replicate, totalling 1800 embryos): **Control**, **Copper 1** (Cu 1), **Copper 2** (Cu 2), **Polyethylene** (PE), **Mixture 1** (Mix 1) and **Mixture 2** (Mix 2). The Control was maintained in embryo water.

The PE treatment consisted of a suspension of ground PE particles (size <100) prepared at a concentration of 1 mg/L in deionized water and manually homogenized. The **copper treatments** were prepared at two concentrations: 15 µg/L (**Cu 1**) and 30 µg/L (**Cu 2**). The mixture treatments combined the PE suspension with the respective copper concentrations: **Mix 1** (1 mg/L PE + 15 µg/L Cu^{2+}) and **Mix 2** (1 mg/L PE + 30 µg/L Cu^{2+}).

Embryos were initially maintained in 50 mL glass beakers (three replicates per treatment). At 6 dpf, surviving larvae were transferred to 700 mL glass containers. All groups were kept in a thermostatically controlled water bath at 26 ± 2 °C and the solutions were changed every day. The experimental design is illustrated in Figure 4.

Mortality was recorded daily, and the hatching rate was determined at 48, and 96 hpf. Heart rate was assessed at 24 hpf. The assay was extended for 40 days, and at the end, a sampling was made for histologic analysis. The larvae were conserved in formaldehyde 3.4-4.0% w/v buffered to pH 3.4 (PanReac AppliCheam, Barcelona, Spain) for a period of 24h, followed by immersion 70% alcohol (AGA, Portugal) for an undefined period.

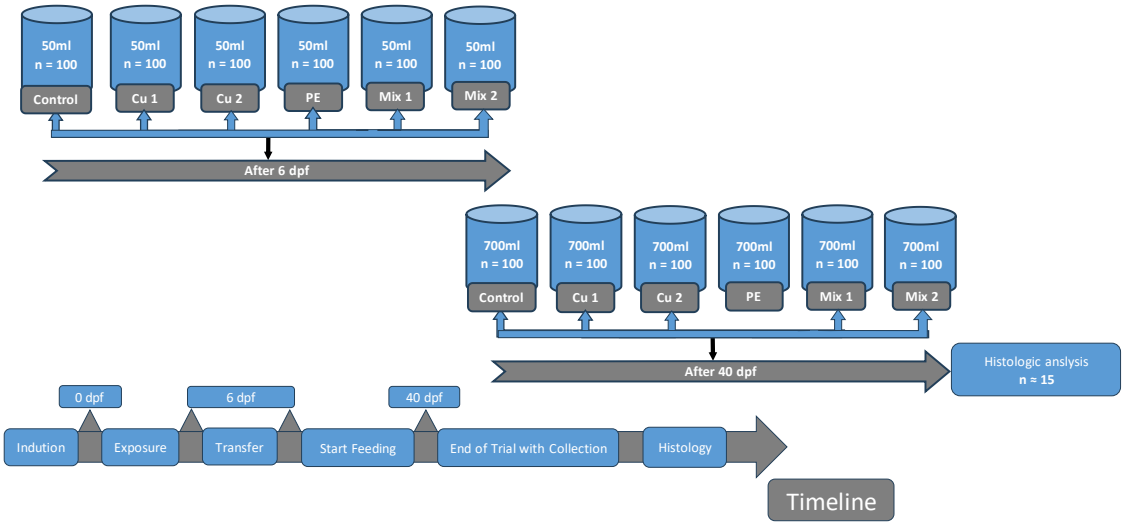

Figure 4: Graphical drawing of the exposure trial. Treatments groups: Control, Copper 1, 15µg/L, (Cu 1); Copper 2, 30µg/L, (Cu 2); Polystyrene, 1mg/L, (PE); Mixture 1, Cu 1 + PE, (Mix 1); Mixture 2, Cu 2 + PE, (Mix 2). Embryos per replica, n = 100. Sampling for histology, n ≈ 15.

5. Analysis

5.1. Heart rate

At 24 hpf, approximately 10 embryos per replicate were recorded for ≈15 s using a stereomicroscope (Olympus SZX7, Japan) equipped with a digital camera (Olympus EP50, Japan) after the procedure the embryos were return to the respective exposure groups. Heartbeats were manually counted from the video recordings and expressed as beats per minute (bpm). For each replicate, mean values were calculated. All measurements were performed in E3 medium at 26–28 °C without anaesthesia, to avoid interference with cardiac function. The hatching rate was also registered between 48 and 96 hpf.

5.2. Histologic Analysis

The histological samples were then processed on a Rotary Tissue Processor (SAKURA Tissue-Tek 4640, Japan), to perform the dehydration, clearing and embedding. The dehydration step aims to completely remove water from the samples after fixation, preparing them for clearing and wax embedding. The removal of water happens because paraffin wax is hydrophobic. Usually, the samples pass through a graded series of alcohols (mostly used is Ethanol), 70%, 80%, 95% and 100%. The concentration was gradually increased to avoid cellular shrinkage or damage from osmotic shock. The clearing aims to remove all the alcohol from the tissue and make it miscible with paraffin, which is not soluble in alcohol. All the samples were then immersed in a clearing agent, Xylol (Carlo Erba, France). Overclearing for too long can harden tissues and make sectioning

difficult. The paraffin embedding aims to saturate and infiltrate the molten Paraffin Wax (Scharav, Spain), which hardens upon cooling and supports the sample structure for sectioning. Samples were immersed in two consecutive baths of liquid paraffin wax at around 58/60ºC, each lasting from 60 minutes to several hours (depending on tissue size). The samples were then included in paraffin wax to form a block (Inclusion equipment; Leica EG1160, Germany). The next step involved the sectioning of the blocks with a thickness of 3 µm, on a rotative microtome (Leica RM 2135, Germany). The sections are then distended in a water bath (Leica H1210, Germany), at around 53°C. After that, they are placed on a microscope slide to verify the state of the cut (Nikon Alphaphot-2 YS2, Japan).

The staining process comprised 6 steps, two 15 minutes baths of xylol followed by 3 baths of 10 minute descending concentration of alcohol baths (100%, 95% and 70%) which was followed by an eight minute bath of Haematoxylin (Fisher Scientific, Belgium), passing by tap water to remove the excess, a 1 minute bath of Eosin (Atom Scientifics, UK), removing the excess by washing with water, a quick 10 second bath on ascending alcohol concentration baths (70%,95% and 100%) and, in the end, two 10 minutes baths in xylol. Then, a coverslip was placed over the stained section on the slide for preservation and posterior examination. Entellan (Merck RGaA, Germany) was used as a mounting medium.

7. Data analysis of adult zebrafish (*Danio rerio*) assays

To build a comprehensive understanding of the effects of microplastics and copper, the study was conducted at two complementary biological levels. First, early-life stage assays were performed, where endpoints such as hatching success, larval survival, and sex ratio were evaluated. These parameters provide essential information on the direct impact of the contaminants on population sustainability and reproductive balance. However, to elucidate the underlying mechanisms that might explain such developmental and reproductive alterations, oxidative stress biomarkers were subsequently assessed in key organs (gonads, intestine, and brain) of adult zebrafish. Although this component of the trial was not performed by myself, we consider that this data would contribute to a broader understanding, as an integrative approach, allowing linking observable phenotypic outcomes in larvae with subcellular and biochemical responses in adults, thus offering a broader perspective on how combined exposure to microplastics and copper can compromise both individual health and population-level dynamics.

7.1. Adult *Danio rerio* assay Procedures

Adult zebrafish (*Danio rerio*), comprising both sexes, were randomly distributed into six experimental groups, each in triplicate, with five fish per tank (total n =

90). The exposure period lasted 21 days, following OECD guidelines. Experimental groups were composed of polystyrene (PS, 1 mg/L), polyethylene (PE, 1 mg/L), copper (25 µg/L), a mixture of copper and polystyrene (Mix 1), and a mixture of copper and polyethylene (Mix 2). A control group was maintained in clean water without the addition of contaminants. Microplastics used were ground and sieved to approximately 100 µm to allow ingestion. The concentrations applied were selected based on their environmental relevance and according to previous studies addressing the toxicity of microplastics and copper in zebrafish, as like Luzio et al. (2021) and Santos et al. (2022).

Before the necropsy, fish were fasted for 24 h. Euthanasia was performed by an overdose of tricaine methanesulfonate (MS-222), after which the fish were rinsed with distilled water to remove any external plastic particles. Subsequently, gonads, brain and intestine, were aseptically dissected under a stereomicroscope (Leica ZOOM 2000 Stereo Microscope, Ireland) for further biochemical analysis.

7.2. Biochemical Analysis

7.2.1. Reactive Oxygen Species (ROS):

ROS levels were determined following Deng et al. (2009), through the oxidation of 2',7'-dichlorofluorescein diacetate (DCFH-DA) to form fluorescent dichloroflurescein (DFC) and reading the excitation (485nm) and emission (530nm) spectrum. The results were expressed in µml DCF/mg of protein.

7.2.2. Superoxide Dismutase (SOD):

SOD activity was measured according to Durak et al. (1993), based on the inhibition of nitroblue tetrazolium (NBT) reduction by superoxide radicals that were generated in the reaction mixture. The reduction of NBT was monitored spectrophotometrically (560nm), and enzyme activity was expressed in U/mg of protein.

7.2.3. Catalase (CAT):

CAT activity was determined following the method of Claiborne (1985) by monitoring the decomposition of hydrogen peroxide (H_2O_2) at 240 nm. The decrease in absorbance was proportional to the enzymatic activity and expressed as U/mg protein.

7.2.4. Glutathione Peroxidase (GPx):

GPx activity was quantified according to Massarsky et al. (2016) by a coupled assay in which the oxidation of NADPH during the reduction of peroxides in the presence of GR. The decrease in absorbance was measured and the activity expressed as µmol NADPH/min.mg protein.

7.2.5. Glutathione Reductase (GR):

GR activity was measured using the same method as before by recording the NADPH oxidation during the reduction of GSSG to GSH. The rate of NADPH oxidation was monitored at 340 nm, and activity was expressed as µmol NADPH/min.mg of protein.

