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Doctoral School of Natural Sciences

CHRONIC EFFECTS ASSESSMENTS OF MICROPOLLUTANTS APPLYING BIOCHEMICAL MARKERS OF FISH

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LIST ABBREVIATIONS

AB	AB wildtype (zebrafish strain)
AChE	Acetylcholinesterase
AhR	Aryl hydrocarbon receptor
ALP	Alkali-labile phosphate
BCF	Bioconcentration Factor
CA	Concentration Addition model
CBZ	Carbamazepine
CDNB	1-chloro-2,4-dinitrobenzene
DO	Dissolved oxygen
DNAsb	DNA strand breaks
DTT	Dithiothreitol
EC_{50}	Effective Concentration 50%
ECx	Effective Concentration x%
EDCs	Endocrine-Disrupting Chemicals
EEC	European Economic Community
EE2	17α-ethinylestradiol
ELISA	Enzyme-Linked Immunosorbent Assay
EPA	Environmental Protection Agency
ERA	Environmental Risk Assessment
EROD	7-ethoxyresorufin O-deethylase
ETS	Electron Transport System
GSH	Reduced glutathione
GSSG	Oxidized glutathione
GPxSe	Selenium-dependent Glutathione peroxidase

GPxTOT	Total Glutathione peroxidase
GR	Glutathione reductase
GST	Glutathione-S-transferase
IA	Independent Action
ISO	International Organization for Standardization
LC_{50}	Lethal Concentration 50%
LDH	Lactate dehydrogenase
LOEC	Lowest Observed Effect Concentration
LPO	Lipid peroxidation
MDA	Malondialdehyde
MoA	Mode of Action
NADH	Nicotinamide adenine dinucleotide (reduced)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced)
NOEC	No Observed Effect Concentration
OECD	Organization for Economic Cooperation and Development
p-INT	p-iodonitrotetrazolium
PAHs	Polycyclic aromatic hydrocarbons
P4	Progesterone (4-pregnene-3,20-dione)
PCBs	Polychlorinated biphenyls
PCR	Polymerase Chain Reaction
ROS	Reactive oxygen species
S12	Supernatant after 12,000×g centrifugation
SDS	Sodium dodecyl sulfate
SOD	Superoxide dismutase
TBARS	Thiobarbituric Acid Reactive Substances
TCS	Triclosan
Tris	Trimethylol aminomethane

TU	Toxicity Unit
USEPA	United States Environmental Protection Agency
UV	Ultraviolet
VTG	Vitellogenin-like proteins
WWTPs	Wastewater Treatment Plants

1. INTRODUCTION

Pharmaceuticals and their metabolites are increasingly recognized as one of the most concerning groups of emerging contaminants in almost every aquatic ecosystem (Wilkinson et al., 2022). These compounds are continuously released into surface waters mainly through wastewater treatment plants (WWTPs), which are generally not effective in their removal or biodegrading efficiency (Conley et al., 2008; de Jesus Gaffney et al., 2015). In surface water, pharmaceuticals are biologically active molecules designed to act on specific molecular targets in humans and animals; however, their occurrence in the aquatic environment may affect non-target species, particularly fish (da Silva Santos et al., 2018; Kreke & Dietrich, 2008; LaLone et al., 2013), through similar biochemical pathways (Martins et al., 2012; Valdés et al., 2016). Therefore, this group of pollutants has received increasing attention in recent years due to its potential ecological and toxicological implications (Santos et al., 2010; Valdés et al., 2016).

Among these compounds, the anticonvulsant carbamazepine (CBZ) is widely prescribed. It is one of the most frequently detected pharmaceuticals in rivers worldwide due to its high persistence and poor removal during WWTPs (Breton et al., 2005a; H. Chen et al., 2014; Wilkinson et al., 2022). Although CBZ toxicity has been investigated in several aquatic species, including algae, cladocerans, and fish (da Silva Santos et al., 2018; Z. Li, Li, et al., 2010; Oropesa et al., 2016; Xin et al., 2017). Most research has focused on short-term exposure, leaving its chronic biochemical and physiological effects largely unexplored (Deblonde et al., 2011).

Progesterone (4-Pregnene-3,20-dione, P4) and related progestins are another group of emerging micropollutants of concern. They are generally used in combination with estrogens as an oral contraceptive and in hormone replacement therapy. Due to their widespread medical use, they are commonly found in surface waters and are known to act as endocrine disruptors, affecting reproduction, metabolism, and development in aquatic organisms (Cardoso et al., 2019; Fent, 2015; Y. Liang et al., 2015; Orlando & Ellestad, 2014; Santos et al., 2010; Zeilinger et al., 2009).

Pharmaceuticals rarely occur alone; they often occur as multi-component mixtures with other micro- and macro-pollutants in aquatic environments (Kasprzyk-Hordern et al., 2008; López-Serna et al., 2012; Vulliet & Cren-Olivé, 2011; Zrinyi et al., 2017). Evidence indicates that mixtures frequently exhibit greater or unpredictable toxicity compared with individual compounds (Backhaus & Karlsson, 2014; Cleuvers, 2003a; Flaherty & Dodson, 2005; Kortenkamp et al., 2009). However, investigations into the combined effects of these compounds on non-target organisms, particularly fish, at environmentally relevant concentrations remain scarce. Furthermore, standard acute toxicity tests lack sufficient sensitivity to detect subtle biochemical or developmental disturbances caused by such low-level exposures (Aguirre-Martínez et al., 2015). Therefore, more sensitive molecular and biochemical endpoints are needed to evaluate the potential risks of pharmaceutical mixtures to aquatic biota.

2. RESEARCH OBJECTIVES

Carbamazepine (CBZ) and progesterone (P4) often co-exist in aquatic environments, raising concerns about their potential combined toxicological effects on aquatic organisms; however, their combined effects remain poorly understood. The main objective of this study was to assess the chronic toxicological impacts of these two pharmaceuticals, both individually and in binary mixtures, at environmentally relevant concentrations, using two ecotoxicologically important fish species: *Cyprinus carpio* (common carp) and *Danio rerio* (zebrafish).

To achieve this goal, the common carp experiment focused on single-compound exposure to CBZ to simulate chronic pharmaceutical stress, whereas the zebrafish experiment examined the combined exposure to CBZ, P4, and their mixtures to evaluate potential additive or synergistic effects.

A set of key biochemical biomarkers was assessed to characterize endocrine disruption, neurotoxicity, oxidative stress, and biotransformation processes, providing a comprehensive understanding of how chronic CBZ and P4 exposures affect aquatic vertebrates at environmentally relevant levels. By examining these indicators, the study aimed to provide a comprehensive understanding of how CBZ alone (in the alternative fish model *C. carpio*) and CBZ/P4 exposures (in *D. rerio*) chronically affect aquatic organisms at environmentally relevant concentrations, offering insights into the potential risks of CBZ and P4 to freshwater ecosystems.

3. LITERATURE OVERVIEW

3.1. Micropollutants

As emerging pollutants, though micropollutants occur in different environmental matrices in trace levels, ranging from a few ng/L to several μg/L in water bodies, they have become a global concern due to their persistence, bioaccumulation potential, and biological activity. Micropollutants may derive from a variety of sources, including industrial and agricultural activities, as well as daily life activities. Micropollutants include industrial chemicals (plasticizers, fire retardants), pesticides (insecticides, herbicides, and fungicides), trace metals (Nickel, Mercury, and Lead), pharmaceuticals (non-steroidal and steroid anti-inflammatory pharmaceuticals, anticonvulsants, antibiotics, lipid regulators, and stimulants), personal care products (UV filters, disinfectants, and fragrances), steroid hormones (estrogens), micro-/nano-plastics, and persistent organic pollutants (fluoranthene, benzene, and alachlor) (Abbasi et al., 2022; Directive 2008/105/EC of the European Parliament and of the Council of 16 December 2008 on Environmental Quality Standards in the Field of Water Policy, Amending and Subsequently Repealing., 2013; Luo et al., 2014).

Micropollutants may enter surface waters mainly from wastewater treatment plants (WWTPs), as WWTPs have limited efficiency in removing micropollutants (Martins et al., 2012). The regulations about the limitation of micropollutants are not enough to fully protect the natural environment against micropollutants. Meanwhile, not all micropollutants have been listed with limitations in the environment; worse still, not all countries have well-established and comprehensive legislation about micropollutants in the environment. Some areas and countries have well-developed legislation about micropollutant standards regarding water bodies. For example, Directive 2008/105/EC is an Act of the European Parliament and of the Council on environmental quality standards in the field of water policy. Limitations of certain micropollutants are listed on the directive, such as Nonylphenol, Bisphenol-A, Dichlorodiphenyltrichloroethane (DDT), Polyaromatic hydrocarbons (PAH), Ethylene Diamine Tetraacetic Acid (EDTA), and so

on. In China, the main standard that specifically lists water pollutant indicators is the "Surface Water Environmental Quality Standard" (GB 3838-2002). This standard classifies surface water quality and establishes target quality levels for various water bodies. It also provides detailed limits for different types of pollutants, ensuring a comprehensive framework for water quality assessment and management (Surface Water Environmental Quality Standards (GB 3838-2002), 2002).

Micropollutants that cannot be completely removed from WWTPs are likely to enter the natural environment (Valdés et al., 2016). Pharmaceuticals and other micro and macro contaminants are usually present in environmental compartments in the form of multi-component mixtures (Kasprzyk-Hordern et al., 2008; López-Serna et al., 2012; Vulliet & Cren-Olivé, 2011; Zrinyi et al., 2017). Although micropollutants have been discharged into nature at low concentrations with invisible single effects, their components are complex, and the amounts are huge (Escher & Fenner, 2011; Luo et al., 2014), and the mixture effects of the micropollutants on aquatic organisms may induce synergistic and antagonistic effects, and the mechanisms of action on non-target wildlife are often complex (Luo et al., 2014; E. Silva et al., 2002). Cleuvers (2003a) stated that pharmaceutical residues found in the aquatic environment usually appear in a mixture instead of isolated compounds. His research highlighted that although the toxicity of a single substance is low, there may be considerable combined effects between substances in mixtures. Notably, his study reported a synergistic interaction between the combination of ethynylestradiol and diclofenac, which, at low concentrations, were individually non-toxic; however, their mixture led to adverse effects on *Daphnia magna* (Cleuvers, 2003a). Meanwhile, short- or long-term exposure of aquatic organisms to mixtures of these trace micropollutants is highly likely to result in unforeseen adverse effects, including acute and chronic toxicity, like endocrine-disrupting effects, and the development of microbial resistance (Luo et al., 2014; Martins et al., 2012; Valdés et al., 2016).

Endocrine-disturbing chemicals (EDCs) have been shown to interfere with endocrine functions. These chemicals interfere with hormonal signalling pathways, potentially leading to adverse effects on humans' and wildlife's reproductive, developmental, and metabolic health (Ács et al.,

2022; Galus et al., 2014; Runnalls et al., 2015). This issue has raised concern since the late 1990s, as the feminization of male fish has been reported in aquatic environments worldwide. Reports from various regions highlight the global issue of male fish feminization in aquatic environments. Studies have identified estrogens and estrogen-like molecules, such as 17α-ethinylestradiol (EE2), as key contributors, disrupting fish reproduction and sexual development even at trace concentrations (Sumpter & Jobling, 2013). Additionally, the antibiotic ciprofloxacin has been shown to exert chronic toxic effects on *D. magna* at low concentrations (Martins et al., 2012). The persistence of these contaminants may further affect organisms at higher trophic levels through food chains. Another contaminant, the biocide triclosan (TCS), has been associated with embryotoxicity, hatching delays, and altered biomarker levels in zebrafish (*D. rerio*) (Oliveira et al., 2009).

3.2. Pharmaceuticals in the Environment

Pharmaceuticals are used by humans and in veterinary medicine, and some pose a risk to the environment (Fent et al., 2006). Pharmaceutical residues in the environment have become the subject of numerous studies (Santos et al., 2010). The pharmaceuticals began commercialization in the late 1980s. Human beings and pharmaceuticals have been closely linked for hundreds of years (Hughes et al., 2013; Staszny et al., 2021a). With the wide consumption and improper disposal, more than 4,000 different pharmaceuticals have been found in the environment over the past decades. Due to population explosion, urbanisation, industrialization, and improper agricultural activities, pharmaceuticals have become a major environmental contaminant since the 2000s (Hughes et al., 2013; Kümmerer, 2009). The main source of pharmaceutical residues in different waterbodies is pharmaceutical residues excreted by humans and entering via WWTPs (Hughes et al., 2013) due to WWTPs' limited efficiency in removing micropollutants, including pharmaceuticals (Martins et al., 2012). In addition, hospital wastewater also contains many pharmaceuticals (Brown et al., 2006).

Pharmaceuticals are compounds with biological activity, intended to interact with specific

pathways and processes in humans and animals. Pharmaceutical residues and their metabolites can have potential impacts on non-target organisms following similar metabolic pathways (Valdés et al., 2016). Inadequately treated pharmaceuticals circulate into other water bodies (e.g., surface water, groundwater, agricultural water) (Vernouillet et al., 2010), which may bioaccumulate (Valdés et al., 2016) and/or exert noxious effects on living organisms (Kodom et al., 2021; Vernouillet et al., 2010), especially fish (da Silva Santos et al., 2018; Runnalls et al., 2015).

The potential impairment caused by pharmaceuticals in water bodies remains relatively less understood than other pollutants. Recent studies (Hughes et al., 2013; Lindberg et al., 2014; Wilkinson et al., 2022) have reported that huge amounts of pharmaceuticals co-occur in the aquatic environment simultaneously, especially in WWTPs and agricultural runoffs (Chauveheid & Scholdis, 2019). A deeper understanding and evaluation of the ecological risks posed by pharmaceuticals is needed. Therefore, more data and testing are required for the proper assessment of their effects on the environment (Ankley et al., 2007).

3.3. General information of compounds investigated in this work

3.3.1. Carbamazepine

Carbamazepine (5H-dibenz/bf/azepine-5-carboxamide) (CBZ) is an anticonvulsant drug prescribed worldwide for the treatment of bipolar disorder, trigeminal neuralgia, and psychomotor epilepsy (Breton et al., 2005b). The chemical structure depiction of CBZ is displayed in Figure 1.

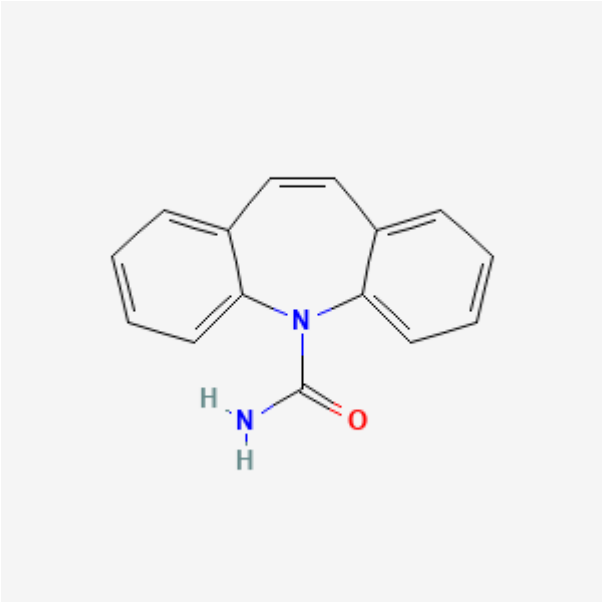


Figure 1: Chemical Structure Depiction of CBZ. Cited from National Center for Biotechnology Information (2024). PubChem Compound Summary for CID 2554, Carbamazepine. Retrieved August 30, 2024 from https://pubchem.ncbi.nlm.nih.gov/compound/Carbamazepine.

In 1997, the consumption of carbamazepine in Austria reached 6,334 kg (Umweltbundesamt, 1999). Among psychiatric pharmaceuticals, CBZ is one of the most common pharmaceuticals found in municipal WWTPs' effluents and urban-impacted surface waters, leading to its frequent detection in aquatic ecosystems, even in drinking water (Björlenius et al., 2018; Breton et al., 2005b; Clara et al., 2004; Santos et al., 2010). It is known that CBZ is absorbed almost entirely in the human gastrointestinal tract, and 72% of the received dose is discharged into the sewage system through urine (Cunningham et al., 2010). The residuals are mainly metabolised in the liver into carbamazepine 10,11-epoxide and other derivatives. These metabolites have antiepileptic properties as well as the CBZ itself (Van Rooyen et al., 2002). However, only 7 to 10% of the CBZ entering wastewater can be removed in WWTPs (Björlenius et al., 2018; H. Chen et al., 2014). Like other trace pollutants that are not fully eliminated, CBZ is released into the natural environment and has a relatively slow degradation process in the environment, typically lasting about 82 days in surface water (Brandão et al., 2013). Therefore, CBZ is widely distributed globally in various water bodies, with concentrations ranging from 3.3 to 128.2 ng/L and an

average of 23.3 ng/L reported globally (Wu et al., 2022). Regionally, significant variations in CBZ concentrations have been observed. For example, in the Nansi Lake basin, China, concentrations were detected as high as 150 μg/L (Sim et al., 2011). In South Korea, surface waters showed concentrations up to 12 μg/L (Loos et al., 2009), while in Europe, levels reached 0.8 μg/L within the Danube River in Hungary (Kondor et al., 2021; Staszny et al., 2021b). These variations may be influenced by factors such as population distribution. Research indicates that CBZ levels tend to be higher among populations aged over 70 years and below 5 years (Ebrahimzadeh et al., 2021).

In humans, CBZ as an anticonvulsant drug affects neurotransmission at the molecular level through a variety of mechanisms. Affects the potassium and sodium channels in nerve cells and signalling pathways. Stabilizing these sodium channels' activity helps reduce the abnormal activity of nerve cells (Ayano, 2016; Brodie et al., 1995). CBZ plays an important role in medicine; however, researchers found it has adverse impacts on non-target organisms in the environment, especially when it is present as an environmental pollutant (Brandão et al., 2013; da Silva Santos et al., 2018; Galus et al., 2014; Yan et al., 2018).

Many studies about the lethal and sublethal effects of CBZ have assessed the biota freshwater habits including organisms such as algae, cladocerans, and fish (LaLone et al., 2013; Z. Li, Li, et al., 2010; Oropesa et al., 2016; Xin et al., 2017). As an emerging pollutant, CBZ was found to damage the cell membranes of green algae *Chlorococcum sp.*, but developed resistance to CBZ after longer exposure (Xin et al., 2017). Environmentally relevant concentrations of CBZ (200 μg/L) can significantly reduce the reproductive yield and slow the rate of ecdysis rate of *Daphnia magna* (Oropesa et al., 2016). Many studies have shown that CBZ can cause short and chronic toxicity to zebrafish, including causing eating disorders, affecting the growth and development of zebrafish embryos and larvae, decreasing egg producing activity, affecting the maturation of gonadal follicles in female fish, affecting sperm activity in male fish, and various molecular biochemical changes (da Silva Santos et al., 2018; Fraz et al., 2018; Gasca-Pérez et al., 2019; LaLone et al., 2013; Z. Li, Li, et al., 2010; Pohl et al., 2019; Qiang et al., 2016).

The bioconcentration factor (BCF) of CBZ in zebrafish plasma was reported to range from 0.83 to 1.45, indicating limited bioaccumulation potential (Z. Li et al., 2011). The total elimination half-life of CBZ in zebrafish is approximately 0.48 days (Z. Li et al., 2011), suggesting rapid clearance from the organism. CBZ has been shown to exert multiple toxic effects in zebrafish. It can act as an endocrine-disrupting compound by altering sex steroid hormones, leading to decreased reproductive capacity, reduced embryo production, and irregular oocytes (Deblonde et al., 2011). In addition, CBZ has also been reported to prolong feeding time and reduce food-intake efficiency, indicating behavioral disturbances in zebrafish. Furthermore, it was also shown that CBZ has neurotoxic potential to aquatic organisms and can inhibit the activity of acetylcholinesterase (AChE), thereby affecting neurotransmission function. For example, 100 µg/L CBZ exposure to zebrafish decreases AChE activity, which in turn affects their behaviours and survival (Jia et al., 2020; LaLone et al., 2013). CBZ also altered liver antioxidant and detoxification enzymes, such as glutathione S-transferase (GST), reduced the activity of catalase (CAT) and lactate dehydrogenase (LDH), and caused DNA damage under continuous exposure to 1, 10, and 100 µg L^{-1} CBZ for 63 days (da Silva Santos et al., 2018). Exposure to a high dose of CBZ (100µg/L) for 45 days inhibited superoxide dismutase (SOD) activity of zebrafish (Jia et al., 2020). Moreover, Fraz et al. (2018) found that CBZ can impact steroidogenic enzyme expression or function in zebrafish after chronic exposure to 10 µg/L CBZ for 67 days. Changes in steroidogenic enzymes may lead to disruption of the endocrine system. This may affect the balance of sex hormones, increase estrogens, and decrease androgens which further affects reproductive ability and development (Tõugu & Kesvatera, 1996; Yan et al., 2021).

Besides zebrafish, in common carp (*C. carpio*), high concentrations of CBZ (0.2 to 2 mg/L) reduced the activity of SOD, glutathione peroxidase (GPx), glutathione reductase (GR), increased lipid peroxidation (LPO) and protein carbonylation content in the sperm of common carp after two hours of *in vitro* exposure. This suggests that CBZ has significant effects on the antioxidant system, resulting in increased production of reactive oxygen species (ROS), which may cause oxidative stress. This can damage cell structure and function (Li et al., 2010). These changes were observed

not only in common carp but also in the brain tissue of rainbow trout (*Oncorhynchus mykiss*). CBZ increased lipid peroxidation, meanwhile decreasing SOD and GR, however, the changes of GPx and CAT showed a nonlinear reaction over time, and their activity increased first and then decreased (Chen et al., 2017). In a previous study, Gasca-Pérez et al. (2019) reported that after 7 days of subacute treatments with 2 mg/L CBZ, increased the LPO, hydroperoxide and protein carbonyl content, while the activities of antioxidant enzymes (SOD, GPx and CAT) were decreased (Galus et al., 2014). These authors suggested that CBZ has a wide range of adverse effects on freshwater ecosystems, especially fish, including oxidative stress, endocrine disruption, toxicant biotransformation alteration, and organ and tissue damage.

3.3.2. Progesterone

Steroid hormones are a class of lipophilic small molecules. They play an important regulatory role in the body, including regulating physiological processes such as growth, development, reproduction, and metabolism (Li, 2004). They enter the environment at a low level while posing potential risks (Christen et al., 2010). Among the pharmaceuticals, steroid hormones are considered strong endocrine disruptors (Fent, 2015), of which progestins have been the least studied, although P4 and synthetic progestins are frequently detected (Fent, 2015). Steroids with progesterone activity are referred to as gestagens, progestins, or progesterone. The term "progestins" is used specifically referring to synthetic progesterone (Paulos et al., 2010; Zeilinger et al., 2009). Natural progesterone (4-pregnene-3, 20-dione, P4), is commonly used in combination with estrogens as oral contraceptives and hormone replacement therapy. Endogenous P4 is an important regulator which is secreted by the *corpus luteum* of the ovary, and it plays a key role during oocyte maturation and pregnancy in vertebrates (Orlando & Ellestad, 2014; Zeilinger et al., 2009). As a natural steroid hormone, P4 is involved in the female menstrual cycle, pregnancy, and embryogenesis in humans and vertebrates (Fent, 2015). P4 and synthetic progestins are often detected in surface water due to their widespread use and discharge into aquatic environments through human and animal faeces, and urine, from paper mill wastewater, wastewater treatment plant wastewater and agricultural runoff (Orlando & Ellestad, 2014) containing P4 concentrations

ranging often from 0.07 to 22.2 ng/L(Fent, 2015; Santos et al., 2010), however, the concentration can reach several μg/L around animal farm waste and runoffs (Fent, 2015). Kolpin et al. (2002) found P4 at low ng/L concentrations at half of the 139 test sites in U.S. streams, including progesterone and norethindrone.

Environmental gestagens are regarded as emerging pollutants (Orlando & Ellestad, 2014), for example, some synthetic progestins may interfere with the reproductive system of freshwater species (Cardoso et al., 2019; Y. Liang et al., 2015; Orlando & Ellestad, 2014; Zeilinger et al., 2009; Zrinyi et al., 2017). For instance, when *Lymnaeas* were exposed to a mixture (10 ng/L) of P4 with three other progestine types (levonorgestrel, drospirenone, and gestodene), fewer eggs were laid in the first week, and the eggs were in low quality (Zrinyi et al., 2017). Another study found that the reproductive time of wild *Xenopus laevis* was altered by environmental gestagens (Ogawa et al., 2011).

