Final report

Identifier: OTKA FK128705

Principal investigator: dr. András, Ács

Title:

„Evaluation of combined effects of antibiotics and endocrine disrupting chemicals on fish growth”

Project start: 01 September 2018
Closing date: 31 August 2022

1. Introduction

The fish production in semi-extensive aquaculture facilities is partially based on the natural planktonic production, which is usually enhanced by fertilizers, most commonly by different types of manure [1]. Manure deriving from intensive livestock farms (most commonly cattle, pig, or poultry) may contain a number of undesirable chemicals, such as significant amounts of antibiotics excreted by animals (17-90 % from livestock) [2–4], and endocrine disrupting chemicals (EDCs), particularly estrogenic hormones [5–9]. Similarly, pharmaceuticals and their metabolites are found in almost every river worldwide [10]. These pharmaceuticals are released into the environment in high amounts, among others through wastewater treatment plants (WWTP), which have very limited removal or biodegrading efficiency [11,12]. In surface waters, these chemicals may undergo bioaccumulation [13] and/or exert harmful effects in living organisms [14], particularly fish [15–17].

Antimicrobials and hormones can be referred as micropollutants. The term micropollutant refers to organic or mineral substances present in waters at trace concentrations, ranging from few ng/L to several µg/L [18]. Pharmaceuticals and other micro- and macro-pollutants often occur as multicomponent mixtures in an environmental compartment [19–22]. Until now, studies on the toxicity of these drugs on non-target organisms, such as fish, particularly as mixtures at environmentally relevant concentrations, have been very limited. Moreover, the joint toxic effect of mixtures is typically higher than the toxicity of the compounds individually [23,24]. Even binary mixtures of different compounds often show a similar effect [25,26]. In the last decade, it has become evident that organisms exposed to a complex mixture of pollutants in the nature, can show unexpected dose-responses, and subsequent synergistic and antagonistic effects, as well as additive or non-monotonic dose responses [27–29]. Such unknown joint-action of chemicals could significantly worsen the energy balance of fish, and subsequently, further decrease the growth rate.

Therefore, our main aim was – through the characterization of toxic alterations, assessment of food utilization efficiency (by means of metabolic enzymes and status), and subsequent energetic stress – to evaluate the potential growth rate reducing effect caused by relevant antimicrobials, endocrine disrupting chemicals (EDCs) and joint-action of their mixtures. By using fish species of toxicologic (zebrafish, *Danio rerio*) and economic (common carp, *Cyprinus carpio*) significance, results of the presented work here, subserves both, aquaculture and environmental protection. Also, the use of juvenile fish has particular relevance to growth rate investigations as them being in a rapid growth period.

2. Experimental design

2.1. FET tests

Zebrafish embryos were obtained from the spawning adults (adult male and female ratio 2/1), placed in breeding chambers the day before embryos were needed. The spawning was induced in the morning, by turning the lights on. Acute toxicity tests on bendiocarb were carried out according to the OECD guideline 236 (fish embryo acute toxicity test (FET), OECD 2013). The fish embryo acute (96 h) toxicity tests were performed using fertilized and apparently healthy zebrafish eggs. Before the embryos reached 8-cell stage, three replicates were set up for each test concentration. All exposure experiments were carried out in plastic 24-well plates equipped with covers. The test was repeated twice on different treated groups at same exposure concentrations. Detailed description of the procedures is described in our previous paper [30].

2.2. Subacute tests

For the subacute, 28 days fish tests, males and females (juvenile carp: weight 7.37 ± 1.35 g; zebrafish: 9–12-month-old) were randomly distributed into fifteen experimental tanks, each containing 50 L (carp) or 3 L (zebrafish) of the test solution in four concentrations and control in three replicates. Fifteen fish (15 fish each replicate) were used per treatment. The lowest and highest test solution concentrations tested were selected based on recent literature data. To ensure agreement between nominal and actual compound concentrations in the aquaria, water samples were analysed during the experimental period by LC–MS/MS. Water samples were collected from the test aquaria after 1 h and 36 h of renewing the test solutions. The mean concentration of CBZ in the water samples was consistently within 20% of the intended concentration.

To evaluate the non-linear response produced by the joint effect of the selected test chemicals in binary mixtures, the EC_{50} was calculated based on VTG results. VTG was selected due to the expected effect of the test chemicals. Based on toxicity units (TU; 1 TU = concentration of a compound in the mixture per the compound's EC_{50}), mixtures were composed to equal 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)). Mixtures were tested in the same setup as described previously (0, MIX1, MIX2, MIX3, 3 replicates, 15 fish each). In all mixtures, the theoretical toxic effect was expected to be 50%. In this setup, non-linear mixture effects (synergistic or antagonistic) were easily identifiable, and the effect of different concentration ratios were observable [31].

