HUNGARIAN UNIVERSITY OF AGRICULTURE AND LIFE SCIENCES

CHRONIC EFFECTS ASSESSMENTS OF MICROPOLLUTANTS APPLYING BIOCHEMICAL MARKERS OF FISH

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1. BACKGROUND AND RESEARCH OBJECTIVES

1.1. Pharmaceuticals

Pharmaceuticals and their metabolites are increasingly recognized as one of the most concerning groups of emerging contaminants in almost every aquatic ecosystem. 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. 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, through similar biochemical pathways. The potential impairment caused by pharmaceuticals in water bodies remains relatively less understood than other pollutants. Recent studies have reported that huge amounts of pharmaceuticals co-occur in the aquatic environment simultaneously, especially in WWTPs and agricultural runoffs. Therefore, this group of pollutants has received increasing attention in recent years due to its potential ecological and toxicological implications.

Among these compounds, the anticonvulsant carbamazepine (CBZ) is widely prescribed. CBZ is an anticonvulsant drug prescribed worldwide for the treatment of bipolar disorder, trigeminal neuralgia, and psychomotor epilepsy. It is one of the most frequently detected pharmaceuticals in rivers worldwide due to its high persistence and poor removal during WWTPs. 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. Although CBZ toxicity has been investigated in several aquatic species, including algae, cladocerans, and fish. Most research has focused on short-term exposure, leaving its chronic biochemical and physiological effects largely unexplored.

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. 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 containing P4 concentrations often ranging from 0.07 to 22.2 ng/L. 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.

1.2. Combined effects of pharmaceutical mixtures

Pharmaceuticals rarely occur alone; they often occur as multi-component mixtures with other micro- and macro-pollutants in aquatic environments. Evidence indicates that mixtures frequently exhibit greater or unpredictable toxicity compared with individual compounds. Mixtures of pharmaceuticals can induce unexpected effects even when individual compounds show no significant effects. 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. Therefore, more sensitive molecular and biochemical endpoints are needed to evaluate the potential risks of pharmaceutical mixtures to aquatic biota.

1.3. Environmental toxicology methods

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.

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. Typically, single-species tests in acute cases are often conducted, where the endpoint is most often survival, growth, reproduction, or related physiological features. 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. Meanwhile, it is important to make sure that the test species adequately represent the studied biota, also a sensitive indicator. 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.

However, 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. 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.

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. The hierarchical biological responses to environmental stress within the biological system are shown in Figure 1, 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.

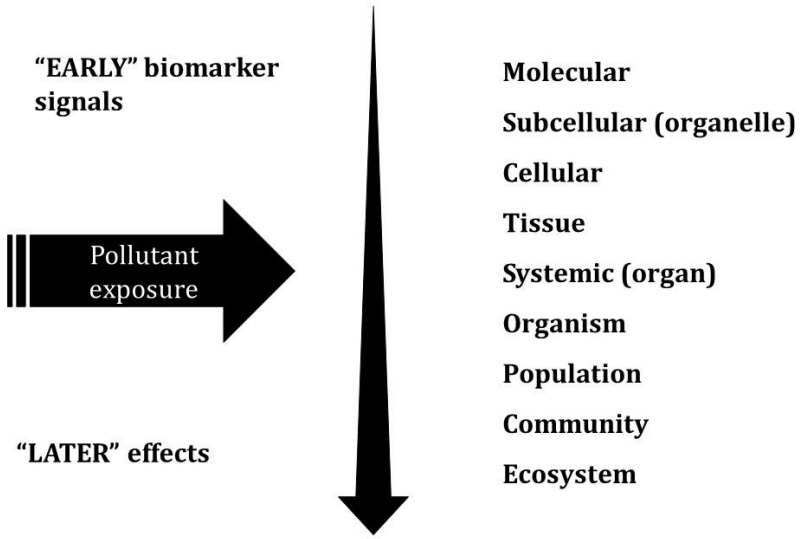

Figure 1: The response orders to environmental stress within a biological system with adjustments.

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.