In zebrafish, exposure to 25 ng/L P4 for 21 days (Blüthgen et al., 2013) and to 33–666 ng/L megestrol acetate (MTA), which is another synthetic progestins for 21 days, with a significant reproductive decline observed at 666 ng/L (Han et al., 2014) have been demonstrated to adversely affect reproduction. The BCF levels of different progestins range from 7 (Dienogest) to 128 (Medroxyprogesterone acetate) (Rocha & Rocha, 2022). More recently, Liang et al. (2015) found that exposure to zebrafish to 4, 33, and 63 ng/L P4 from 20 to 60 days post fertilization resulted in a significant increase in the proportion of females, with the strongest effect observed at 63 ng L^{-1}. The sex hormone levels of zebrafish were altered; sex differentiation may have been affected, and gene expressions were also affected. Cardoso et al. (2019) exposed female zebrafish to 10 and 1000 ng/L levonorgestrel (a synthetic progestin) for 21 days and observed significant hepatic responses at the lower concentration. Specifically, VTG immunostaining was reduced, and catalase showed a hormetic response at 10 ng/L

3.4. Combined effects of pharmaceuticals

As mentioned before, pharmaceuticals and other micro- and macro-contaminants are usually

present in environmental compartments in the form of multi-component mixtures (Kasprzyk-Hordern et al., 2008; López-Serna et al., 2012; Vulliet & Cren-Olivé, 2011; Zrinyi et al., 2017). Two common mathematical models are used to describe the joint effect of toxic mixtures: Concentration Addition (CA) and Independent Action (IA) models. The CA model is one of the most used models in mixture toxicity research, and it can be dated back to the early works of Loewe and Muischnek in 1926 (Loewe & Muischnek, 1926), who introduced it in pharmacology. Later, the CA model was formalized mathematically by Berenbaum in 1985 using the following equation:

$$\sum_{i=1}^{n} \frac{Ci}{\mathrm{EC}xi} = 1$$

In the equation, C_i (Individual concentration of each substance in the mixture) represents the actual concentration of substance *i* in the mixture, and ECx_i (Effect concentration of each substance at *x%* effect level) is the concentration of the single chemical that would induce the same *x%* effect when acting alone. The CA model describes the combined effects of chemicals that share the same mode of action. It assumes that the toxic effects of all chemicals can be summed, and their combined effect is equivalent to the sum of their individual effects. Importantly, even when all individual substances are below their no observed effect concentration (NOEC), they may still contribute to the total effects of the mixture. To quantify the contribution of each chemical, concentrations can also be expressed in terms of Toxicity Units (TU), where $TU_i = C_i / ECx_i$, If the sum of toxicity units ΣTU is less than or equal to 1, no toxic effect is expected; if it exceeds 1, toxicity may occur (Cleuvers, 2003b; Iwasaki & Gauthier, 2016). The CA model has been widely applied in ecotoxicology to predict the joint toxicity of environmental pollutants, including pharmaceuticals and pesticide residues. Additionally, Backhaus and Faust (2012) expanded the use of the CA model in environmental sciences, making it an essential tool for ecological risk assessment (ERA) (Backhaus & Faust, 2012).

On the other hand, Bliss introduced the concept of Independent Action (IA) in 1939 (Bliss, 1939).

The IA model is also known as the Response Addition model. Some compounds in their mixture have different modes of action, and these compounds act on different biological pathways and have different modes of action, meaning their toxic effects are not additive (Iwasaki & Gauthier, 2016). Unlike the CA model, if the concentration of each substance in the mixture is below its respective NOEC, these substances will not contribute to the overall effect of the mixture; in other words, there will be no toxic effects of the mixture if the concentrations of each substance are below their NOEC. The IA model assumes that the substances in the mixture contribute independently to the total toxicity. (Bliss, 1939; Cleuvers, 2003a).

The mathematic equation of IA for a binary mixture can be expressed as:

$$E(c_{mix}) = 1 - \left[\left(1 - E(c_1)\right)\left(1 - E(c_2)\right)\right]$$

The mathematic equation of IA for general mixtures can be expressed as:

$$E(c_{mix}) = 1 - \prod_{\{i=1\}}^{n} (1 - E(ci))$$

Where $E(c_1)$ and $E(c_2)$ are the effects of the single chemicals, and $E(c_{mix})$ represents the total effects of the chemical mixture. $E(c_i)$ is the effect induced by the individual chemical *i* at its concentration c_i.

Until recently, there have been very limited studies on the toxic effects of the mixtures on non-target organisms like fish, especially when they are present as mixtures at environmentally relevant concentrations. Moreover, the toxic effects of mixtures in combination were generally higher than the toxicity of the compounds on their own (Backhaus & Karlsson, 2014; Kortenkamp et al., 2009). Mixtures of pharmaceuticals can induce unexpected effects even when individual compounds show no significant effects. For example, Flaherty and Dodson (2005) found that specific pharmaceutical mixtures, such as a combination of fluoxetine (36 µg/L) and clofibric acid (100 µg/L), caused significant mortality and deformities (36 µg/L fluoxetine and 10 µg/l clofibric acid) of *D. magna*, although clofibric acid (10 µg/l) showed a positive effect which was significantly

increased the fecundity of *D. magna*. The ternary mixture of erythromycin, triclosan, and trimethoprim caused a significant change in the sex ratio of *D. magna* (with 20% fewer male offspring compared to the control) at the mixture concentrations of 30 μg/l, while the individual components did not cause significant acute effects at 10 μg/L (Flaherty & Dodson, 2005). Even binary mixtures of different compounds tend to show a similar phenomenon (Cleuvers, 2003a; Flaherty & Dodson, 2005). Mixtures such as fluoxetine (36 μg/L) and clofibric acid (100 μg/L) killed more than 50% of the *Daphnia magna* population after 6 days of exposure, while the individual components did not cause significant effects (Flaherty & Dodson, 2005). The interaction of drug mixtures (roxithromycin-fluoxetine and propranolol-fluoxetine) at 4, 20, and 100 μg/L fluoxetine for 7 days induced a stronger antioxidant response than the single one to the fish liver (Ding et al., 2016). Runnalls et al. (2015) observed that a mixture of the synthetic estrogen ethinylestradiol (EE2) and levonorgestrel reduces egg production of fathead minnow (*Pimephales promelas*) in a concentration-dependent manner, while the model of the combined effects is not defined in this study. Cleuvers (2003a) found that the combined effect of clofibrate and CBZ follows the concept of concentration addition in the mixture test on daphnia. The mixture resulted in more immobilization on *Daphnia magna* than its individual component. Jia et al. (2020) found that combined exposure to CBZ (1, 10, and 100 μg/L) and Cu (0.5, 5, and 10 μg/L) for 45 days led to more reactive oxygen species production, more severe effects on the antioxidant system, and more severe damage to zebrafish liver cells. The combined environmental-related concentrations of CBZ and Cu can inhibit AChE-related by inhibiting the expression of the AChE-realted gene, leading to neurotoxic effects in zebrafish.

3.5. Environmental toxicology methods

3.5.1. Standardized methods and species

Aquatic toxicity refers to the effects of chemicals on water-dwelling organisms which contain different trophic levels. The endpoints of a toxic assessment can be acute or chronic. Acute toxicity refers to short-term exposure, and the LC_{50} (lethal concentration for 50% of test organisms) is

often used. Chronic toxicity refers to long-term exposure, and the endpoints include NOEC (No Observed Effect Concentration), LOEC (Lowest Observed Effect Concentration), and ECx values. Acute aquatic toxicity is mandatory in EU chemical legislation (European Commission Joint Research Centre, 2023).

Standard test methods have been developed to assess the risk of a single chemical substance in aquatic environments, guiding for evaluation of their potential toxicity (United States Environmental Protection Agency (EPA), 2024). These methods are not only applied in the laboratory but also applied to natural environmental samples to improve the ecological risk assessment (Anderson et al., 2004). Typically, single-species tests in acute cases are often conducted, where the endpoint is most often survival, growth, reproduction, or related physiological features (Crouau & Moïa, 2006; OECD, 2004, 2006). However, relying on a single species may not fully indicate the complexity of real ecological conditions. Therefore, a battery test system involving multiple species from different trophic levels is recommended (Ács et al., 2013; Repetto et al., 2001). Meanwhile, it is important to make sure that the test species adequately represent the studied biota, also a sensitive indicator (European Commission Joint Research Centre, 2023). Among the standardized zooplankton organisms commonly used in aquatic toxicology, *D. magna*, *Daphnia pulex*, and *Ceriodaphnia dubia* are widely applied due to their sensitivity to pollutants (Versteeg et al., 1997). Endpoints such as the immobilization of *D. magna* belong to standard acute toxicity tests (OECD, 2004).

Over the past decade, it has become evident that standard acute toxicity tests lack the sensitivity needed to evaluate the effects of pharmaceuticals on aquatic organisms. For instance, teratogenic effects were observed in sea urchin (*Paracentrotus lividus*) after exposure to environmental concentrations of carbamazepine and ibuprofen at a concentration as low as 10 ng/L (Aguirre-Martínez et al., 2015). It underscores the importance of incorporating more sensitive response endpoints, such as molecular-level biochemical markers, in toxicology studies to enhance the detection and assessment of toxic effects.

Environmental Risk Assessment (ERA) is a scientific process used to predict the potential adverse effects of pollutants on the environment and human health while also determining whether appropriate risk management measures are required (Depledge & Fossi, 1994; Van der Oost et al., 2003). ERA regulations are typically based on short-term ecotoxicological studies across various species (Aguirre-Martínez et al., 2015). The Organization for Economic Cooperation and Development (OECD) guidelines for the Testing of Chemicals are a unique tool for assessing chemical impacts on health and the environment. Internationally recognized as standard safety testing methods, these guidelines are extensively adopted by industry, academic researchers, and regulatory agencies for the assessment of a broad range of chemicals from industrial chemicals and agricultural pesticides to personal care products. Their wide applicability ensures consistency and reliability in chemical safety evaluations across diverse sectors, facilitating the identification of potential risks to both human health and environmental systems (OECD, 2024). For example, the acute fish test (OECD, 2019) involves exposing fish to varying concentrations of a test substance over a short period (typically 96 hours) to determine the concentration that causes mortality in 50% of the fish (LC_{50}), thereby assessing the substance's acute toxic effects. In addition to the OECD, the United States Environmental Protection Agency (USEPA, 1995) contributes to the development of relevant standards and guidelines for ERA. Similarly, the International Organization for Standardization (ISO) has established various standards for toxicological testing. For instance, ISO 21115 provides guidelines for assessing the acute toxicity of water samples and chemicals using a fish gill cell line (ISO, 2019).

Aguirre-Martínez et al. (2015) reported that carbamazepine and ibuprofen significantly reduced embryo-larval development at a concentration of 10 ng/L compared to the control group. However, no toxic effects were observed when using three other common standardized acute toxicity tests—bioluminescence inhibition in *Vibrio fischeri*, growth inhibition in *Isochrysis galbana* and *Pseudokirchneriella subcapitata*, and fertilization tests in sea urchins—at much higher concentrations in the mg/L range. The bioluminescence inhibition assay evaluates the effects of pharmaceuticals on *V. fischeri* by measuring changes in bacterial luminescence following exposure

to a range of test concentrations. *V. fischeri* luminescence serves as an indicator of its response to toxic substances (Aguirre-Martínez et al., 2015; Azur Environmental, 1998; OECD, 2006). However, the results demonstrate that bioluminescence intensity alone is insufficient for evaluating pharmaceutical toxicity. While bioluminescence inhibition assays were included in the study to assess pharmaceutical effects, the findings underscore the limitations of standard acute toxicity tests. Specifically, the current endpoints defined in existing guidelines may lack the sensitivity required to detect the subtle toxic effects of pharmaceuticals on aquatic organisms. This calls for the integration of more sensitive biomarkers and refined endpoints in future toxicological assessments.

3.5.2. Alternative methods and test species

Toxicology testing has traditionally relied on mammals, particularly in regulatory assessments. However, in recent years, increasing public and scientific interest in reducing vertebrate use has led to the exploration of alternative model organisms in toxicology testing. According to the White Paper, European Commission (2001) and EU Directive 86/609/EEC (1998), the number of animal tests should be minimized where it's possible. Current efforts focus on integrating non-vertebrate species, such as bacteria, fungi, algae, invertebrates, and early-stage vertebrate embryos, into standardized test batteries (Repetto et al., 2001).

A variety of pollutants and their derivatives create a more challenging situation for risk assessment due to their interactions and delayed toxic effects (Van der Oost et al., 2003). These pollutants often present complex risks because their effects on wildlife populations are not immediately apparent. Conventionally, mortality is regarded as a standard endpoint in ecotoxicology, but mortality is an irreversible and non-specific indicator that may fail to indicate the sublethal effects of low chemical substances, whose effects cannot be detected by mortality tests and may also pose an ecological risk. On the other hand, harmful effects are typically difficult to detect on time, as they may appear much later after exposure, and the damage can be difficult or even impossible to reverse once it is evident. This delayed detection emphasises the importance of focusing on

sublethal endpoints, providing earlier, more specific insights into the toxic mode of action (MoA) of the exposure material. Sub-lethal biomarkers, such as changes in behaviour, reproduction, and biochemical processes, often serve as early indicators of stress or damage caused by environmental pollutants. These sublethal biomarkers serve as sensitive indicators and are especially valuable for identifying risks early. The absence of these sublethal endpoints may result in undetected risks, with severe damage before conventional methods are capable of detecting (Bayne et al., 1985; Van der Oost et al., 2003).

The hierarchical biological responses to environmental stress within the biological system are shown in Figure 2, starting at the molecular level, and progressing through subcellular (organelles), cellular, tissue, organ, organism, population, community, and finally, ecosystem levels. Each level of biological organization exhibits distinct responses to stressors, which underscores the importance of using biomarkers to reflect early warnings of environmental toxins. Measuring biomarkers at different biological levels allows for the detection of early warnings of environmental stress and offers identification of potential risks before significant damage or death (Aguirre-Martinez, 2021; Bucheli & Fent, 1995). Biomarkers serve as measurable indicators of the presence of toxic substances within an organism. These biomarkers are typically derived from body fluids, cells, or tissues, and they reflect biochemical or cellular changes resulting from the presence of toxic substances or the organism's response to these substances (Committee on Biological Markers of the National Research Council, 1987; Van der Oost et al., 2003).

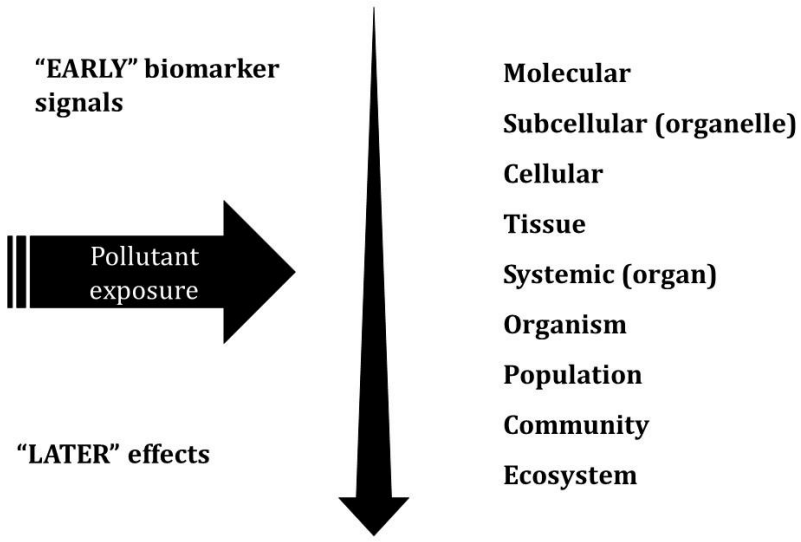


Figure 2: The response orders to environmental stress within a biological system with adjustments (Bayne et al., 1985).

A variety of biochemical and molecular approaches have been developed to evaluate sublethal toxicity, such as Enzyme-based assays (*in vivo*, *in vitro*), immunological methods (e.g., ELISA), mammalian cell line-based assays, and PCR-based molecular biomarker detection. Additionally, biochemical biomarkers serve as indicators of physiological stress and can be categorized, for example, as follows:

Markers of energy reserves

- Lipids
- Proteins
- Carbohydrates
- Electron transport system (ETS) activity

Markers of chemical impact

(a) Biotransformation markers
- 7-Ethoxyresorufin-O-deethylase (EROD)
- Glutathione-S-transferase (GST)
- Metallothioneins (MT)
- Benzo[a]pyrene hydroxylase (BaP)

- Glutathione (GSH & GSSG)

(b) Antioxidant enzymes
- Catalase (CAT)
- Glutathione peroxidase (GPx)
- Superoxide dismutase (SOD)

(c) Markers of Cellular Damage
- Lipid peroxidation (LPO)
- DNA strand breaks (DNAsb)
- Chromosomal aberrations (MN)
- Acetylcholinesterase (AChE)
- Endocrine disruption markers (e.g., Vitellogenin (VTG))

Various biochemical biomarkers have been selected to screen for and identify mechanistic effects. For example, biomarkers of oxidative stress, such as catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidases (GPxSe and GPxTOT), can provide early indications of oxidative damage. These biomarkers offer sub-lethal means of assessing pollutant-induced stress and can detect changes in the organism's physiology before more severe damage occurs. Other biomarkers include those related to xenobiotic metabolisms, such as 7-ethoxyresorufin O-deethylase (EROD) and glutathione-S-transferase (GST), which reflect the organism’s ability to detoxify harmful substances and alterations in xenobiotic metabolization processes. Additionally, biomarkers related to nervous system effects, DNA damage (such as DNA strand breaks (DNAsb)), and endocrine disruption (e.g., vitellogenin-like proteins (VTG)) provide further insights into the toxicological effects of pollutants (Cardoso et al., 2019; Ding et al., 2016; X. Liang et al., 2022; Nkoom et al., 2020).

To reduce conventional vertebrate models, alternative test species are increasingly utilized in ecotoxicological assessments. For instance, *Dreissenid* mussels in Lake Balaton are one of the most abundant macroinvertebrates in the littoral zone, making them ideal candidates for pollution monitoring and bioaccumulation studies. Given their filter-feeding nature, these mussels effectively accumulate pollutants, providing insights into contaminant levels in aquatic environments (Ács et al., 2016; Farkas et al., 2017).

Fish have received considerable attention as sentinel species in aquatic ecosystems in aquatic

pollutant studies (Powers, 1989). They are valuable for assessing the health of aquatic ecosystems because they are sensitive to a wide range of pollutants and can provide early warning signs of contamination. Despite species-specific limitations, fish biomarkers remain an effective tool for pollution monitoring. These biomarkers help assess a wide range of pollutants and their impacts, although there are variations in how biomarkers are expressed across different fish species (Van der Oost et al., 2003).

In recent years, a variety of alternative tests have been developed using biochemical markers as endpoints to assess environmental stress in a more specific and sensitive manner (Gagné, 2014). For example, to assess oxidative stress caused by a particular substance, antioxidant enzymes such as SOD are measured. These assays can be performed in high-throughput 96-well microplates, allowing for large-scale screening of pollutant effects. In a non-enzymatic superoxide generation system, p-iodonitrotetrazolium (p-INT) (Ewing & Janero, 1995) is used as a substrate to detect the presence of superoxides. When superoxides are generated, p-INT undergoes a reduction reaction, resulting in a colour change. This colour change can be measured to detect the production of superoxides and assess the associated biochemical reactions. Such assays provide efficient and effective means of monitoring oxidative stress in aquatic organisms (Gagné, 2014).

For instance, the assay for measuring the activity of CAT is based on the time-dependent elimination of H_2O_2 by CAT. There are different assays to assess the CAT activity. The assay used in this study is based on the measurement of the decay rate of H_2O_2 catalyzed by CAT using a multimode spectrophotometer (Aebi, 1984). The concentration of H_2O_2 was followed by measuring absorbance at 240 nm, specific for H_2O_2. Alternatively, a fluorescence-based assay can also be employed, where fluorescence intensity decreases over time due to CAT activity, using a fluorescent dye (Gagne & Francois, 2014). Both methods measure the CAT activity ultimately, but they differ in the techniques and parameters they measure (absorbance vs. fluorescence).

In addition to oxidative stress, the biotransformation of xenobiotic substances is another critical area of research. Cytochrome P450-associated activity is commonly assessed to understand how

organisms metabolize and detoxify pollutants. Specific substrates are used for different cytochrome P450 subtypes (e.g., P4501A1, P4501A2, P4503A4), and enzyme-linked immunoassays or targeted mRNA gene expression can be used to measure enzyme activity and gene expression (Gagné, 2014). These assays provide valuable information on the mode of action of pollutants, making it possible to track the biochemical pathways through which pollutants exert their toxic effects.

Vitellogenin (VTG) is another important biomarker used to assess endocrine disruption in aquatic organisms VTG is synthesized in the liver of vertebrates and gonads of invertebrates, and its production is regulated by estradiol and other neuropeptides (Gagnaire et al., 2009). The presence of VTG is an indicator of estrogenic activity and can be measured using direct and indirect assays. Indirect assays measure VTG levels by assessing the levels of alkali-labile phosphates, while direct detection involves quantitative polymerase chain reaction or enzyme-based immunoassays. However, VTG levels can vary significantly between species, so species-specific assays are required. A VTG immunoassay developed for one fish species may not be suitable for detecting VTG in another species, highlighting the need for tailored approaches when assessing endocrine disruption across different organisms (Gagne & Francois, 2014a).

In addition to biochemical biomarkers, physiological, histological, and morphological biomarkers are also measured in ecotoxicology to assess the impact of environmental pollutants at multiple biological levels (Van der Oost et al., 2003). Such as survival, reproduction (Kuperman et al., 2018), changes in locomotion (Da Luz et al., 2004), feeding activity, heart rate, and light preference, can serve as early warning indicators. For instance, exposure to *Cylindrospermopsis raciborskii* has been shown to cause significant feeding inhibition and increased lethality in crustacean neonates (Ács et al., 2013). Similarly, exposure to Avobenzone, a common sunscreen ingredient, has been reported to increase reproductive output, heart rate, and filtration rate in *D. magna*, while simultaneously reducing swimming activity (Németh et al., 2024). To assess the behavioural and physiological biomarkers, feeding inhibition assays can be conducted on *D. magna*. Their ability to ingest food particles was measured. *D. magna* acute immobilization assay can be applied to

measure the swimming, determining the physiological stress effect. The heart rate measured according to the number of beats per minute of *D. rerio* embryos can examine the sublethal effect (Ács et al., 2013). The filtration rate can be assessed by measuring the optical density (OD650 nm) of an algal suspension before and after a 4-hour exposure using a spectrophotometer (Németh et al., 2024).

3.6. Model species

Zebrafish (*D. rerio*) (Figure 3) is a small tropical freshwater fish that originated from northern India (Briggs, 2002; Howe et al., 2013). The important beginning of the formal use of zebrafish as a laboratory model began in 1981 with George Streisinger (Streisinger et al., 1981). As a vertebrate, the zebrafish is one of the most popular animal models for the analysis of host-pathogen interactions and infectious diseases (H. Meijer & P. Spaink, 2011; Llamas & van der Sar, 2014). There are many advantages to using zebrafish as a test model, for example, zebrafish have strong reproductive ability, a short growth period, are easy to feed, are low-cost to manage, and can meet the requirements of the experiment on the number of samples and the experiment period (Veldman & Lin, 2008). The most important reason is that zebrafish share a high genetic similarity with humans; they have direct homologues of 82% of the genes associated with human disease (Howe et al., 2013). Also, zebrafish take advantage of the optical transparency of the embryos (Briggs, 2002; H. Meijer & P. Spaink, 2011). Therefore, zebrafish have been applied frequently in the field of toxicology (MacRae & Peterson, 2015). Besides, because zebrafish are relatively small (3-4 cm) and have high efficiency in reproduction (Finley & Zon, 2004), it's easier and cheaper to use zebrafish than other bigger animal models (Veldman & Lin, 2008). Zebrafish usually lay eggs in the early morning, and spawning behaviour is influenced by the light cycle. When breeding, male, and female fish can be put into the breeding tank in a certain proportion, and they will mate and lay eggs (Westerfield, 2000).

Figure 3: Adult male and female AB strain of zebrafish, picture adapted from (Teame et al., 2019) Available at https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6951987/ at 2024.09.05.

