Assessment of immunohematological, hematological and biochemical responses in cultivable fish *Cyprinus carpio* exposed to an antibiotic sulfamethoxazole (SMX)

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ABSTRACT

Sulfamethoxazole (SMX) is a member of the sulfonamides group of antibiotics which is used extensively in aquaculture throughout the world. In this study, common carp (*Cyprinus carpio*) was used as the bioindicator to assess the toxicity potential of SMX. Effects were based on chronic toxicity of environmentally relevant dosages of 25, 50, 100, and 200 μg/L of SMX for 28 days. Cytotoxicity through hematology and biochemistry showed a dose–response relationship. Numerous variations were recorded in blood profile and biochemical parameters in SMX-exposed groups when compared to control. Hemoglobin, platelet, and erythrocyte levels were significantly decreased. Leukocyte level was significantly increased with values ranging from 131 to 303 ($\times 10^3/\mu L$). Changes in biochemical indices: glucose, total protein, and triglycerides showed biphasic trend, but alanine transaminase secretion was significantly increased from 25.13 to 204 U/L at higher concentration compared to control, suggesting liver damage. Spectrophotometric nitroblue tetrazolium reduction assay showed that respiratory burst activity increased as a function of SMX dose and exposure time (0.48–1.33 absorbance) ultimately leading to reduction in immunity. The present study highlights that prolonged exposure of SMX affects biochemistry, hematology, and immunohematology of fish and these biomarkers act as an effective tool for environmental risk assessment of drugs in the aquatic environment.

Key words | antibiotic, biochemical analysis, fish toxicity, hematology, respiratory burst activity

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HIGHLIGHTS

- Antibiotics are an evolving pollutant due to their occurrence at high levels and potential risk to ecosystem.
- This study investigates the toxicity potential of sulfamethoxazole using common carp as bio-indicator.
- Many changes were found in hematological profile, biochemical and anti-oxidant status in exposed groups when compared to control suggesting strict monitoring of antibiotics release in the aquatic environment.

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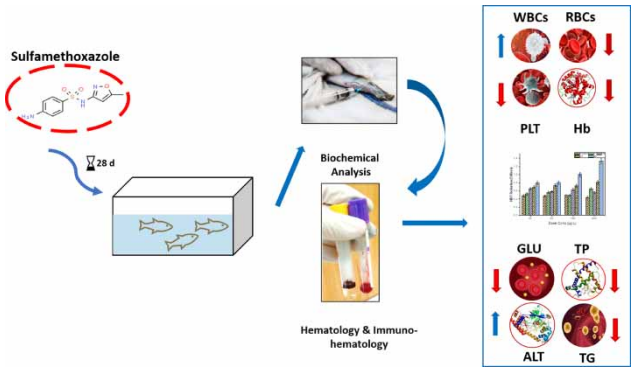
GRAPHICAL ABSTRACT



INTRODUCTION

Industrialization, urbanization, and growth in human population has resulted in a proportionate increase in environmental pollution with numerous inorganic and organic pollutants. Among these contaminants, pharmaceutical products are considered as evolving pollutants due to their numerous uses, occurrence in different environmental compartments at high levels, and possible risk to living organisms (Khan & Nicell 2015). Pharmaceuticals are intricate molecules, mostly carbon-based in nature with different physicochemical and healing properties. These pharmaceutical compounds (PCs) ultimately become part of the environment via various point sources such as manufacturing facilities, hospitals, agricultural and land runoff, household use, and improper disposal (Dorival-García *et al.* 2013).

Pharmaceuticals are biologically active compounds that may interact with specific biological systems or generically act all over the body depending on their chemical properties (Isidori *et al.* 2005). Among all the pharmaceutical classes, antibiotics have the highest consumption rate (Dorival-García *et al.* 2013).