Fish have received considerable attention as sentinel species in aquatic ecosystems in aquatic pollutant studies. 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.

1.4. Objectives

Pharmaceuticals such as carbamazepine (CBZ) and progesterone (P4) are frequently detected in aquatic environments, often co-occurring at environmentally relevant concentrations. Their potential chronic and combined toxicological effects on aquatic organisms, however, remain poorly understood.

The main objective of this study was to investigate the chronic biochemical responses induced by CBZ and P4, both individually and in binary mixtures, using two model 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.

2. MATERIALS AND METHODS

2.1. Fish maintenance

Fish maintenance was conducted in the Department of Environmental Toxicology at the Hungarian University of Agriculture and Life Sciences (Gödöllő, Hungary). Juvenile common carp (*Cyprinus carpio*) were kept in an individually designed recirculating system (10 m^3 tanks) under controlled water conditions (22 ± 2 °C, pH 7.8 ± 0.2, redox 230 ± 2 mV, dissolved O_2 6.8 ± 1 mg/L) with a 14 h:10 h light–dark cycle. The fish were fed AquaGarant Aquastart pellets (1.2–1.5 mm; 10 g kg^{-1} body weight) twice daily.

AB wild-type zebrafish (*Danio rerio*) were maintained in a Tecniplast ZebTec recirculating housing system (Buguggiate, Italy) under similar light–dark conditions (14 h:10 h). Water parameters were kept constant (25 ± 0.5 °C, pH 7.0 ± 0.2, conductivity 500 ± 50 µS, hardness < 0.5° dH, DO > 90%). Zebrafish were fed twice daily with ZEBRAFEED (Sparos, 400–600 µm) and supplemented with brine shrimp (Ocean Nutrition, > 230,000 NPG) twice a week.

2.2. Experimental Design

The exposure experiments for common carp and zebrafish followed similar 28-day protocols with minor differences. Both species were exposed to carbamazepine (CBZ) at nominal concentrations of 0, 1, 5, 50, and 100 µg/L in triplicate tanks (15 fish per tank). Fish were sampled after 7, 14, and 28 days (five fish per tank) and euthanised with an overdose of MS-222 (0.04%) for tissue collection (brain, liver, gonads, or intestine). Samples were stored at −80 °C for biochemical analyses.

For common carp, juveniles (7.37 ± 1.35 g) were kept in 50 L tanks, and the test medium was renewed every three days. Water quality and chemical concentrations were monitored by LC–MS/MS, remaining within ± 20% of nominal levels throughout the experiment.

For zebrafish, adult AB wild-type fish (9–12 months old) were kept in 3 L tanks under similar conditions. In addition to CBZ, progesterone (P4) was tested individually (at nominal concentrations of 0, 1, 5, 50, and 100 µg/L) and in mixtures to evaluate combined effects. Mixtures were prepared according to toxicity unit (TU) ratios of CBZ: P4 (MIX1: 0.75:0.25, MIX2: 0.5:0.5, MIX3: 0.25:0.75), corresponding to a total of 1 TU each. Water quality and chemical stability were confirmed by LC–MS/MS analysis (within 20% of nominal concentrations). From each replicate, five zebrafish were sacrificed per sampling period for tissue collection and storage at −80 °C.

2.3. Biomarker Determinations

Biochemical biomarkers were determined in homogenised liver, intestine, and brain tissues from both fish species. Samples were prepared under cold conditions using appropriate phosphate or Hepes buffers, and protein concentrations were determined by the Bradford method. The following biomarkers were analysed: lipid peroxidation (LPO) as an indicator of oxidative damage, based on the thiobarbituric acid reactive substances (TBARS) assay; acetylcholinesterase (AChE) activity, following, to assess neurotoxicity; and lactate dehydrogenase (LDH) activity, measured according to, to evaluate cytotoxicity and tissue damage. DNA strand breaks (DNAsb) were quantified using the alkaline precipitation assay, and vitellogenin (VTG) was determined through the alkali-labile phosphate method as an indicator of endocrine disruption. The activities of major antioxidant enzymes, including catalase (CAT), superoxide dismutase (SOD), glutathione reductase (GR), glutathione peroxidase (GPx), and glutathione S-transferase (GST) were determined spectrophotometrically according to established protocols, while 7-ethoxyresorufin-O-deethylase (EROD) activity was assessed as a marker of cytochrome P4501A (CYP1A)-mediated phase I metabolism. All enzymatic activities were measured in triplicate using a Thermo Varioskan™ LUX multimode microplate reader, and results were normalised to protein content.