Common carp (*C. carpio*), a teleost species native to the Black, Caspian, and Aral Sea basins, is one of the most widely recognized freshwater fish species due to its adaptability and economic significance. Over time, this species has been successfully introduced to numerous regions worldwide, becoming a significant role in commercial aquaculture because of its fast growth, resilience, and high market demand. Mature carp typically measure between 25 and 36 cm in length, although this can vary depending on environmental conditions and local aquaculture practices. Known for their hardy nature, common carp can thrive in various aquatic habitats, from natural rivers and lakes to artificial ponds and reservoirs. Importantly, common carp have emerged as a valuable species in environmental and toxicological research. Their ability to tolerate diverse environments while maintaining physiological sensitivity to xenobiotics makes them particularly useful for studying the impacts of environmental contaminants. These fish exhibit measurable adaptive responses when exposed to pollutants, making them effective bioindicators of aquatic ecosystem health. Additionally, they are easy to handle and could acclimate well to laboratory

conditions, further supporting their use as test models in toxicological assays. The combination of their ecological adaptability, economic importance, and scientific relevance underscores the important role of common carp in both applied and experimental contexts, particularly in assessing the risks of exposure to contaminants in freshwater environments (Gasca-Pérez et al., 2019; Karan et al., 1998; Kawamura & Freyhof, 2008; Özcan Oruç & Üner, 2002; Saucedo-Vence et al., 2015).

3.7. Biochemical Markers of Chemical Stress in Fish

3.7.1. Endocrine Disruption Biomarker

Vitellogenin (VTG) is a glycolipophosphoprotein synthesized by the liver of fish. It plays a key role in the formation of the yolk in female fish. VTG serves as the precursor of yolk proteins, and it provides essential nutrients to the eggs and helps the embryos develop. In sexually mature females, VTG synthesis is naturally regulated by endogenous estrogens, particularly 17β-estradiol. When estrogen levels in the fish are elevated, VTG production increases to meet the needs of egg development (Emmersen et al., 1979).

Although the VTG gene is present in both male and female individuals, under normal circumstances, the VTG gene is usually silenced in males. However, exposure to exogenous estrogen compounds (e.g., natural estrogens: estrone, estradiol; Synthetic steroid: ethinylestradiol; Industrial chemicals: bisphenol A, alkyl phenols, phthalates; and agrochemicals: pesticides, polychlorinated biphenyls, heavy metals) can induce abnormal synthesis of VTG in males and immature females. This phenomenon becomes a key marker of environmental hormone interference, making VTG a useful biomarker for evaluating estrogen activity in environmental pollution. At present, the application of VTG in fish is widely studied (Jackson et al., 1977; Matozzo et al., 2008).

Municipal and industrial wastewater, agricultural runoff and animal husbandry waste are the main sources of exogenous estrogens in aquatic ecosystems. These substances can exist in the water environment for a long time and accumulate in organisms, interfering with the endocrine function

of multiple populations. With the widespread existence of exogenous environmental endocrine disruptors (EDCs), VTG monitoring has become an important method of environmental pollution assessment. (Gagné & Blaise, 1998; Sumpter, 1995).

3.7.2. Neurological Biomarker

Acetylcholinesterase (AChE) is a key enzyme, mainly found in the nervous system, responsible for breaking down the neurotransmitter acetylcholine (ACh). During neural signalling, ACh is released at the synapses between neurons, transmitting signals. The function of AChE is to rapidly hydrolyze ACh, terminate signal transmission, and ensure the normal function of the nervous system (Pfeifer et al., 2005).

The activity of AChE is essential for the balance of the nervous system. Too high or too low AChE activity will lead to neurological dysfunction. For example, certain toxins and chemicals (such as organophosphorus and carbamate insecticides) cause ACh accumulation by inhibiting AChE activity, causing overstimulation of the nervous system, and even a fatal outcome. Therefore, determination of AChE activity is often used to assess the toxicity of these chemicals. In addition, AChE activity has also been used as a biomarker of environmental pollution. Changes in AChE activity in aquatic organisms (such as fish) can reflect the presence and influence of pollutants in the environment (such as heavy metals and pesticides) (Galgani & Bocquené, 2000).

3.7.3. Antioxidant Enzymes

3.7.3.1. Catalase (CAT)

Oxidative stress is a common toxicity response that can cause tissue injury and threaten an organism's health. In respiration, uncoupled high-energy electrons released by mitochondria and chloroplasts are one of the main sources of intracellular reactive oxygen species (ROS). ROS include superoxide anions, hydrogen peroxide, hydroxyl radicals and so on. These substances are highly oxidizing and attack biological molecules such as proteins, lipids, and DNA. Many xenobiotics may lead to more uncoupled electrons leaking from mitochondria, producing ROS,

and further causing oxidative damage (Gagné, 2014). Antioxidants can delay or prevent oxidation even at low concentrations (Godin et al., 2010).

CAT is an enzyme belonging to the antioxidant defence system and holds significant importance. It serves a crucial function in protecting organisms against the harmful effects of ROS through hydrolysis and mitigation. As a catalyst, CAT is responsible for breaking down hydrogen peroxide (H_2O_2) into water (H_2O) and oxygen (O_2), thereby preventing the accumulation of H_2O_2, which is a type of ROS. The ROS can harm cells because it can potentially cause oxidative damage to genetic material, lipids, and other cell components (Gagne & Francois, 2014; Imlay, 2008).

Here is the chemical equation of how CAT breaks the hydrogen peroxide:

$$2\ H_2O_2 \rightarrow 2\ H_2O + O_2$$

3.7.3.2. Superoxide dismutase (SOD)

SOD is an antioxidant enzyme that is in both the mitochondria and cytoplasm of cells. These enzymes are responsible for dismutating superoxide anions, converting them into hydrogen peroxide. This step is crucial because superoxide anions are highly reactive and can cause damage to cells if not neutralised. After the hydrogen peroxide is produced, CAT takes over and breaks it down into water and oxygen, which are harmless to the cell (Deeth, 2021; Gagne & Francois, 2014a).

3.7.3.3. Glutathione reductase (GR)

GR is a flavoprotein that catalyses the reduction of oxidised glutathione (GSSG) to its reduced form (GSH) using NADPH as a cofactor. This process is essential for regenerating GSH, which is a critical molecule in cellular antioxidant defence. GR plays a key role in detoxifying peroxides and neutralizing free radicals, particularly within mitochondria, where oxidative stress is often high. By maintaining adequate levels of GSH, GR helps protect cells from oxidative damage and supports the overall balance of the redox potential. Its function is vital for cellular health and for defending against damage caused by reactive oxygen species (ROS). As a result, GR is considered

one of the most important antioxidants in cells (Meister, 1988; Serracarbassa, 2019).

3.7.3.4. Glutathione peroxidase (GPx)

GPx is an enzyme composed of three amino acids—glutamic acid, cysteine, and glycine—forming a tripeptide structure. It plays a crucial role in cellular defence by converting hydrogen peroxide (H_2O_2), a harmful byproduct of metabolism, into harmless molecular oxygen and water. During this process, glutathione (GSH) is oxidised into glutathione disulfide (GSSG). To maintain the balance of reduced glutathione in the cell, GR then reduces GSSG back into GSH using NADPH as a cofactor (Koju et al., 2019). This continuous cycle ensures that GPx can effectively prevent oxidative damage to cells by neutralising reactive oxygen species (ROS) and protecting cellular components from harm (Torzewski et al., 2007).

3.7.4. Markers of oxidative cell damage

3.7.4.1. Lactate dehydrogenase (LDH)

LDH is an important enzyme involved in anaerobic glycolysis, where it primarily catalyses the redox reaction between lactic acid and pyruvate. The conversion process can be summarized as follows (Alegre et al., 2015):

Pyruvate + NADH (reduced Nicotinamide Adenine Dinucleotide) + H ↔ Lactate + NAD^+ (oxidized Nicotinamide Adenine Dinucleotide)

LDH is found in the cytoplasm of cells and plays a key role in energy production, especially under low-oxygen conditions. However, its activity changes significantly during oxidative stress. When oxidative stress occurs, lipid peroxidation damages the cell membrane, compromising the integrity of cells. As a result, LDH leaks from the cells into the surrounding environment, including the bloodstream, leading to elevated extracellular LDH activity. This increase in LDH levels serves as a marker of cellular damage caused by oxidative stress. Monitoring LDH levels is therefore a useful indicator for assessing the extent of cell injury and the impact of oxidative stress on tissues (Jovanović et al., 2010).

3.7.4.2. Lipid peroxidation (LPO)

Lipids play a vital role in living organisms, serving as fundamental components of cell membranes and acting as efficient energy storage molecules. Beyond these primary functions, lipids are also involved in crucial biological processes such as cell signalling, insulation, and maintaining structural integrity. However, they are highly susceptible to damage caused by high levels of free radicals or reactive oxygen species (ROS) (Ayala et al., 2014).

Lipid peroxidation is a damaging process triggered by ROS, where lipid molecules undergo a chain reaction of oxidation. This process leads to the formation of lipid peroxides, which contain unstable oxygen-oxygen (O-O) bonds. The reaction typically starts with the attack of ROS on polyunsaturated fatty acids, initiating a cascade of reactions that continuously oxidise lipid molecules. Over time, this chain reaction generates various reactive intermediates that further amplify oxidative stress and contribute to significant cellular damage. The disruption caused by lipid peroxidation can impair membrane structure and function, leading to loss of cell viability and overall tissue damage (Cai, 2005).

LPO level can be measured according to the TBARS assay, it is based on the reaction between malondialdehyde (MDA), a pink by-product of lipid peroxidation resulting from the peroxidation of polyunsaturated fatty acids, and thiobarbituric acid (TBA) under acidic conditions. The assay is conducted under acidic conditions because an acidic environment is essential for the efficient progression of the reaction and generation of a spectrophotometrically measurable coloured complex (Wills, 1987). The heating process promotes the reaction so that malondialdehyde fully reacts with thiobarbituric acid.

3.7.4.3. Acetylcholinesterase (AChE)

As a neurotransmitter-regulating enzyme, AChE plays a critical role in the nervous system. It catalyses the hydrolysis of acetylcholine, a neurotransmitter, breaking it down into choline and acetic acid. This process is essential for terminating nerve impulses at synapses and ensuring proper communication between neurons. AChE is predominantly found in neuromuscular

junctions and cholinergic synapses within the central nervous system. It is also present in red blood cell membranes, where it performs similar regulatory functions. Because of its sensitivity to certain chemicals, AChE measurement serves as an early biomarker for detecting the effects of organophosphorus and carbamate compounds on the nervous system. These compounds, often found in pesticides and industrial chemicals, can inhibit AChE activity, leading to an accumulation of acetylcholine and potential disruption of normal nerve function (Lionetto et al., 2013).

3.7.4.4. DNA strand breaks (DNA sb)

DNA damage serves as an indicator of the impact of environmental stress and endogenous factors on cells. For instance, ROS can harm critical cellular components and subsequently lead to DNA damage. This type of damage is considered a reliable and accurate biomarker for assessing the effects of various risks on organisms. Different mechanisms can induce DNA damage, and oxidative stress is one of the primary causes. It can result in strand breaks, where the DNA double helix is disrupted, compromising its integrity. These breaks not only reflect the extent of oxidative stress but also highlight potential risks to cell function and survival, making DNA damage a valuable tool for evaluating environmental and toxicological effects (Nikitaki et al., 2015).

3.7.5. Xenobiotic metabolizing enzymes

3.7.5.1.Ethoxyresorufin-o-deethylase (EROD)

EROD has been widely recognized as an environmental biomarker, particularly in fish, for monitoring exposure to pollutants (Ducrotoy, 2024; Whyte et al., 2000). EROD is an enzyme involved in both phase I and phase II biotransformation processes, where it plays a key role in metabolizing exogenous substances such as polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), and dioxins. EROD activity is regulated by the aryl hydrocarbon receptor (AhR) and is part of the P450-dependent monooxygenase system, specifically within the CYP1A enzyme family. Cytochrome P450 CYP1A is directly responsible for catalysing EROD, making its activity closely linked to the functional status of CYP1A. Changes in EROD activity provide a reliable measure of CYP1A induction and can reflect the

organism's response to environmental toxicants. This makes EROD a valuable tool for assessing the impact of chemical pollutants on aquatic ecosystems (Jönsson et al., 2006; Zamaratskaia & Zlabek, 2009).

3.7.5.2. Glutathione s-transferase (GST)

Detoxification refers to the metabolic process wherein detoxification enzymes play a role in eliminating exogenous toxic compounds such as carcinogens, pesticides, herbicides, oxidative stress products, and pharmaceuticals. The detoxification process consists of two phases: phase I and phase II. Toxic substances, in the form of metabolites after phase I, can be dissolved and excreted by detoxification enzymes (such as glucuronosyltransferase and GST enzymes) in phase II (M. Silva & Da Gloria Carvalho, 2018).

GST is a homologous or heterodimer protein that plays a crucial role in phase II metabolization with the participation of reduced glutathione (GSH). GST can defend the cell against potential damage. Homologous GST can form dimers with itself. Heterodimeric GST can interact with different proteins during detoxification. GST acts as a catalyst for the binding of GSH with various toxic compounds. These compounds can be naturally present in the body or come from external sources. In phase II, this catalytic binding forms highly soluble complexes that can be easily eliminated (Dasari et al., 2018; M. Silva & Da Gloria Carvalho, 2018; Vernouillet et al., 2010).

Habig and his team used microplates to measure GST activity by following the conjugation process of 1-chloro-2,4-dinitrobenzene (CDNB) in the presence of the co-substrate GSH, catalysed by GST. The hydrolysis rate of CDNB is used to determine the activity of GST (Habig et al., 1974; Summer & Wiebel, 1981).

4. MATERIALS AND METHODS

4.1.Fish maintenance

We have used the fish housing systems in the Department of Environmental Toxicology at the Hungarian University of Agriculture and Life Sciences (Gödöllő, Hungary) for fish maintenance. As for the common carp, the juveniles were kept in an individually designed recirculating system. Each fish recirculating tank is 10 m^3 of water. The quality parameters of water were constantly maintained, (22 ± 2 °C, pH 7.8 ± 0.2, redox potential, 230 ± 2 mV, the level of dissolved O_2, 6.8 ± 1 mg/L) and the ratio of light and dark period was set to 14 h:10 h. The carp were fed 10 g/kg body weight AquaGarant Aquastart (Aqua Garant, Pöchlarn, Austria) pelleted feed (1.2–1.5 mm) twice a day.

As for zebrafish, AB wildtype was selected as the exam type. As mentioned above, the fish-maintained system (Tecniplast ZebTec (Buguggiate, Italy) recirculating zebrafish housing system) was also in the Department of Environmental Toxicology. The water quality parameters were recorded constantly too (25 ± 0.5 °C; pH 7.0 ± 0.2; conductivity 500 ± 50 µS; alkalinity < MDL, 0 mM carbonate ion (CO_3^{2-}), 0.4 mM bicarbonate ion (HCO_3^{2-}); hardness < 0.5° German degrees of hardness(dH); Dissolved Oxygen (DO) > 90%; system water). The ratio of light and dark time was 14 h:10 h. The fish were fed twice a day with ZEBRAFEED (Sparos, 400–600 µm) and two times a week with brine shrimp (Ocean Nutrition > 230,000 NPG).

4.2. Experimental Design

The two fish experiments were performed roughly in the same way, with some minor differences. In both assessments, fish were exposed for 28 days to test chemicals. The CBZ exposure concentrations were identical (0, 1, 5, 50, or 100 µg/L). The lowest (1 µg/L) and highest (100 µg/L) CBZ concentrations applied were based on the paper by da Silva Santos et al. (2018). Exposures were run in triplicate (three tanks for every exposure concentration), and in all tanks, 15 fish were

placed. After 7, 14, and 28 days five fish from every exposure tank (15 fish for every concentration) was selected and sacrificed after being given an anaesthetic overdose (0.04% MS-222, also known as tricaine methane sulphonate, obtained from Sigma-Aldrich, Darmstadt, Germany) and dissected for brain, liver, muscle, and gonad tissues. The collected samples were placed in round-bottom Eppendorf tubes and stored at −80 °C until biochemical measurements.

In tests with common carp, juvenile fish were used with randomly distributed males and females (weight 7.37 ± 1.35 g). Each tank contained a 50 L test solution. During exposure, fish were fed a 10 g/kg body weight AquaGarant Aquastart (Aqua Garant, Pöchlarn, Austria) pelleted feed (1.2–1.5 mm) twice daily. The test media was completely renewed every three days, and the water quality parameters were recorded as described in the "Fish Maintenance" section. To ensure agreement between nominal and actual compound concentrations in the solution, water samples were collected from the test aquaria at 1 hour and 36 hours after renewing the solutions. To ensure the stability of the exposure concentrations described below, the water samples were analysed during the experimental period by Liquid Chromatography with tandem mass spectrometry (LC–MS/MS). The measured average concentration of CBZ in the water samples remained within ± 20% of the planned nominal concentration throughout the experiment.

As for the 28-day exposure experiments of zebrafish, adult fish aged 9-12 months were randomly assigned to 15 experimental tanks, each containing 3L of test solution (containing CBZ and P4, respectively or merged in ratios presented in the Experimental Design section), and there were three replicates per treatment, with 15 fish per replicate. Fish were fed once daily with a quantity of ZEBRAFEED (Sparos, 400–600 μm) corresponding to 2% of the fish weight in the aquarium. Nominal concentrations of CBZ and P4 were set at 0, 1, 5, 50, and 100 μg/L, respectively. These concentration ranges were carefully selected to align with recent studies investigating the environmental and toxicological effects of these compounds (da Silva Santos et al., 2018). Corresponding concentrations of P4 were derived from the study by Liang et al. (2015). These studies helped guide the selection of a range of concentrations that include both levels commonly found in the environment and higher levels to test stronger effects. Unlike previous studies, which

often focused on the individual effects of CBZ or P4, our experiment was designed to assess their joint effects. By evaluating the combined impact of CBZ and P4 across this concentration range, the experiment aims to provide a more comprehensive understanding of their potential risks, particularly in aquatic ecosystems where such mixtures are commonly found. To analyse the non-linear response produced by the joint effect of CBZ and P4, the EC_{50} value was calculated based on VTG results. VTG was selected due to the expected effect of the test chemicals. Different concentrations of the mixtures of CBZ and P4 were set based on their toxicity units (TU; 1 TU = concentration of a compound in the mixture per the compound's EC_{50}), and mixtures were established to result the sum of 1 TU (CBZ: P4 ratios were: 0.75 TU:0.25 TU (MIX1), 0.5 TU:0.5 TU (MIX2), and 0.25 TU:0.75 TU (MIX3)). The composition of the mixtures is presented in Table 1. As in the previous setting, 45 fish (3 replicates) were placed in each mixture solution (0, MIX1, MIX2, MIX3). The toxic effect was expected to be 50% in all the mixtures. According to the paper by Berenbaum (1985), the non-linear effects (synergistic or antagonistic) of the mixtures were readily identifiable in this setup, moreover, the effect of different concentration ratios could be observed. The test solutions were completely renewed every three days, and the water quality parameters that were mentioned in the previous section were recorded constantly. Also, the water samples were collected from the test solutions at 1 and 36 h, respectively, after renewing the test medium. Water samples were analysed by the LC-MS/MS during the exposure period to identify the nominal and actual test concentrations, and the water samples' mean concentrations of CBZ and P4 consistently stayed within 20% of the intended concentrations.

T**able 1**. The preset TU ratio and the composition of mixtures applied in the experiment.

	CBZ		P4		STU
	TU	µg/L	TU	ng/L	
MIX1	0.75	11.5	0.25	4.375	1
MIX2	0.5	5.75	0.5	8.75	1
MIX3	0.25	2.875	0.75	17.5	1

From each test solution replicate, 5 zebrafish were sacrificed, and the brain, liver, gonad and

intestine of each fish were carefully dissected and collected. These tissues were then placed in microtubes and immediately stored at −80 °C to preserve them for later biochemical analyses.

4.3. Biomarker Determinations

Prior biochemical assessments of the different tissues collected from both species were homogenised using a small bead mill (TissueLyser LT, Qiagen, Germantown, MD, USA). Correspondent enzymatic activities were evaluated in triplicate (technical replicate) with a Thermo Varioskan™ LUX multimode microplate reader at 25 °C (Thermo Fisher Scientific, Waltham, MA, USA). The intestines of fish and approximately the half of the liver tissues were homogenized in a general buffer consisting of 25 mM Hepes-NaOH, 130 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, with a pH of 7.4, at a weight-to-volume ratio of 1:5. Then those subsamples of the homogenates were frozen at -80 °C for analyses of LPO, DNAsb, EROD, and VTG (intestines) . After, the remaining liver tissues were homogenised in 100 mM phosphate buffer with a pH of 7.4, which contains 100 mM KCl, 1 mM EDTA, dithiothreitol (DTT), 0.5 M sucrose, and 40 µg/mL aprotinin. Then, they were centrifuged at 12,000× *g* for 30 minutes at 4 °C. The supernatants (Supernatant after 12,000×g centrifugation(S12)) of the homogenate were collected and aliquots were stored at -80 °C for further analyses of GR, GPx, GST, CAT, LDH, and SOD. As for the brain tissues, they were homogenised in 0.1 M phosphate buffer (pH = 7.2) and the weight-to-volume ratio was 1:10. Subsequently, they were centrifuged at 6000× *g* for 3 minutes at 4 °C. According to the Bradford method (Bradford, 1976), the protein concentration of the samples was determined in triplicate with bovine serum albumin as a standard by the microplate reader. The absorbance of the samples was recorded at 595 nm after an incubation period of 15 min.

According to the thiobarbituric acid reactive substance (TBARS) assay method described by Wills (1987), the LPO process can be a measure of the amount of malonaldehyde (MDA) produced through lipid oxidation in tissue homogenates. The procedure began with the collection of 150 µL of raw tissue homogenate, which was then mixed with 300 µL of a reagent containing 10% trichloroacetic acid and 1 mM $FeSO_4$, along with 150 µL of 0.67% thiobarbituric acid. The mixture

was then heated to 80°C for 10 minutes to allow the reaction to occur. Following the reaction, the produced sediment was then separated by centrifugation (10,000 ×*g* centrifugation for 10 seconds). The resulting supernatant, which contained the reaction products, was then collected and used for subsequent measurements to evaluate LPO levels by measuring fluorescence of the samples in black microplates at excitation: 516 nm; emission: 600 nm. Results were expressed as µmoles of thiobarbituric acid reactants (TBARS) per milligramme of homogenate protein.

AChE activity was determined based on the method of Ellman et al. (1961). The 96-well clear microplates were filled with three replicates of 50 µL of homogenate supernatant for each sample, and 250 µL of the reaction solution was added to each well of the plate. The reaction solution consisted of 0.075 M acetylthiocholine iodide and 10 mM 5,5 dithio-bis (2-nitrobenzoic acid) as a colour agent in phosphate buffer (0.1 M, pH = 7.2). In the blank controls, the sample was replaced with phosphate buffer. Meanwhile, electric eel acetylcholinesterase was used as a positive control. The absorbance was measured at 414 nm every minute for 15 minutes total. The enzyme activity was calculated according to the slope of the absorbance curve, and the result was expressed in units (U) corresponding to the protein content per milligram. 1 unit (U) was 1 µmol substrate hydrolysed per minute.

LDH activity was measured according to the methodology described by Vassault (1983), and adapted to the microplate by Diamantino et al. (2001). A 96-well microplate was filled with 25 µl samples, 125 µL of reduced nicotinamide adenine dinucleotide solution (NADH) with a concentration of 300 µM, and 20 µL of pyruvate solution with a concentration of 4.5 µM were successively added to the microwells. The readings were then taken at a wavelength of 340 nm. The data were read every 40 seconds over 5 minutes to observe the decrease of absorbance due to oxidation of NADH. LDH activity was expressed in unit (U) per mg of protein. One unit (U) was defined as 1 µM of NADPH hydrolysed per minute. The activity of LDH can be calculated by recording the absorbance changes.

To quantify the DNAsb, the alkaline precipitation assay from Olive (1988) was used in this

experiment. Firstly, 25 µL raw tissue homogenates were mixed with 200 µL of a specific reaction solution. The reaction solution contained 2% sodium dodecyl sulfate (SDS), 10 mM ethylenediamine tetraacetic acid (EDTA), 10 mM Trimethylol aminomethane base (Tris-base), and 40 mM NaOH. Then the mixed solution was shaken well for 1 minute. Then 200 µL of 0.12 M KCl solution was added, heated at 60 °C for 10 minutes, and reversed mixed at 4 °C for 30 minutes cooling down. Subsequent, the mixture was centrifuged at 4 °C at a centrifugal force of 8000×*g* for 5 minutes. Then 50 µL was added from the centrifuged supernatant to 150 µL of Hoechst dye (1 µg mL^{-1}, in buffer containing 0.4 M NaCl, 4 mM sodium cholate and 0.1 M Tris-acetate, pH 8.5–9). Mixed them well on a flat shaker for 5 minutes to fully combine the dye with the ingredients in the supernatant. Fluorescence intensity was measured using 360 nm excitation / 450 nm emission wavelengths. The sample blanks contained the same constituents as the above experimental steps, but the tissue homogenate was replaced with 25 µL Hepes buffer. DNA calibration was performed using the salmon sperm DNA standard. The result was expressed as µg DNA_sb mg^{-1} protein (the number of µg of DNA strand breaks per mg of protein).