At the 7^{th}, 14^{th}, and 28^{th} days of exposure, 5 fish from each exposure concentration and replicate were sacrificed after an anaesthetic overdose (0.04% MS-222 (tricaine-methane-sulphonate) (Sigma-Aldrich, Darmstadt, Germany)). After weight scaling the brain, liver, and intestine of each fish was isolated and stored in microtubes at −80 °C for later bio-chemical analyses.

Biochemical analyses included the assessment of biotransformation enzymes 7-ethoxyresorufin O-deethylase (EROD) and gluta-thione-S-transferase (GST), antioxidant enzymes catalase (CAT), superoxide dismutase (SOD), glutathione peroxidases (GPxSe and GPxTOT), and glutathione reductase (GR), and markers of damage, such as DNA strand breaks (DNAsb), lactate dehydrogenase (LDH), lipid peroxidation (LPO), and vitellogenin-like proteins (VTG). Homogenization and biochemical determinations were performed as described in our previously appeared papers [29,30,32].

An integrated biomarker response (IBR) index was calculated for biomarkers and ranged clockwise according to their hierarchy of biological organization, from the subcellular to the individual level, using a star plot calculation, for each compound, or each mixture. Individual

areas Ai connecting the i^{th} and the $(i + 1)^{th}$ radius coordinates of the star plot will be obtained according to the formula:

$$Ai=1/2\text{-}\sin((2\pi/n)\ Si\ Si+1))$$

where Si and Si + 1 represent the individual biomarker scores and their successive star plot radius coordinates and n represent the number of radii corresponding to the biomarkers used in the present work plan. The final IBR values will be presented by dividing the IBR values by the number of biomarkers used (n) and are expressed as IBR/n.

2.3. Fishpond cultured field samples

Fishpond cultured specimens of common carp (*Cyprinus carpio*) were collected in April 2022. Fish were dissected at site and brain, liver, and intestine of each fish were collected, frozen at -20°C for biochemical analyses.

2.4. Test chemical selection

Test chemicals were selected based on previous literature data and own analyses of field samples (e.g. Kondor et al. [33]). Based on analytical data of samples collected from manure used for fishpond fertilization, water and sediment samples, and preliminary FET and subacute tests, antimicrobials proved to be irrelevant micropollutants in fishponds. The threshold concentrations of detectable adverse effects of these compounds are beyond environmental relevant concentrations, particularly in fishponds fertilized by organic manure. Manure before used as fertilizer is often stored at site, thus the active antibiotics content decreases greatly, because the natural degradation time of antimicrobials commonly used in intensive livestock farms is relatively short (DT_{50} =4-43 days) [34]. In manure, where often higher temperatures may appear because of decomposition processes, this time could more shorten significantly. The antibiotics amount entering the fishpond via manure is way too low to maintain a concentration in water sufficient to cause any effects in fish.

However, other pharmaceuticals occur often in manure and fish pound water body in a concertation sufficient to produce measurable effects in fish including some steroid hormones and other EDCs or compounds potentially maintaining endocrine disrupting effects. Based on own unpublished analytical measurements, carbamazepine (CBZ) was selected as test chemical, which was later confirmed as the most often the most frequently detected pharmaceutical in rivers worldwide [10]. Due to its high level of consumption, human healthcare serves as a continuous source of CBZ release to the environment [35] since the majority (72%) of the received amount enters sewage within urine [36]. The highest reported concentrations were up to 150 µg/L in South Korea [37]. In Europe, the average amount of CBZ detected was 12 µg/L [38], while in Hungary (river Danube), 0.8 µg/L was detected [33,39]. CBZ was suggested earlier also to have endocrine disrupting effects in fish [17,40].

Due to its widespread usage as a malaria vector control and pest control agent, and potential endocrine disrupting effects according to our preliminary studies, also bendiocarb, a carbamate compound was selected for testing in this project.

Common EDCs, found by our analytical measurements and most likely present in surface waters, and manure like steroid hormones, particularly estrogenic compounds 17β-estradiol (E2), the synthetic ethinyl-estradiol (EE2) and progesterone (P4) were also tested in this project.

3. Sub-lethal effects of bendiocarb in zebrafish embryos

Our results obtained for zebrafish embryos after sub-lethal exposure to bendiocarb inflicted– like other carbamates – adverse effects causing embryonal deformities, reduces body- and notochord length, and oxidative stress in aquatic vertebrates. However, a disruption of

endocrine system was not proved, thus this chemical was tested further in 28 days tests. Results were presented by Gazsi et al. [30].