2.4. 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.

3. RESULTS AND DISCUSSION

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

3.1.1. 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. By day 14, VTG levels declined to control values without clear dose dependence. After 28 days, slight increases were detected at 1 and 5 µg/L, followed by decrease at 50 and 100 µg/L, however, these decreases were not statistically significant.

3.1.2. 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. 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.

3.1.3. Damage Markers (DNAsb, LDH, LPO)

The DNAsb measured in liver samples was slightly decreased as compared to controls during the first two weeks of exposure to CBZ, with differences in measured levels diminishing by the fourth week of exposure. Differences (decrease) in experimental DNAsb levels compared to the control groups proved significant ($p < 0.05$) only with 1 µg/L CBZ concentration exposure after 7 days of exposure. Statistically, there was no time- or concentration dependence. LDH activity decreased slightly at 1 µg/L after 7 days and showed no significant change at later stages. LPO remained stable up to day 28, when a sharp and significant increase appeared at 100 µg/L ($p < 0.05$). 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).

3.1.4. Antioxidant Defence (CAT, SOD, GR)

The CAT activity slightly increased at 50 µg/L and sharply increased at 100 µg/L during the first week, both with significant differences ($p < 0.05$), and CAT activity remained elevated throughout the 28-day CBZ exposure, showing a clear time- and concentration-dependent pattern. SOD and GR activities displayed similar trends, significant ($p < 0.05$) increases at 5 and 50 µg/L after 7 days, followed by declines to near-control levels after 14 and 28 days. The early activation and subsequent reduction of these enzymes suggest an initial antioxidant response that diminished under prolonged exposure.

3.1.5. Biotransformation Enzymes (EROD, GST)

After 7 days of exposure to CBZ, the hepatic EROD significantly increased as a function of exposure concentration. 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. 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 ($p < 0.05$). GST activity showed only a moderate rise during the 28-day exposure, with a significant ($p < 0.05$) increase detected solely at 100 µg/L.

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

3.2.1. Endocrine Disruption Biomarker (VTG)

Exposure to CBZ caused a concentration-dependent increase in VTG levels after 7 and 14 days, with significant elevation observed only at 100 µg/L. 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. In contrast, P4 exposure produced a decreasing trend in VTG levels at all test concentrations

after 7 and 14 days, with statistically significant reductions detected only at 50 and 100 ng/L after 28 days. All the binary mixtures did not significantly ($p < 0.05$) affect VTG levels after 7 days of exposure. For the binary mixtures, VTG levels remained unchanged after 7 days and showed a slight, non-significant decrease after 14 days. However, after 28 days, VTG increased progressively with the proportion of P4 in the mixture, reaching significant ($p < 0.05$) elevations in MIX2 (CBZ: P4 = 0.5:0.5 TU) and MIX3 (0.25:0.75 TU).

3.2.2. Neurological Biomarker (AChE)

After 7 days of exposure to CBZ, AChE of zebrafish slightly increased 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. As for P4, AChE activity increased at higher concentrations (50 and 100 ng/L) after 7 days, with a slight but significant ($p < 0.05$) elevation persisting at 100 ng/L after 14 and 28 days ($p < 0.05$) compared with the activity values measured in control groups. In exposures to mixtures, a significant ($p < 0.05$) drop was only 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.