Owing to the high demand of food security the aquaculture sector has grown very rapidly all over the world. In the recent decade, antibiotics have attracted significant public attention as a prevalent pollutant and their potential risk to human health and the ecological environment. In aquaculture industry antibiotics play a key role in inhibiting terrible infectious diseases. For the last decade, it is calculated that 200,000 tons of antibiotics have been used all over the world (Gao *et al.* 2012). Due to their high consumption, increasing detection of pharmaceuticals has been found largely all around the world including North America, China, the UK, and Europe (Yang *et al.* 2017). In the total of antimicrobials utilized, sulfur drugs occupy 6%. Among them, sulfonamide groups are widely used as a veterinary antibiotic (Perlovich *et al.* 2014).

Sulfamethoxazole (SMX) is predominantly used in aquaculture and rigorous livestock farming and is considered as heavily used antibiotics in the European Union (Białk-Bielińska *et al.* 2014). In the early 21st century, 16,000 tons of antibiotics were consumed each year in the USA, of which, 2.3% of the total amount of antibiotics used in veterinary medicine were sulfonamides. This value varied from 11 to 23% in Europe (Mahmoud *et al.* 2013). SMX prevents the enzymatic pathway implicated in bacterial folate synthesis. Long-acting SMX obstructs the conversion of para-aminobenzoic acid to di-hydrofolic acid by controlling the dihydrofolate synthetase enzyme resulting in bactericidal effect (Romero *et al.* 2012).

The highest quantified concentration of SMX in freshwater lakes is 4 ng/L, and in hospital effluents 49 μg/L in Pakistan (Khan *et al.* 2013). Reported concentrations of SMX in the USA and Thailand are up to 19 and 40 μg/L, respectively (Schwab *et al.* 2005; Van Doorslaer *et al.* 2014).

Fish occur at the top of the food chain hierarchy in the aquatic environment and are valuable bio-monitors of aquatic pollution. Several risks have been linked to the

disproportionate usage of antibiotics in fish, including immunosuppression and emergence of antibiotic resistance (Saglam & Yonar 2009). However, scarce data exist on sublethal effects (e.g., genotoxicity, hematology, and oxidative stress) that could result from the biochemical action of SMX.

Chronic effects, which may occur over a long period of time, are more likely. Long-term exposure to pharmaceuticals may result in anomalous physical processes and reproductive disorders and increased chances of cancer (Kolpin *et al.* 2002). Many studies have reported on the toxic effects of SMX on marine organisms (Migliore *et al.* 1993; Yildiz & Altunay 2011; Anskjaer *et al.* 2013). However, studies on the toxicity of SMX in freshwater fish, particularly in Pakistani cultivable fish, are limited and data about its long-term exposure at environmentally relevant concentrations is still scare and further research is needed.

MATERIALS AND METHODS

Purchase and maintenance of experimental fish (common carp)

Healthy common carp were purchased from Punjab Hatchery Rawal Town (Aquaculture and Fisheries Program and Research Centre), Islamabad. The purchased specimens were transferred carefully to Environmental Toxicology Laboratory (NUST) in aerated polyethylene bags and then kept in experimental tanks having dimensions of $3 \times 1.5 \times 1.5$ ft.

Acclimatization of fish

Fish were acclimatized to laboratory conditions for a period of 14 days and fed with commercial food pellets containing soybean, rice, bran, corn, wheat, and other agricultural by-products, on a daily basis. To avoid fouling of tanks, dead fish were removed immediately during the acclimatization period and tank water was renewed on alternate days.

Morphometric parameters of experimental fish were determined immediately after shifting to the laboratory.

Water parameters of fish tanks

The physicochemical parameters of experimental tanks and lake water were assessed by following standard OECD guideline method, 203 (OECD 1992). pH and temperature were measured using multi-parameter analyzer, Consort-C1020. Dissolved oxygen (DO) was measured using Winkler method, whereas titration method was followed to measure total hardness. Tap water was provided to fish to avoid any damage to tissues or organs.