4. Chronic effects of carbamazepine at environmentally relevant concentrations in zebrafish

VTG concentration in zebrafish was increased following CBZ exposure for one and two weeks, with a significant elevation being detected in fish subjected to the 100 μg/L concentration. Conversely, VTG levels decreased slightly after 28 days, as compared to the values in control groups. Increasing AChE activity after CBZ exposure found in this study is in agreement with other zebrafish studies. In this study, only 1 and 5 μg/L CBZ concentrations caused a significant increase after 28 days, with time and concertation dependency being support-ed by statistics. The decrease at higher exposure concentrations may be from other toxic effects. After seven days of exposure to CBZ, increasing EROD activity shows that CYP1A enzymes were biosynthesized to detoxify and metabolize CBZ. This result agrees with the findings of a previous study with *Carassius carassius,* where 2 and 10 μg/L CBZ concentrations were proven to elevate hepatic EROD activity after 1, 4, and 7 days [40]. The subsequent decrease after 14 and 28 days of exposure to CBZ may be attributed to adaptations to the chemical stressor or changing metabolism of CBZ. GST did not show increased activity after the first seven days of CBZ exposure, then it increased significantly after the second and remained significantly elevated during the fourth exposure week. In previous studies, GST was also shown to increase in *Cyprinus carpio*, *Carassius carassius,* and *Danio rerio* after exposure to environmentally relevant concentrations (1–100 μ/L) of CBZ [17].

CBZ stimulated SOD, GR, and GPxSe activity after the first exposure week, resulting in a significantly high activity of SOD, GR, and GPxSe. Subsequently, SOD and GR activities dropped to control levels at 100 μg/L, even at the first week, and following the second week, they remained slightly suppressed until the 28th day of exposure, as seen by EROD activities. Conversely, GPxTOT and CAT activities were not altered until the second exposure week. After the second week, GPxTOT, and then after the fourth week both CAT and GPxTOT activities were increased significantly at 5 and 50 μg/L CBZ concentrations. After exposure to 100 μg/L of CBZ for 28 days, GPxTOT and CAT activities decreased to control levels. The first week results suggest that antioxidant enzyme activities were increased as a consequence of inorganic ROS produced by EROD (or other phase I metabolites) and/or by SOD activity, which were neutralized by GPxSe and GSH (as indicated by increased GR activity). The decreasing GST activity also reflects the diminishing amount of glutathione after the first week. After the second exposure week, most probably, EROD and SOD were failing to perform their functions, and organic ROS were becoming predominant. There is evidence that antioxidant enzyme activities may decrease under excess ROS production, if, for example, superoxide radicals not eliminated by SOD are able to inhibit CAT or GPxSe, and proteins inhibiting other antioxidant enzymatic activities [41,42]. It is also important to note that CAT and GPxSe have complementary roles in H_2O_2 elimination [42], with each having different subcellular localizations, such as peroxisomal (GPx) versus mitochondrial and cytosolic fractions (CAT) [43], as well as different target molecules (reduction of H_2O_2 by CAT and GPxSe, while selenic-independent GPx is able to reduce toxic hydroperoxides) [44]. In the present case, a failure of phase I metabolism, including EROD which should eliminate organic xenobiotics, may have led to excess ROS, and thus resulted in the observed increased GPxTOT and CAT activity, and the inhibition of SOD, GPxSe, and GR. It is also plausible that an energy (NADPH) shortage following exposure to CBZ was causing the observed effects [45,46] (Fig. 1). This shortage shows that a significant energetical stress appeared during the exposure to CBZ. Results were presented by Ács et al. [29]