3.2.3. Damage Markers (DNAsb, LPO, LDH)

Exposure to CBZ caused a concentration- and time-dependent fluctuation in DNA strand break levels. 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 ($p < 0.05$). 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. Exposure to P4 caused a significant elevation of DNAsb after one week at the concentrations tested. In P4-treated fish, DNAsb increased significantly ($p < 0.05$) after 7 days across all concentrations, reaching the highest level at 5 ng/L. After 14 days, a significant ($p < 0.05$) increase persisted only at 100 ng/L, while after 28 days, no significant differences were found. For the binary mixtures, only MIX3 significantly ($p < 0.05$) decreased DNAsb values as compared to controls after 7 days. 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.

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. 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. According to the results, binary mixtures did not significantly induce LPO changes in zebrafish during the four-week exposure time.

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. 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. For the mixtures, MIX1, MIX2, and MIX3, the LDH activity significantly increased after one week. 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.

3.2.4. 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. 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. 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. In the binary mixtures of CBZ and P4, only MIX3 induced a significant increase in CAT activity after 14 days of exposure.

Exposure to CBZ produced distinct and time-dependent changes in glutathione peroxidase (GPx) activity. The selenium-dependent GPx (GPxSe) increased significantly ($p < 0.05$) at 50 and 100 µg/L after 7 days, but its activity decreased sharply at all concentrations after 14 and 28 days. In contrast, total GPx (GPxTOT) activity remained stable during the first week but rose significantly ($p < 0.05$) between 5 and 100 µg/L after 14 days. At 28 days, GPxTOT remained elevated ($p < 0.05$) at 5 and 50 µg/L but declined ($p < 0.05$) at 100 µg/L compared with the value measured at 50 µg/L. 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 of P4. 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. 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. 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. 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. 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).

In zebrafish exposed to CBZ, GR activity increased significantly ($p < 0.05$) at low to medium concentrations (1–50 μg/L) after 7 days, showing a concentration-dependent trend, while the activity at 100 μg/L declined slightly to control levels. No significant differences were observed after 14 or 28 days. Following P4 exposure, GR activity showed consistent and significant ($p < 0.05$) increases across most concentrations throughout the 28 days. The elevation was concentration-dependent after 7 days and remained stable at 5–100 ng/L after 14

and 28 days. 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.

Exposure to CBZ caused significant ($p < 0.05$) increases in SOD activity after 7 days at 1–50 µg/L, peaking at 50 µg/L, while activity at 100 µg/L declined to control levels. After 14 and 28 days, SOD activity decreased across all treatments and returned to baseline, but without any significant differences. 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. 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. 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.

3.2.5. Biotransformation Enzymes (EROD, GST)

After the first week of exposure to CBZ, EROD activity increased continuously and significantly ($p < 0.05$), peaking at 5 µg/L before declining toward control levels at higher concentrations (50–100 µg/L). During prolonged exposure (14 and 28 days), EROD activity declined to near-control levels and did not differ significantly among the CBZ treatments. 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 ($p < 0.05$). After 14 days, EROD activities increased significantly ($p < 0.05$) at lower concentrations (1, 5 ng/L). After 28 days, EROD activity was significantly ($p < 0.05$) reduced only at 100 ng/L.

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).

GST activity at different exposure concentrations did not cause a significant change after the first week of CBZ exposure. After 14 and 28 days, GST levels showed a concentration-dependent increase: 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. 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. 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.

3.3. Discussion

Carbamazepine (CBZ) is among the most frequently detected pharmaceuticals in surface waters worldwide and is known to exert chronic sublethal effects on aquatic organisms even at environmentally relevant concentrations. In this study, CBZ exposure caused multiple biochemical alterations in the common carp (*Cyprinus carpio*) and zebrafish (*Danio rerio*), reflecting oxidative stress, adaptive detoxification, and possible endocrine disruption.

VTG, a glycolipophosphoprotein synthesized in the liver, serves as a sensitive biomarker for detecting estrogenic substances in aquatic environments. Increased VTG levels of the common carp at 50, 100 µg/L after seven days point to possible estrogenic-like effects of CBZ, supporting previous findings that link CBZ exposure with reproductive disruption in fish. As for the zebrafish, CBZ exposure

elevated VTG concentrations during the first two weeks, with a significant rise detected at the 100 µg/L concentration, this pattern was like 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. P4, in contrast, reduced VTG production after 28 days of exposure to 50 and 100 ng/L, indicating an inhibitory effect at higher concentrations. Whereas mixtures of CBZ and P4 caused significant VTG increases after prolonged exposure, suggesting potential synergistic endocrine disruption at higher P4 ratios.