Experimental design

Experimental tanks were filled with 50 L of tap water and changed daily during the exposure period of 28 days. Stock solution was prepared by dissolving 1 mg of SMX in 1,000 mL of tap water. From this stock solution, further dilutions were made to get the desired concentration of toxicity solution. Air stones were placed in all the experimental tanks and the concentration of SMX was renewed on a daily basis. Using random selection method healthy fish with desired morphometric characteristics (Table 1) were divided into experimental and control groups. A set of eight fish in each tank was exposed to SMX for 28 days at the following environmentally relevant concentrations: 25, 50, 100, and 200 μg/L. All parameters were assessed at 7, 14, 21, and 28 days, respectively. Residual SMX concentrations in experimental and control tanks were analyzed after 1 and 24 h of renewing the test solutions through HPLC (Agilent 1260 Infinity II LC System) (Supplementary material, Table S1). Toxicity in fish was assessed by different types of analysis following OECD guidelines 204 (OECD 1984). During the exposure period (28 days) no mortality of fish was witnessed.

Chemicals used

All the chemicals used in the current research were of analytical grade. SMX >98% purity was purchased from Sigma-Aldrich for the preparation of stock solution. Nitrotetrazolium blue chloride (Bioworld, USA) and

Table 1 | Morphometric parameters of experimental fish

Fish species	Total length (cm)	Total weight (g)	Age (months)
Common carp (*Cyprinus carpio*)	15 ± 0.2	40 ± 0.3	3

N,N-dimethylformamide (Sigma-Aldrich, USA) were used for nitrotetrazolium blue chloride (NBT) reduction assay. Biochemical parameters (glucose, total protein, alanine transaminase (ALT), and triglycerides (TG)) were analyzed using reagents kits purchased from AMP Diagnostic, Austria.

Biochemical analysis

Biochemical analysis of fish blood samples was done according to the method stated by Perveen *et al.* (2019) to assess toxic impacts of SMX. Biochemical indices: glucose, triglyceride, total protein, and ALT were selected for the present study. For biochemical analysis the blood samples were withdrawn with the help of a syringe in gel activators for the preparation of blood serum. To produce serum, samples were centrifuged at 4,000 rpm for 10–20 min and then run through AMP Piccos II Chemistry analyzer.

Respiratory burst activity (NBT assay)

Immunohematological changes were assessed by using NBT reduction assay by following the method listed by Zanuzzo *et al.* (2015) with slight modifications. 0.1 mL of both heparinized blood and 0.2% of NBT (in phosphate-buffered saline solution) were co-incubated at room temperature for 45 min. Fifty μL from the resultant suspension was added to 1 mL of N,N-dimethylformamide and centrifuged at 2,000 rpm for 10 min. The optical density (OD) of supernatant was measured on UV-Visible spectrophotometer at 540 nm. The blank consisted of similar steps and components, excluding blood that was replaced with distilled water.

Hematological parameters

To determine the effect of applied doses on hematological parameters of exposed fish blood samples, a test was carried out for 28 days of exposure duration and weekly blood samples were collected Sample preparation and analysis was done according to methodology stated by Perveen *et al.* (2019). The blood was collected through cardiac puncture from the caudal vein below the dorsal fins using a 5 mL heparinized syringe. The blood was collected in sterile purple topped EDTA vials containing anticoagulant. After collecting blood, the vials were gently shaken by hand to dissolve anticoagulant agent properly. Before the commencement of hematological analysis, blood samples were centrifuged on Platform shaker LABCON-SPo-MP3 for 10–15 min at 300 rpm to avoid formation of any clots. Finally, red blood cells (RBCs), white blood cells (WBCs), hemoglobin (Hb) count, and platelets (PLT) were measured using Sysmex blood analyzer XP-100.

Statistical analysis

The results of the current study were subjected to two-way analysis of variance (ANOVA) to measure significant difference between control and SMX-exposed groups. Significant difference was defined by using the criterion, $p < 0.05$ as significance level.