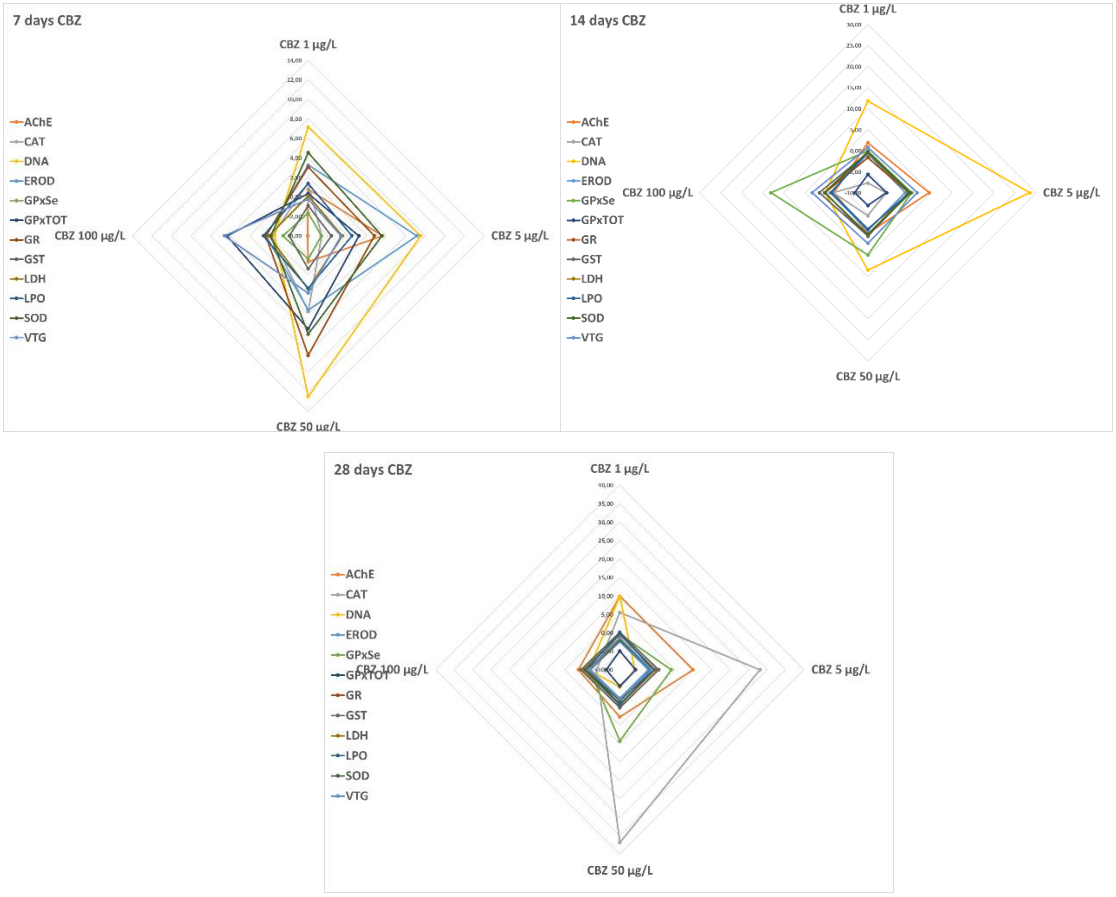

Figure 1. BRI indices calculated for fish exposed to CBZ for 7, 14 and 28 days.

5. Chronic effects of progesterone at environmentally relevant concentrations in zebrafish

A time- and concentration-dependent decrease in VTG concertation was demonstrated. A significant drop in VTG was observed after 28 days of exposure to 50 and 100 ng/L of P4. After 28 days, P4 also increased the level of AChE activity at the 100 ng/L exposure concentration. Previous animal studies have shown a relationship between increased AChE activity, oxidative stress [47], the production of free radicals, and apoptotic processes [48,49]. Physiologically, AChE breaks down the neurotransmitter acetylcholine, resulting in decreased acetylcholine receptor stimulation and affecting an organism's cognitive function. EROD activity was significantly (50, 100 ng/L) increased after the first and second weeks of exposure to P4, then later significantly decreased by the 28th exposure day. Regarding the effect of P4 on GST, only the lowest applied exposure concentration (1 ng/L) triggered a significant change in GST activity, most probably due to a hormetic response. The observed pattern of the mixtures in GST and EROD activity may suggest an altered metabolic route for xenobiotics. Antioxidant system enzymes' results of P4-exposed fish, meant to neutralize inorganic ROS, were following the pattern of EROD activity changes: GR and GPxSe were in-creased significantly after one week of exposure (50 and 100 ng/L of P4), and remained significantly higher than control values during the second (1, 5, 50, 100 ng/L of P4) and fourth weeks (1, 5, 50, 100 ng/L of P4). CAT showed significantly elevated activity at the 100 ng/L P4 concentration after 28 days. SOD was not affected by P4 exposure. These results also suggest that phase I metabolism or other processes producing inorganic ROS were mainly causing the measured enzyme activity changes. After four weeks of exposure to P4, TBARS levels were found to be significantly higher at 5, 50, and 100 ng/L concentrations, supporting the finding that exogenic P4 causes oxidative stress in zebrafish. In this study,

significantly increased levels of LDH activity in fish exposed to P4 for one week at 50 and 100 ng/L, and for two weeks at 100 ng/L, together with the AChE activity results, indicate structural damage to the liver cells (Fig. 2). Results were presented in by Ács et al.[29].