To determine the level of VTG, the alkali-labile phosphate (ALP) method developed by Blaise et al. (1999) was applied. First, 200 µL of the sample homogenate was mixed with 54 µL of acetone, which gives a final acetone concentration of 35%. After mixing for 10 minutes, the samples were centrifuged at 10000×*g* for 5 minutes. After centrifugation, the remaining precipitated particles were dissolved in 50 µL of 1M NaOH and mixed at 60°C for 30 minutes. Then, total phosphate was determined by the colorimetric phosphate-molybdenum method developed by Stanton (1968). The steps were to add 125 µL of H_2O, 5 µL of 100% trichloroacetic acid (TCA), 25 µL of molybdate reactive and 25 µL of 1% ascorbate to the 20 µL sample, mixing for 10 minutes, and reading the absorbance at 815nm and 444nm. In the experiment, the rainbow trout VTG was used for calibration, and 1M NaOH equal samples were used as blank controls. Ultimately, VTG levels were expressed as µmoles of ALP per mg of protein.

The measurement of CAT activity was performed in triplicate based on Aebi's method (Aebi, 1984). 50 mM H_2O_2 ($\varepsilon = -0.0436\ mM^{-1}\ cm^{-1}$) solution was added to 50 mM phosphate buffer (pH 7.8).

To this solution, 10 µL of tissue supernatant (S12) was added. Then, the absorbance change of the solution was recorded continuously at 240 nm every 10 seconds for a duration of 1 minute. The results were expressed as U/mg protein. One unit of CAT was defined as the amount of CAT capable of catalysing the breakdown of 1 mmol of H_2O_2 in one minute.

The total SOD activity was measured according to the xanthine oxidase/cytochrome c method proposed by Crapo et al. (1978). The reaction of xanthine oxidase with hypoxanthine produced superoxide anions. The superoxide anions can react with cytochrome c, reducing cytochrome c and this reduction reaction can be measured by recording the absorbance change at a wavelength of 550nm. While the main function of SOD is to catalyze the disproportionation of superoxide anions and convert them into H_2O_2 and O_2. therefore, the degree of reaction between superoxide anions and cytochrome c was influenced by SOD. The reaction mixture contained 46.5 mM KH_2PO_4/K_2HPO_4 (pH = 8.6), 0.1 mM EDTA, 195 mM hypoxanthine, 16 mM cytochrome c, and 2.5 mU xanthine oxidase. Then, the enzyme activity of SOD was calculated by measuring the slope of the recorded absorbance curve.

GR activity was measured according to Carlberg & Mannervik (1975a). In the reaction system, the relevant reaction of GR can lead to the consumption of NADPH, meanwhile, NADPH can be recorded at 340 nm wavelength. Therefore, GR activity can be measured by monitoring the reduction of NADPH (reduced nicotinamide adenine dinucleotide phosphate) at 340 nm for 1 minute. So, the decrease in absorbance means a decrease in NADPH, and the GR activity can be inferred. The reaction medium contained 100 mM phosphate buffer (pH = 7.4), 0.1 mM NADPH and 30 µL of the prepared supernatant (S12). GR activity was expressed as U per mg of protein (one U corresponding to 1 µM NADPH hydrolysed/min).

GPx activity was measured according to the method proposed by Paglia & Valentine (1967), modified by Lawrence and Burk (Lawrence & Burk, 1976), and further adapted for 96-well microplates (Faria et al., 2009). The reaction mixture contained 30µL sample (S12), 100 mM phosphate buffer (pH 7.5), 2 mM GSH, 2 U glutathione reductase, 0.12 mM NADPH, sodium

azide (0.5 mM), 0.2 mM H_2O_2 (for Selenium dependent GP_X activity) or 3 mM cumene hydroperoxide (CHP) (for total GPX activity). GP_X activity was measured by monitoring the decrease in NADPH concentration at 340nm wavelength. During a reaction involving GP_X, reduced glutathione (GSH) was oxidised to oxidised glutathione (GSSG), using H_2O_2 (Se-dependent activity), or cumene hydroperoxide (total GPX) as substrate. GPx activity was expressed as U per mg of protein. (a U corresponding to 1 μM NADPH hydrolysed/min).

The GST activity was measured using the method proposed by Habig et al. (1974) and adapted for use in the microplate. A 100 M glutathione (GSH) solution in phosphate buffer (pH = 6.5) was prepared. At the same time, a solution of 60 mM 1-chloro-2,4-dinitrobenzene (CDNB, ε = 9.6 mM−1 cm−1) in ethanol was prepared just before the assay. The reaction mixture consisted of phosphate buffer, GSH solution, and CDNB solution in a ratio of 4.95 mL (phosphate buffer):0.9 mL (GSH):0.15 mL (CDNB). Then, in the microplate, 0.2 mL of the reaction mixture was added to 0.1 mL of the sample (S12), which contained GST. Following, GST activity was immediately detected at a wavelength of 340 nm at intervals of 20 seconds for 5 minutes. GST from the equine liver was used as a positive control. During the reaction process, the absorbance of the reaction mixture at 340 nm changed, followed by the substrate produced by GSH and CDNB. The enzyme activity of GST was calculated by the slope of the absorbance curve because the slope of the absorbance curve reflects the reaction rate. The result was expressed in units (U) per mg of protein content, where 1 unit (U) is defined as the amount of enzyme that can hydrolyse 1μmol of substrate per minute.

EROD activity was determined according to the method described by Burke & Mayer (1974). First, the subsamples of tissue homogenates were centrifuged at 12,000× *g* at 4°C for 30 min to separate the components. 50 μl of the resulting supernatant was incubated in a reaction mixture of 150 μl at 30°C for 60 minutes. This reaction mixture contained 100 mM phosphate buffer (pH = 7.4), 100 μM reduced NADPH, and 10 μM 7-ethoxyresorufin. The reaction was initiated by the addition of NADPH, and when the reaction had been going on for 60 min, the reaction was terminated by adding 100 μL of 0.5 M NaOH. The product, 7-hydroxyresorufin, was detected using fluorometry

at an excitation wavelength of 520 nm and an emission wavelength of 590 nm. Calibration was performed with serial dilutions of 7-hydroxyresorufin. In the end, the results were expressed in picomoles (pM) of 7-hydroxyresorufin produced per mg of protein per minute.

4.4. Statistical Analysis

The software package OriginPro, version 2019 (OriginLab Corporation, Northampton, MA, USA) was used to do all the statistical data analysis. A two-way analysis of variance (ANOVA) was performed to assess the interactive effects of different CBZ concentrations and exposure duration on biochemical markers with exposure time (7, 14, and 28 days) and treatment (control, 1, 5, 50, and 100 μg/L) as categorical factors. The interactions between time and treatment were defined as categorical predictor factors, and the measured biomarkers were considered dependent variables. After the interaction between the time and treatment was determined, a one-way ANOVA was conducted to examine the effects of one main factor at a specific level of another main factor. To clarify the significant difference under a certain factor, a post-hoc Tukey test was used for multiple comparisons, and the significance level was set at $p < 0.05$. Before statistical analyses, all the raw data were diagnosed for normality of distribution and homogeneity of variance with the Kolmogorov-Smirnov test and Levene's test, respectively.

5. RESULTS

5.1. Biochemical alterations elicited by chronic CBZ exposure in Common carp

5.1.1. Endocrine Disruption Biomarker (VTG)

VTG levels increased in a concentration-dependent manner after the first 7 exposure days. There were significant ($p < 0.05$) increases in VTG levels at 50 and 100 µg/L after 7 days of exposure. After 14 days of exposure, the VTG level decreased and returned to a similar level as in the control fish, while no significant exposure dose dependency was observed. After 28 days of exposure, there were slight increases in VTG levels at 1 and 5 µg/L, followed by a decrease at 50 and 100 µg/L, but no significant ($p < 0.05$) differences were observed compared to the control group (Figure 4).

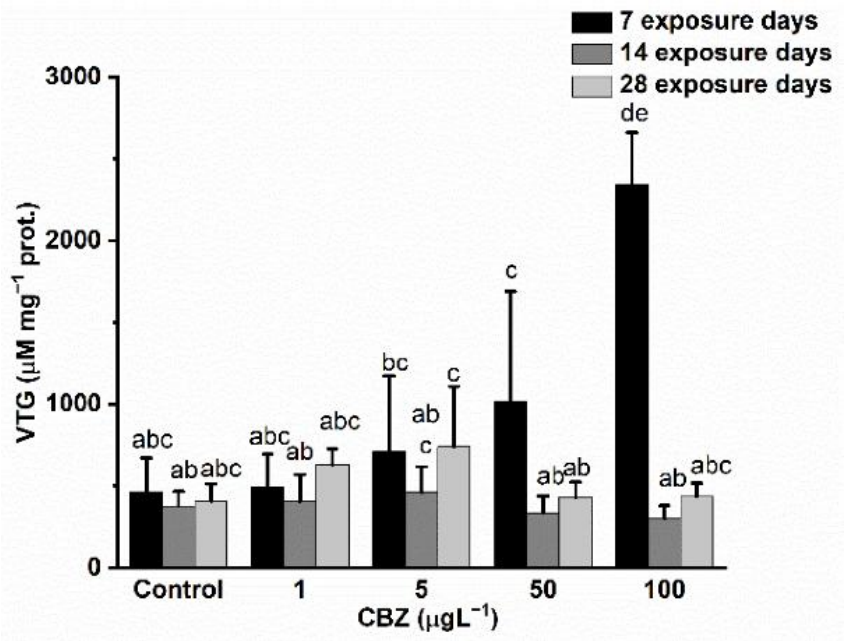


Figure 4: Changes in the VTG levels in the gonads of *Cyprinus carpio* exposed to CBZ for 7, 14, and 28 d. Data are expressed as mean ± standard deviation of three replicates (n = 3). Different letters designate significant differences at $p < 0.05$ after a two-way ANOVA followed by Tukey's post-hoc test.

5.1.2. Neurological Biomarker (AChE)

Compared with the control group, exposure to CBZ induced clear time-dependent changes in AChE activity in common carp. After seven days of exposure, AChE activity significantly decreased ($p < 0.05$) at 100 µg/L CBZ. (Figure 5). In contrast, AChE activity increased as the CBZ concentration increased after 14- and 28-day exposures. This increasing trend in AChE activity after the 14 days was statistically ($p < 0.05$) confirmed to be time-dependent and not concentration-dependent.

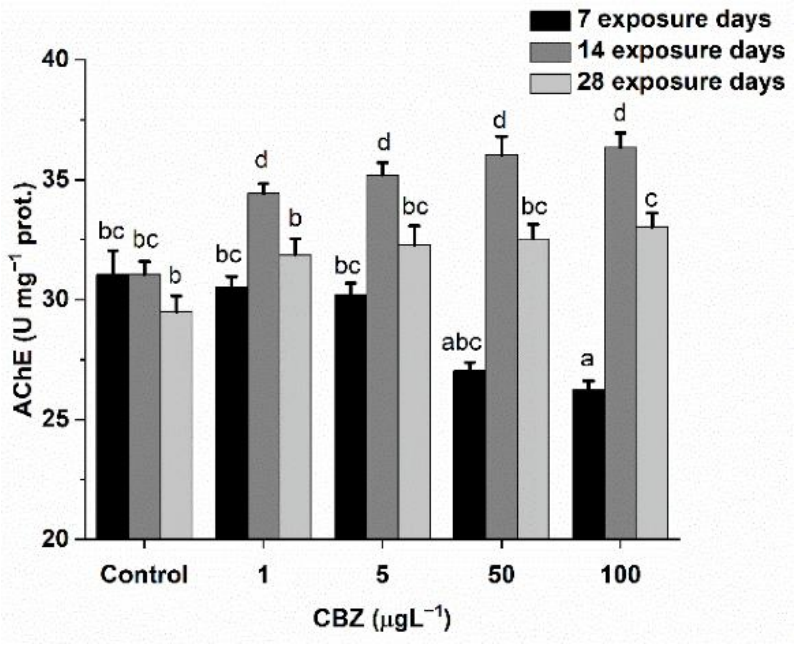


Figure 5: Changes in AChE activity in the brain of *Cyprinus carpio* exposed to CBZ for 7, 14, and 28 d. Data are expressed as mean ± standard deviation of three replicates (n = 3). Different letters designate significant differences at $p < 0.05$ after a two-way ANOVA followed by Tukey's post-hoc test

5.1.3. Damage Markers (DNAsb, LDH, LPO)

The DNAsb measured in liver samples in the first two weeks (7- and 14-day exposure) was slightly decreased compared to the control, a significant ($p < 0.05$) difference was only found at the concentration of 1 µg/L. By the end of 28 days of exposure, the DNAsb levels showed an

increasing trend with diminishing differences. However, there was a significant ($p < 0.05$) decrease at the concentration of 5 µg/L. Statistically, the level of DNAsb did not reveal any in time- or concentration-dependent patterns (Figure 6A).

LDH activity in the CBZ-exposed fish showed a significant ($p < 0.05$) decrease at 1 µg/L compared with the activity measured in the control group after 7 days of exposure. On the other hand, no significant differences were detected at the other concentrations (5, 50, and 100 µg/L) after 7 days of exposure to CBZ. After 14 days of exposure to CBZ, there were no notable changes of LDH activities at all test concentrations compared with the one measured in the control. Although there was an increasing trend of LDH activity after 28 days at every concentration except 100 µg/L, the changes were not statistically significant (Figure 6B).

The level of TBARS, representing the level of LPO, did not significantly change as compared to the controls up to the 28th day of exposure. A sharp increase was measured at 100 µg/L after 28 days with a significant difference ($p < 0.05$). Although a concentration-dependent pattern was visible for the groups measured after the 28-day exposure, statistically significant differences when compared to controls were only confirmed in the highest exposure concentration (100 µg/L CBZ) (Figure 6C).

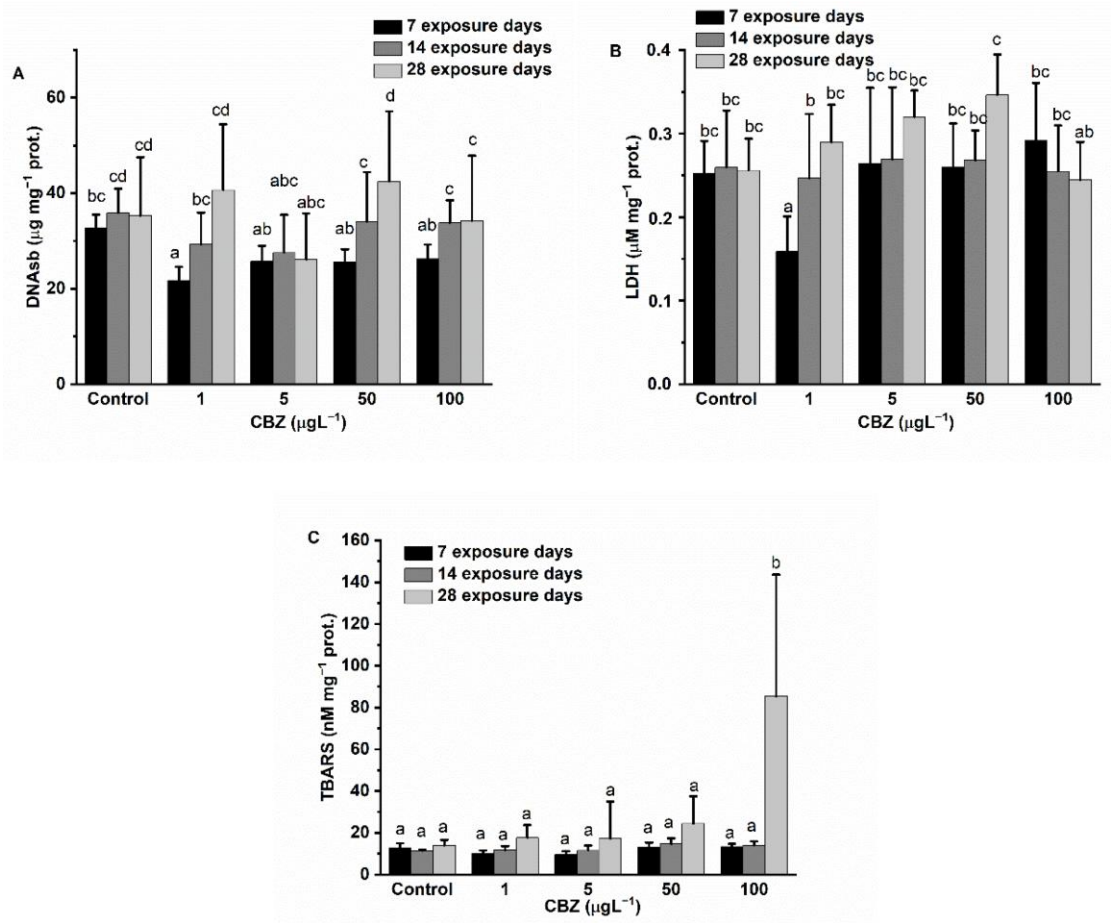


Figure 6: Changes in the DNA strand breaks (A), LDH activity (B), and level of lipid peroxidation (C) in the liver of *Cyprinus carpio* exposed to CBZ for 7, 14, and 28 d. Data are expressed as mean ± standard deviation of three replicates (n = 3). The same letters designate no significant differences at $p < 0.05$ after a two-way ANOVA followed by Tukey's post-hoc test.

5.1.4. Antioxidant Defence (CAT, SOD, GR)

The CAT activity slightly increased at 50 µg/L and sharply increased at 100 µg/L after 7 days of CBZ exposure, both with significant differences ($p < 0.05$). After 14 days, the CAT activity increased with the increasing concentration, while statistically significant differences only appeared in the 50 µg/L and 100 µg/L CBZ concentrations. After 28 days, a significant increase in CAT activity was measured at all CBZ exposure concentrations (1, 5, and 100 µg/L). According to the whole trends, CAT activity changes followed a time- and concentration-dependent pattern

during the 28-day CBZ exposure (Figure 7).

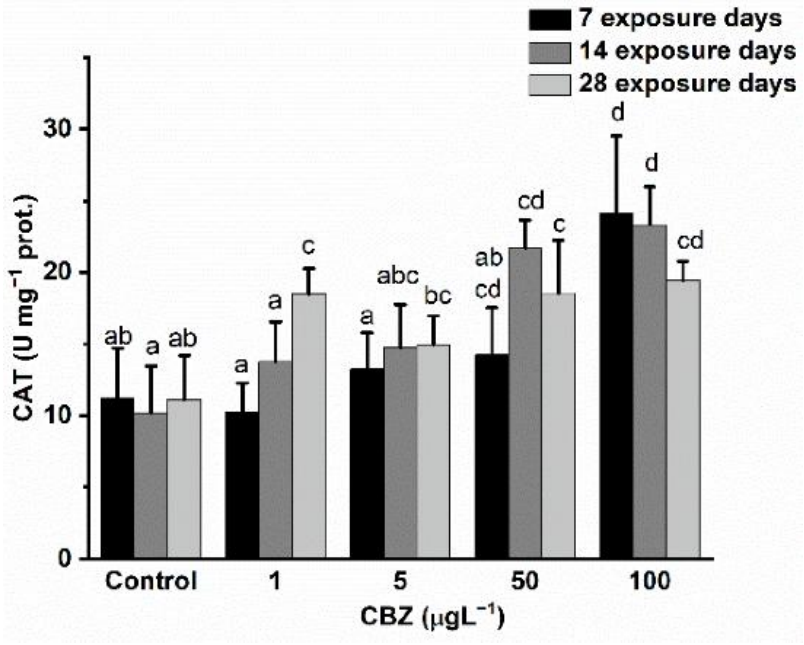


Figure 7: Changes in the CAT activity in the liver of *Cyprinus carpio* exposed to CBZ for 7, 14, and 28 d. Data are expressed as mean ± standard deviation of three replicates (n = 3). The same letters designate no significant differences at $p < 0.05$ after a two-way ANOVA followed by Tukey's post-hoc test.

Significant alterations in SOD activity were detected in carp following 7 days of exposure to 5, 50, and 100 µg/L CBZ ($p < 0.05$), manifested in a concentration-dependent increase. At 100 µg/L CBZ concentration exposure, SOD activity was significantly lower compared to the values measured at 5 and 50 µg/L CBZ concentration exposure, but still significantly higher than control values. After 14- and 28-day exposure in all groups treated with CBZ, decreases in SOD activity were detected as compared to control values, but those decreases were not significant ($p < 0.05$) (Figure 8).

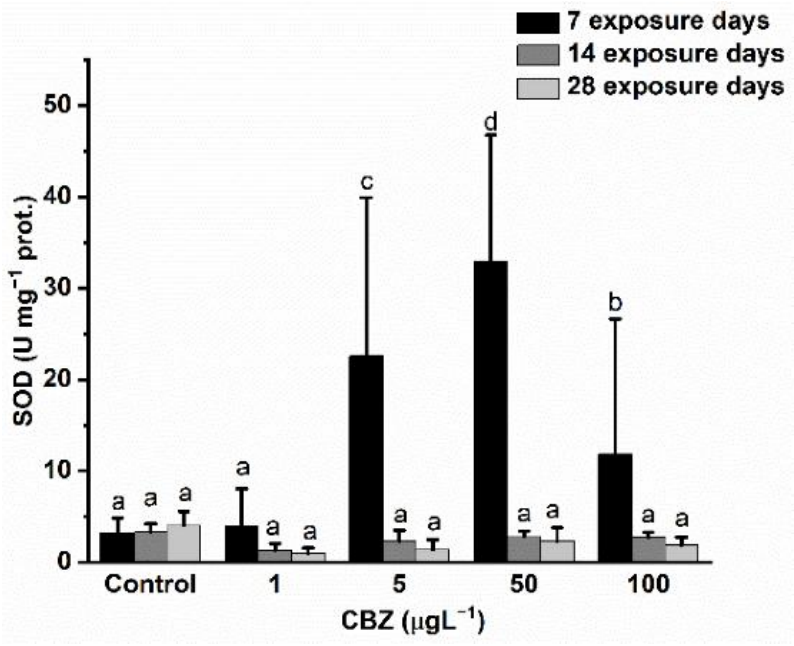


Figure 8: Changes in the SOD activity in the liver of *Cyprinus carpio* exposed to CBZ for 7, 14, and 28 d. Data are expressed as mean ± standard deviation of three replicates (n = 3). Different letters designate significant differences at $p < 0.05$ after a two-way ANOVA followed by Tukey's post-hoc test.

The GR activity changes exhibited a very similar response pattern to SOD activity. After 7 days, a significant ($p < 0.05$) and concentration-dependent increase was detected at 5 and 50 µg/L CBZ exposure. However, the GR activity at 100 µg/L was significantly lower compared to 5 and 50 µg/L CBZ concentration exposure, but higher than control values. However, the GR activity dropped to a level similar to that of the control group and remained depressed throughout the 14 and 28 days of exposure at all the CBZ concentrations applied, but the decrease in GR activity remained insignificant compared to the control levels (Figure 9).

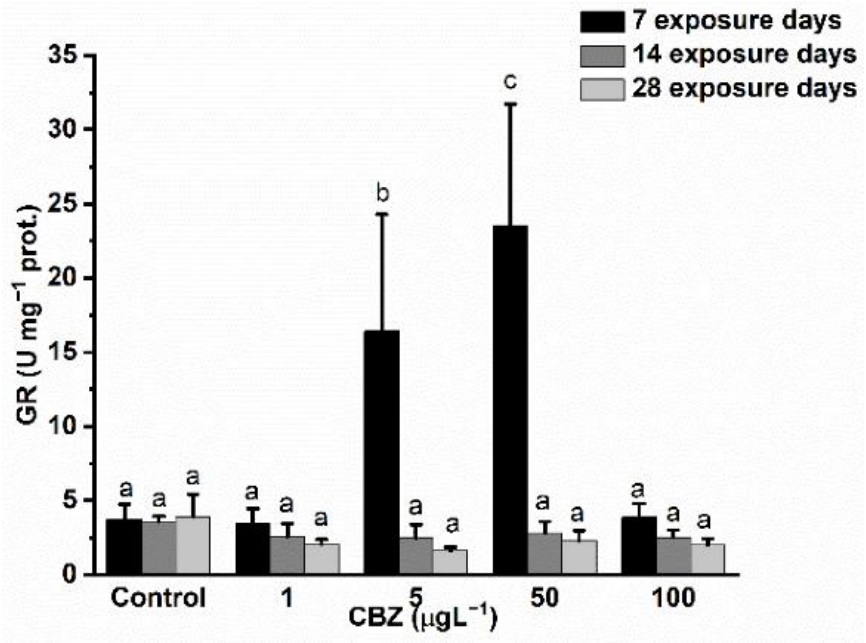


Figure 9: Changes in the GR activity in the liver of *Cyprinus carpio* subjected to CBZ for 7, 14, and 28 d. Data are expressed as mean ± standard deviation of three replicates (n = 3). Different letters designate significant differences at $p < 0.05$ after a two-way ANOVA followed by Tukey's post-hoc test.