RESULTS AND DISCUSSION

Physicochemical parameters of experimental tank and lake water

Water quality of experimental tanks was determined by investigating different parameters as prescribed by OECD guidelines 203 for toxicity test (OECD 1992). Water quality was analyzed at the start of the experiment and compared with physicochemical values of Rawal Lake hatchery water from where fish samples were procured.

The physicochemical analysis of water parameters is presented in Table 2. The results showed that mean values of temperature, dissolved oxygen, and hardness (28.5 °C, 6.9 and 230.7 mg/L) increased in lake water in comparison to experimental tanks (23.4 °C, 5.5 and 220 mg/L). The probable reason for the significant ($p < 0.05$) increase in temperature and hardness may be due to the entry of pollutant load from the nearby areas. Further, increase in pollution load may be due to excessive use of pesticides, improper disposal of poultry and domestic waste coming from the nearby tributaries such as Bhara Kahu and Noorpur, etc. Similar results were reported by Malik & Nadeem in 2011; they found that the quality of lake water deteriorated adjacent to populated areas, whereas water was found to be relatively clean and free of organic waste at the sites which were less impacted by nearby settlements.

Table 2 | Physiochemical parameters of the experimental tanks and lake water

	Parameters			
	Mean values (minimum–maximum)			
	Temperature (°C)	pH	Dissolved oxygen (mg/L)	Hardness (mg/L)
Experimental tank	23.41 ± 3.6 (19.5–27.5)	7.82 ± 0.3 (7.5–8.3)	6.9 ± 1.6 (4.5–8.2)	220.25 ± 68.4 (139–304)
Rawal Lake	28.55 ± 2.3 (24–30.3)	7.89 ± 0.4 (7.2–8.4)	6.9 ± 0.9 (7–7.9)	230.75 ± 45.4 (211–298)
OECD guidelines	20–24	6–8.5	80% of air saturation	10–250

Ayaz *et al.* (2016) conducted a study to evaluate water quality of Rawal Lake and quantified that most of the physicochemical parameters exceed the permissible limits prescribed by the World Health Organization.

Effects of SMX on hematological profile

Alterations in hematological count have been extensively used as a powerful tool for the determination of health and physiological status of fish (Gabriel *et al.* 2011) and allow fast and rapid evaluation of sub-acute toxicity of xenobiotics on target organs. This results in assessing the pathophysiological status of fish and parameters to help in diagnosing structural and functional changes in fish due to chemical exposure. Figures 1–4 show variation in RBCs, Hb, WBCs, and PLT count after SMX exposure. The levels

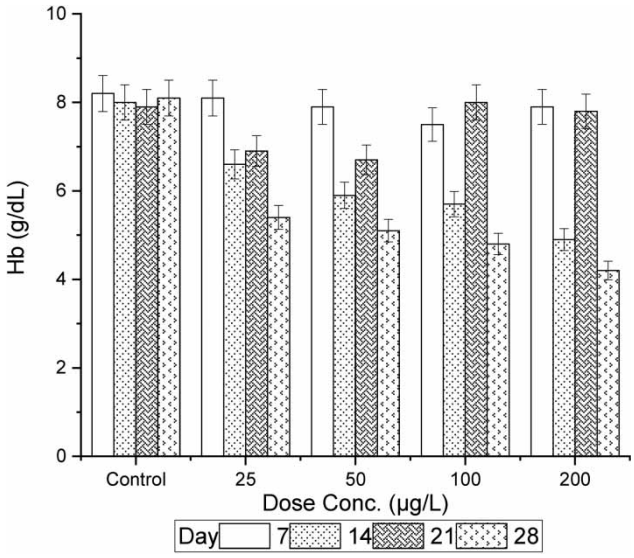


Figure 2 | Hb level of control and SMX-exposed fish for 28 days (values are expressed as mean ± SE, n = 8).