7.2.6. Glutathione S-Transferase (GST):

GST activity was determined following Habig and Jakoby (1981) by measuring the conjugation of GSH with the substrate 1-chloro-2,4-dinitrobenzene (CDNB). The formation of the conjugated product was quantified spectrophotometrically at 340 nm and expressed as CDNB/min.mg protein.

7.2.7. Lactate Dehydrogenase (LDH):

LDH activity was measured according Domingues et al. (2010) by monitoring the conversion of pyruvate to lactate, coupled with the oxidation of NADH to NAD^+. The decrease in absorbance at 340 nm reflected enzyme activity and was expressed as µmol NADH/min.mg protein.

7.2.8. Reduced Glutathione (GSH) and Oxidized Glutathione (GSSG):

Levels of GSH and GSSG were determined using the method described in Gartaganis et al. (2006). The GSH was quantified using derivatization with o-phthalaldehyde (OPT) that produced a fluorescent signal measured at excitation (320 nm) and emission (420 nm) spectrum and expressed as µmol GSH/mg protein. The GSSG was blocked with N-ethylmaleimide (NEM), followed by derivatization with OPT in the same detection conditions. The results were expressed as µmol GSSG/mg protein

7.2.9. Oxidative Stress Index (OSI):

OSI was calculated as the ratio of GSSG to total glutathione (GSH + GSSG), providing an integrated indicator of oxidative balance versus oxidative stress.

7.2.10. Lipid Peroxidation (TBARS):

Lipid peroxidation was estimated according to Wallin et al. (1993) by measuring thiobarbituric acid reactive substances (TBARS). Malondialdehyde (MDA), a byproduct of lipid peroxidation, reacts with thiobarbituric acid under high temperature and acidic conditions, forming a pink chromogen detected spectrophotometrically between 530-600 nm, 532 nm and was expressed as µmol MDA/mg protein.

7.2.11. Protein Carbonyls:

Protein carbonyl content can be quantified through measurement of carbonyl groups using the 2,4-dinitrophenylhydrazine (DNPH) assay. Following Mesquita et al. (2014), the samples were derivatized with DNPH to form hydrazones that were measured at 450nm. An alternative to this procedure can also be used the method described by Reznick and Packer (1994), in which the samples were precipitated with trichloroacetic acid after DNPH derivation, followed by a wash of cold acetone and the resulting pellet was solubilized in guanidine hydrochloride. This pellet was detected spectrophotometrically at 370 nm. The results were expressed as nmol DNPH/min.mg protein.

7.3. Statistical analysis

Statistical analysis was performed using the Sigmaplot program for Windows, version 16 (Grafiti LLC, Palo Alto, California, United States of America). The results were expressed as a mean ± standard deviation (SD), and the differences between experimental groups were considered statistically significant at the significance level p-value < 0.05. All data were first assessed for compliance with ANOVA assumptions, including normality (Shapiro-Wilk test) and homogeneity of variances (Brown-Forsythe test). Whenever the assumptions of normality or homogeneity of variances were not met, data were transformed using a square root transformation before analysis. Two-way ANOVA with interaction terms was performed, followed by Student-Newman-Keuls post-hoc test for multiple comparisons. This approach was used to identify statistically significant differences among treatments and to evaluate whether the presence of microplastics influenced copper toxicity.

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Results

1. Exposure Trial

During the assay, the mortality was registered daily. An unusually high mortality was observed across both types of compounds and concentrations used. The results are presented as mean cumulative mortality (%) for each treatment group calculated from multiple independent replicates. This was particularly evident in treatments Cu 1 and Cu 2, as well as Mix 1 and Mix 2, where mortality rates during the first 24 hpf reached nearly 50%. On average, groups Mix 1 and Mix 2 did not survive beyond 8 days, while Cu 1 and Cu 2 remained viable only until 18 dpf. The only treatments that reached the end of the trial were the control and PE groups, in the first three essays, with the control group completing one additional assay compared to the PE group. Figure 5 shows the mean cumulative mortality rate for each treatment group as a function of time.

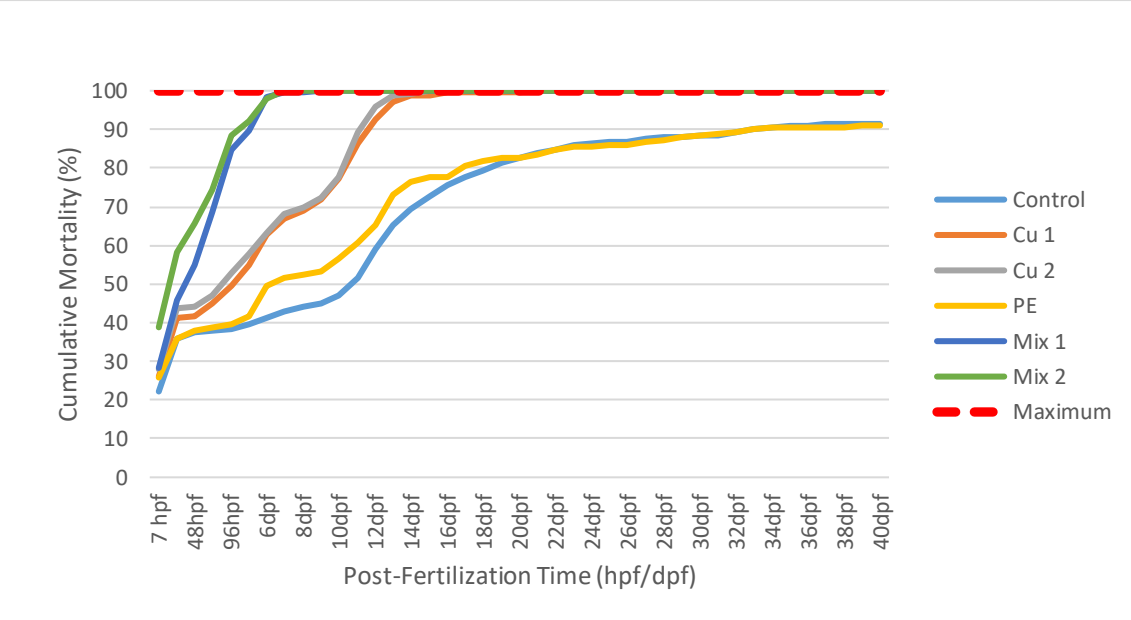


Figure 5: Mean cumulative mortality (%) of zebrafish embryos and larvae across all treatment groups and developmental stages. Data represent the average of all multiple independent assays. Treatments groups: Control, Copper 1 (Cu 1); Copper 2 (Cu 2); Polystyrene (PE); Mixture 1, Cu 1 + PE (Mix 1); Mixture 2, Cu 2 + PE (Mix 2).

For a better interpretation of the mortality results, graphics of each treatment group comparing each replica are shown in the appendix.

2. Embryogenic Study

The embryogenesis assay allowed the simultaneous evaluation of three critical developmental endpoints: cumulative mortality, hatching success, and cardiac function (heart rate). These endpoints provide complementary insights into the toxicological impact of the different treatments, during early development.

2.1. Mortality

The mortality data revealed increased lethality in several treatment groups, like Cu 1, Cu 2, Mix 1 and Mix 2, where cumulative mortality reached higher levels than the control, especially after 72 hpf (Figure 6).

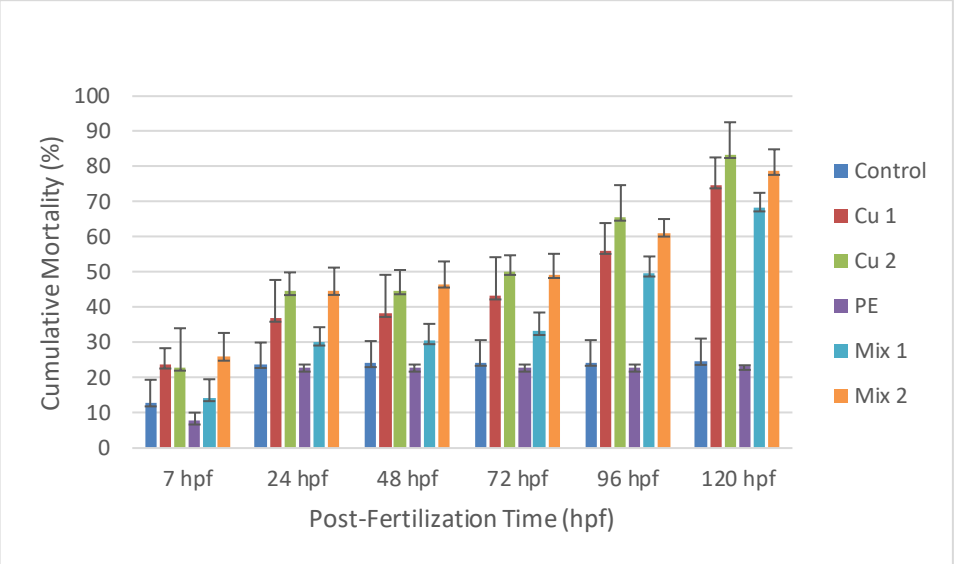


Figure 6 - Cumulative mortality (%) of zebrafish embryos across different treatment groups at 7, 24, 48, 72, 96, and 120 hpf. Mean values were calculated from multiple replicates per group. Treatments groups: Control, Copper 1 (Cu 1); Copper 2 (Cu 2); Polystyrene (PE); Mixture 1, Cu 1 + PE, (Mix 1); Mixture 2, Cu 2 + PE, (Mix 2).

2.2. Hatching

The hatching rate (Figure 7) was significantly affected by treatments, particularly in the Mix and Cu groups, which showed both delayed and reduced hatching rates. By 120 hpf, the control group reached 71% hatching, whereas Mix 1 peaked at 35% and Cu 1 at 31%. In contrast, Cu 2 and Mix 2 remained below 22%.