5.1.5. Biotransformation Enzymes (EROD, GST)

After 7 days of exposure to CBZ, the hepatic EROD significantly increased as a function of exposure concentration (Fig. 10A). Following a slight, insignificant decrease at 1 µg/L exposure compared with the control group, EROD activity significantly increased along the tested concentrations, peaking at a concentration of 100 µg/L CBZ ($p < 0.05$). After 14 days, no significant differences in EROD activity were detected as compared to the control fish irrespective of CBZ. Then, after 28 days of CBZ exposure, a significant ($p < 0.05$) dose-dependent increase in EROD activity was measured in up to 50 µg/L CBZ exposure. EROD activity levels measured after 28 days of exposure to 100 µg/L CBZ appeared lower than those recorded in fish treated with 50 µg/L CBZ; however, this difference was not statistically significant ($p < 0.05$) (Figure 10A).

A slight increase in GST activity, as a function of exposure concentration, was apparent during the 28-day CBZ exposure; however, there was only one significant elevation in GST activity detected at the concentration of 100 µg/L after 28 days of exposure. (Figure 10B)

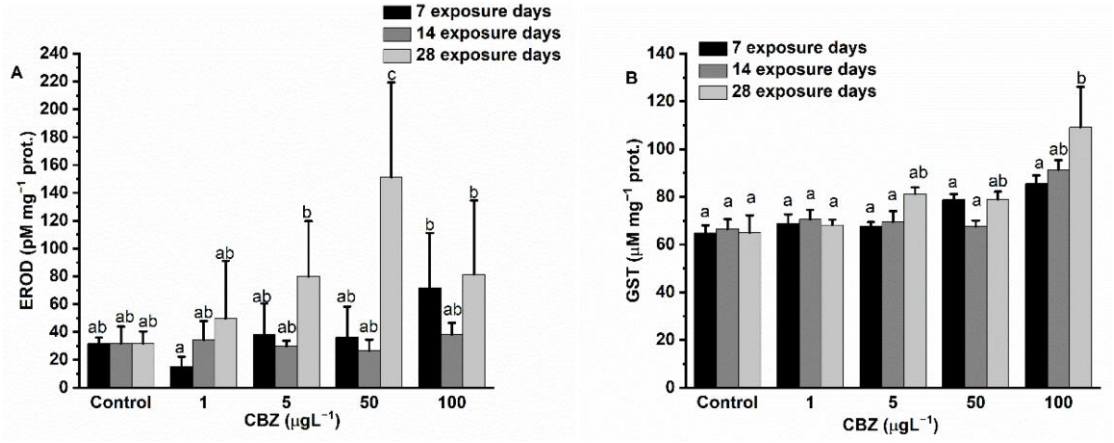


Figure 10: Effect of CBZ on the level of hepatic EROD (A), and hepatic GST (B) in the liver of *Cyprinus carpio*. Data are means ± S.D., n = 3. Columns sharing the same superscript letter indicate no significant differences after a two-way ANOVA followed by Tukey's post-hoc test ($p > 0.05$).

5.2. Biochemical alterations elicited by chronic CBZ, P4 and their exposure in Zebrafish

During the experimental assay, no zebrafish died, and no visible sublethal effects were detected (control, CBZ-, P4-, or mixture-exposed) at any of the tested conditions.

5.2.1. Endocrine Disruption Biomarker (VTG)

The results of VTG levels after exposure to the CBZ, P4, and their binary mixtures were displayed in Figures 11A, 11B, and 11C, respectively. Regarding the results of exposure to CBZ, VTG levels increased following a concentration-dependent pattern after 7 and 14 days; however, significant ($p < 0.05$) differences in VTG content in the samples were detected only in fish exposed to 100 μg/L of CBZ, both for 7- and 14-day exposure. After 28 days, VTG levels decreased in a concentration-dependent manner, but this decrease was not statistically significant ($p < 0.05$) at any exposure concentration (Figure 11A). Exposure to P4 caused a decreasing trend of VTG level for each test concentration compared with the control after 7 days, while no significant differences were shown. After 14 days, P4 also caused a decrease in the VTG content after exposure to all

applied concentrations (1, 5, 50, 100 ng/L) with no significant differences. After 28 days, all the applied concentrations caused a moderate decrease in the VTG levels, with statistically significant ($p < 0.05$) differences only observed at 50 and 100 ng/L of P4 (Figure 11B). Exposure to the binary mixtures did not significantly ($p < 0.05$) affect VTG levels after 7 days of exposure to MIX1, MIX2, or MIX3. However, after 14 days, the three mixtures caused a non-significant drop in VTG levels, and VTG concentrations decreased with growing proportions of P4 in mixtures compared to control group levels. After 28 days, in contrast, VTG levels followed an increasing pattern with the growing proportion of P4 and reached a significant ($p < 0.05$) increase in the case of MIX2 and MIX3 (Figure 11C).

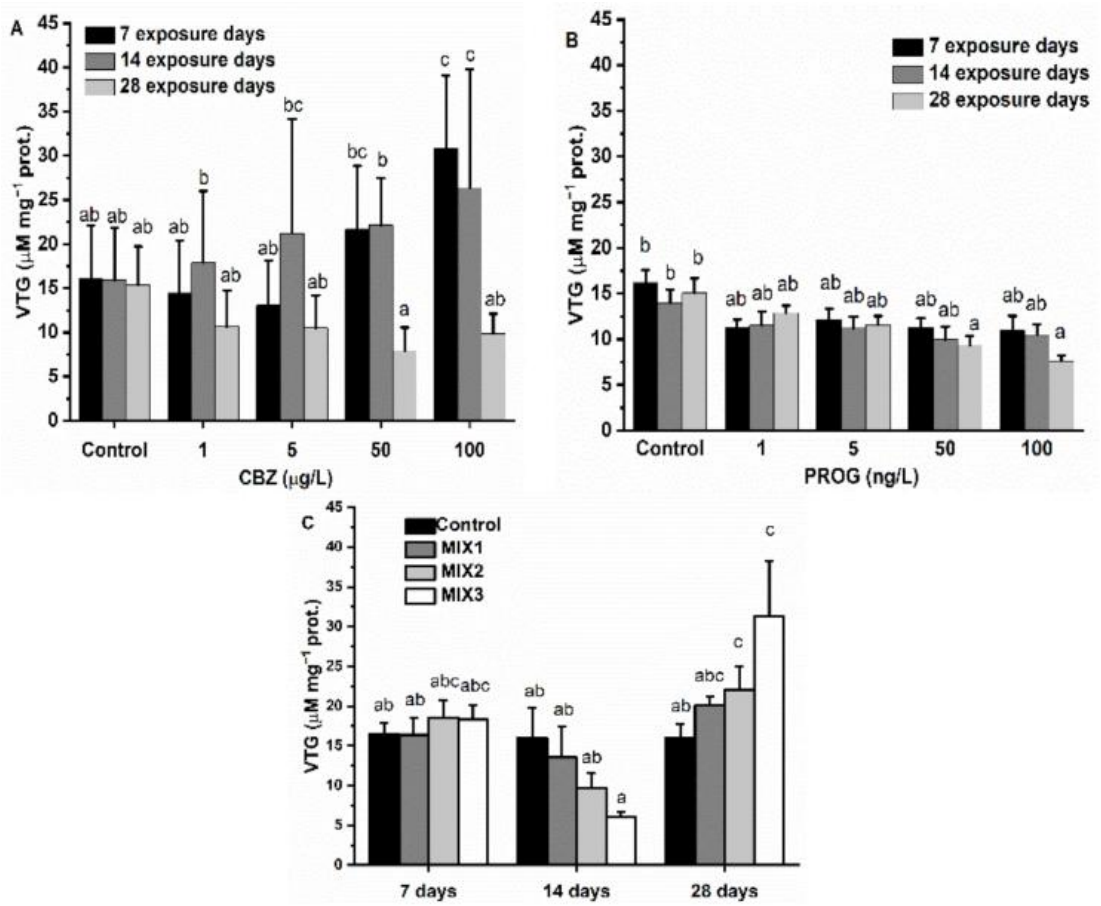
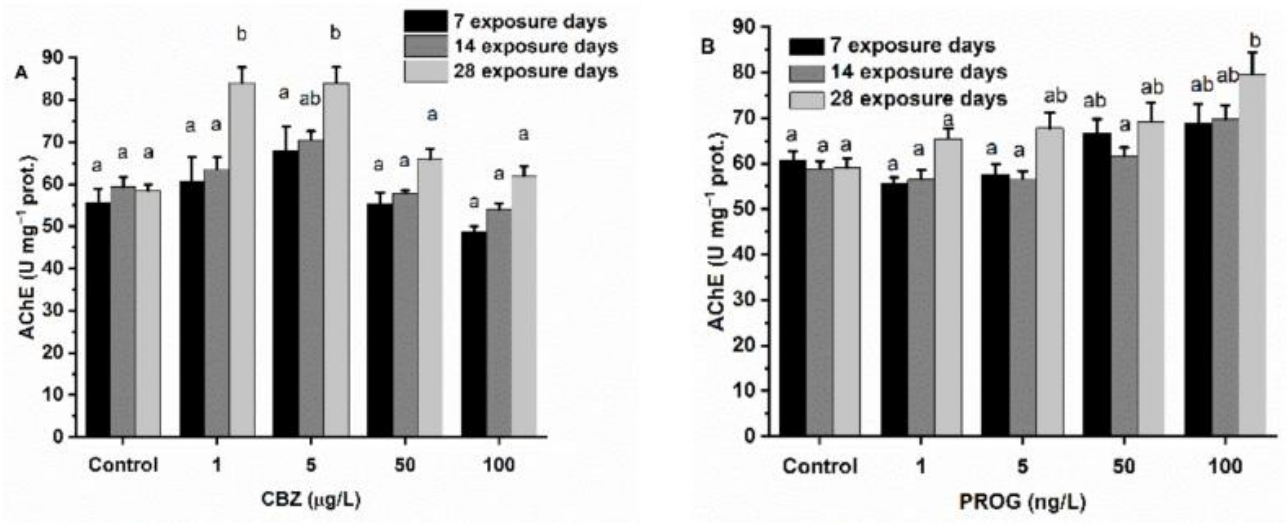


Figure 11: Changes in VTG content in the gonads of *Danio rerio* exposed to (A) CBZ, (B) P4, and (C) binary mixtures of CBZ and P4 for 7, 14, and 28 days. Data are expressed as mean ± standard deviation of fifteen replicates (n = 15). Different letters indicate significant differences at $p < 0.05$ after a two-way ANOVA followed by Tukey's post hoc test.

5.2.2. Neurological Biomarker (AChE)

The results of AChE activities measured in the brain tissues of zebrafish after exposure to the CBZ, P4, and their binary mixtures in different proportions are displayed in Figure 12, from A to C, respectively. After 7 days of exposure, there was a slight increase until 5 ng/L CBZ, then decreased from 50 to 100 ng/L, but these alterations were statistically not significant. Similarly, after 14- and 28- days of exposure, an apparent increase occurred until 5 ng/L CBZ, followed by a drop to the 100 ng/L dose. Statistically significant differences were only detected at the concentrations of 1 and 5 µg/L after 28 days of CBZ exposure (Figure 12A). After 7 days, concentrations of 50 and 100 ng/L PROG induced increases in AChE activity (Fig. 12 B). After 14 days of exposure, a slight statistically significant elevation was recorded at 100 ng/L P4. After 28 days, a concentration-dependent increase in AChE activity was evidenced, with statistical significance ($p < 0.05$) found only at the concentration of 100 ng/L P4 compared with the activity values measured in control groups (Figure 12B). In exposures to mixtures, a significant ($p < 0.05$) drop was observable in the case of MIX1 after 7 days of exposure, while MIX2 and MIX3 did not cause any significant change in AChE activity compared to control group values. After 14 and 28 days of exposure, although there were some slight decreases and increases in AChE activities of treated fish, no significant difference was found in AChE activity compared to control fish (Figure 12C).



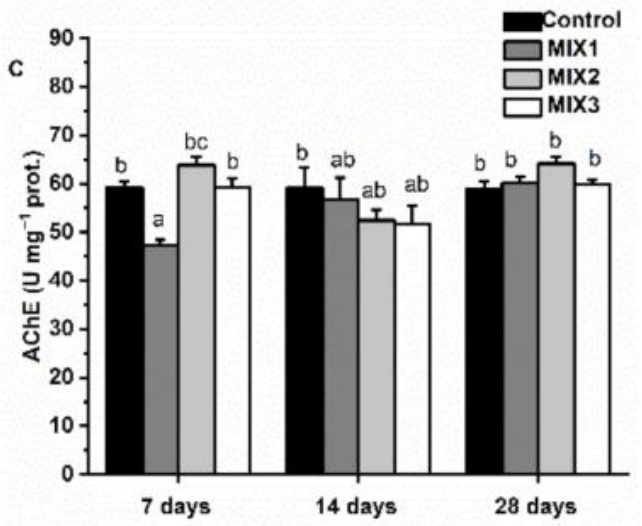


Figure 12: Changes in AChE activity in the brain of *Danio rerio* exposed to (A) CBZ, (B) P4, and (C) binary mixtures of CBZ and P4 for 7, 14, and 28 days. Data are expressed as mean ± standard deviation of fifteen replicates (n = 15). Different letters indicate significant differences at $p < 0.05$ after a two-way ANOVA followed by Tukey's post hoc test.

5.2.3. Damage Markers (DNAsb, LPO, LDH)

After one week, DNAsb values increased at 1, 5, and 50 µg/L of CBZ exposure, with a significant ($p < 0.05$) increase at 50 µg/L. Then, the value decreased significantly, even below the control level at 100 µg/L ($p < 0.05$) (Fig. 13A). Compared with the control groups, 14 days of exposure to CBZ caused significantly ($p < 0.05$) increased DNAsb values at concentrations of 1, 5, and 50 µg/L of CBZ, with the highest levels appearing at 5 µg/L of CBZ; while a sharp and statistically significant drop was shown at 100 µg/L of CBZ ($p < 0.05$). After 28 days of exposure to CBZ, elevated DNAsb levels were significant ($p < 0.05$) only at 1 µg/L, while in contrast, a significant ($p < 0.05$) decrease was observed at 5, 50, and 100 µg/L, respectively, compared with the control (Figure 13A). Exposure to P4 caused a significant elevation of DNAsb after one week at the concentrations tested. The highest DNAsb values were measured in 5 ng/L of P4-exposed fish. After 14 days, there was a significant increase only detected at the concentration of 100 ng/L compared with the control. Following 28 days of exposure to P4, none of the tested concentrations

caused any statistically significant changes in DNAsb concentrations (Figure 13B). For the binary mixtures, after 7 days, only MIX3 significantly ($p < 0.05$) decreased DNAsb values as compared to controls (Fig. 13C). After 14 days, although there were slight increases in DNAsb of fish exposed to MIX2 and MIX3, no statistical differences were recorded. After 28 days of exposure, MIX2 and MIX3 significantly ($p < 0.05$) increased the level of DNAsb in fish (Figure 13C).

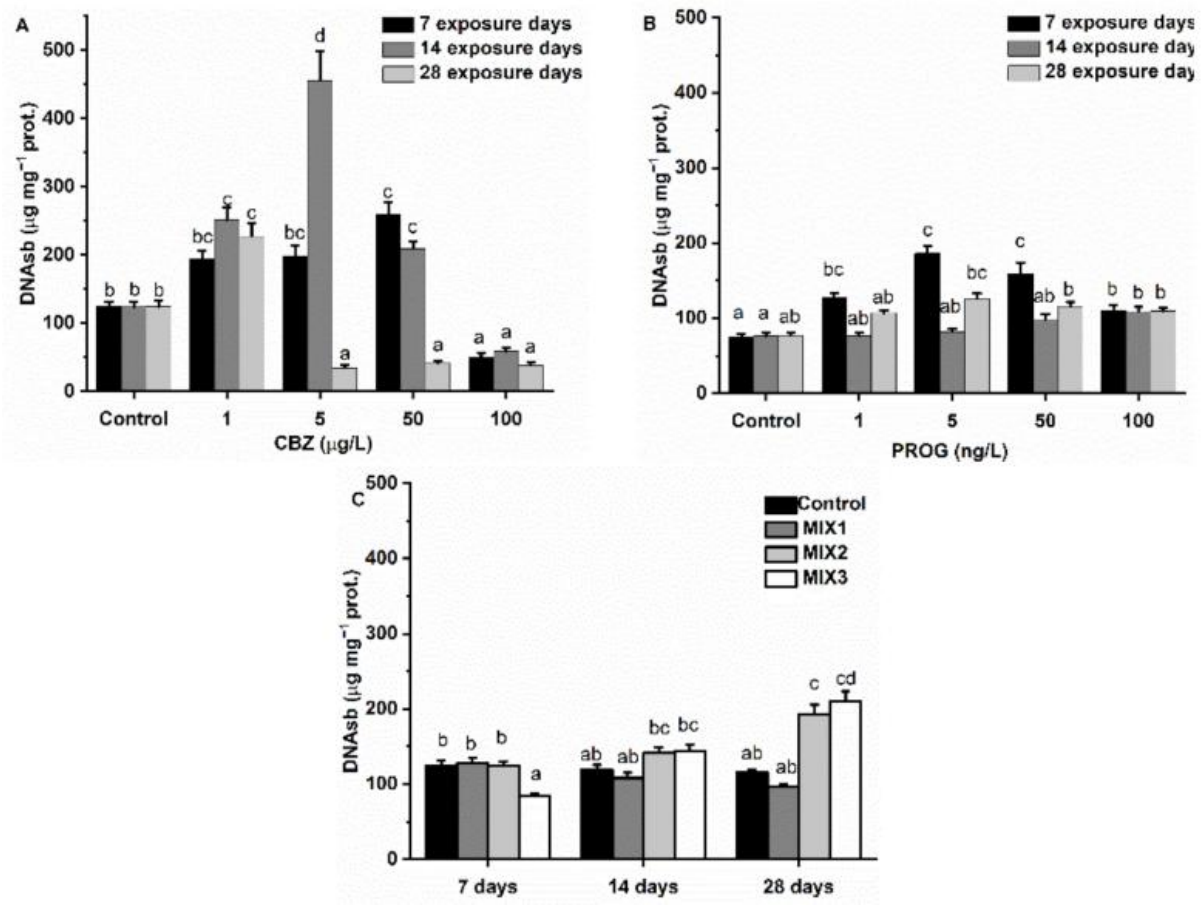


Figure 13: Changes in DNAsb concentration in the liver of *Danio rerio* exposed to (A) CBZ, (B) P4, and (C) binary mixtures of CBZ and P4 for 7, 14, and 28 days. Data are expressed as mean ± standard deviation of fifteen replicates (n = 15). Different letters show differences at $p < 0.05$ after a two-way ANOVA followed by Tukey's post hoc test.

As for the results of LPO after 7 days of exposure, although the LPO levels measured at all applied concentrations were higher than the value measured in the control group, no significant differences were detected. However, the LPO value at 100 ng/L was significantly ($p < 0.05$) lower than that at 50 ng/L. After 14 days, CBZ significantly ($p < 0.05$) decreased the LPO content of the samples

at a concentration of 100 µg/L of CBZ compared to control samples. There were no other significant changes during the exposure time of four weeks, while a decreasing tendency was observable in LPO values after two and four weeks of CBZ exposure (Figure 14A). Concerning the P4, the LPO values at 1, 5, and 50 ng/L were higher than those measured in the control, while there were no statistical differences. Similarly, after 14 days of exposure, 1, 5, and 50 ng/L of P4 increased the LPO content with no significant difference. Compared to control values, P4 significantly ($p < 0.05$) increased the LPO content in samples exposed to 5, 50, and 100 ng/L concentrations for four weeks (Figure 14B). According to the results, binary mixtures did not significantly induce LPO changes in zebrafish during the four-week exposure time (Figure 14C).

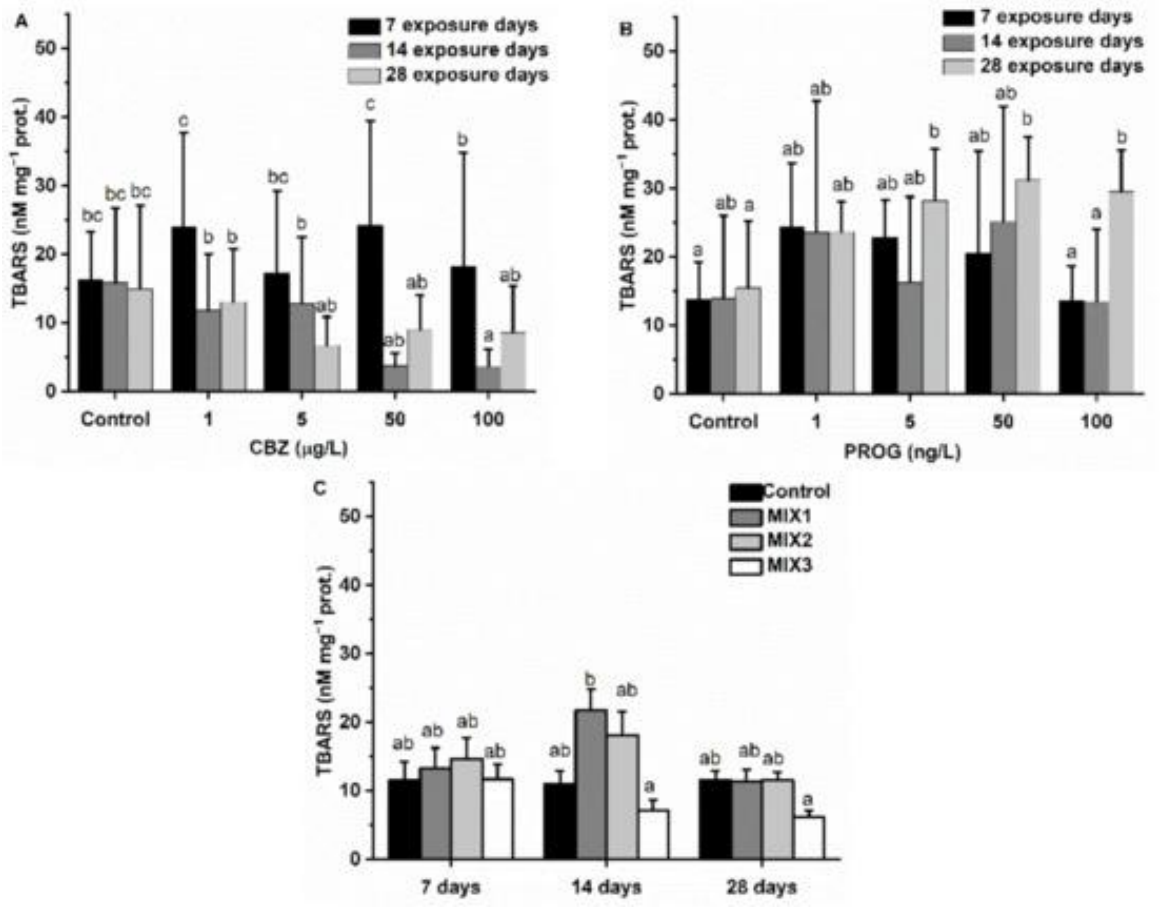


Figure 14: Changes in LPO concentration in the liver of *Danio rerio* exposed to (A) CBZ, (B) P4, and (C) binary mixtures of CBZ and P4 for 7, 14, and 28 days. Data are expressed as mean ± standard deviation of fifteen replicates (n = 15). Different letters show significant differences at $p < 0.05$ after a two-way ANOVA followed by Tukey's post hoc test.

During the four weeks of exposure to CBZ, the LDH level measured only decreased at 1 ng/L compared with the control after 28 days (Figure 15A). P4 significantly ($p < 0.05$) increased LDH activity in samples after one week of exposure to 50 and 100 ng/L (with a sharp elevation), and after two weeks in 100 ng/L P4 treatments (with a sharp increase). There were no significant changes found after 28 days of exposure to P4 (Figure 15B). For the mixtures, MIX1, MIX2, and MIX3, the LDH activity significantly increased after one week (Fig. 15C). After two weeks of exposure to MIX1 and MIX3, the LDH levels were slightly increased with statistical significance ($p < 0.05$). After 28 days, only MIX3 caused a moderate but statistically significant increase (Figure 15C).