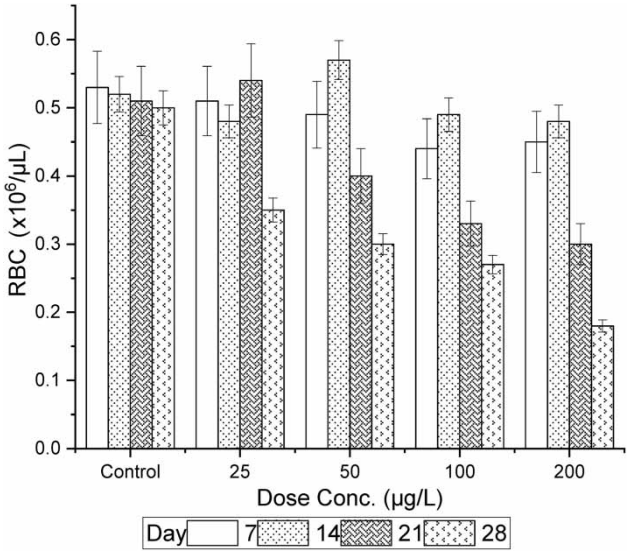


Figure 1 | RBC level of control and SMX-exposed fish for 28 days (values are expressed as mean ± SE, n = 8).

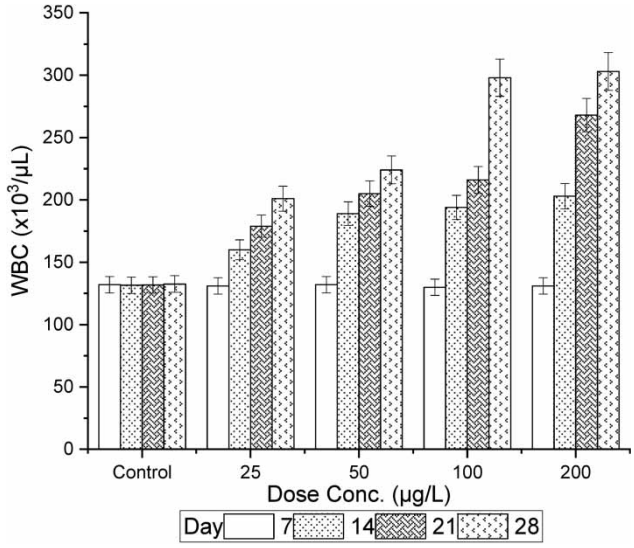


Figure 3 | WBCs level of control and SMX-exposed fish for 28 days (values are expressed as mean ± SE, n = 8).

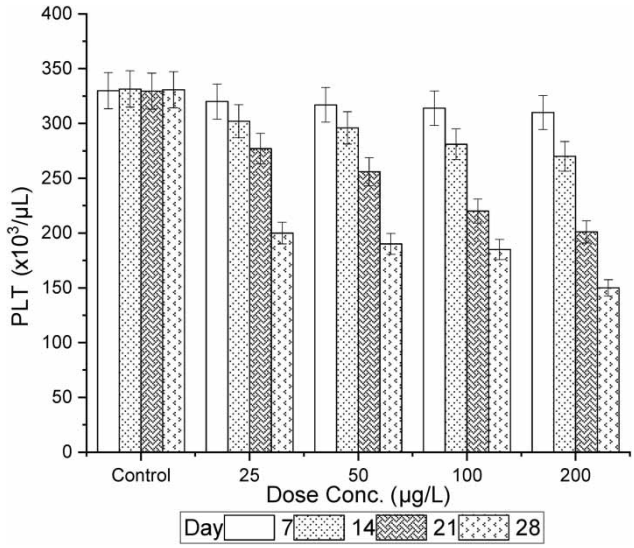


Figure 4 | PLT count of control and SMX-exposed fish for 28 days (values are expressed as mean ± SE, n = 8).