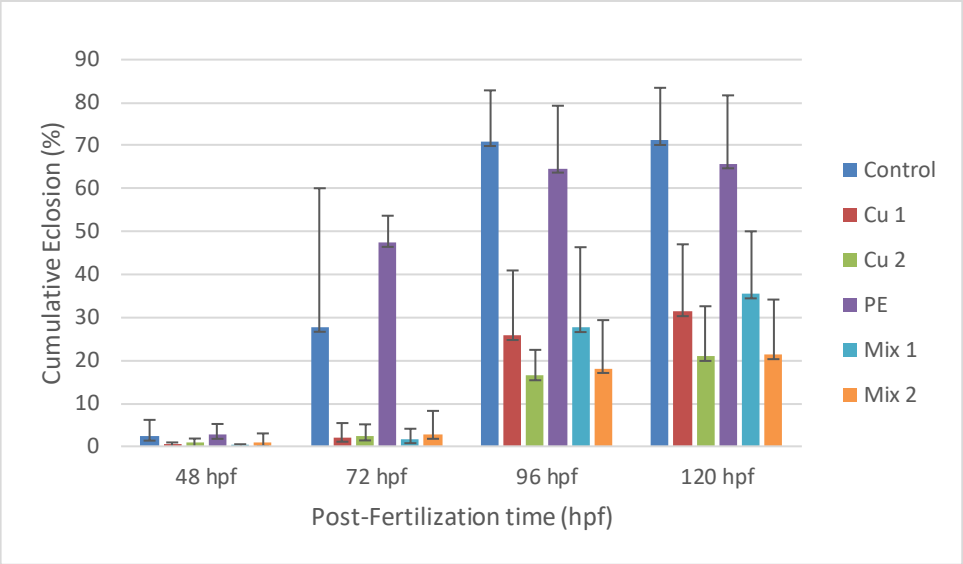


Figure 7 - Mean cumulative hatching success (%) of zebrafish embryos exposed to different treatments at 48, 72, 96, and 120 hpf. Treatments groups: Control, Copper 1 (Cu 1); Copper 2 (Cu 2); Polystyrene (PE); Mixture 1, Cu 1 + PE, (Mix 1); Mixture 2, Cu 2 + PE, (Mix 2).

2.3. Cardiac Function

Heart rate measurements at 24 hpf provided an early physiological indicator of developmental stress. While natural variability in embryonic heart rate is expected, treatments such as Mix 1 and Mix 2 resulted in lower average heart rates compared to control, suggesting potential bradycardia or disruption of early cardiac activity; however, a similar value was observed between all the groups (Figure 8).

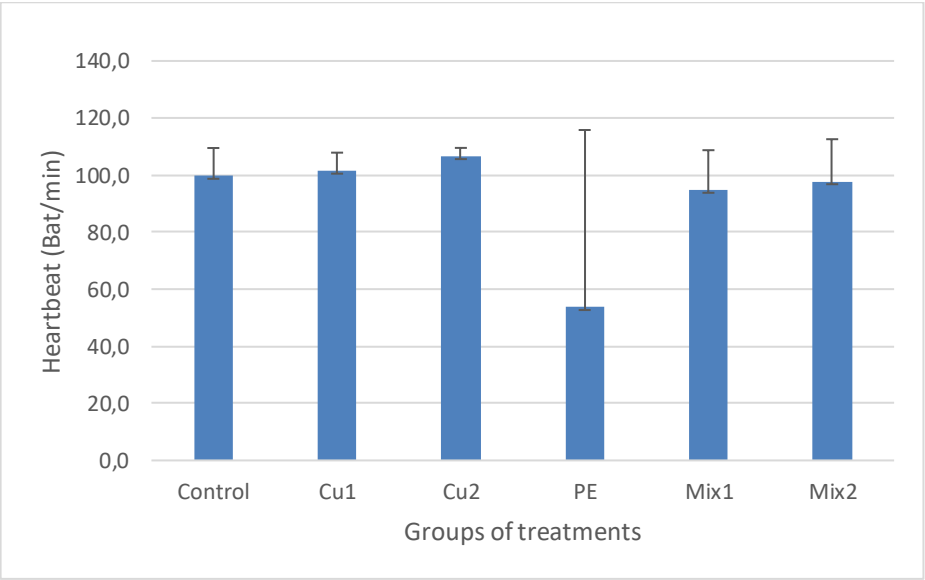


Figure 8 - Mean heart rate (beats per minute) of zebrafish embryos at 24 hpf for Control, Cu 1, Cu 2, PE, Mix 1 and Mix 2 treatment group. Treatments groups: Control, Copper 1 (Cu 1); Copper 2 (Cu 2); Polystyrene (PE); Mixture 1, Cu 1 + PE, (Mix 1); Mixture 2, Cu 2 + PE, (Mix 2).

3. Histologic Analysis

A histologic study was conducted in two treatment groups, at the end of 35dpf, to assess gonad development and sexual differentiation. All the evaluated samples presented gonads. Regarding sexual differentiation, most individuals showed clear differentiation: in the Control group (n = 25), 72% were differentiated and 28% remained undifferentiated, while in the PE group (n = 10), 80% were differentiated and 20% undifferentiated (Figure 9).

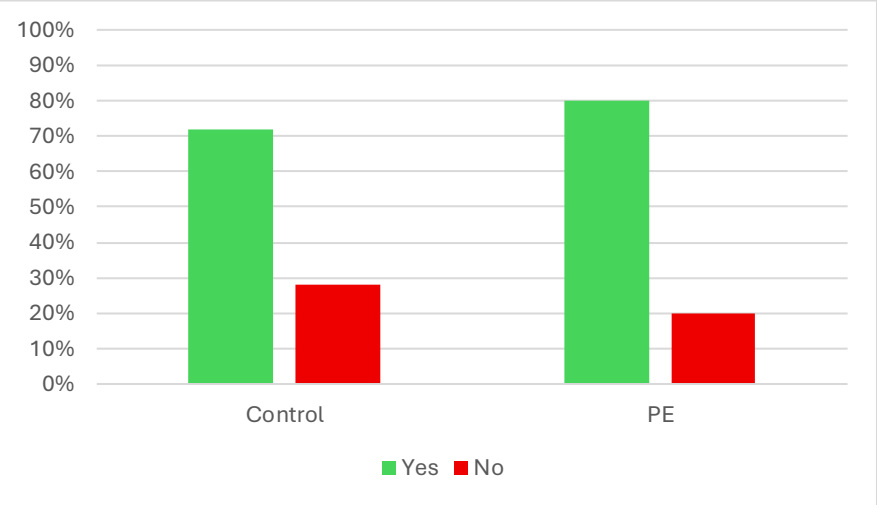


Figure 9 - Sexual Differentiation Rate of zebrafish at 35dpf for each treatment group. Treatments groups: Control (n = 25); Polystyrene (PE) (n = 10).

Analysis of the sex ratio revealed differences between groups. In the control group, 52% of fish were female 20 % were male, with the remaining individuals undifferentiated. In contrast, in the PE group, 80% were female, and no males were observed (Figure 10).

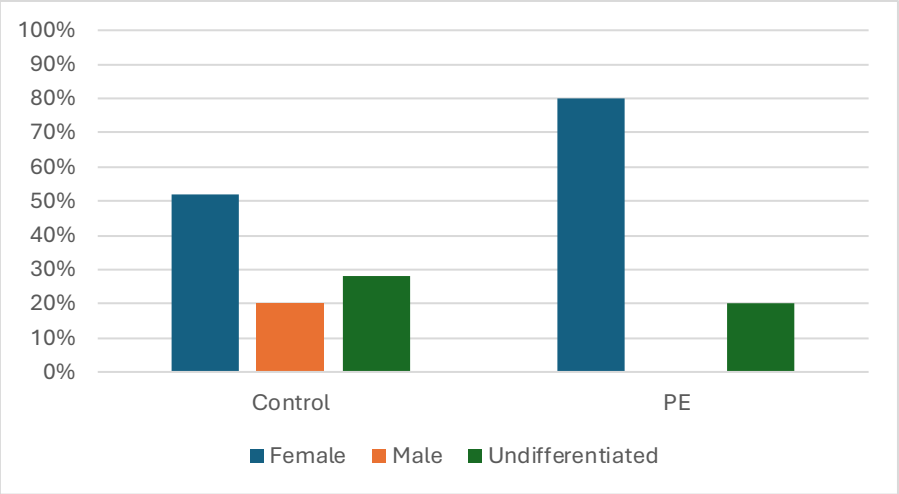
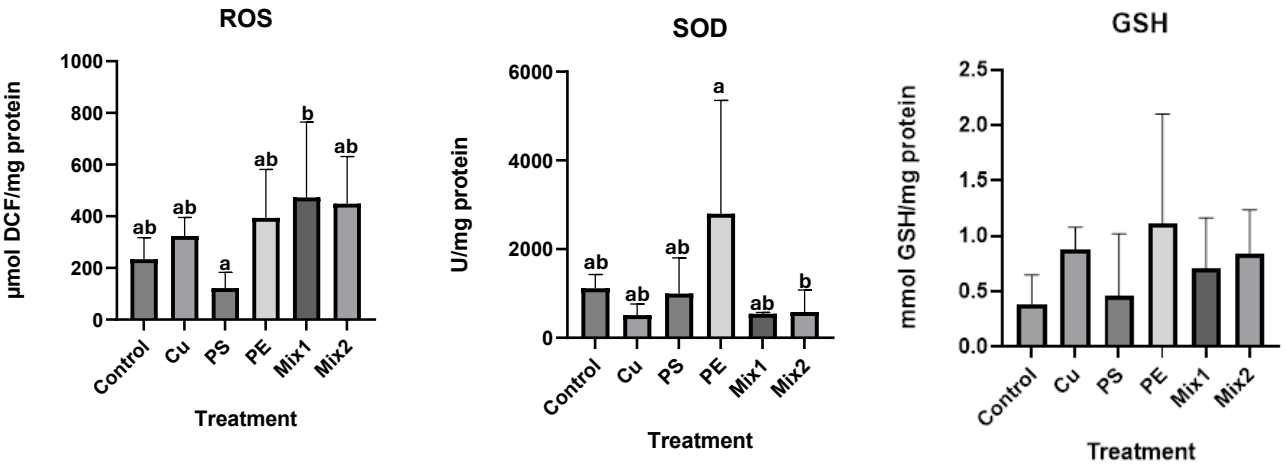


Figure 10 - Sex Ratio of zebrafish at 35dpf for each treatment group. Treatments groups: Control (n = 25); Polystyrene (PE) (n = 10).