Reduced AChE activity is attributed to neurotoxic agents, and it is a commonly applied biochemical marker of neurotoxic environmental pollutants. The inhibition of AChE activity in the common carp after short-term exposure and its subsequent increase during longer exposure periods may indicate an initial neurotoxic effect followed by compensatory or apoptotic processes. Similar patterns were reported in other fish species (e.g., *Brachionus koreanus, Carassius Carassius*) exposed to CBZ. An increase in AChE activity is often a consequence of tissue alterations (e.g., apoptosis). For example, a previous study found that CBZ at environmentally relevant concentrations (1, 10, and 100 µg/L) caused apoptosis in the liver cells of *Gobiocypris rarus*. As for zebrafish, CBZ exposure leads to time- and concentration-dependent changes in AChE activity, which is consistent with findings from other zebrafish studies. However, at higher concentrations, such as 50 µg/L and 100 µg/L, AChE activity decreased. This reduction at higher concentrations may be due to the cumulative toxic effects of CBZ. Similarly, after 28 days of exposure to P4, a significant increase in AChE activity was observed at the highest concentration (100 ng/L). Previous animal studies have linked increased AChE activity with oxidative stress, the production of free radicals, and apoptosis.

As for the common carp, our study found a slight decrease in DNAsb levels during the 28-day exposure period. This lower level of DNA strand breaks may be explained by the inhibitory effect of CBZ on cell division during longer exposure times. In zebrafish, DNAsb levels initially increased after CBZ exposure, likely

due to oxidative damage caused by ROS. This suggests that oxidative stress was a key factor contributing to genetic material damage. However, the subsequent decrease in DNAsb levels might indicate the inhibition of cell division, which could have limited the division of DNA strands.

LPO is the result of the process where ROS react with polyunsaturated fatty acids in cell membranes, causing damage to the structure of the membranes. The TBARS levels in our tests with common carp exposed to CBZ showed a significant elevation only at the highest exposure 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. It highlights that prolonged CBZ exposure can lead to oxidative stress in different fish. As for zebrafish, CBZ alone did not elevate lipid peroxidation except the decrease at 100 μg/L CBZ concentration after 14 days, which may be attributed to the protective effect of the antioxidative system. 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, suggesting a deterioration in the condition of fish.

LDH is a common indicator of tissue and organ damage, reflecting both metabolic activity and structural alterations. In common carp, a transient decrease occurred at 1 μg/L after 7 days, possibly reflecting an adaptive response to low-level stress. P4 exposure led to significantly elevated LDH activity at 50–100 ng/L after one week and at 100 ng/L after two weeks, indicating hepatic stress and potential cellular damage. In the binary mixtures, all treatments caused early increases in LDH activity, suggesting acute metabolic stress, but only MIX3 (the P4-dominant mixture) maintained this elevation after four weeks. The stronger response in MIX3 is consistent with its greater impact on other biomarkers, implying enhanced toxicity from the combined action of CBZ and P4.