of RBCs, Hb, and PLT count were decreased with time in all the exposure groups whereas the number of WBC count was found to be increased. Figure 1 indicates the number of RBCs of control and exposed groups to SMX for a period of 28 days. RBC count of 0.53, 0.52, 0.51, 0.53×10^6/µL was observed in the control group for the 7th, 14th, 21st, and 28th days, respectively. Initially at the 7th day the RBC count did not change significantly for all the exposure groups but as time passed there was significant ($p < 0.05$) decrease in the RBC count on the 14th, 21st, and 28th days, where the values were found to range from 0.54 to 0.78×10^6/µL. The maximum decline (0.18×10^6/µL) was detected for the 200 µg/L group at the end of the 28th day. RBCs are a very important component of the blood and help in the circulation of oxygen that is important for normal bodily functions, hence, their measurement is vital and any slight deviation may provide information about the health of an organism. Lipophilic properties of SMX enables it to cross the membranes of RBCs and make them fragile and prone to disruption. Further more, the ion and gas exchange during excessive energy demands may also affect the membrane of RBCs. In the current study, the alterations in the number of RBCs may be due to a redeeming response of fish towards SMX toxicity (Cicha *et al.* 2003).

A similar pattern to RBCs was observed in the Hb, as shown in Figure 2. It showed a decrease in value over the passage of time. Hb content when compared with control was found to have declined by the end of the exposure period, showing values of 5.4, 5.1, and 4.8 g/dL for 25, 50, and 100 µg/L groups, respectively, on day 28. A notable reduction in Hb content was observed on the 28th day, displaying a value of 4.2 g/dL for the highest dosage group (200 µg/L). Lower Hb concentration in this study is a sign of hypochromic microcytic anemia. A slight increase in Hb content was observed on the 21st day for all the exposure groups, showing fish under stress conditions. Hb in blood is a good reflection of oxygen level in blood. During internal and external stress, the body undergoes anoxic conditions which disturb the body's alternative energy synthesis process within the organism. A similar response to clofibric and diclofenac acids was also noticed in common carp (Saravanan *et al.* 2011). Decrease in the Hb content may be attributed to occult blood loss (Goldstein *et al.* 2011). High Hb content during the exposure period occurs due to increase in oxygen transport during stress. In the current study, the escalation in Hb level is an indication of a condition known as erythrocytosis, or SMX may have replaced denatured Hb (Nussey *et al.* 1995). In the present study, effects of SMX to inhibit erythropoiesis in the target fish have caused lower levels of Hb in the highest concentration exposure group (200 µg/L) by the end of the exposure time. Our results are in accordance with Ramesh *et al.* (2018), who reported Hb values of 6.7 and 5.1 g/dL for day 21 and 28, respectively, for fish exposed to sulfamethazine (SMTZ) at 10 mg/L concentration.

White blood cells play a major role in providing defense to the body and any alteration in WBC count may indicate an infection in the organism. These infections may be caused by stress or damage to tissues. Figure 3 shows the number of WBCs of control and exposed fish to SMX for 28 days. When compared to control, the exposed group shows a sharp increase in WBC count. The WBC count started to increase with the passage of time and continued until the 28th day with values ranging from 131 to 303 ($\times 10^3$/µL) for all the exposure groups. Maximum increase was seen for the highest concentration exposure group (200 µg/L) on the 28th day with a value of 303×10^3/µL. Basically, the process of phagocytosis in fish is carried out by mononuclear phagocytes and WBCs that produce large quantities of superoxide anion (O_2^-) upon stimulation with

a range of xenobiotic compounds. This leads to the increased consumption of oxygen to produce hydrogen peroxide (H_2O_2) by dismutation of O_2^-. SMX inhibits catalase activity happening within fish body and results in the accumulation of H_2O_2. That ultimately increases the number of WBCs in the fish body. This immunostimulatory effect may have happened due to the probable increase in H_2O_2 levels by SMX (Saglam & Yonar 2009). Lunden & Bylund (2002) verified that WBC count is related to the immune response of the body, thus, in the presence of any foreign stress the spleen produces new WBCs. Proliferation in WBC count indicates production of antibodies in the organism. The immune system has a critical role in sustaining proper biological processes and protection against diseases. The observed leucocytosis depicts the reaction of fish to SMX toxicity.