4. Biochemical Analysis

To gain further insight into the physiological responses underlying the effects of combined exposure to MPs and copper, oxidative stress biomarkers were evaluated in three target organs of adult zebrafish: the brain, gonads, and intestine. These organs were selected due to their central roles in vital functions—neurological activity, reproduction, and nutrient assimilation, respectively—and their known sensitivity to contaminants. The analysis of enzymatic and non-enzymatic biomarkers in these organs may provide information on how co-exposure influences cellular homeostasis and defence mechanisms. The results are shown in figures 11, 12 and 13.



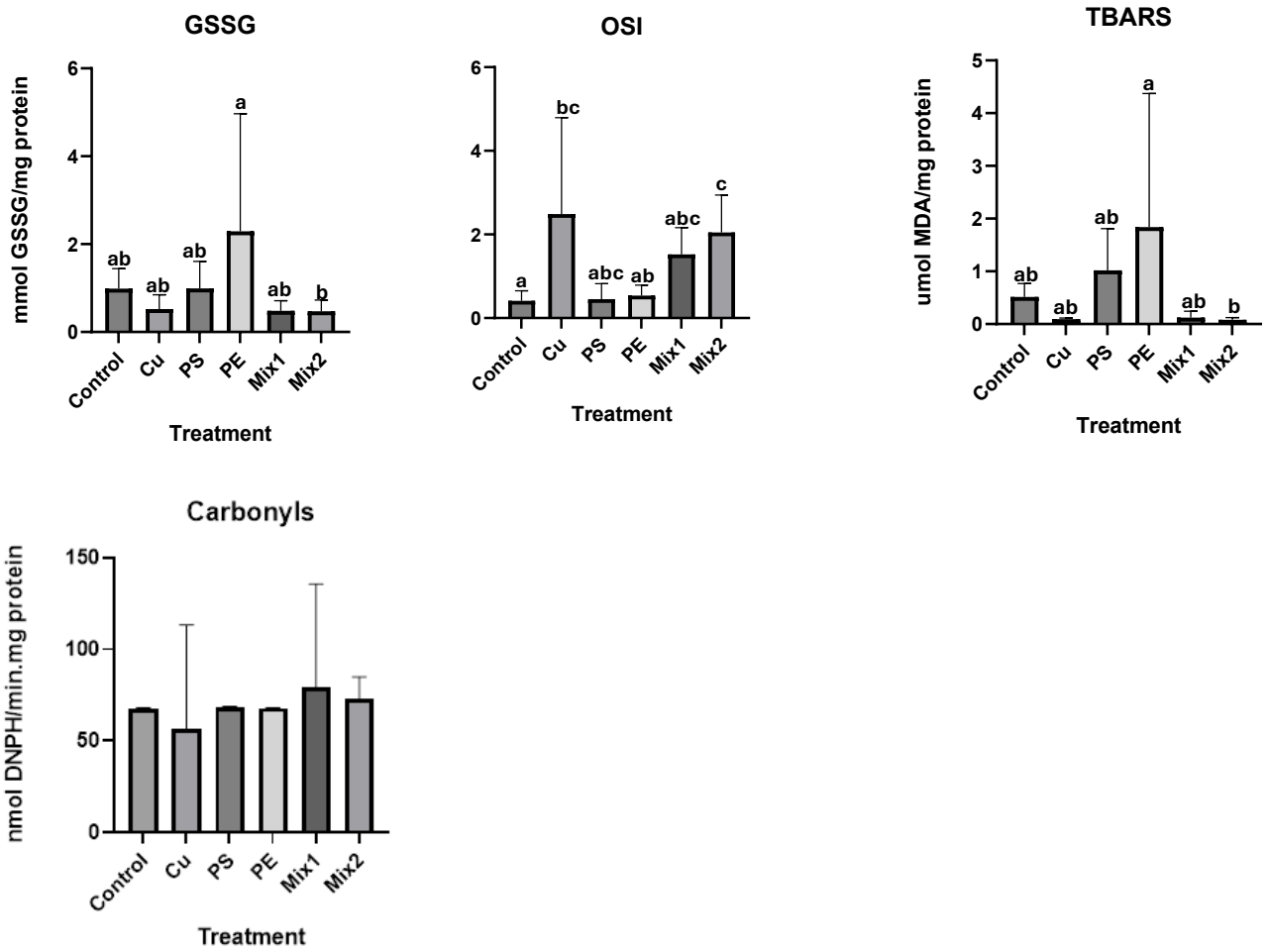


Figure 11 - Biochemical parameters in zebra fish, Danio rerio, brain exposed to different contaminants. Reactive Oxygen Species (ROS); Superoxide Dismutase (SOD); Reduced Glutathione (GSH); Oxidized Glutathione (GSSG); Oxidative Stress Index (OSI); Thiobarbituric Acid Reactive Substances (TBARS); Carbonyls. Data are expressed as mean ± SD (Control = 5, Polystyrene (PS) = 4, Polystyrene (PE) = 5, Copper (Cu) = 6, Mixture 1 (Cu + PS) = 4, Mixture 2 (Cu + PE) = 6). Statistically significant differences were identified by two-way ANOVA followed by Student-Newman-Keuls post hoc test ($p < 0.05$). Different letters indicate significant differences between treatments.

In the brain, exposure to copper revealed significant effects across several parameters. At the level of ROS, a significant increase was observed in response to copper ($p = 0.01$), while exposure to MPs, either alone or in combination, did not induce relevant changes. Nevertheless, the combined exposure to copper and PS led to a significant increase in ROS compared to the group exposed to PS alone ($p = 0.005$).

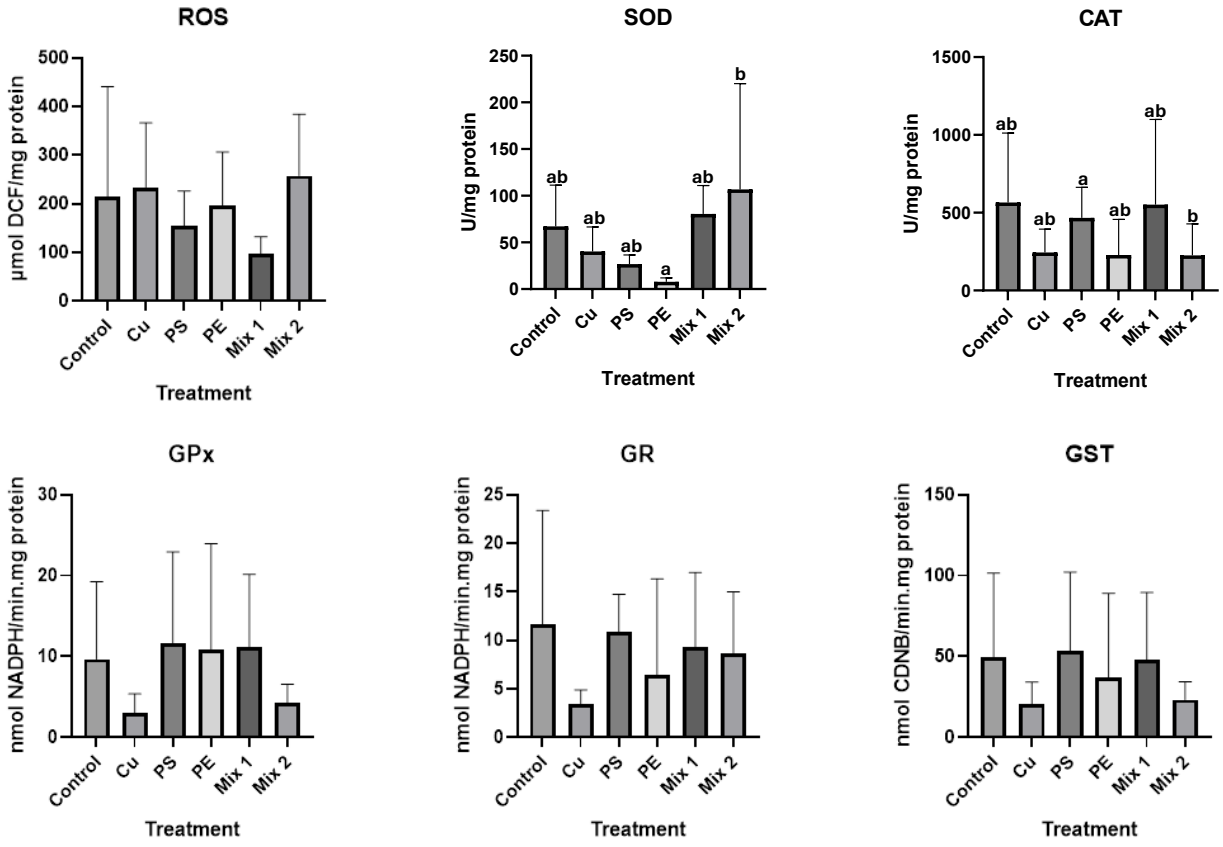
Regarding SOD activity, the presence of copper also exerted a significant effect ($p = 0.006$). However, the group exposed to copper alone did not significantly differed from the control. However, combined exposure to copper and PE resulted in a significant decrease in SOD activity compared with the PE-only group ($p = 0.005$).

No significant differences were found for GSH levels between treatments. In contrast, GSSG showed a significant effect of copper ($p = 0.038$), and the Cu +

PE combination led to a significant decrease in GSSG compared to the PE-only group ($p = 0.015$).

