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EFFECTS OF SULFAMETHOXAZOLE ON ANTIOXIDANT DEFENSE SYSTEM IN *CYPRINUS CARPIO* FISH

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Introduction

Sulfonamide antibiotics are extensively used in human and veterinary treatments and also agriculture as growth livestock promoter. Sulfamethoxazole (SMX) it a member of sulfonamides group with a low removal yield in WWTPs (aprox. 50-60%), with an incidence of 87% in Romanian water (median concentration of 7.9 ± 2.4 ngL^{-1} and a maximum concentration about 15.7 ngL^{-1}). The presence of this compound in surface water do not have acute toxic effects on aquatic life taking into considerations laboratory data (*Daphnia magna* $EC50_{48h}$ >100mg/L; *Selenastrum capricornutum* $EC50_{72h}$ 24.77 mg/L; *Cyprinus carpio*, $LC50_{96h}$ >100 mg/L; *Heterocypris incongruens*, $EC50_{6\ days}$ 72 mg/L; risk coefficients (Danube River) <0.1 - insignificant). The ecotoxicological evaluation of SMX was performed based on visible symptoms of organisms (mortality, growth or behaviour) without any data concerning the sub-lethal effects. In the above context, the paper aims to investigate the effects and action mode of SMX exposure in fish at translational level through the investigation of physiological indices, hepatic / gonad-somatic indices and enzyme activity changes (hepatic transaminases and oxidative stress enzymes).

Materials and methods

Acclimatized *Cyprinus carpio* fish (10 individuals, weight 25-28 g) were exposed to SMX (MM=253.28, CAS 723-46-6, 99.74%, LGC Labor GmbH, Germania, in theoretical concentrations: 10µg/L and 100µg/L), in 100 L aquariums, for 14 days in semi-static conditions using the OECD no. 305 experimental methodology and taking in to consideration ethical principles regarding the animal suffering. The test medium was 80% renewed every 48 h. The SMX analytical concentrations were determinate using LC-MS/MS Agilent 1260/6410, column Zorbaz 300Extend-C18, narrow-bore 2.1x150mm, 3.5µm, 300A, quantification limit 2.6 ng/L. The average analytical concentration of SMX in the testing period were: 6.39 ± 2.04 µg/L (n=20) in case of 10 µg/L and 48.46 ± 12.90 µg/L in case of 100 µg/L. The analytical concentrations variations were 30% for the low concentration and 26% for the high concentration. 10 fish in absence of SMX (0 µg/L) in free chlorine tap water represented the test control. The fish were fed daily with 1% dry food from the weight of each fish lot. The water quality parameters were constantly monitored

such as organic loading, pH dissolved oxygen, temperature, total purity, suspended matter, in the experimental tanks, control and SMX testing solutions. The monitored abiotic parameters assured the survival conditions of fish according to test criteria. After 14 days of exposure 5 fish were sacrificed on ice in order to reduce the animal distress. Liver, gills, intestines and gonads were collected, individual weighing, homogenized per organ type (n=5) and frozen at -80°C for biochemical analyses. Before and after exposure the fish lots were characterized in terms of weight, length, height, to obtain biometrical data for physiological indices calculation (weight - W, food consumption - C, growth coefficients - G, efficiency of food utilization - K%, production - P, biomass B) according to internal procedure. The liver and gonads were individual weight for hepatic and gonad somatic indices calculation (HIS/IGS). HSI = (liver weight/body mass) x100; GSI = (gonad weight/body mass) x100.
After tissue homogenate preparation (1/10 weight/volume in ice-cold 0.1M Tris buffer (pH 7.4) containing 5 mM EDTA, the supernatants were used for biochemical determinations. The protein contents from tissues were spectrophotometric measured at 660 nm according to Lowry's 1951 method using bovine serum albumin as a standard. All of the enzymatic activities were normalized to protein concentrations in order to be expressed in terms of units of activity/mg of protein and as a percentage from control. The enzymes activities were quantified using kinetic method according to following methods: hepatic enzyme (alanine aminotransferase - ALT and aspartate aminotransferase – AST) with the kit LiquiUV Tests, Human Gesellschaft, Germany; Oxidative stress enzymes Superoxide dismutase (SOD) – kit Sigma –Aldrich; Catalase (CAT) – method of Aebi H, 1984; Glutathione reductase (GRED) - method of Goldberg and Spooner, 1983; Glutathione peroxidase (GPX) - kit Sigma, USA; Glutathione S-transferase (GST)- kit Cayman Chemical, USA; Glucose 6-phosphate dehydrogenase (G6PDH)-method of Lohr and Waller, 1974).

Results and conclusions

After 14 days of fish exposure to SMX, no mortality was recorded in all experimental tanks. Also, no visible behavior alterations were observed. At the end of test, a decrease of 15-17 g per lot (<10%) in both controls and tests was observed, probably due to the stress to which the fish were subjected. Given that weight loss was observed in both control and tests, the effect cannot be related to SMX intoxication. Lengths and heights did not show changes compared to the control group. The instantaneous growth coefficient (G) showed negative values in both cases (control and tests), correlated with the decrease of <10% in weight of the lots at the end of the test. The instantaneous mortality coefficient (Z) did not changed, because no mortality was recorded during the tests. The initial and average biomasses (Bi and B) did not varied. The production (P) influenced by the instantaneous growth coefficient (G), was negative both in the control and in the tests. The efficiency of food use for production (K%) was correlated with the instantaneous growth coefficient (G) and production (P), which indicated a slight insignificant negative effect on the growth of the lots used in the experiment. No significant differences in body mass and length between control and exposed groups were observed. The HSI and GSI were unchanged indicating that SMX did not influenced vitellogenesis in the liver and vitellogenin endocytosis in ovarian follicles during the 14 days of exposure. Additionally, no mimetic estrogen activity caused by SMX exposure was evidenced.

Contrary to the above data, at translational level, SMX could induce oxidative stress responses in all studied organs the (gill>intestines>liver) of *Cyprinus carpio* after 14 days of exposure. The effects were strongly observed in case of 100 µg/L (analytic conc. 50 µg/L), but some effect were also observed in case of 10 µg/L (analytic conc. 6 µg/L). The effects on enzymatic antioxidant defence systems were related to the overexpression of some oxidative stress enzyme activities especially in case of the high SMX concentration as follow: gill – SOD, CAT, GPX, GRED, GST and G6PDH – significant effects; liver – SOD, CAT, GRED – moderate effects and hepatic enzyme AST and ALT – significant effects; intestine – SOD, CAT, GPX and GRED – significant effects. In case of the low tested concentration of SMX the enzymatic activities of CAT and SOD slightly increased. Over expression of GST activity was observed also for this concentration. Inhibitions of GPX, GRED and G6PDH were observed. At gills level the oxidative stress was more strongly because this tissue is the first organ in contact with testing media and the entrance of SMX in the body. In the liver, the detoxified organ only some antioxidant enzyme activities were detected (SOD-CAT–GRED) and also the overexpression of transaminase enzyme involved in detox processes suggesting the hepatocytes alterations. Intestine was affected through the over expression of SOD-CAT – GPX. The results could be compared with literature. Environmental concentration (2.90 µg/L in 2021 from surface water – Danube River) indicated a continued pollution with SMX compared to 2018 that could induce metabolic changes in fish body as a results of long exposure and bioaccumulation. Enzymatic investigation in the fish organs of *Cyprinus carpio*, could be used as biomarkers in the water quality monitoring and assessment of the ecotoxicological risk of chemical pollutants.

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