Figure 4 shows the number of PLT count of control as 330, 331.2, 329.5, 330.75 × (10^3/μL) for 7, 14, 21, and 28 days, respectively. When compared to control, the exposed group shows a gradual decrease in PLT count with values ranging from 330 to 150 × (10^3/μL) indicating onset of thrombocytopenia; a type of disorder in which there is a lower number of PLTs. Drug-induced thrombocytopenia occurs when certain drugs rescind PLTs or inhibit the body's ability to make a sufficient number of them. Some drugs result in the production of antibodies in the body, which destroy PLT and this process is known as drug-induced immune thrombocytopenia (Van den Bemt *et al.* 2004). Sulfonamides have been proven as the cause of thrombocytopenia (Warkentin 2018). Numerous infective mechanisms have been correlated with drug-induced thrombocytopenia, of which SMX is found to be associated with quinine-type drug-dependent antibodies (DDAbs). In this class, DDAbs affix rigidly to PLTs in the presence of antibiotics and specifically target two genes: GPIIb/IIIa or GPIb/IX, leading to a drastic decrease in the PLT count in the blood of the target organism (Bakchoul & Marini 2018).

Biochemical parameters

Biochemical parameters are studied to assess the physiological systems of an organism in routine examination. Glucose and total protein level are important parameters used to determine the normal bodily function in toxicology studies (Vutukuru 2003). Figure 5 shows the effect of SMX on glucose levels of the fish. When compared with control (57.5, 58.0, 59.0, 58.5 mg/dL), values for the exposed group show a fluctuating trend for glucose levels. An overall decrease was observed in values with an increase on the 14th day of all the exposures. The values range from 73 to 35 mg/dL with a lowest value recorded on the 28th day for the 200 μg/L exposure group. The breakdown of glucose in the body is a major process for energy production; glucose level in the body may vary if the fish is under stress and requires high energy needs. The conditions may occur if the fish is under external or internal stress that may be caused by antibiotics which lead to a natural stress response in which the body secretes hormones such as corticosteroids, epinephrine and dopamine as a primary component to reactivate glycogenesis to overcome the high energy demand. The increase and decrease in glucose levels due to the exposure of fish to antibiotics may be due to the influence of carbohydrate metabolism which may cause an increase or decrease in cortisol production which is a primary stress response. In the present study, an initial increase followed by decrease in glucose level may be due to high metabolic demand caused by SMX. A similar biphasic trend was also observed for rohu (*Labeo rohita*) exposed to 80 mg/L of oxytetracycline, where serum biochemical

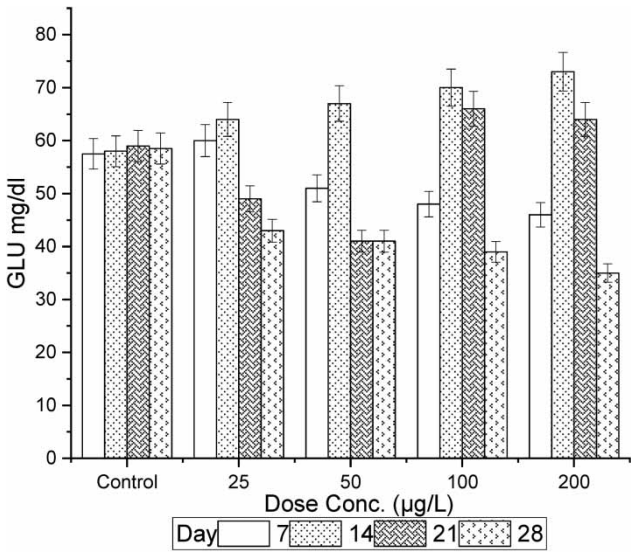


Figure 5 | Glucose level of control and SMX-exposed fish for 28 days (values are expressed as mean ± SE, n = 8).

profile showed increasing values for the first ten days and then subsequent decrease was observed at the end of the exposure period (25th day) (Ambili *et al.* 2013).