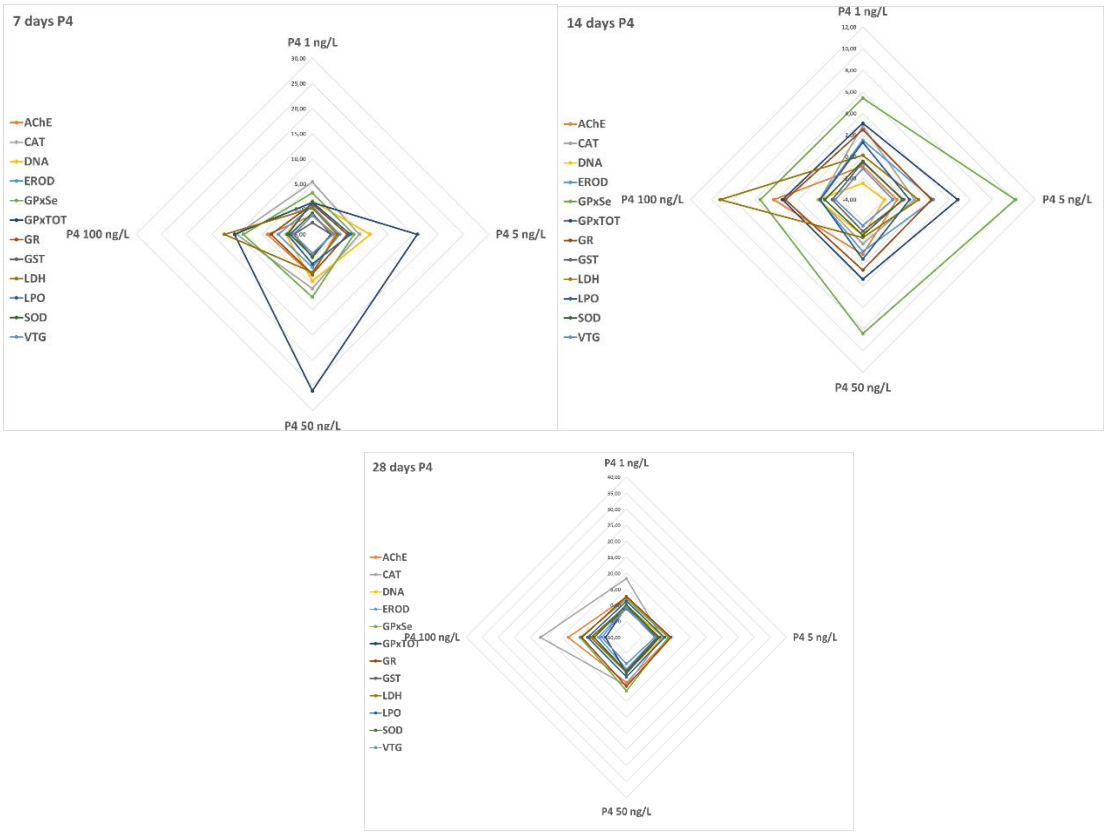

Figure 2. BRI indices calculated for fish exposed to P4 for 7, 14 and 28 days.

6. Chronic effects of binary mixtures of carbamazepine and progesterone at environmentally relevant concentrations on zebrafish

Binary mixtures of CBZ and P4 significantly altered VTG production after 28 days and VTG production was increased after exposure to MIX1 and MIX2, but MIX1 had no significant effect. After two weeks, a non-significant decrease in VTG content was observed following an increasing proportion of P4 and a decrease in CBZ concentration. An initial drop, or increase, in VTG concentration may be attributed to a hormetic effect, which often appears in endocrine signalling [42,50]. Increasing VTG concentrations in fish after long-term exposure to mixtures of CBZ and P4 may support the suggestion that CBZ and P4 are acting together as synergic compounds. The increase in the proportion of P4 seemed to increase and prolong the CBZ effect at the tested concentration range. None of the assessed mixtures caused a significant effect in EROD activity after 1 week of exposure, but MIX2 and MIX3 caused a significant increase after 14 and 28 days. In the case of EROD activity, mixtures seemed to shift significant effects in time, mitigating short-term effects and causing a significant increase in chronic effects. Chronic effects were absent in single-compound exposures. For GST, the observed effect was increasing in relation to a growing P4 ratio (MIX1 < MIX2 < MIX3). These observations suggest that mixtures of P4 and CBZ may have a synergistic effect on a chronic timescale, becoming more pronounced with the proportion of P4. Binary mixtures caused significant alterations of antioxidant enzymes GR, GPxSe, and GPxTOT. After the first exposure week, an inhibition in the activity of GPxSe, GPxTOT, and GR was observed. At the fourth week, GPxSe and GPxTOT showed significantly

higher activity as compared to control group values. After 28 days, MIX1 caused a significant decrease in SOD activity. This result may suggest that the metabolism, or mode of action of the mixture of these compounds may differ from the single chemical's effect, as also suggested by xenobiotic metabolization enzymes' results, and the mode of action of the antioxidant system depends not only on exposure time, but also on the proportion of CBZ and P4. Mixtures seem to mitigate, or even reverse the short- and long-term effects of all assessed antioxidant enzymes predicted from the single-solution results. It is notable that MIX3, containing the highest proportion of P4, had the most significant effects. Binary mixtures did not cause increased TBARS levels in zebrafish during our assessments. The results obtained for mixtures also showed increased LDH activity in the liver, however the LDH activity was in the same range as single-component solutions. After one week of exposure, MIX1, MIX2, and MIX3 had a significantly increased effect on LDH activity. After two weeks, MIX1 and MIX3, and after four weeks only MIX3, resulted in a significant increase. Additionally, MIX3 had the most pronounced effects on LDH, as seen in the other markers. The results of the biochemical markers assessed in this study indicate a synergistic dose-ratio-dependent effect of CBZ and P4 on xenobiotic metabolization enzymes and VTG in zebrafish after chronic exposure [51]. Differences in the mixture combinations revealed a non-linear response of zebrafish to the assessed mixtures. After short-term exposure to binary mixtures, oxidative stress enzyme activities were lower than expected based on single-component results. The observed marker responses to binary mixtures showed that not only time but also the proportion of the components determines the main toxicological effect of CBZ and P4. Changes of the response to oxidative stress depending on single-component solutions or binary mixtures helps to better understand the toxic effect mechanism of multiple chemical stressors. The results of VTG concentration changes also confirm the risk concerning alterations in reproductive success caused by pharmaceuticals co-appearing in surface waters [17].