Reactive oxygen species (ROS) imbalance is a key driver of oxidative stress. In

common carp, CBZ exposure induced typical antioxidant responses: SOD and GR activities increased during the first week, then declined below control levels after 14–28 days, while CAT activity remained significantly elevated among all the concentrations. This pattern suggests an initial activation of antioxidant defenses followed by partial exhaustion under prolonged stress. The sustained CAT elevation likely reflects ongoing H_2O_2 detoxification, whereas the later decrease in SOD and GR may result from enzyme inhibition or NADPH depletion, consistent with previous studies reporting transient activation and subsequent suppression of antioxidant enzymes during chronic CBZ exposure. In zebrafish exposed to CBZ, SOD, GR, and GPxSe activities increased significantly during the first week, reflecting an early antioxidant defense against ROS. However, at 100 μg/L, SOD and GR rapidly returned to control levels and remained suppressed through 28 days, indicating enzyme inhibition or exhaustion. GPxTOT and CAT activities rose later, peaking at moderate concentrations (5–50 μg/L) after two weeks, but declined again at 100 μg/L after 28 days, suggesting that prolonged oxidative pressure overwhelmed the antioxidant system. The early activation of enzymes was likely triggered by inorganic ROS from phase I metabolism, while the later decline corresponded to excessive ROS and NADPH depletion. Complementary roles of CAT and GPx in H_2O_2 detoxification explain their parallel upregulation, whereas the observed GST decrease suggests GSH consumption during detoxification. Overall, these findings indicate that sustained CBZ exposure disrupted redox process by exhausting the enzymatic antioxidant capacity over time. P4 exposure in zebrafish induced sustained increases in GR and GPx activities, indicating continuous antioxidant activation against inorganic ROS, while CAT rose later under high oxidative pressure. In CBZ–P4 mixtures, antioxidant responses were initially suppressed but recovered over time, with the strongest effects in P4-dominant mixtures, suggesting complex, non-additive oxidative interactions.

EROD and GST are key biotransformation enzymes. Both common carp and zebrafish showed a concentration- and time-dependent increase in GST activity during chronic exposure to CBZ. As for the common carp, the induction of EROD and GST activities confirmed the activation of hepatic phase I and II detoxification pathways. EROD activity increased at early exposure stages and GST at later stages, suggesting metabolic adaptation to xenobiotic stress. As for

the zebrafish, EROD and GST showed opposite time-dependent patterns: EROD induction appeared early and declined over time, whereas GST increased during later exposure stages and under P4 co-exposure. In mixture treatments, both enzymes showed altered time-dependent patterns, with the early detoxification being delayed or suppressed, suggesting that combined CBZ and P4 exposure modified the normal phase I and II metabolic responses.

4. 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.

5. 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.

6. PUBLICATIONS IN THE RESEARCH FIELD

Peer-reviewed journal articles

1. Ács, A., **Liang, X.**, Bock, I., Griffitts, J., Ivánovics, B., Vásárhelyi, E., Ferincz, Á., Pirger, Z., Urbányi, B., & Csenki, Z. (2022). Chronic effects of carbamazepine, progesterone and their mixtures at environmentally relevant concentrations on biochemical markers of zebrafish (*Danio rerio*). Antioxidants, 11(9), 1776. DOI: 10.3390/antiox11091776
2. **Liang, X**., Csenki, Z., Ivánovics, B., Bock, I., Csorbai, B., Molnár, J., Vásárhelyi, E., Griffitts, J., Ferincz, Á., Urbányi, B., & Ács, A. (2022). Biochemical marker assessment of chronic carbamazepine exposure at environmentally relevant concentrations in juvenile common carp (*Cyprinus carpio*). Antioxidants, 11(6), 1136. DOI: 10.3390/antiox11061136
3. Tóth, G., Háhn, J., Szabó, G., Bakos, K., Volner, C., **Liang, X.**, Göbölös, B., Bock, I., Szoboszlay, S., Urbányi, B., Kriszt, B., Kaszab, E., Szabó, I., & Csenki, Z. (2024). In vivo estrogenicity of glyphosate, its formulations, and AMPA on transgenic zebrafish (*Danio rerio*) embryos. Environmental Pollution, 342, 123113. DOI: 10.1016/j.envpol.2023.123113

Conference abstracts/presentations

4. **Liang, X.** (2022). Chronic Effects of Carbamazepine, Progesterone and Their Mixtures at Environmentally Relevant Concentrations on Biochemical Markers of Zebrafish (Danio rerio).
   Scientific Conference of PhD Students of FAFR, FBFS and FHLE SUA, Nitra, Slovakia.
   ISBN: 978-80-552-2538-8. DOI: DOI: 10.15414/2022.9788055225388
   (Based on: Ács, A., Liang, X., et al. 2022. Antioxidants 11(9):1776.
   DOI: 10.3390/antiox11091776)