The OSI index exhibited marked alterations: copper induced a significant increase ($p = 0.001$), and the Cu + PE combination also resulted in significantly higher values compared to both the control ($p = 0.023$) and the PE-only group ($p = 0.043$).

For TBARS, copper exerted a significant effect ($p = 0.010$), associated with a decrease in lipid peroxidation. Specifically, the Cu + PE group showed significantly lower TBARS levels than the PE-only group ($p = 0.010$). Conversely, no significant differences were observed in protein carbonyl levels across treatments.



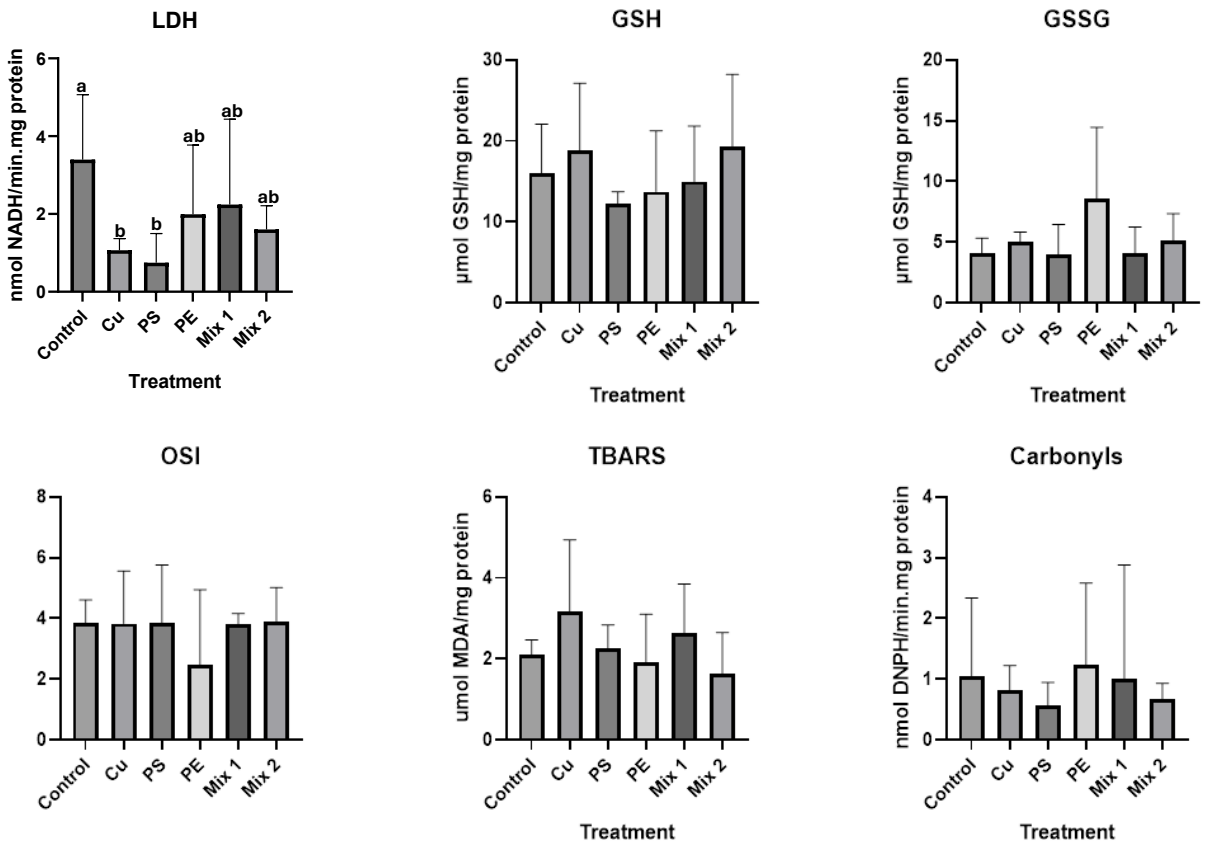


Figure 12 - Biochemical parameters in zebra fish, Danio rerio, gonad exposed to different contaminants. Reactive Oxygen Species (ROS); Superoxide Dismutase (SOD); Catalase Activity (CAT); Glutathione Peroxidase (GPx); Glutathione Reductase (GR); Glutathione S-Transferase (GST); Lactate Dehydrogenase (LDH); Reduced Glutathione (GSH); Oxidized Glutathione (GSSG); Oxidative Stress Index (OSI); Thiobarbituric Acid Reactive Substances (TBARS); Carbonyls. Data are expressed as mean ± SD (Control = 5, Polystyrene (PS) = 4, Polystyrene (PE) = 5, Copper (Cu) = 5, Mixture 1 (Cu + PS) = 5, Mixture 2 (Cu + PE) = 5). Statistically significant differences were identified by two-way ANOVA followed by Student-Newman-Keuls post hoc test ($p < 0.05$). Different letters indicate significant differences between treatments.

In the gonad, ROS levels did not differ significantly between treatments. Neither copper nor PS or PE exposure, whether alone or combined, induced relevant changes in this parameter.

SOD activity showed a significant interaction between copper and microplastics ($p = 0.037$). While copper exposure alone did not cause differences relative to the control, and PS or PE alone had no significant effect, the Cu + PE group presented a significant increase in SOD activity compared to PE alone ($p = 0.008$).

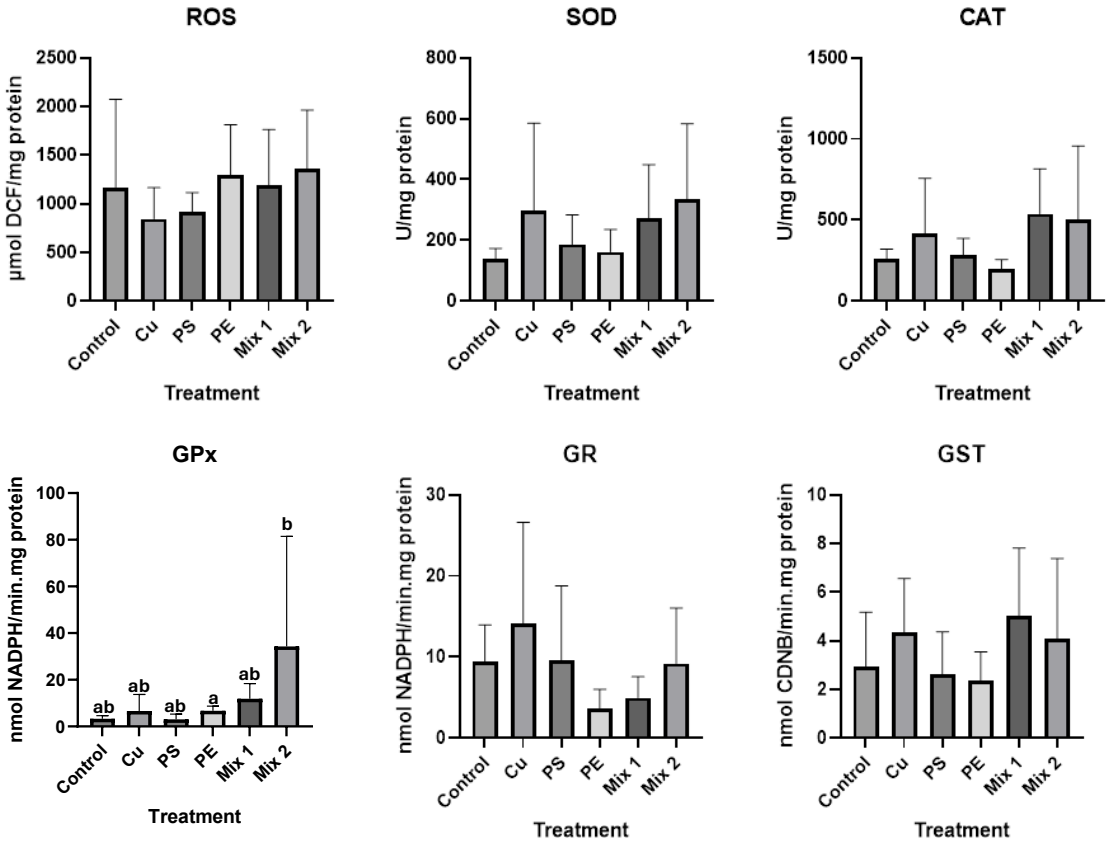
Similarly, CAT activity revealed a significant interaction between copper and microplastics ($p = 0.037$). Neither copper nor microplastics alone induced significant alterations, but the Cu + PS group exhibited a significant increase in CAT activity compared to PS alone ($p = 0.025$).

In contrast, GPx, GR and GST activities were not significantly affected by any of the treatments or their interaction.

LDH activity followed a different pattern, showing a significant decrease in response to copper alone ($p = 0.017$) and PS exposure ($p = 0.028$), while no relevant changes were observed in the remaining groups.

For GSH, GSSG, TBARS, carbonyl levels and the OSI index, no significant differences were detected across treatments, indicating no marked effects of these factors on these parameters.

Overall, the gonad results highlight increases in SOD and CAT under specific combinations of copper and microplastics, as well as reductions in LDH activity induced by copper alone and PS exposure, while other parameters, including ROS, remained unaffected.