Chronic exposure to environmentally relevant concentrations of CBZ, P4, and their mixtures inflicted significant biochemical alterations, and to mixtures biochemical markers in zebrafish showed non-linear strengthening responses. These synergistic effects on VTG production suggests a high risk to the reproductive success of fish, if these chemicals are present simultaneously. In addition to the mixture effects on reproduction, xenobiotic metabolizing enzymes (EROD, GST) and the oxidative stress marker (DNAsb) were also significantly altered as compared to the results of single-chemical exposure after 28 days (Fig. 3). These results suggest that fish production efficiency is decreased in two ways by this micropollutants. Thought oxidative stress food utilization of fish decreases, and through endocrine disrupting effects, the reproductive success worsens in fish. Results were presented by Ács et al. [29].

Figure 3. BRI indices calculated for fish exposed to mixtures of CBZ and P4 for 7, 14 and 28 days.

7. Chronic effect of carbamazepine exposure at environmentally relevant concentrations in juvenile common carp

AChE inhibition observed after 7 days of CBZ exposure. Reduced AChE activity is attributed to neurotoxic agents, and it is a commonly applied biochemical marker of neuro-toxic environmental pollutants [52]. In this study, following 14- and 28-day exposure to CBZ, a time and concentration dependent increase was observed in AChE activity. Increased AChE activity is often associated with the production of free radicals and oxidative stress [47] and ongoing apoptotic processes in the test organisms [48,49]. The elevated AChE activity measured in this study may be a consequence of apoptotic processes.

A significant increase of EROD activity was found after 28 days exposure to 50 and 100 µg/L CBZ concentrations indicates that CYP1A enzymes were biosynthesized to detoxify and metabolize CBZ. These results are in accordance with a previous study with *Carassius carassius*, where 2 and 10 µg/L CBZ concentrations were shown to induce hepatic EROD activity after 1, 4 and 7 days [53]. Our observation of a decrease in EROD activity after 28 days exposure to a concentration of 100 µg/L CBZ, as compared to fish exposed for 28 days to 50 µg/L CBZ, may be attributed to liver damage, as proposed in the case of AChE.

A significant increase in GST activity measured during our study suggests an oxidative stress-induced adaptive response, or, alternatively, the conjugation and excretion processes of carbamazepine CBZ in the liver of common carp.

After an initial increase in the activity of SOD and GR during the first seven days of CBZ exposure, their activity dropped below levels measured in the control group after 14 days and remained sup-pressed in the 28-day treatment group. CAT activity increased significantly through the 28 days of exposure compared to the activity levels measured in the control group.

These results are in accordance with preceding studies. For instance, several previous studies were reporting significantly increased SOD, CAT and GR activities after short term (< 7days) exposure to CBZ in rainbow trout [54] and *Carassius carassius* [53]. The initial increase in the activity of antioxidant enzymes could be explained by elevated ROS concentrations actuating the antioxidant system and SOD activity in the tissues of fish in order to initiate the dismutation of ROS derived from drugs (such as superoxide anion radical $O_2^{-\bullet}$) to molecules which are less toxic (like H_2O_2). The increase in the activity of GR subserves the conversion of oxidised glutathione (GSSG) to reduced GSH; reduced GSH can directly scavenge ROS and is subsequently reduced to GSSG in an energy demanding process utilizing NADPH [45]. The detected drop after an initial increase in SOD and GR enzymes may be attributed to: lipid peroxidation and the direct attack of reactive oxygen species, proteins decreasing ROS [58], or an energy (NADPH) shortage following pro-longed exposure to CBZ [57,58]. CAT activity may have remained at a higher level due to H_2O_2 originating from sources other than SOD activity.

Low intensity oxidate stress can induce cells to produce antioxidant enzymes that are able to eliminate ROS, while severe oxidate stress can overwhelm these protective enzymes, resulting in oxidative damage of cell components like lipids, proteins and even DNA [55]. TBARS is the most widely used indicator of lipid peroxidation (LPO) triggered by oxidative stress in fish [59]. Our results showed a significant elevation of TBARS only at the end of the tests in the case of the highest applied CBZ concentration (100 µg/L); however, the continuous elevation of TBARS levels in our data as compared to control values hints at an ever-growing oxidative stress pressure due to a malfunction of the antioxidant defence system. This finding corresponds to other studies, for example Li et al. [58], that reported oxidative stress and elevated TBARS levels in rainbow trout after 21- and 48-days exposure to 20 and 200 µg/L CBZ.