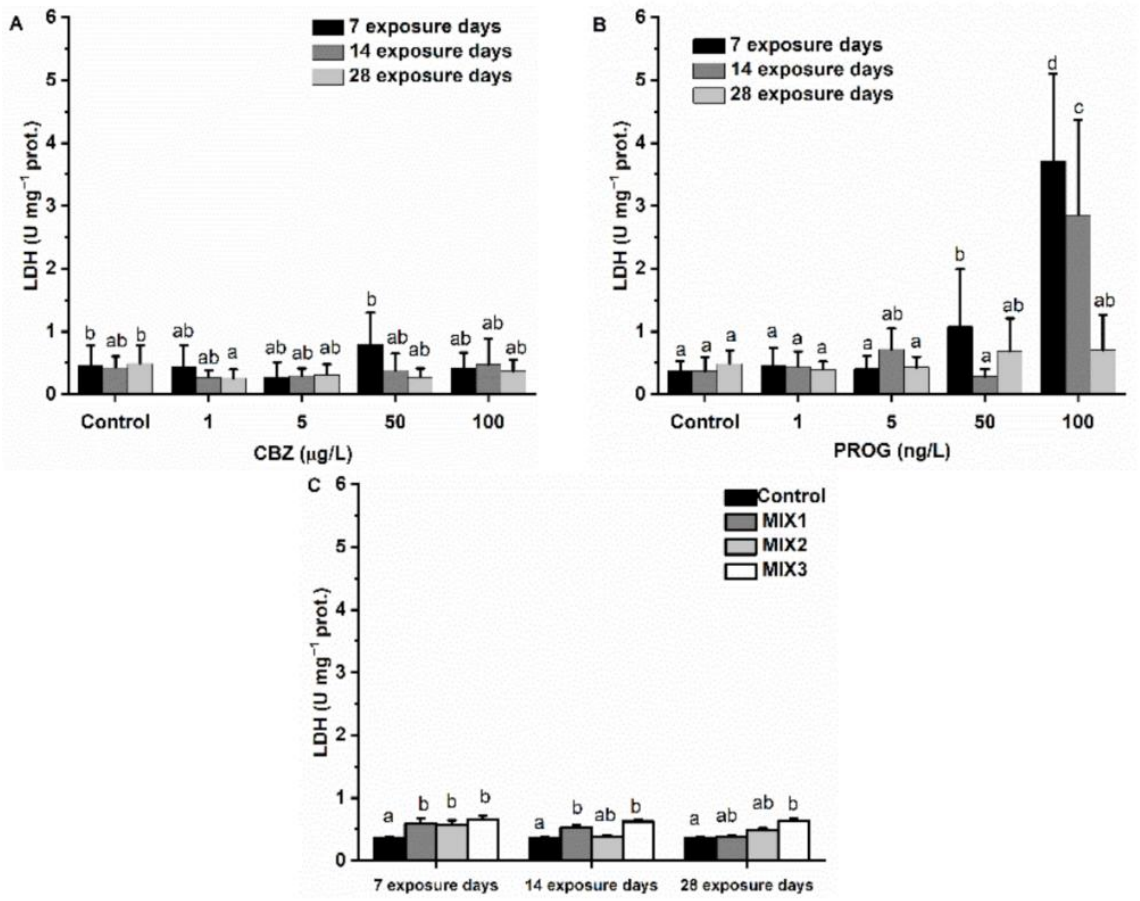
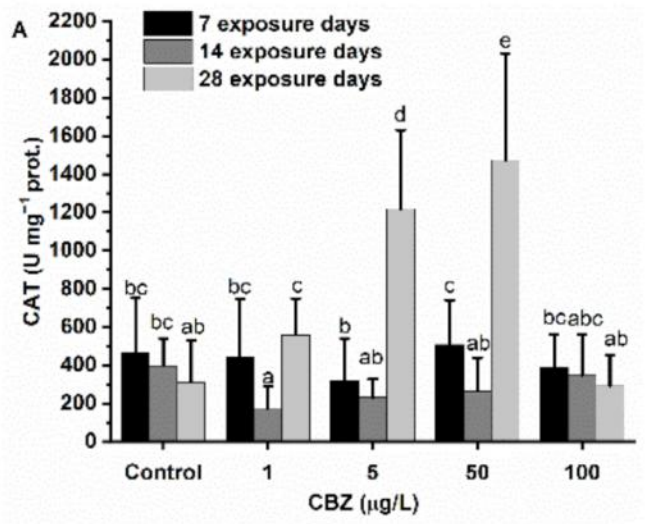
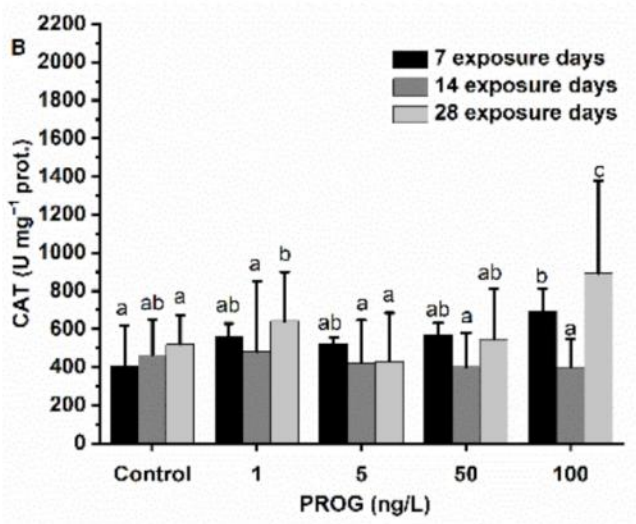


Figure 15: Changes in LDH activity in the liver of *Danio rerio* exposed to (A) CBZ, (B) P4, and (C) binary mixtures of CBZ and P4 for 7, 14, and 28 days. Data are expressed as mean ± standard deviation of fifteen replicates (n = 15). Different letters indicate significant differences at $p < 0.05$ after a two-way ANOVA followed by Tukey's post hoc test.

5.2.4. Antioxidant Defence (CAT, GPx, GR, SOD)

During the first two weeks of treatment with CBZ (7 days and 14 days), the change in CAT activity at each concentration (1, 5, 50, 100 µg/L) was relatively small and did not show significant changes with the increasing concentration (Fig. 16A). A significant ($p < 0.05$) decrease was only observed at the 1 µg/L CBZ concentration after 14 days. By the fourth week, however, an increase in CAT activity began to appear concentration-dependent, and was observed in the 1, 5, and 50 µg/L CBZ exposure groups with significant differences ($p < 0.05$). However, the highest concentration of 100 µg/L of CBZ didn't induce any significant alteration in CAT activity as compared to the levels measured in the control group (Figure 16A). P4 significantly ($p < 0.05$) increased CAT activity after exposure to 100 ng/L of P4 in the first 7 days, as compared to the control groups. There were no significant differences in CAT activity detected in all the tested groups as compared to the levels measured in the control group after 14 days. After 28 days of exposure, the CAT activity was statistically increased at 1 and 100 ng/L; however, no time- or concentration-dependent pattern was detected (Figure 16B). In the binary mixtures of CBZ and P4, only MIX3 induced a significant increase in CAT activity after 14 days of exposure (Figure 16C).





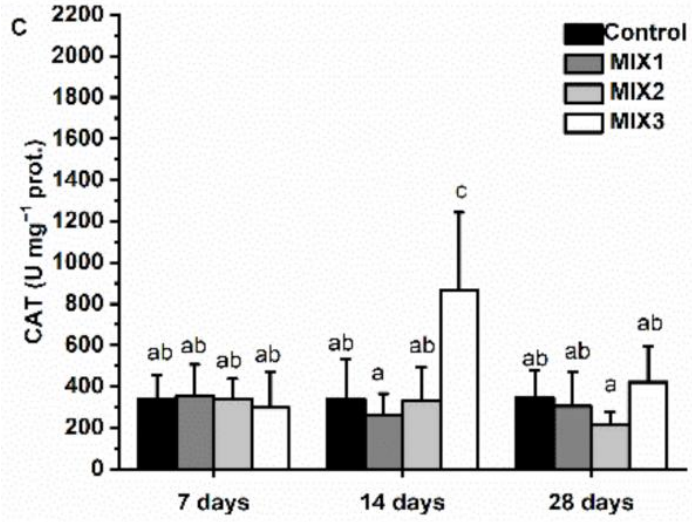


Figure 16: Changes in CAT activity in the liver of *Danio rerio* exposed to (A) CBZ, (B) P4, and (C) binary mixtures of CBZ and P4 for 7, 14, and 28 days. Data are expressed as mean ± standard deviation of fifteen replicates (n = 15). Different letters indicate significant differences at $p < 0.05$ after a two-way ANOVA followed by Tukey's post hoc test.

The results of selenium-dependent GP (GPxSe) activities and the total GP (GPxTOT) activities are displayed in the figure below (Figure 17). According to Figure 17A, the GPxSe increases followed a concentration-dependent pattern after 7 days of exposure to CBZ, with significant ($p < 0.05$) elevations at 50, 100 µg/L CBZ concentrations. After 14 and 28 days of exposure to CBZ, all applied concentrations caused strong decreases in a time- and concentration-dependent manner on GPxSe activity, with significant decreases ($p < 0.05$) at all exposure concentrations. As for the GPxTOT, CBZ did not affect its activity after seven days of exposure. While after 14 days of exposure, GPxTOT increased in a concentration-dependent manner, growing significantly ($p < 0.05$) within the concentration range of 5-100 µg/L CBZ. After 28 days of exposure, 5 and 50 µg/L of CBZ exposure significantly ($p < 0.05$) elevated the activity of GPxTOT, then, the GPxTOT value dropped sharply with a significant difference from the value measured at 50 µg/L, but not different from the control ($p < 0.05$) (Figure 17A).

Concerning the exposure to P4, GPxSe was increased in a time- and concentration-dependent manner at all applied concentrations and time points except the lowest treatment of 1 µg/L. After

the first week of P4 exposure, GPxSe increased significantly ($p < 0.05$) at 50 and 100 ng/L. After 14 days of exposure, the increase reached a significant ($p < 0.05$) level at exposure concentrations of 1, 5, 50, and 100 ng/L of P4, and reached the highest point at 5 ng/L of P4, then the activity decreased slightly at 50 and 100 ng/L P4 concentrations. A similar pattern was observed after 28 exposure days. GPxSe activity peaked at 50 ng/L of P4, then slightly reduced at the 100 ng/L exposure concentration. (Figure 17B).

For the mixtures, there were no observed changes during the first two weeks; only MIX3 induced a significant ($p < 0.05$) difference in the GPxSe activity compared with the control after 28 days of exposure (Figure 17C). As for the results of GPxTOT, there were no changes in the GPxTOT activity after the first week. The activity showed an increasing trend with the increase of CBZ concentration after 14 days. A significant difference ($p < 0.05$) was confirmed at 50 and 100 ng/L CBZ concentrations, and peaked at the highest value at 100 ng/L. Similarly, the activity increased with the increasing concentration of CBZ except at 100 ng/L after 28 days, with significant ($p < 0.05$) changes at 5, 50 ng/L. However, the activity measured at 100 ng/L was significantly ($p < 0.05$) lower than that measured at 50 ng/L (Figure 17D). GPxTOT activity showed an initial increase in activity, peaking at 50 ng/L of P4 after one week of exposure, followed by a slight decrease at 100 ng/L. Significant differences ($p < 0.05$) were only confirmed at 5 and 50 ng/L P4 concentrations. Similarly, the GPxTOT activity after 14 days of P4 exposure increased initially and peaked at 5 ng/L of P4, then was followed by a slight reduction at 50 and 100 ng/L; a statistical significance ($p < 0.05$) was confirmed in the 5 ng/L P4 concentration only. After 28 days of exposure, there were no changes in the GPxTOT activity until a minor decrease at 100 ng/L, with no statistical significance (Figure 17E). Binary mixtures caused significant ($p < 0.05$) changes in GPxTOT activity in MIX3 after one week of exposure (decrease), and after four weeks of exposure (increase) (Figure 17F).

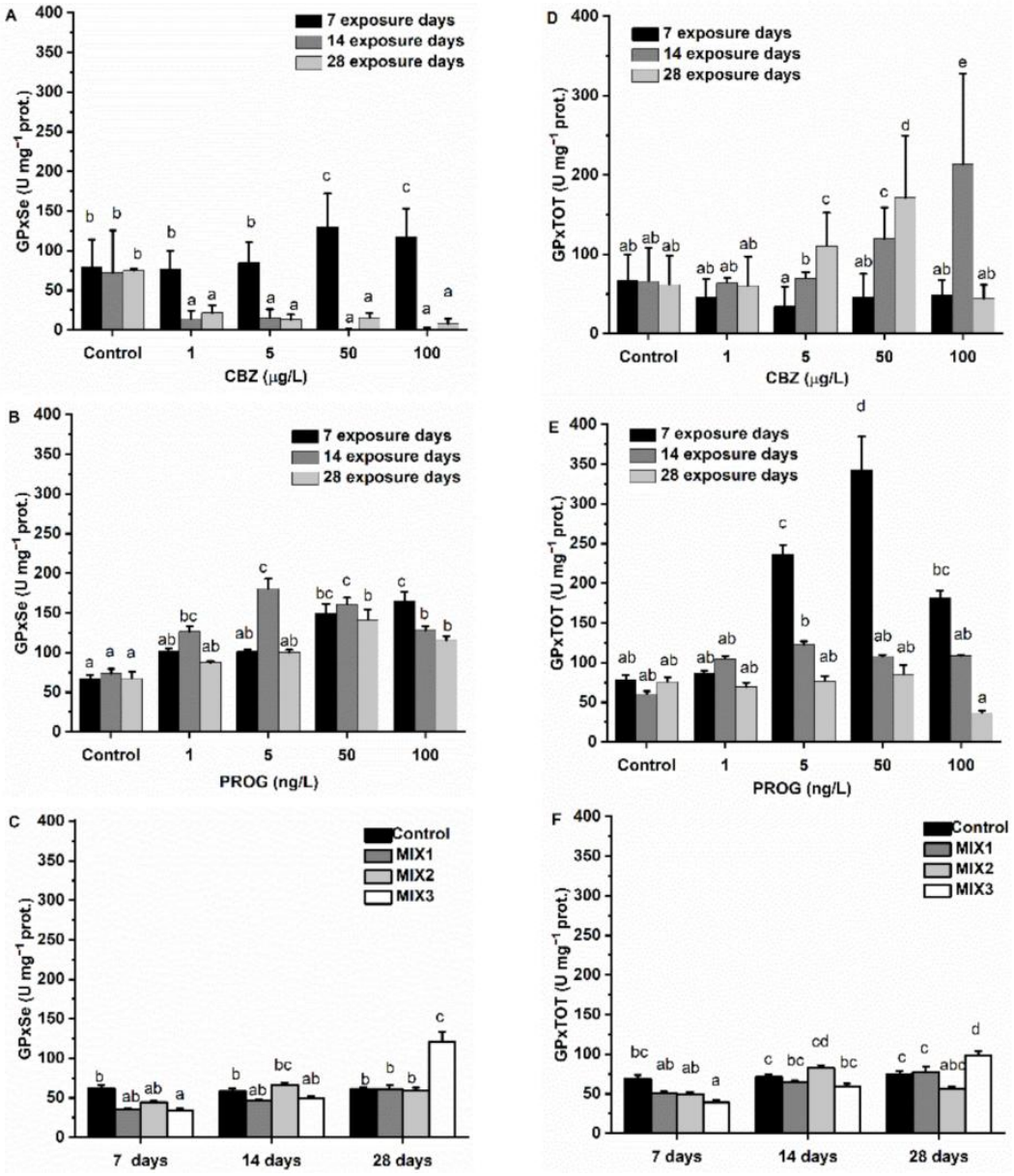


Figure 17: Changes of biotransformation enzymes' activity—GPxSe (A–C) and GPxTOT (D–F) in the liver of *Danio rerio* exposed to (A, D) CBZ, (B, E) P4, and (C, F) binary mixtures of CBZ and P4 for 7, 14, and 28 days. Data are expressed as mean ± standard deviation of fifteen replicates (n = 15). Different letters indicate significant differences at $p < 0.05$ after a two-way ANOVA followed by Tukey's post hoc test.

Figure 18 shows the results of GR activities of zebrafish upon treatments. After 7 days of exposure to CBZ, the GR activity elevated significantly ($p < 0.05$) at the concentrations of 1, 5, and 50 µg/L, and the value increased with the increasing concentration. However, the activity decreased at 100 µg/L CBZ concentration with no significant difference compared to the control group activity

values. For the longer exposure time of 14 and 28 days, there was no significant change in GR activity detected in any treatment condition with CBZ (Figure 18A). Concerning P4, after the first week, P4 induced a stable and significant ($p < 0.05$) increase at 5, 50, and 100 µg/L in a concentration-dependent manner, which was proved by statistics. After two weeks of exposure, similarly, P4 induced stable and significant ($p < 0.05$) elevations at all the applied concentrations (1, 5, 50, 100 µg/L). After 28 days, the increase pattern was the same as the pattern shown after the first week; GR activity increased significantly ($p < 0.05$) at exposure concentrations above 1 ng/L (5, 50, 100 µg/L), also in a concentration-dependent pattern. It was proved that the GR activity in fish exposed to P4 showed a time-dependent pattern during the 28-day exposure (Figure 18B). For the mixtures, only MIX3 decreased the activity of GR significantly ($p < 0.05$) after 7 days of exposure, whereas other mixtures showed no impact (Figure 18C).

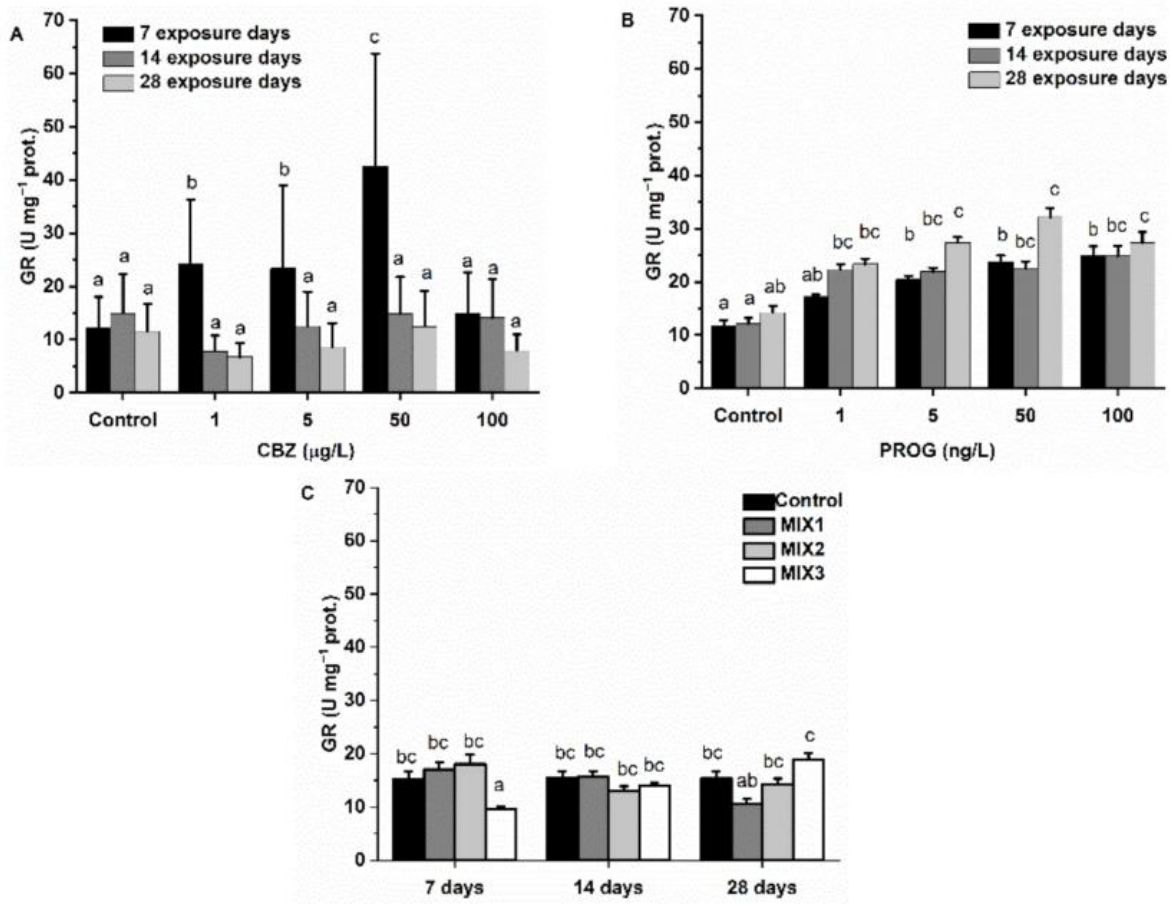


Figure 18: Changes in GR activity in the liver of *Danio rerio* exposed to (A) CBZ, (B) P4, and (C) binary mixtures of CBZ and P4 for 7, 14, and 28 days. Data are expressed as mean ± standard deviation of fifteen replicates (n = 15).

Different letters show significant differences at $p < 0.05$ after a two-way ANOVA followed by Tukey's post hoc test.

The activities of SOD assessed under different CBZ concentrations are displayed in Figure 19, respectively. After 7 days, significant ($p < 0.05$) increases in SOD activity were observed at 1, 5, and 50 µg/L CBZ concentrations compared to control group values, and the activity peaked at 50 µg/L, showing the highest value (Fig. 19A). The SOD activity in fish treated with 100 µg/L CBZ, dropped to levels measured in control fish. After 14- and 28-day exposure durations, SOD activity became much lower than the values measured in the first week, without any significant differences among the applied concentrations. In contrast, P4 elicited decreases in SOD activity at all the applied concentrations with significant ($p < 0.05$) differences after the first week of exposure, and the activity reached the lowest level at the 5 ng/L P4 treatment concentration (Fig. 19B). After a longer exposure time (14 and 28 days), the SOD activities didn't change compared to the values measured in the control groups, respectively; however, the activities measured after 14 days and 28 days were higher than those measured in the first 7 days within all applied concentrations (Figure 19B). The binary mixture Mix1 caused a significant ($p < 0.05$) reduction in the SOD activity after 28 days of exposure, while no other changes were observed in any other treatment condition (Figure 19C).

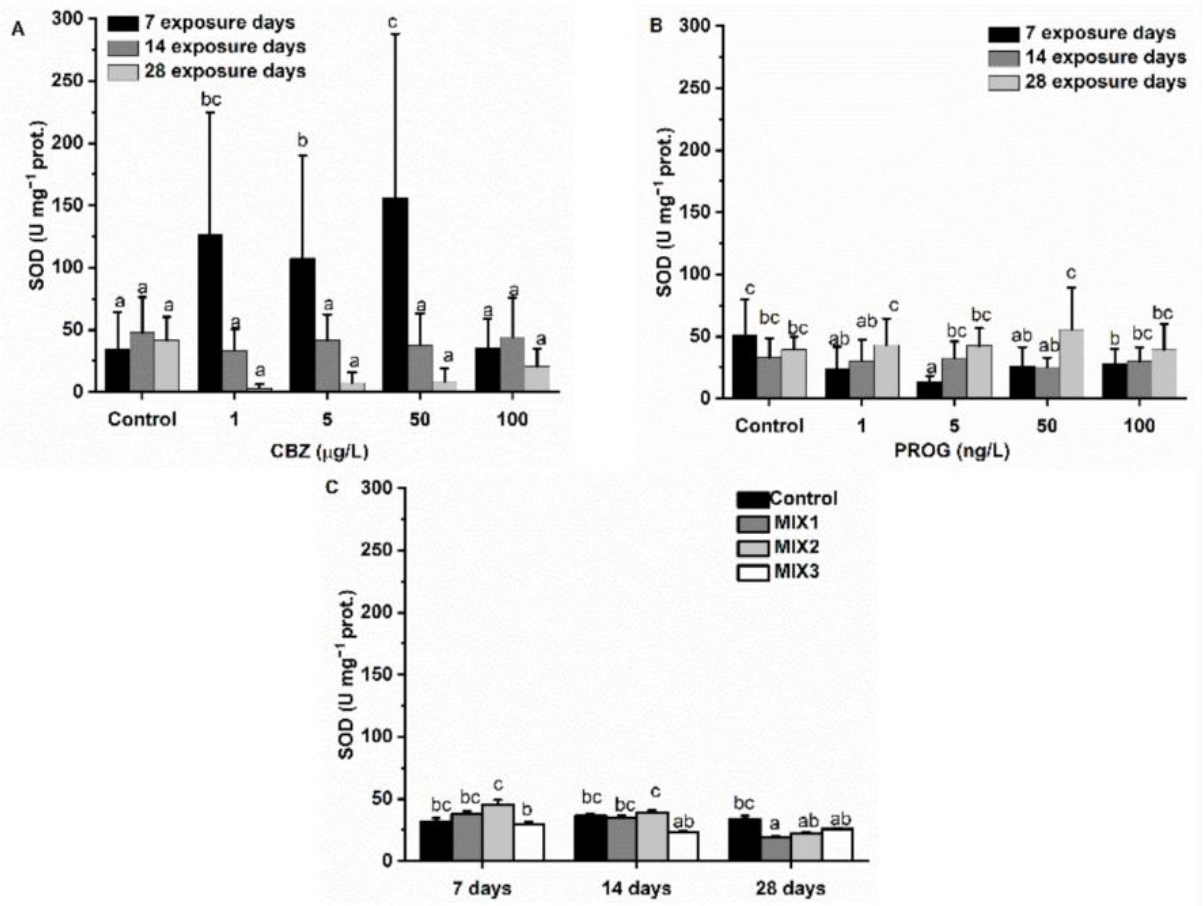


Figure 19: Changes in SOD activity in the liver of *Danio rerio* exposed to (A) CBZ, (B) P4, and (C) binary mixtures of CBZ and P4 for 7, 14, and 28 days. Data are expressed as mean ± standard deviation of fifteen replicates (n = 15). Different letters show significant differences at $p < 0.05$ after a two-way ANOVA followed by Tukey's post hoc test.