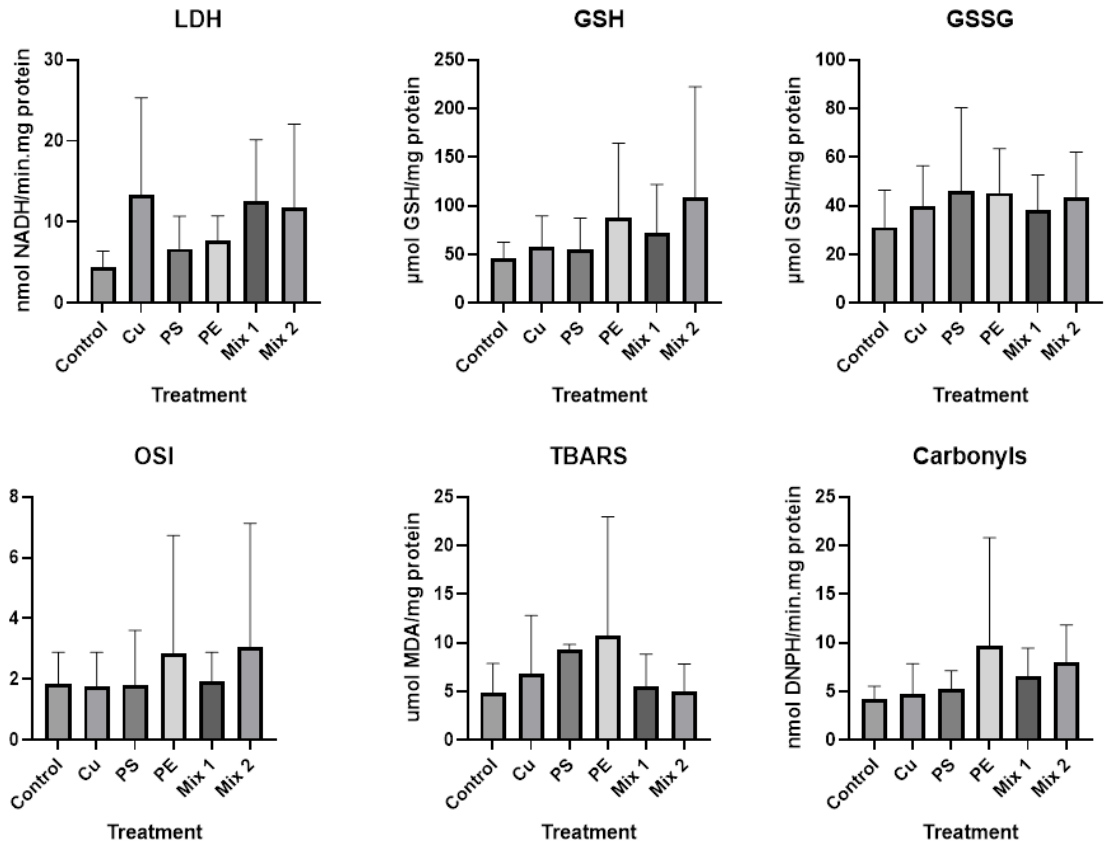


Figure 13- Biochemical parameters in zebra fish, Danio rerio, gonad exposed to different contaminants. Reactive Oxygen Species (ROS); Superoxide Dismutase (SOD); Catalase Activity (CAT); Glutathione Peroxidase (GPx); Glutathione Reductase (GR); Glutathione S-Transferase (GST); Lactate Dehydrogenase (LDH); Reduced Glutathione (GSH); Oxidized Glutathione (GSSG); Oxidative Stress Index (OSI); Thiobarbituric Acid Reactive Substances (TBARS); Carbonyls. Data are expressed as mean ± SD (Control = 5, Polystyrene (PS) = 5, Polystyrene (PE) = 5, Copper (Cu) = 5, Mixture 1 (Cu + PS) = 5, Mixture 2 (Cu + PE) = 5). Statistically significant differences were identified by two-way ANOVA followed by Student-Newman-Keuls post hoc test ($p < 0.05$). Different letters indicate significant differences between treatments.

In the intestine, ROS levels did not differ significantly between treatments. Neither copper ($p = 0.974$), microplastics ($p = 0.410$), nor the interaction between the two ($p = 0.490$) induced relevant changes.

For most other parameters, no significant differences were observed in response to exposure to copper, MPs or their interaction. However, some parameters showed sporadic effects associated with copper exposure.

GPx activity revealed a significant effect of copper ($p = 0.021$), although post-hoc comparisons did not confirm consistent differences between groups. A similar trend was observed for LDH, with a significant copper effect ($p = 0.041$) that was not confirmed by multiple comparisons.

GSH levels also displayed a significant copper effect ($p = 0.032$). GR activity, showed a statistically significant increase in the Cu + PE group compared to PE alone ($p = 0.012$), indicating a specific effect of the interaction between copper and polyethylene.

For the remaining parameters — SOD, CAT, GST, GSSG, TBARS, carbonyls and OSI — no statistically significant differences were detected, with values remaining similar among groups.

Overall, intestinal results suggest that copper alone may induce alterations in certain antioxidant biomarkers, although these were not consistently supported by post-hoc analyses. Only GR exhibited a clear response associated with the Cu + PE combination, reinforcing the idea that interactions between copper and polyethylene may play a relevant role in modulating intestinal oxidative stress.

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Discussion

Zebrafish (*Danio rerio*) has become an important model species in ecotoxicology and aquaculture-related research due to its well-characterised development, genetic tractability and sensitivity to environmental contaminants (Chahardehi et al., 2020). In recent years, growing concern has arisen regarding emerging pollutants such as microplastics and heavy metals, which frequently co-occur in aquatic systems, posing significant risks to early development and reproductive success (Liu et al., 2021).

In light of these concerns, the findings from the exposure trial, followed by the embryogenic, histological and biochemical analyses, allow for an integrated interpretation of the effects of these contaminants.

1. Exposure Trial

An unexpectedly high cumulative mortality was observed during the exposure trial, especially in the groups exposed to copper (Cu 1 and Cu 2) and to the mixtures (Mix 1 and Mix 2). In these treatments, approximately 50% of embryos died within the first 24 hpf, and the survival rate rarely surpassed 18 dpf. These data confirm the toxicity of copper at environmentally relevant concentrations, a phenomenon previously described in several fish species. Such effects have been associated with structural damage to the liver, gills and kidney, leading to hypoxia, necrosis and metabolic alterations (Wang et al., 2018; Tesser et al., 2019). At the larval stage, copper exposure can also induce ROS generation and apoptosis of macrophages and neutrophils, decreasing phagocytosis that compromises the response of the immune system and reduces viability (Chen et al., 2019). Moreover, the combined presence of copper and MPs can produce synergistic effects. Microplastics can act as heavy metal vectors, enhancing their bioavailability and promoting their accumulation in organs such as the liver and brain (Santos et al., 2021; Rong et al., 2024). The greater stability observed in the Control and PE-only groups suggests that, while MPs may cause sublethal alterations, their interaction with copper poses the most severe threat to population viability.

2. Embryogenic Study

In the embryo development study, three parameters were evaluated: mortality, hatching rate, and heart rate. These results revealed that copper and the mixtures had drastically reduced hatching success, with rates below 35%, in contrast to >70% in the control group. This decrease is consistent with the literature, which associates heavy metals with embryo malformations and delayed development (Hu et al., 2023). Moreover, the observation of bradycardia in embryos exposed

to Mix 1 and Mix 2 suggests early physiological dysfunctions, often linked to oxidative stress and alterations in energy metabolism (Pitt et al., 2018).

Under normal conditions, the heart rate of *Danio rerio* embryos is comparable to that of human's embryos, ranging between 120 and 180 bpm, and a progressive increase throughout development. Studies have reported a significant rise in heart rate between 48, 72, and 96 hpf, reflecting the physiological maturation of the cardiovascular system (De Luca et al., 2014).

3. Histologic Study

Histological analysis revealed that both the control and PE groups exhibited differentiated and developed gonads at 35dpf. However, a marked difference in sex ratio was observed: while the control group presented a relatively balanced distribution of males (20%) and females (52%), the PE group showed a prevalence of 80% females and a complete absence of males. Such an unbalanced sex ratio suggests the potential action of endocrine disruption mechanisms.

In zebrafish, sex differentiation depends heavily on hormonal and environmental factors. All individuals initially develop an undifferentiated gonad. The presence of oestrogenic compounds in the environment may inhibit the normal reproductive development and lead to feminised populations (Kidd et al., 2007). This phenomenon is consistent with the results obtained in the present study, suggesting that PE Mps may have acted as oestrogenic modulators during the critical period of sex differentiation, a mechanism that has been widely associated with endocrine disruption processes induced by microplastics and plastic additives capable of mimicking natural hormones and interfering with reproductive development, (Rochman et al., 2014; Chen et al., 2019).

Although some studies have reported sex ratio shifts associated with modulation of oestrogenic signalling, the current literature does not directly confirm an extreme feminisation effect induced exclusively by PE. For example, Peng et al. (2024) observed a marked male bias in esr2b knockout zebrafish, demonstrating that disruptions in oestrogen receptor signalling can drastically alter sex ratios, but not necessarily towards feminisation. Similarly, Wu et al. (2024) found that female zebrafish exposed to PS were more vulnerable in terms of metabolism and reproduction, including reduced oocyte production, although without a clear alteration of sex ratio. These findings indicate that MPs may affect males and females differently, but the direction and magnitude of sex ratio alterations depend on polymer type, exposure conditions, and interactions with other contaminants.

Adverse reproductive outcomes observed in response to other environmental contaminants with oestrogenic activity further support this hypothesis. For instance, Tao et al. (2023) showed that life-cycle exposure to the UV filter 2-

phenylbenzimidazole-5-sulphonic acid (PBSA) impaired ovarian development and reduced egg production, with effects persisting into subsequent generations. Such evidence highlights the relevance of chronic environmental exposures in compromising population sustainability.

Mechanistically, alterations in key steroidogenic genes such as aromatase (cyp19a1a) have been identified as central mediators of endocrine disruption. Inhibition of aromatase reduces the conversion of androgens into oestrogens, thereby altering hormonal balance and modulating gonadal differentiation (Wu et al., 2024; Peng et al., 2024). It is therefore plausible that PE exposure in the present study induced similar molecular changes, resulting in the observed sex ratio.

Taken together, the results suggest that exposure to PE may interfere with critical endocrine processes, promoting feminisation of zebrafish populations. Although current evidence does not conclusively confirm such an extreme effect as being caused solely by PE, it is clear that MPs and other oestrogenic contaminants disrupt hormonal homeostasis, steroidogenic gene expression, and gonadal function, ultimately threatening reproductive success and long-term population viability.