CBZ was shown to cause alterations in the genetic material of Chinese rare minnows (*Gobiocypris rarus*) after 28 days of 1, 10 and 100 µg/L CBZ exposure [52]. In the present study, strand break levels showed a slight decrease during the 28-day tests in common carp compared to control groups. The observed lower level of DNA strand breaks could be attributed to repair or recovery mechanisms [60] initiated by oxidative stress effects. An inhibitory effect on cell division may also be involved in the observed responses [61].

LDH is a widely used marker of organ and tissue damage reflecting metabolic activity (e.g. carbohydrate metabolism), as well as structural and morphological alterations of tissues that are closely associated with pathological processes [62]. In our study, LDH activity was slightly increased in 50 µg/L CBZ concentration experimental groups after 28 days exposure, and decreased in 100 µg/L CBZ groups, although these changes were not significantly different from control activity values. Increased LDH activity in the liver and gills of common carp caused [46] by 5700 µg/L CBZ exposure from 7 to 28 days was previously attributed to metabolic changes and tissue hypoxia due to the disruption of respiratory epithelium resulting in a decrease in oxidative metabolism [55]. Observed drop in LDH activity in the 100 µg/L, 28-day CBZ exposure group as compared to the LDH activity peak in the 50 µg/L CBZ group may also provide evidence for apoptotic damage in liver tissues as is also suggested in our results from the AChE, EROD and antioxidant enzyme analyses.

Vitellogenin (VTG) from fish is a glycollipophosphoprotein produced in the liver and its production is induced by 17β-estradiol or compounds which are capable of interacting with the estrogen receptor [56]. In this study, significant increases in VTG levels were measured in fish subjected to 100 µg/L CBZ after seven days as compared to control values. In addition, our findings showed a statistically significant concentration dependence on VTG levels. This

finding also corroborates the suggestion that CBZ's toxic mechanistic routes may be similar to estrogenic compounds.

In summary, chronic exposure to environmentally relevant concentrations of CBZ inflicted biochemical, and, presumably, physiological effects in common carp. In this study, fish subjected to 5 µg/L of CBZ exhibited a significant change in hepatic antioxidant status. With increasing CBZ concentrations, enzymatic and non-enzymatic biomarkers of oxidative defence (CAT, SOD, GR, DNAsb), toxicant biotransformation (EROD, GST), and organ and tissue damage (LDH, AChE) were altered. The AChE, LDH and LPO results are suggestive of apoptotic processes and tissue damage after 28 days exposure to CBZ. The energetic stress caused by these micropollutants did not result in reduced weight of test animals, most probably in a longer time frame, such effects would also appear. The findings of the present study suggest significant adverse effects of CBZ on common carp at concentrations often found in surface waters (Fig. 4). Results were presented by Liang et al. [32].

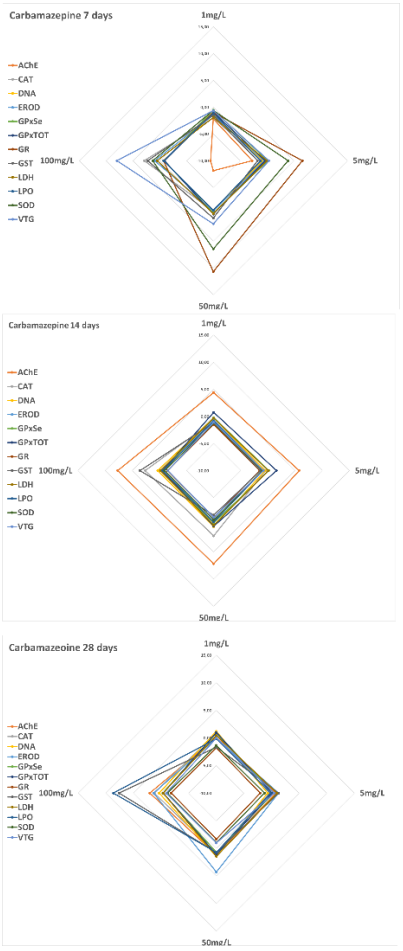

Figure 4. BRI indices calculated for fish exposed to CBZ for 7, 14 and 28 days.

8. Other pharmaceuticals and their binary mixtures' effect

Fish, both, carp and zebrafish were also exposed to E2, EE2 and their binary mixtures with CBZ and P4 in all combinations for 28 days. However, the results of these experiments were not evaluated until the project closing date.

9. Fishpond cultured fish results

Common carp specimens were collected in April 2021-2022. Biomarker analyses showed significant oxidative stress of the collected fish. SOD is the first line of antioxidant defense against ROS, and second-line enzyme like CAT break down H_2O_2 occurring as a result of SOD activity. [57,58]. As a result of elevated oxidative stress, biochemical markers of cellular damage, lipid peroxidation and hepatic LDH were observable (Fig. 5).