5.2.5. Biotransformation Enzymes (EROD, GST)

The results of hepatic EROD activity changes are shown in Figure 20. After the first week of exposure to CBZ, EROD activity increased continuously and significantly ($p < 0.05$) with the increasing CBZ concentration, peaking at 5 µg/L, and then decreased at 50 and 100 µg/L, up to the baseline EROD activity measured in control fish (Figure 20A). After 14 and 28 days of exposure, EROD activity declined to near-control levels and did not differ significantly among the CBZ treatments compared to the control group, and between different CBZ exposures.

EROD activity in fish exposed to P4 after 7 days of treatment increased in a concentration-

dependent manner, with significant differences detected at 50 and 100 ng/L P4 concentrations ($p < 0.05$) (Figure 20B). After 14 days of exposure to P4, EROD activities increased significantly ($p < 0.05$) at lower concentrations of P4 (1, 5 ng/L), and the value decreased slightly at 50 ng/L, followed by the inhibition of EROD activity at 100 ng/L P4 concentration, but no significant differences. After 28 days of P4 exposure, the changes at 1, 5, and 50 ng/L were not notable, EROD activity was significantly ($p < 0.05$) reduced only at 100 ng/L (Figure 20B).

After the first week of exposure to the binary mixture, a non-significant decrease in EROD activity was shown (Figure 20C). After 14 days of exposure to the mixtures, the overall EROD activities were higher than those measured after 7 days of exposure. The highest elevation in EROD activity was detected in fish subjected to the MIX2 and MIX3 treatments ($p < 0.05$). After 28 days of exposure, MIX1 did not alter EROD activity, but MIX2 and MIX3 still increased EROD activity, with MIX3 eliciting the significantly ($p < 0.05$) highest increase in enzymatic activity as compared to control levels (Figure 20C).

The results of GST are also shown in Figure 20. There were no significant changes at any of the applied concentrations of CBZ compared with the control group after 7 days of exposure. After 14 and 28 days, GST levels showed a concentration-dependent increase (Figure 20D). A significant ($p < 0.05$) elevation of the activity was observed after 14 days of exposure to 100 µg/L CBZ. After 28 days of exposure, there were significant ($p < 0.05$) increases in GST activity at 5, 50, and 100 µg/L of CBZ (Figure 20D). In zebrafish exposed to P4, significant ($p < 0.05$) concentration-dependent changes in GST activity were observed only after 28 days at 50 and 100 ng/L. After one week of exposure, GST activity decreased significantly at 1 and 100 ng/L P4, whereas no significant changes were detected at other concentrations after one or two weeks. (Figure 20E). For the binary mixtures of CBZ and P4, all three mixtures (MIX1, MIX2, MIX3) caused a significant ($p < 0.05$) decrease in GST activity in a concentration-dependent manner. An increasing inhibitory effect was observed in proportion to the increasing amount of P4 (MIX1 < MIX2 < MIX3) present in the mixtures. The inhibitory effect of the mixtures was still detectable after two and four weeks of exposure, but differences as compared to the control group were not so

pronounced. Specifically, a significant ($p < 0.05$) inhibition was only found in MIX2 after 28 days, and in MIX3 after 14 days and 28 days (Figure 20F).

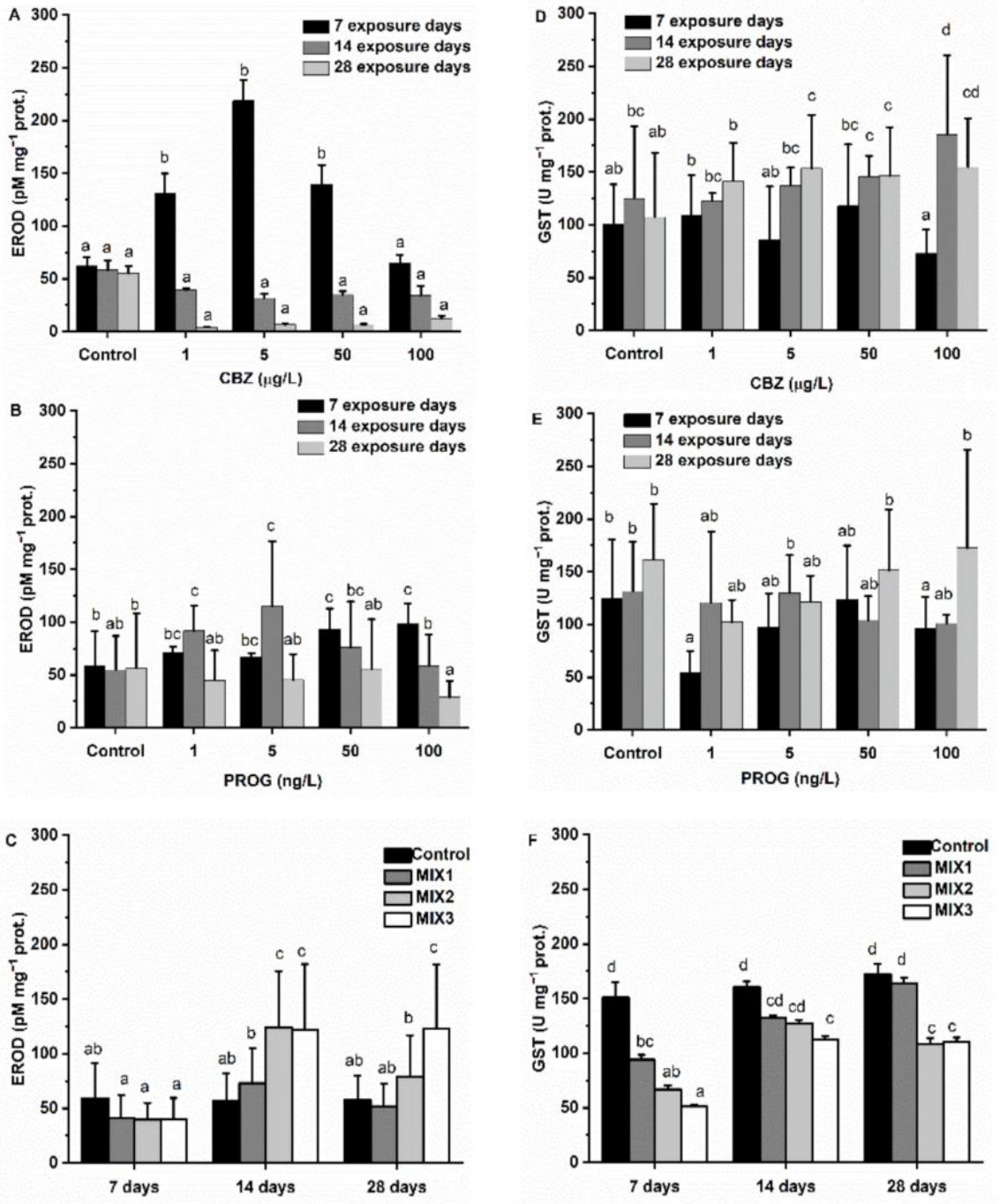


Figure 20: Changes of biotransformation enzymes' activity—hepatic EROD (A–C) and GST (D–F) in the liver of *Danio rerio* exposed to (A, D) CBZ, (B, E) P4, and (C, F) binary mixtures of CBZ and P4 for 7, 14, and 28 days. Data are expressed as mean ± standard deviation of fifteen replicates (n = 15). Different letters show significant differences at $p < 0.05$ after a two-way ANOVA followed by Tukey's post hoc test.

6. DISCUSSION

Our findings from experiments on zebrafish and common carp revealed the sub-chronic effects, as well as the combined impacts of CBZ and P4, highlighting the importance of investigating the sub-chronic and further chronic influences of emerging pollutants at environmentally relevant concentrations.

6.1. Endocrine disruption indicator (VTG)

Results on common carp exposure to CBZ revealed significantly increased VTG levels in fish exposed to 100 µg/L CBZ after the first seven days, indicating an estrogenic-like response. The effective concentration used in this study (100 µg/L) is higher than the current median CBZ concentrations typically detected in surface waters, which are usually in the ng/L to low µg/L range (Dang et al., 2024; Jeon et al., 2024). Previous studies have also highlighted the reproductive toxicity of CBZ. For instance, Li, et al. (2010) reported that 2 hours of 2 and 20 mg/L CBZ exposure caused oxidative stress in common carp spermatozoa, impairing sperm quality. Our findings further showed a clear concentration-dependent pattern, strongly suggesting that CBZ disrupts normal endocrine functions in fish. Similarly, da Silva Santos et al. (2018) exposed adult zebrafish to CBZ at 10 µg/L and 10,000 µg/L for 63 days, representing environmentally relevant and supra-environmental concentrations, respectively. Compared with our study (1, 5, 50, and 100 µg/L for 28 days in *C. carpio*), the higher dose tested by da Silva Santos et al. caused similar endocrine and hepatic responses with an increase in vitellogenic follicles, emphasizing CBZ's potential to disrupt reproductive functions in fish, similar to other non-steroidal pharmaceuticals. Their histopathological analysis also suggested that CBZ acts through estrogenic-like pathways.

VTG, a glycolipophosphoprotein synthesized in the liver, serves as a sensitive biomarker for detecting estrogenic substances in aquatic environments (Emmersen et al., 1979; Gagné & Blaise, 1998; Miracle et al., 2006; Runnalls et al., 2015; Shilling & Williams, 2000). Since its vitellogenin production is typically triggered by estrogens such as 17β-estradiol or chemicals that interact with

estrogen receptors (Emmersen et al., 1979). Our results further support the hypothesis that CBZ may mimic estrogenic effects in fish. This could explain the observed increase in VTG levels of common carp. Notably, the rise in VTG levels after only seven days of exposure to 100 µg/L CBZ indicates a rapid and pronounced biochemical response, raising concerns about the long-term reproductive effects on fish populations exposed to environmentally relevant concentrations of CBZ.

As for the experiments on zebrafish, the VTG concentrations in zebrafish increased after CBZ exposure for one and two weeks, with a significant rise detected at the 100 µg/L concentration, this pattern was similar to the trend observed in common carp, but the peak response occurred later, at 14 days instead of 7 days. However, after 28 days of exposure, VTG levels showed a slight decline relative to the control in both zebrafish and common carp. This may be because of the hormetic effects, where low-dose exposure can stimulate, and high-dose or prolonged CBZ exposure can suppress the VTG response (Prehn & Berd, 2006; Sumpter, 1995).

P4 is known to alter VTG levels in fish, with either inhibition or induction depending on the concentration and the type of progestogen (Maasz et al., 2017; Zucchi et al., 2013). In our results, a time- and concentration-dependent decrease in VTG levels was observed after 28 days of exposure to 50 and 100 ng/L of P4, indicating an inhibitory effect at higher concentrations.

When zebrafish were exposed to binary mixtures of CBZ and P4, after two weeks, a slight but non-significant drop in VTG levels was noted with an increased proportion of P4 and reduced CBZ levels. VTG levels showed significant changes after 28 days of exposure. VTG levels increased after exposure to MIX1, MIX2 and MIX3, though MIX1 did not cause a statistically significant effect. These findings suggest that the changes in VTG concentration may follow a hormetic pattern, which often occurs in endocrine responses, where low doses may stimulate and higher doses may cause inhibition (Prehn & Berd, 2006). The prolonged exposure to combinations of CBZ and P4 showed that the two compounds may act synergistically. Our results indicate that increasing P4 concentrations may amplify and extend the estrogenic-like effects of CBZ,

particularly at environmentally relevant concentrations. This synergism points out the importance of studying the long-term effects of mixed pharmaceutical contaminants in aquatic ecosystems.

6.2. Modulation of AChE activity in fish

In common carp exposed to CBZ, an initial decrease in AChE activity was observed after 7 days, aligning with previous research. Similar effects have been reported in the clam *Ruditapes philippinarum* (Aguirre-Martínez et al., 2016), monogonont rotifer (*Brachionus koreanus)* (Rhee et al., 2013), and crucian carp (*Carassius carassius*) (Nkoom et al., 2020). The study by Nkoom et al. (2020) evidenced that short-term exposure (< 7 days) to CBZ also caused significant inhibition of AChE activity. This reduction may have occurred because CBZ, as a neurotoxic agent, interferes with normal neurotransmission by preventing the breakdown of acetylcholine (ACh) in synapses, leading to neuron overstimulation and potentially impaired movement and cognitive functions (Pfeifer et al., 2005).

However, after 14 and 28 days of exposure, AChE activity increased depending on the exposure time and concentration. This result suggested that the fish's body may have activated a compensatory response to long-term CBZ exposure. Higher AChE activity can be linked to oxidative stress and the production of free radicals (Ferreira et al., 2012), which trigger an increase in enzyme production. In addition, the ongoing apoptosis process may also be one reason (B. Zhang et al., 2012; X. Zhang et al., 2002). For example, Yan et al. (Yan et al., 2021) found that CBZ at environmentally relevant concentrations (1, 10, and 100 µg/L) caused apoptosis in the liver cells of *Gobiocypris rarus*. Therefore, the elevated AChE activity measured on common carp in this study may be linked to apoptotic processes.

An increase in AChE activity is often a consequence of tissue alterations (e.g., apoptosis) (Zhang et al., 2002). Higher AChE levels cause a faster breakdown of neurotransmitter ACh, leading to reduced stimulation of ACh receptors. Earlier, it was suggested that reduced stimulation of Ach receptors in turn may lead to poorer cognitive functions in fish (Tõugu & Kesvatera, 1996). A similar increase in AChE activity was observed in zebrafish after 63 days of CBZ exposure (da

Silva Santos et al., 2018), indicating that this response may be common in different fish species. In this study, the observed changes in AChE activity (an early decrease followed by an increase) may therefore reflect the complex physiological adjustments to CBZ exposure

In zebrafish exposed to CBZ, P4, and their binary mixtures, CBZ induced time- and concentration-dependent changes in AChE activity, a finding consistent with other studies on this species(da Silva Santos et al., 2018). After 28 days, significant increases in AChE activity were observed at 1 µg/L and 5 µg/L CBZ concentrations. However, at higher concentrations, such as 50 µg/L and 100 µg/L, AChE activity decreased, which may be attributed to the cumulative toxic effects of CBZ. In zebrafish, AChE activity first decreases during short-term exposure, indicating an early toxic effect, but then increases over time, likely as a compensatory response to stress. In contrast, zebrafish AChE activity initially increases at low concentrations, suggesting an early stress response, but drops at higher concentrations for prolonged exposure, possibly due to severe damage that disrupts enzyme production.

Similarly, after 28 days of exposure to P4, a significant increase in AChE activity was observed at the highest concentration (100 ng/L). Previous studies have linked increased AChE activity with oxidative stress, the production of free radicals (Ferreira et al., 2012), and apoptosis (Zhang et al., 2012; Zhang et al., 2002). As mentioned before, physiologically, AChE plays an important role in breaking down the ACh, when AChE activity increases, ACh levels decrease more rapidly, reducing stimulation at acetylcholine receptors, which can impair cognitive function (Yan et al., 2021).

AChE activity in response to the mixtures followed distinct patterns over time.A significant decrease after 7 days was observed in MIX1. Although slight variations in AChE activity were observed following 14 and 28 days of exposure, no statistically significant differences were found for any of the mixture treatments. These results highlighted the complexity of mixture effects and suggested that some combinations of contaminants may have less pronounced impacts on AChE activity. Although AChE is a key enzyme in neurotransmission, moderate changes (<50%) in its

activity are generally interpreted as modulation rather than direct neurotoxic impairment

The two fish species exhibited opposing trends in AChE activity following CBZ exposure.In the common carp, AChE activity decreased during short-term exposure and later increased, which may reflect an initial stress response followed by compensatory adaptation. Conversely, in zebrafish, AChE activity initially increased at low concentrations, suggesting an early stress response, but dropped at higher concentrations or prolonged exposure, possibly due to severe damage that disrupts enzyme production. These findings indicate that different fish species may respond differently to the same chemical exposure. Carp are larger in size, long-lived fish with a slower metabolism, while zebrafish are small and have a faster metabolic rate. For example, van de Pol et al. (2017) indicated that there are significant differences in energy metabolism between different fish species. A study on the activity of metabolic enzymes in carp muscle indicated that carp might have a relatively slow metabolic characteristic (Johnston et al., 1977)

6.3. Damage markers (DNAsb, LPO, LDH)

6.3.1. DNAsb

Previous studies have shown that CBZ may cause alterations in the genetic material of Chinese rare minnows (*Gobiocypris rarus*) after 28 days of exposure to 1, 10, and 100 μg/L of CBZ (Yan et al., 2021). Here, in common carp, a slight decrease in DNAsb levels was observed over the 28-day exposure period compared to the control groups.. The decreased level of DNA strand breaks may be explained by the inhibitory effect of CBZ on cell division during longer exposure times (Oliveira et al., 2007).

In zebrafish, DNAsb levels initially increased after CBZ exposure, likely due to oxidative damage caused by ROS, as suggested by ROS defence enzyme results. This suggests that oxidative stress was a key factor contributing to genetic material damage (Birben et al., 2012). However, the subsequent decrease in DNAsb levels might indicate the inhibition of cell division, which could have limited the division of DNA strands (Bolognesi & Hayashi, 2011; M. Oliveira et al., 2007).

By using EdU (5-ethynyl-2′-deoxyuridine) or BrdU (5-bromo-2′-deoxyuridine) incorporation assays, it can be directly counted how many cells are actively replicating DNA. A significant reduction in EdU/BrdU-positive cells compared to controls would provide clear evidence that cell division has been inhibited (Verduzco & Amatruda, 2011). Notably, in the group exposed to progesterone (P4), DNAsb levels were significantly elevated after one week but returned to control group levels after two and four weeks, implying that the antioxidant defence system may be capable of mitigating the effects of oxidative stress over time. Both, zebrafish and common carp showed initial DNA damage following CBZ exposure, linked to oxidative stress. Zebrafish demonstrated greater sensitivity to the exposure than common carp, as evidenced by a sharp increase in DNAsb levels, whereas common carp showed only a slight decrease.The results of tests with binary mixtures showed a more complex action pattern. After 7 days, DNAsb levels decreased in the MIX3 group compared to the control and other mixtures, suggesting the initial DNA repair mechanisms may have been activated upon MIX3 treatment. However, significantly higher DNAsb levels were observed after four weeks of exposure to both MIX2 and MIX3. This increase likely occurred because prolonged exposure suppressed the antioxidant defense systems, leading to an accumulation of DNA damage under the stress of the mixtures. The overall response appears to be biphasic. The initial decrease in DNA damage might be linked to low-intensity oxidative stress inducing a protective response, where cells produce antioxidant enzymes to eliminate ROS. In contrast, the subsequent increase in DNAsb suggests that severe oxidative stress eventually overwhelmed these protective mechanisms, resulting in damage to cellular components such as lipids, proteins, and DNA (Maasz et al., 2017).

6.3.2. LPO

Lipid peroxidation (LPO) results from the reaction of reactive oxygen species (ROS) with polyunsaturated fatty acids in cell membranes, which damages their structure and compromises membrane integrity (Ayala et al., 2014; Oakes & Van Der Kraak, 2003). A variety of assays are available to quantify lipid peroxidation. The thiobarbituric acid reactive substances (TBARS) assay is a well-established method for assessing lipid peroxidation, and the resulting TBARS level

serves as a good indicator of oxidative damage to lipids. (Oakes & Van Der Kraak, 2003). Malondialdehyde (MDA) is commonly used as a biomarker for oxidative stress because its production increases in response to the oxidation of lipids by reactive oxygen species (ROS). (Gaweł et al., 2004).

CBZ exposure induced a significant elevation in TBARS levels in common carp only at the highest concentration (100 µg/L) after 28 days. However, the gradual rise in TBARS levels throughout the experiment compared to the control group suggests increasing oxidative stress caused by a malfunctioned antioxidant defence system. This observation aligns with findings from previous studies, such as Li et al. ((2010), who reported elevated TBARS levels and oxidative stress in rainbow trout after 21 and 48 days of exposure to CBZ concentrations of 20 and 200 µg/L. These similarities highlight that prolonged CBZ exposure can lead to oxidative stress in different fish.

In zebrafish, CBZ exposure did not induce statistically significant changes in LPO levels, with the notable exception of a decrease at 100 µg/L after 14 days, which may be attributed to a protective antioxidant response. Elevated TBARS levels found at 5, 50, and 100 ng/L P4 concentrations after four weeks of exposure supported the finding that exogenic P4 causes oxidative stress in zebrafish. Binary mixtures did not cause significantly increased TBARS levels in zebrafish during our assessments. However, significant reductions of LPO levels were observed after two and four weeks of exposure to MIX3, which may be attributed to the lower lipid content of the cells (Gagné, 2014), suggesting a deterioration in the condition of fish. The observed reduction may also contribute to the antioxidative protection of SOD because another biomarker SOD value in the same test on zebrafish increased significantly with exposure to MIX3 after four weeks.

6.3.3. LDH

LDH is a key biomarker for organ and tissue damage, reflecting both metabolic shifts, such as in carbohydrate metabolism, and pathological structural changes. (Osman et al., 2010). In carp, CBZ exposure elicited a time-dependent response in LDH activity. After 7 days, a significant decrease was observed in the 1 µg/L group, suggesting a possible adaptive response at low concentrations.

In contrast, after 28 days, LDH activity showed a non-significant increasing trend at concentrations of 1, 5, and 50 µg/L compared to the control.Previous studies have reported increased LDH activity in the liver and gills of common carp after exposure to 5700 µg/L CBZ for 7 to 28 days, which was linked to metabolic disruption and tissue hypoxia caused by respiratory epithelium disruption, leading to reduced normal oxidative metabolism (Malarvizhi et al., 2012). In another study, da Silva Santos et al. (da Silva Santos et al., 2018) reported elevated LDH levels in zebrafish after 63 days of 10,000 µg/L CBZ exposure and no change after 10 µg/L CBZ concentration. In contrast, no significant dose-dependent increase in LDH activity was observed after 28 days of CBZ exposure, although minor fluctuations were noted among the treatment groups.In zebrafish, P4 caused significant increase in LDH activity after exposure to at 50 and 100 ng/L for one week and at 100 ng/L for two weeks. The concurrent alterations in both AChE and LDH activities suggest structural damage to liver cells. This interpretation is logical given the liver's high detoxification capacity, where altered AChE activity reflects a disruption of physiological processes, and changes in LDH indicate cellular injury.. In the mixture-exposed groups, LDH activity in the liver also increased, with levels comparable to those observed in the single-component exposures. A rapid and significant increase was observed after just one week for all three mixtures (MIX1, MIX2, and MIX3), indicating that these combinations can quickly induce metabolic stress or tissue damage. However, this initial effect evolved differently over time. By week two, only MIX1 and MIX3 still induced a significant effect, suggesting a potential compensatory or adaptive response to the MIX2 treatment. By the end of the fourth week, a significant LDH increase was only persistent in the MIX3 group. Overall, MIX3 consistently elicited the most pronounced and sustained effect on LDH activity. This finding, which aligns with the trends seen for other biomarkers, suggests a more-than-additive or synergistic toxicity between CBZ and P4 in this specific combination.