4. Biochemical Analysis

The present study demonstrated that copper exposure induced significant alterations in oxidative stress biomarkers in the brain, including increases in ROS, GSSG, OSI, accompanied by reductions in TBARS, whereas MPs alone exerted no major effects. Importantly, combined exposure to copper and PE amplified these responses, consistent with synergistic interactions. These findings are consistent with previous studies showing that copper induces neurotoxicity through oxidative stress mechanisms, disrupting enzymatic antioxidant defences and redox homeostasis. In carp, copper exposure has been shown to increase ROS levels, lipid and protein oxidation, while altering antioxidant enzymes such as SOD, CAT, GPx, GR and GST (Jiang et al., 2014). Similarly, recent research has demonstrated that copper combined with PVC MPs intensified ROS production, lipid peroxidation and apoptosis in fish brains, through pathways involving BDNF/miR132/FOXO3a signalling (Bakhasha et al., 2025). Together, these findings highlight that copper is a potent neurotoxicant and that MPs may potentiate its deleterious effects.

In the gonads, ROS levels remained unaltered, but significant interactions between copper and MPs were detected for antioxidant enzymes. SOD and CAT activities increased under Cu + PE and Cu + PS treatments, while LDH activity decreased significantly following copper or PS exposure. These results suggest adaptive modulation of antioxidant defences in reproductive organs, although the

protective value of these changes remains uncertain. Comparable responses have been reported in bivalves, where exposure to MPs and cadmium altered gonadal antioxidant enzyme activities (SOD, CAT, GST) (Parra et al., 2024). In zebrafish, co-exposure to copper and MPs has been associated with suppression of GPx activity and dysregulation of hypothalamic-pituitary-gonadal (HPG) axis genes, reinforcing possible reproductive consequences (Santos et al., 2025). Therefore, the present data support the view that copper, particularly in combination with MPs, modulates redox balance in the gonads, with potential implications for reproductive physiology.

In the intestine, ROS levels were not significantly affected by copper, MPs, or their mixture. Nonetheless, copper exposure alone was associated with alterations in GPx, LDH and GSH, although post-hoc analyses did not consistently confirm group differences. Moreover, GR activity was significantly elevated in the Cu + PE group compared to PE alone, suggesting a specific interaction effect. These results indicate that intestinal oxidative responses are more subtle than those in the brain or gonads, yet still susceptible to modulation by copper and MPs. Supporting evidence from goldfish shows that MPs enhance copper accumulation in the intestine, leading to oxidative stress, inflammation and apoptosis, although in some cases, inflammatory responses were attenuated under co-exposure (Zhang et al., 2022). The significant increase in GR activity in the Cu + PE group may therefore reflect an adaptive mechanism counteracting oxidative challenges.

Overall, the results point towards organ-specific oxidative stress responses. The brain appears particularly vulnerable to copper toxicity, with neurotoxic effects enhanced by PE MPs. The gonads display a dual response, with adaptive increases in antioxidant activity but concomitant decreases in LDH, which could impact reproductive performance. The intestine, while less responsive overall, exhibited parameter-specific changes that may reflect both protective and stress related processes. These findings align with the broader literature indicating that MPs enhance copper bioavailability and toxicity in aquatic organisms (Zhang et al., 2022; Parra et al., 2024; Bakhasha et al., 2025; Santos et al., 2025). The observed organ-specific responses underscore the complexity of multi-contaminant exposures, highlighting the importance of integrative mechanistic studies exploring molecular pathways such as Nrf2/ARE, apoptosis regulation and metal accumulation (Jiang et al., 2014).

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Conclusion

This study provides novel insights into the individual and combined effects of copper and MPs, *Danio rerio*, with emphasis on embryonic development, gonadal differentiation, and oxidative stress responses in different organs. The findings demonstrate that copper at environmentally relevant concentrations carries out pronounced toxicity, leading to increased embryonic mortality, developmental impairments, and biochemical alterations. Microplastics alone elicited sublethal but significant changes, particularly in sex differentiation, where PE exposure resulted in a striking feminisation of the population. Importantly, co-exposure to copper and microplastics amplified adverse outcomes, confirming a synergistic interaction that enhances bioavailability and toxicity.

Histological analyses revealed potential endocrine-disrupting effects of MPs, suggesting interference with hormonal regulation and steroidogenic pathways, which may compromise reproductive success and population sustainability. Biochemical assays highlighted organ-specific oxidative stress responses: the brain emerged as highly susceptible to copper-induced neurotoxicity, the gonads exhibited adaptive but possibly insufficient antioxidant modulation, and the intestine displayed subtle, yet measurable responses indicative of compensatory mechanisms.

Taken together, these results underscore the ecological relevance of combined contaminant exposure scenarios, highlighting the urgent need to integrate multi-stressor approaches into ecotoxicological risk assessments, as single-contaminant studies may underestimate real-world hazards in aquaculture systems. Furthermore, the zebrafish model proved to be a powerful tool for mechanistic investigations into the toxicological impacts of emerging pollutants.

Future research should expand on molecular pathways such as Nrf2/ARE signalling, apoptosis regulation, and endocrine gene expression to elucidate long-term and transgenerational consequences. Ultimately, the evidence presented reinforces the necessity of stricter environmental policies addressing microplastic pollution and heavy metal contamination to safeguard aquatic ecosystems and the sustainability of aquaculture.

In this context, it is crucial to clarify how the interaction between heavy metals and MPs disrupts metabolic and developmental processes during the early life stages, since such alterations may contribute to increased mortality and impaired organ development. A deeper understanding of these mechanisms will reinforce the relevance of ecotoxicological assessments and help anticipate potential risks to aquaculture systems and ecosystem health.

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Appendix

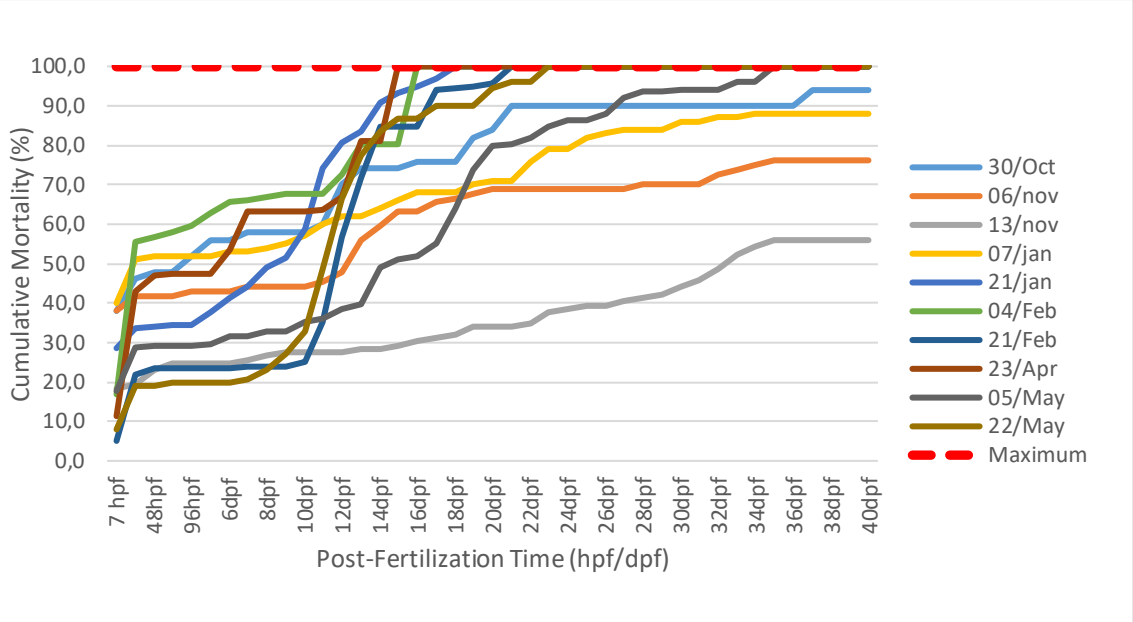

Figure 14 - Mean cumulative mortality (%) of zebrafish embryos and larvae in the Control group across all independent assays.

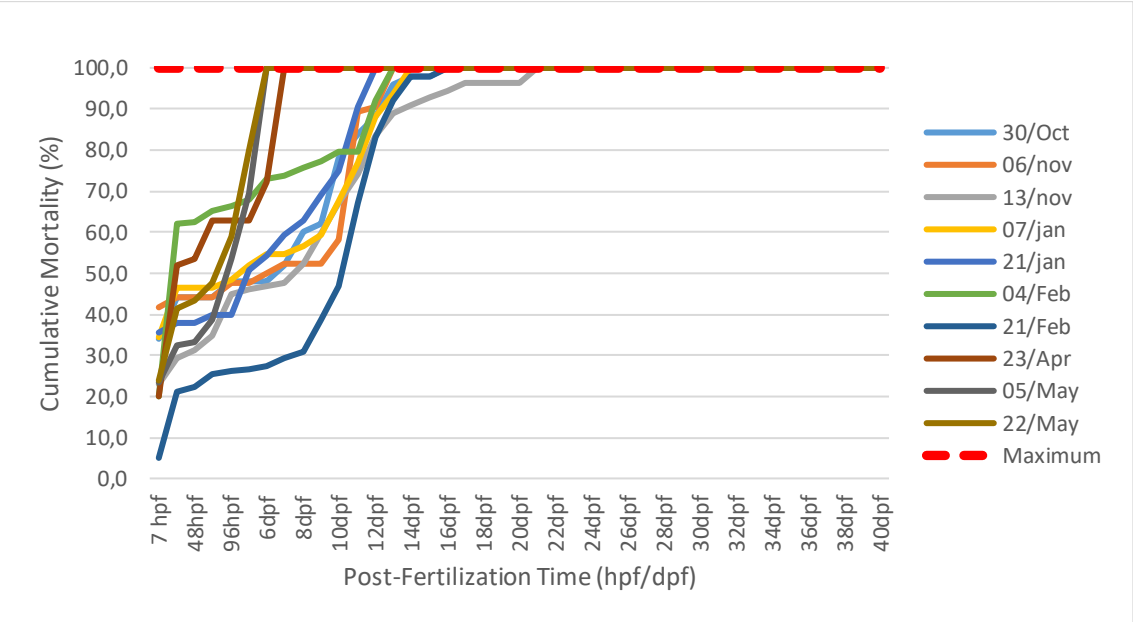

Figure 15 - Mean cumulative mortality (%) of zebrafish embryos and larvae in the Cu 1 group across all independent assays.