Analytical results of water samples showed the presence of CBZ (0.2 µg/L), P4 (1 ng/L), oestron (4 ng/L) and 17β-estradiol (1 ng/L). Other pharmaceuticals were not detectable. Results of fish pound cultured carps are supposed to be published in late 2022.

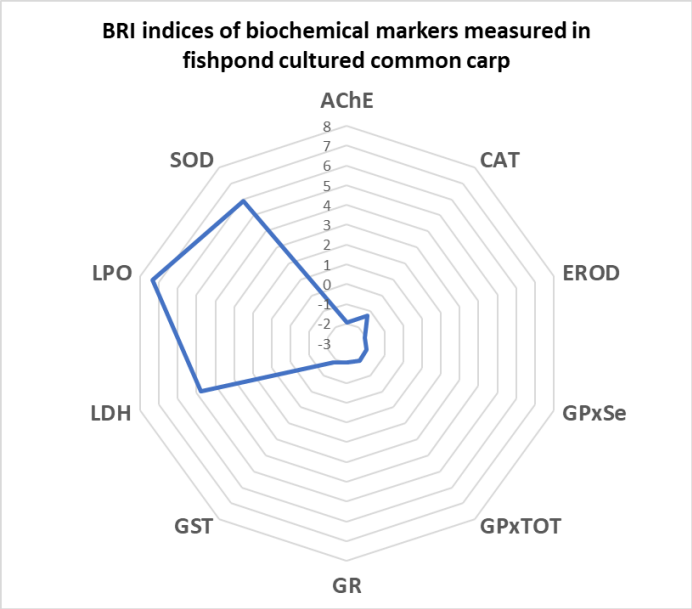

Figure 5. BRI indices calculated for fishpond collected carp.

10. Metabolic analyses in fish

Metabolic activation and /or modification plays an important role in toxicology. By applying a microinjection method, which is a simple way to introduce polar and nonpolar materials as well as organic matter-rich test substances into newly fertilised fish eggs [59–61], we could find a way to better understand the metabolic processes of fish. With the appropriate settings, the technique enables the administration of exact amounts and ensures the reliability of results [61]. As such, the microinjection of fish embryos could be an alternative method to test materials treated/activated with S9 mix, representing an absent metabolic enzyme complex in fish. As such, this method enables to find differences in toxic metabolism and activation between fish and higher vertebrates. Results were presented by Csenki et al. [62].

Summary

In semi-extensive aquaculture facilities, the fish production is partially based on the natural planktonic production, which is usually intensified by fertilizers, most commonly by different types of manure. Intensive livestock farm manure may contain a number of undesirable chemicals, entering the waterbody via manuring process. These micropollutants occur as multicomponent mixtures, and their joint effect may greatly differ from the effect suggested single compounds. This project aimed to unveil such unexpected dose-responses, and subsequent synergistic and antagonistic effects, which may affect fish growth and production,

by assessing a toolset of biochemical markers of zebrafish, the commonly applied test species in environmental toxicological studies, and common carp, which is of great economic significance. We have identified synergistic combinations of micropollutants and previously unknown potential endocrine disrupting effect of carbamazepine. We found that some micropollutants, even in environmental relevant concentrations may cause alterations to such an extent in fish, which may result in energy loss and decreasing fish production. These results suggest that fish production efficiency is decreased in two ways by this micropollutants. Thought oxidative stress food utilization of fish decreases, and through endocrine disrupting effects, the reproductive success worsens in fish.

Published scientific papers

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Publications under preparation

András Ács, Xinyue Liang, Illés Bock, Jeffrey Griffitts, Bence Ivánovics, Erna Vásárhelyi, Zsolt Pirger, Béla Urbányi, Zsolt Csenki, Árpád Ferincz. Biochemical marker effects of fishpond cultured common carp *(Cyprinus carpio).*

Conference presentations

András Ács, Xinyue Liang, Illés Bock, Jeffrey Griffitts, Bence Ivánovics, Erna Vásárhelyi, Árpád Ferincz, Zsolt Pirger, Béla Urbányi, Zsolt Csenki. 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 in Nitra. 10. November 2022.

Ivánovics Bence, Gazsi Gyöngyi, Póliska Szilárd, Tóth Márta, Staszny Ádám, Ács András, Kovács Róbert, Baska Ferenc, Domokos Apolka, Varga Zsófia, Boda Zsófia, Bácsi Attila,

Urbányi Béla, Csenki-Bakos Zsolt, Czimmerer Zsolt. Egy inszekticid karbamát, a bendiokarb embrionális hatásainak vizsgálata zebradánión. TOX'2022 Tudományos Konferencia. Zalakaros, 2022. október 12–14. (Accepted presentation)

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