6.4. Antioxidant defence (SOD, CAT, GPx, GR)

Reactive oxygen species (ROS) are by-products of normal cellular metabolism and play an important role in cell signalling and physiological functions at baseline levels. Excessive ROS

levels which are often caused by xenobiotics, can damage biological molecules like lipids, proteins, and DNA, leading to oxidative stress. Further, this imbalance between ROS and antioxidants can disrupt cellular functions and contribute to impairment of basic metabolic processes in cells. To maintain the redox balance, organisms have developed antioxidant defence systems, which include enzymatic antioxidants such as SOD, CAT, and GPx, as well as non-enzymatic antioxidants like GSH, vitamins C and E, and carotenoids. These antioxidants work together to neutralize ROS, preventing them from causing cellular damage. However, the antioxidant system may become overwhelmed under prolonged oxidative stress, leading to cell dysfunction and oxidative-related diseases (Birben et al., 2012).

Phase I metabolism in fish, such as the enzymatic activity of EROD, generates ROS as by-products (David R. Livingstone, 1991). To combat ROS, cells utilise non-enzymatic scavengers like reduced glutathione (GSH) and antioxidant enzymes (e.g., SOD, CAT, and GPx) (Dhaunsi et al., 1992; Orbea et al., 2000; Singh et al., 1994). CAT, SOD, GPx, and GR work in a coordinated defence system to neutralise different types of ROS at various stages. SOD serves as the first line of defence by converting superoxide radicals into H_2O_2. CAT and GPx then act as the second line of defence, breaking down H_2O_2 into water and oxygen to prevent its accumulation. GR plays a key role by converting oxidized glutathione (GSSG) back into its reduced form (GSH), ensuring a steady supply of GSH for GPx to act (Birben et al., 2012; Carlberg & Mannervik, 1975b; Faria et al., 2009).

In our study on the liver of common carp exposed to CBZ, the measured alterations in antioxidant defence system enzyme activities illustrate a dynamic oxidative stress response to CBZ. Firstly, a significant increase in SOD activity was observed after 7 days at higher CBZ concentrations (5 and 50 µg/L), indicating an early activation of antioxidant response to against elevated ROS levels, in another words, SOD activity in the tissues of fish to initiate the dismutation of ROS derived from pharmaceuticals (such as superoxide anion radical O2−•) to molecules which are less toxic (such as H2O2). SOD activity decreased to levels comparable to the control, and remained low after 28 days, while lipid peroxidation (LPO) levels increased significantly. This pattern suggests

that prolonged CBZ exposure may have exceeded the compensatory capacity of the antioxidant system, leading to oxidative damage accumulation. The reduction in SOD activity could also result from several detailed factors: lipid peroxidation; direct ROS attacks on proteins, which lead to enzyme inactivation; and an energy (NADPH) shortage (Z. Li, Velisek, et al., 2010; Viarengo et al., 1995).

Regarding CAT levels in common carp exposed to CBZ, a significant increase was observed at the highest concentration after 7 days, suggesting an adaptive antioxidant response to elevated ROS. This activation reflects the upregulation of CAT to detoxify hydrogen peroxide (H_2O_2) into water and oxygen. Our results are consistent with previous studies, where short-term (<7 days) CBZ exposure significantly increased SOD, CAT, and GR activities in *Oncorhynchus mykiss* (Z. Li, Velisek, et al., 2010) and *Carassius carassius* (Nkoom et al., 2020). The initial rise in antioxidant enzyme activity likely resulted from ROS accumulation, which stimulated SOD to catalyse the dismutation of superoxide anion radicals ($O_2^{-}\bullet$) into H_2O_2. In turn, H_2O_2 accumulation, produced both by SOD and other oxidase enzymes (e.g., xanthine oxidase, amino acid oxidase, NAD(P)H oxidase), can further induce CAT activity as a compensatory defence (Birben et al., 2012; Van der Oost et al., 2003; Viarengo et al., 1995). Elevated CAT activity persisted after 14 days, indicating sustained oxidative stress, and remained upregulated at 100 µg/L after 28 days. This persistent increase suggests that continuous H_2O_2 production from multiple metabolic sources maintained CAT activation. In contrast, at lower CBZ concentrations (1 and 5 µg/L), CAT activity showed only moderate elevation, reflecting an activated but not exhausted antioxidant defence system.

In the liver of common carp exposed to CBZ, glutathione reductase (GR) levels followed a similar trend to that of superoxide dismutase (SOD). Both enzymes showed a significant increase at 5 and 50 µg/L concentrations after 7 days, suggesting a coordinated and effective defense response to oxidative stress.The upregulation of GR level aimed at maintaining sufficient levels of GSH to support antioxidant processes. The increase in the activity of GR subserves the conversion of GSSG to reduced GSH; reduced GSH can directly scavenge ROS and is subsequently reduced to GSSG in an energy-demanding process utilising NADPH (Z. Li, Velisek, et al., 2010). However,

the GR levels dropped below control levels at 100 µg/L CBZ. This decline might result from prolonged oxidative stress leading to enzyme inactivation due to lipid peroxidation and direct ROS attacks on proteins. Additionally, NADPH depletion, which is required for GR activity, may further limit its function, contributing to the observed suppression over time. Our results of the antioxidant enzymes indicated that the antioxidant defence system may initially compensate for oxidative stress but eventually become overwhelmed, supporting the hypothesis that prolonged CBZ exposure may induce oxidative stress.

As for the experiment on the zebrafish exposure to the CBZ, SOD, GR, and selenium-dependent GPx (GPxSe) levels had significant increases during the first week, indicating a strong initial antioxidant response to ROS. However, at the highest concentration (100 µg/L), the activities of SOD and GR dropped back to control levels even after just one week and remained slightly suppressed through 28 days of exposure. In contrast, GPxTOT and CAT activities did not change significantly until the second week of exposure. After two weeks, both GPxTOT and CAT activities increased at moderate CBZ concentrations (5 and 50 µg/L), suggesting an effective defence response to sustained ROS exposure. However, at 100 µg/L CBZ after 28 days, the activities of GPxTOT and CAT returned to control levels, indicating that the antioxidant system may have been overwhelmed. The initial elevation in antioxidant enzyme activities appears to be driven by inorganic ROS produced during phase I metabolism, as well as by superoxide radicals neutralized by SOD. The observed decline in GST activity also reflects a reduction in available GSH after the first week, most presumably due to the increased usage of GSH in the detoxification process. After the second week, it was likely that the EROD and SOD enzymes became less effective, resulting in an increase in organic ROS. Excessive ROS may have inhibited antioxidant enzymes like CAT and GPxSe, or led to direct oxidative damage to proteins, causing further suppression of enzyme activity (Halliwell & Gutteridge, 2015; Kono & Fridovich, 1982). It is important to note that CAT and GPxSe play complementary roles in eliminating H_2O_2 (Halliwell & Gutteridge, 2015), however, they operate in different cellular compartments, in which GPx functions in the peroxisomes, while CAT is active in the mitochondria and cytosol (David R. Livingstone, 1991).

Additionally, while CAT and GPxSe both target H_2O_2, selenium-independent GPx can also neutralize toxic lipid hydroperoxides (De Luca-Abbott et al., 2005). In this present study, the phase I metabolism (e.g., EROD) cannot effectively break down progesterone, which may lead to higher levels of ROS. This can trigger an increase in GPxTOT and CAT activities as the cells try to remove excess ROS. At the same time, the high ROS levels may overwhelm antioxidant the system and inhibit the activities of SOD, GPxSe, and GR, reducing their ability to function properly. Meanwhile, an energy (NADPH) shortage caused by prolonged CBZ exposure may also explain the observed reduction in antioxidant enzyme activities over time (Z. Li, Velisek, et al., 2010; Viarengo et al., 1995).

The antioxidant enzyme activity patterns in P4-exposed zebrafish suggested that inorganic ROS, likely produced during phase I metabolism, triggered significant changes in the antioxidant defence system. GR and GPxSe showed significant increases after one week of exposure at higher concentrations (50 and 100 ng/L of P4), indicating an early response to elevated ROS levels. This pattern continued through the second and fourth weeks across all P4 concentrations, showing that the cells kept working to maintain GSH levels and reduce ROS over time. The measured GPxTOT activities were generally higher than GPxSe activities, especially at 5 ng/L, 50 ng/L, and 100 ng/L after the first week of exposure. It suggests that both selenium-dependent and independent enzymes contribute to ROS detoxification. CAT activity remained largely unchanged at lower concentrations but was significantly elevated after 28 days at 100 ng/L P4. This late increase suggested that higher levels of H_2O_2 accumulated implied increased CAT needs. Interestingly, SOD activity was not significantly affected by P4 exposure at any time point. It may be because the primary ROS generated may not include superoxide radicals which are specifically targeted by SOD, but rather inorganic ROS produced in phase I metabolism or other processes. Our findings proved that the observed enzyme activity changes correspond closely to phase I metabolism processes. The absence of significant SOD changes supports the idea that H_2O_2 may be the main ROS involved in the antioxidant response to P4 exposure. Additionally, the strong and sustained increases in GPxSe and GR reflected the necessity to regenerate GSH against oxidative stress over

prolonged P4 exposure.

Among the binary mixtures tested, MIX3 had a particularly pronounced effect on zebrafish, causing significant alterations in the antioxidant enzymes GR, GPxSe, and GPxTOT.After the first week, the activities of GPxSe, GPxTOT, and GR were reduced, showing that the antioxidant defence system was initially suppressed. By the fourth week, GPxSe and GPxTOT activities increased significantly, indicating that the cells were trying to recover from the stress. MIX1 caused a significant drop in SOD activity after 28 days, suggesting that the metabolism or mode of action of the mixture of these compounds may differ from the single chemical's effect; the mixture may affect the removal of superoxide radicals. The data also show that different mixtures had different effects, depending on the proportion of CBZ and P4. It indicated that the combined effect of the chemicals is not simply additive but depends on how the compounds interact. The response of the antioxidant system varies with both exposure time and the concentration of each compound. In some cases, the mixture seemed to reduce the harmful effects predicted from the single chemicals, while in others, it made the effects worse. It is notable that MIX3, which had the highest amount of P4, caused the strongest changes in enzyme activities.

6.5. Biotransformation Enzymes (EROD, GST)

EROD is a highly sensitive indicator of contaminant uptake in fish, providing evidence of receptor-mediated induction of cytochrome P450-dependant monooxygenases (the CYP1A subfamily specifically) by xenobiotic chemicals. It is widely used as a biomarker in fish for screening the uptake of environmental organic pollutants (Jönsson et al., 2006). In our experiments with common carp, the significant increase in EROD activity after 28 days of exposure to 50 and 100 µg/L CBZ concentrations indicated that CYP1A enzymes were biosynthesised to detoxify and metabolise CBZ. Notably, the response was dose- and time-dependent, with the highest induction observed at 50 µg/L after 28 days, indicating that CYP1A-mediated metabolism was progressively upregulated as CBZ accumulates. This result is similar to a previous study on *Carassius carassius*, where lower CBZ concentrations (2 and 10 µg/L) also increased EROD activity after a few days

(Nkoom et al., 2020). However, instead of continuing to increase, EROD activity dropped at 100 µg/L after 28 days, suggesting that excessive CBZ exposure impairs the metabolic function of liver, making it harder to keep producing the enzymes, as proposed in the case of AChE.

GST, a member of the Phase II biotransformation enzyme group, is engaged in the conjugation of organic xenobiotics with glutathione, making them more soluble and easier to eliminate and excrete (Vernouillet et al., 2010). GST activity levels significantly increased in the experiments with common carp, suggesting an oxidative stress-induced defence response, alternatively, an enhanced conjugation and excretion of CBZ metabolites in the liver of fish. This result aligns with findings by Nkoom et al. (Nkoom et al., 2020), where 2 and 10 µg/L CBZ exposure led to a significant increase in GST activity in *Carassius carassius* after 1, 4, and 7 days. While GST activity remained stable at lower CBZ concentrations (1 and 5 µg/L), a noticeable increase was observed at higher concentrations (50 and 100 µg/L), particularly after 28 days of exposure, indicating a stronger detoxification response under prolonged high-concentration CBZ exposure. The combined data on EROD and GST activities in our study suggest that CBZ undergoes both Phase I biotransformation and Phase II metabolism in the liver of common carp.

EROD activity showed a clear time-dependent response to CBZ exposure in zebrafish. A significant increase in EROD activity after 7 days of exposure to CBZ showed that CYP1A enzymes were biosynthesized to detoxify and metabolize CBZ. This pattern is consistent with previous studies on *Carassius Carassius* by Nkoom et al., where EROD activity was elevated within the first few days of exposure to CBZ (Nkoom et al., 2020). However, after 14 and 28 days of exposure to CBZ, EROD activity showed a significant decline across all concentrations, suggesting that the initial induction of EROD may have been a short-term response to CBZ exposure. The subsequent decrease might be attributed to metabolic adaptations to the chemical stressor. Additionally, the diminishing response at later time points might be because the metabolism pathway of CBZ has shifted over time. EROD activity exhibited a fluctuating response to P4 exposure, the early increase after 7 and 14 days of exposure suggesting an initial enzymatic induction, potentially linked to the activation of phase I detoxification. The decline of EROD

activity after 28 days of P4 exposure may be related to metabolic adaptation, enzyme inhibition, or a shift in detoxification over a prolonged exposure period. In mixtures, none of the assessed combinations caused a significant effect in EROD activity after 1 week of exposure. MIX2 and MIX3 caused a notable increase in EROD activity after 14 days of exposure to the mixtures. This increase suggested that prolonged exposure triggers phase I metabolic activation. Also, it indicated that MIX1 had a weaker impact on EROD activity compared to the other mixtures.

Both common carp and zebrafish showed a concentration- and time-dependent increase in GST activity during chronic exposure to CBZ, but the timing and pattern of activation differed. GST activity in zebrafish after CBZ exposure showed no significant alteration across all tested concentrations after the first 7 days, suggesting that phase II detoxification was not immediately activated. This aligns with the suggestion that early-stage CBZ metabolism primarily undergoes Phase I metabolism (via CYP1A activation), which is proven by the initial increase of EROD during our tests. In contrast, common carp demonstrated an earlier activation of GST, as written in the previous text. The subsequent notable increase of GST activity at higher CBZ concentrations indicated that the metabolic response shifted toward phase II detoxification. GST activity levels remaining high until 28 days of CBZ exposure suggest that there was a demand for GST for oxidative stress defence and detoxification over a prolonged exposure period. These findings are consistent with previous studies on *Cyprinus carpio*, *Carassius carassius*, and *Danio rerio*, where GST was upregulated after exposure to environmentally relevant CBZ concentrations (1–100 µ/L) (da Silva Santos et al., 2018; X. Liang et al., 2022; Nkoom et al., 2020). Regarding the effect of P4 on GST activity, only the lowest applied concentration (1 ng/L) caused a significant increase in GST levels. This non-linear response pattern may reflect a hormetic effect, where low P4 exposure levels induce moderate antioxidant activation, while higher concentrations fail to produce a similar response. Such concentration-dependent variations in enzyme activity have also been reported in previous studies (Prehn & Berd, 2006).

Regarding the GST activity to the exposure of mixtures, GST activity in all mixture treatments (MIX1, MIX2, MIX3) was lower than in the control group at every time point, suggesting that the

combined exposure to CBZ and P4 does not enhance GST activity as strongly as individual exposure to either compound alone. It might indicate that CBZ and P4 together interfere with GST enzyme function from the early stage. GST activity remained lower in mixtures than in control, suggesting that phase II detoxification is impaired. Meanwhile, there was a clear pattern where higher P4 ratios (MIX1 < MIX2 < MIX3) resulted in stronger GST inhibition. Furthermore, our results showed that the presence of both CBZ and P4 in mixtures could have a synergistic toxic effect, disrupting normal metabolic processes. The observed pattern of the mixtures in GST and EROD activity may suggest an altered metabolic route for xenobiotics, which means that when CBZ and P4 were present together, the way fish metabolised these chemicals was different from when they were exposed to CBZ or P4 alone. Unlike single exposure, the mixtures do not immediately cause a strong metabolism response; the short-term detoxification was delayed in mixtures. In the case of EROD activity, mixtures of CBZ and P4 seemed to shift significant effects in time, mitigating short-term effects and causing a significant increase in chronic effects. This could mean that the presence of P4 delayed CBZ metabolism, possibly because P4 altered enzyme expression.

7. CONCLUSION AND RECOMMENDATIONS

Our studies provide significant insights into the biochemical responses of juvenile common carp (*Cyprinus carpio*) and zebrafish (*Danio rerio*) to chronic exposure to environmentally relevant concentrations of carbamazepine (CBZ) and progesterone (P4), both individually and in their binary combinations. Our findings indicate that prolonged exposure to these pharmaceuticals significantly disrupts xenobiotic metabolism, detoxification processes, oxidative stress defence, and endocrine function. The observed alterations in biotransformation enzymes (EROD, GST), oxidative stress markers (SOD, CAT, GPx, GR, LPO), reproductive biomarkers (VTG), and organ and tissue damage (LDH, AChE, DNAsb) indicate potential ecological risks associated with pharmaceutical pollution in aquatic environments.

Both studies indicate that CBZ and P4 exposure disrupts Phase I (EROD) and Phase II (GST) metabolism, which play crucial roles in the defence against these of xenobiotics. CBZ exposure initially induced EROD activity, reflecting an effective detoxification response; however, prolonged exposure resulted in a decline in EROD activity, suggesting either metabolic adaptation or enzymatic inhibition. GST activity increased in a concentration-dependent manner, indicating elevated detoxification demands, while P4 exposure further modulated these enzymatic pathways, with synergistic effects observed in CBZ and P4 mixtures. These findings suggest that pharmaceutical mixtures may enhance metabolic disruption beyond the effects of individual compounds, emphasising the need to consider specific mixture toxicity features in environmental risk assessments.

Changes in the activity of antioxidant enzymes (SOD, CAT, GPxSe, GPxTOT, GR) showed that oxidative stress defences were disrupted. The antioxidant enzyme system showed an initial upregulation, followed by a reduction in activity upon extended exposure, indicating potential adaptation of fish to toxic stress. The increased levels of LPO and DNAsb further confirmed oxidative damage, suggesting that chronic pharmaceutical exposure can cause cellular damage. Additionally, the significant increase in LDH activity indicates metabolic stress and potential

tissue damage, reinforcing concerns regarding the sub-lethal effects of these contaminants.

The observed changes in vitellogenin-like protein (VTG) levels further highlight the endocrine-disrupting potential of CBZ and P4. CBZ exposure upregulated VTG expression, suggesting estrogenic-like activity, while P4, known for its endocrine-modulating effects, induced concentration-dependent alterations in VTG levels. Notably, CBZ and P4 mixtures exhibited non-linear, synergistic interactions, exacerbating VTG disruption and indicating potential reproductive toxicity in exposed fish populations. These findings underscore the importance of evaluating pharmaceutical mixtures, as chemical interactions may enhance endocrine disruption beyond individual compound effects.

Given the widespread occurrence of pharmaceutical contaminants in aquatic environments, these results highlight the need for long-term ecological studies to assess the impact of pharmaceutical mixtures on aquatic biota. Most of the biomarkers assessed in this study, such as EROD, GST, LPO, LDH, and VTG, serve as early warning signals at the biochemical or cellular level. Future research should explore the molecular mechanisms underlying metabolic adaptation and oxidative stress responses, and further investigate whether these biochemical and endocrine disruptions may lead to adverse reproductive outcomes in aquatic organisms. Additionally, improved wastewater treatment technologies and stricter regulatory policies are essential to mitigate the risks posed by pharmaceutical pollutants and safeguard the health of the aquatic ecosystem.

8. NEW SCIENTIFIC RESULTS

1. This study provides novel subacute toxicity data on carbamazepine (CBZ) exposure in *Cyprinus carpio*, a freshwater species less studied in pharmaceutical ecotoxicology compared to zebrafish. Using environmentally relevant concentrations and a comprehensive biomarker approach, the study reveals significant oxidative stress, enzymatic disruption, and tissue-level damage after 28 days of exposure.

2. This study is one of the first to report that chronic CBZ exposure at environmental concentrations can trigger apoptotic responses and tissue damage in juvenile *Cyprinus carpio*, as indicated by alterations in LPO, LDH, and DNAsb biomarkers.

3. This is one of the first studies to demonstrate synergistic and dose-ratio-dependent effects of CBZ and progesterone (P4) mixtures in *Danio rerio* at environmentally relevant concentrations. The observed effects on endocrine (VTG), biotransformation (EROD, GST), and damage (DNAsb) biomarkers exceeded the toxicity thresholds observed for individual compounds, suggesting chemical interaction and enhanced mixture toxicity.

4. This study provides new toxicological evidence supporting the use of a multi-biomarker approach to assess sublethal effects of chronic pharmaceutical exposure. The integrated biomarker responses offer early and reliable warning signals, even at low exposure levels.

5. The cross-species comparison between Cyprinus carpio and Danio rerio under harmonized experimental conditions provides a novel dataset for comparative pharmaceutical toxicity assessment. The findings contribute to understanding species-specific biomarker response patterns, supporting broader applications of fish models in environmental risk assessment.

6. The endocrine-disrupting potential of CBZ and P4 was evidenced by altered VTG levels in zebrafish, suggesting reproductive risks associated with long-term exposure.

9. SUMMARY

The objective of this work was to evaluate the chronic ecotoxicological effects of two representative emerging micropollutants, carbamazepine (CBZ) and progesterone (P4), at environmentally relevant concentrations.Two ecologically and environmentally important fish species, *Danio rerio* (zebrafish) and *Cyprinus carpio* (common carp) were selected to systematically evaluate both individual and binary mixture effects of CBZ and P4 under their environmentally relevant concentrations

This study investigated the chronic biochemical responses of common carp and zebrafish by measuring a set of biochemical markers, including antioxidant enzymes (SOD, CAT, GPx, GR), biotransformation enzymes (EROD, GST), endocrine disruption biomarker (VTG), neurological biomarker (AChE) and cellular damage indicators (LPO, LDH, DNAsb) after exposure to CBZ, P4, and their binary mixtures for 28 days.

The results demonstrated that prolonged exposure to CBZ and P4 disrupted Phase I and II detoxification processes, induced oxidative stress, and modulated endocrine function. EROD activity was initially elevated but decreased over time, while GST showed concentration-dependent induction. Antioxidant enzyme activities fluctuated, with early activation followed by inhibition under long-term exposure. Biomarkers of damage, such as LPO, DNAsb, and LDH increased significantly, indicating metabolic and cellular stress.

Notably, CBZ and P4 mixtures exhibited synergistic effects on multiple endpoints, particularly VTG expression, highlighting their potential to disrupt the endocrine system. The findings suggest that pharmaceutical mixtures pose greater risks than individual compounds and emphasize the need to include mixture toxicity in environmental risk assessments.

10. PUBLICATIONS

Peer-reviewed journal articles

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Time flows quietly, and the years slip by. Now, standing at the threshold of my thirties, I look back on my academic journey with deep emotion. Many of those feelings, like the wind, have passed gently through me: soft, yet unforgettable. In the bright summer of 2020, I began my PhD journey without hesitation. I still remember that day: the sky in Gödöllő was bright blue, the breeze was light, and a voice inside me whispered, “Go ahead. Whatever you long to experience, go and embrace it.” As an old Chinese proverb says, “Do good deeds, and do not ask about the future.” So I followed the wind, full of ideals and courage, and stepped into this chapter of my life.

Perhaps the gears of destiny began turning even earlier. At the end of 2013, I learned about the bilateral scholarship program jointly supported by the China Scholarship Council (CSC) and Hungary’s Tempus Public Foundation (TPF). After months of preparation, I arrived in Hungary in 2014 to begin my studies. I was not yet 19. A blue 200E airport bus carried me into the city. I could never have imagined that one day I would develop such a deep and enduring connection with this land.

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In early 2021, I became pregnant. There was a moment of joy, but also of overwhelming uncertainty. As a young PhD student, the decision to continue was not easy. I still remember standing at the entrance of the lab, unsure of the future. It was Zsolti who sat beside me, gently held my hand, and said, “Xinyue, don’t be afraid. Don’t give up. We will support you.” His words became a beacon of strength in the years that followed.

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"Though the road is long and winding, I will keep going." — Qu Yuan, Warring States Period

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12. APPENDICES

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12.2. Supplementary data

12.2.1. Institutional Review Board Statement

All animal experiments in this study were conducted in compliance with the International Guiding Principles for Biomedical Research Involving Animals (EU Directive 2010/63) and the relevant Hungarian laws on animal welfare. The experimental procedures were approved by the National Scientific Ethical Committee on Animal Experimentation (protocol number: KA-2711). The animal use license was issued by the Government Office of Pest County (Permit number: PE/EA/731-7/2019). All procedures were performed by trained and accredited researchers to ensure ethical and professional handling of the fish.