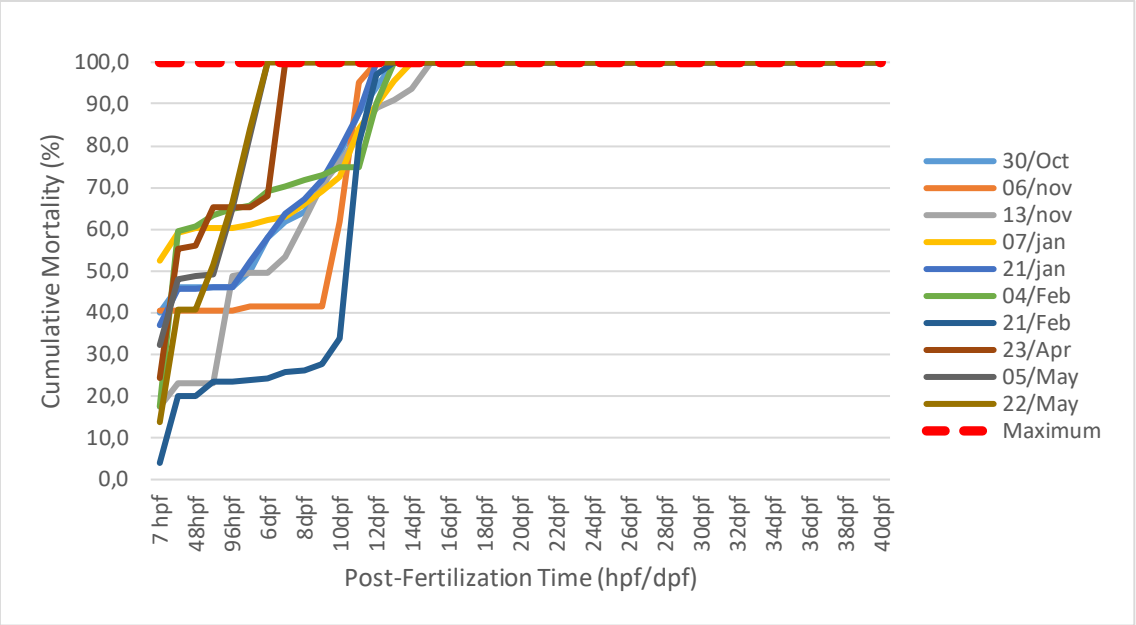

Figure 16 - Mean cumulative mortality (%) of zebrafish embryos and larvae in the Cu 2 group across all independent assays.

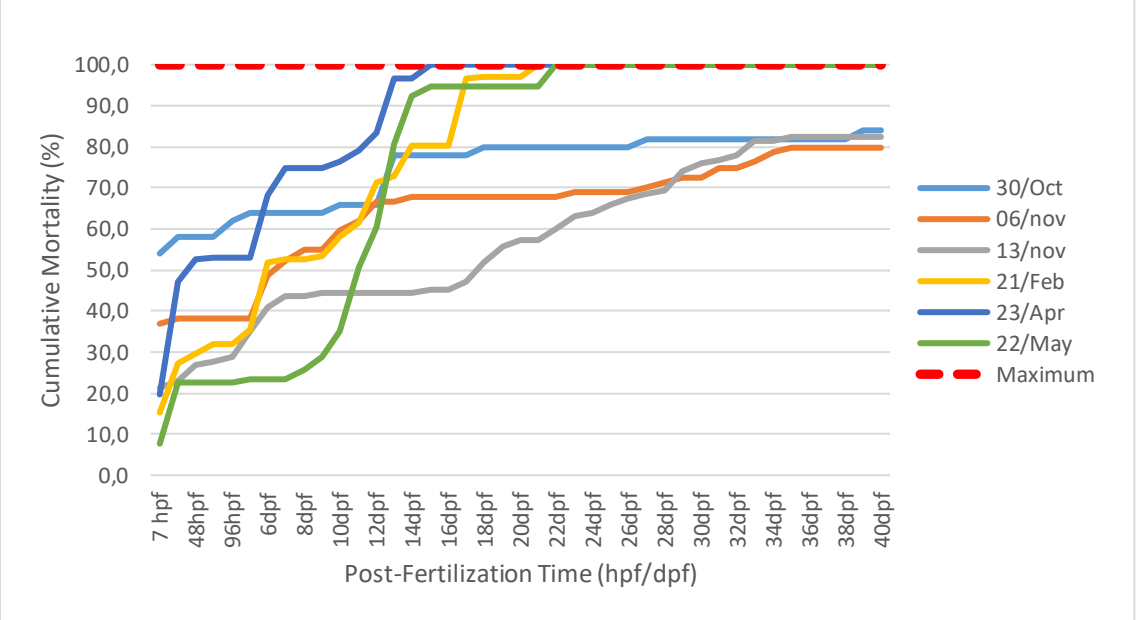

Figure 17 - Mean cumulative mortality (%) of zebrafish embryos and larvae in the PE group across all independent assays.

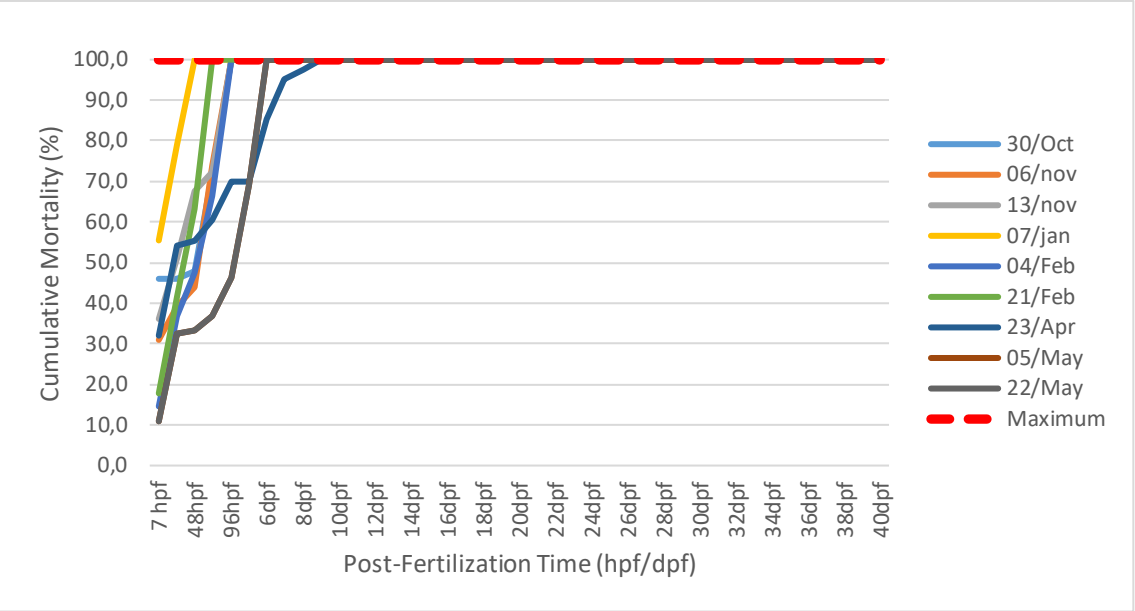

Figure 18 - Mean cumulative mortality (%) of zebrafish embryos and larvae in the Mix 1 group across all independent assays.

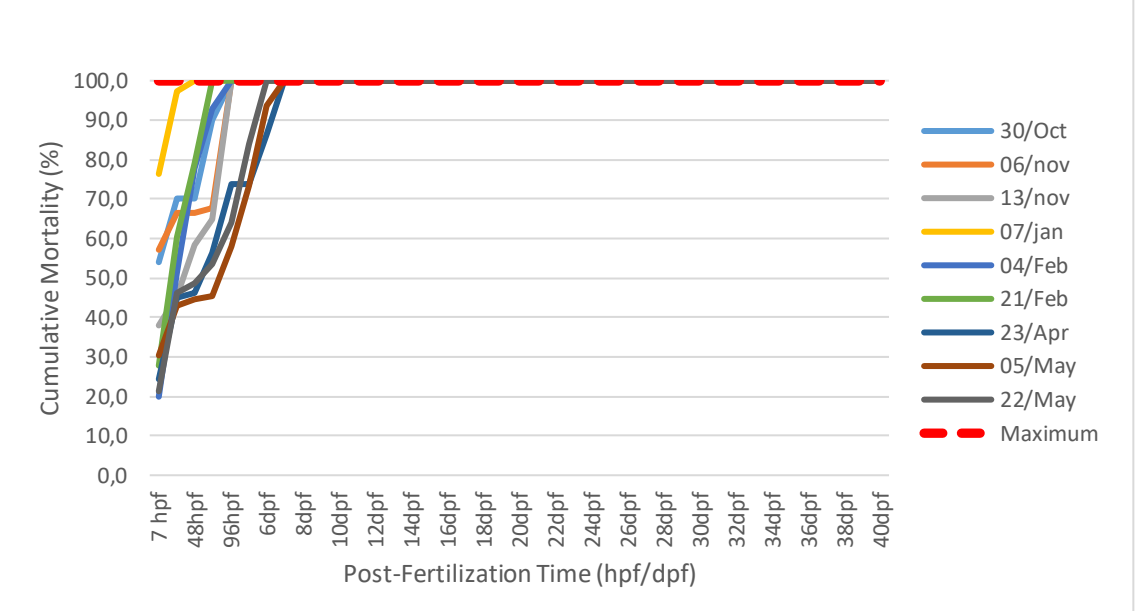

Figure 19 - Mean cumulative mortality (%) of zebrafish embryos and larvae in the Mix 2 group across all independent assays.