A fluctuating trend was also observed in the protein levels as indicted in Figure 6. An overall decrease ranging from 20 to 34 mg/dL was observed in the protein level compared with the control values (7.9, 8.5, 8, and 8.5 mg/dL) for 7, 14, 21, and 28 days, respectively. All exposure groups showed higher protein values on the 7th day, maximum increase (46 mg/dL) was observed on the 7th day of exposure for 200 μg/L, and then it started to decrease until the final week. Protein is a main component of all cells in the body. Proteins help the body to form and repair tissues, to create enzymes/hormones and other body chemicals. Protein is a main constituent of bones, muscles, skin, and blood. Fish when exposed to stress may undergo oxygen-deficient conditions which may affect the protein content of the body (Ramesh *et al.* 2018). The reduction in protein level can indicate the inhibition of protein synthesis and a rise in protein levels may be due to the adaptation of fish towards the contaminant. In this study, the fish exposed to SMX show a decrease in protein level as compared to control, indicating that SMX may have a potential stressful effect on the fish leading to low protein formation in the body. Heat shock proteins may also lower the protein content. *Onchorhynchus mykiss* exposed to 200 mg/kg SMTZ showed a decrease in protein content of 29 mg/dL on day 7 and 28 mg/dL on day 21 which may be attributed to stress caused by the antibiotic or reduced protein synthesis (Saglam & Yonar 2009). Similar results have been reported by Ramesh *et al.* (2015), who found variations in hematological and biochemical parameters of fish with decreased levels of glucose and protein under high toxicity effect. Ejraei *et al.* (2015) reported that many fluctuations were found in hematological and blood plasma indices of fish under the influence of age and hormonal treatments.

Triglycerides (TGs) also showed biphasic fluctuations with maximum level at 0.25, 0.26, 0.28, and 0.29 mg/dL for 25, 50, 100, and 200 μg/L groups, respectively, on day 14, as shown in Figure 7, whereas it decreased after the 21st day as compared to the control group. TGs are a very important source of energy during stress, and an increase in TG level may be due to lipid mobilization to cope with increased energy demand (Tan *et al.* 2018). High TG content in the blood may be due to their transfer from the synthesis site for consequent use by process of oxidation or steady instauration of these molecules. Liver disorders and disruption of lipid metabolism also promote an increase in their level (Gaber *et al.* 2013). The nonpolar lipophilic nature of SMX can degenerate lipid-containing cell membrane leading to elevated levels of TGs in fish body. Decreased TGs at the end of the exposure period may be due to low feed

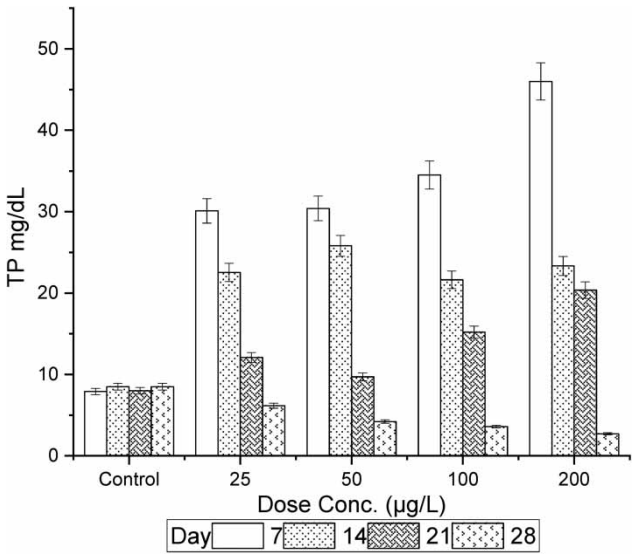


Figure 6 | Total protein level of control and SMX-exposed fish for 28 days (values are expressed as mean ± SE, n = 8).

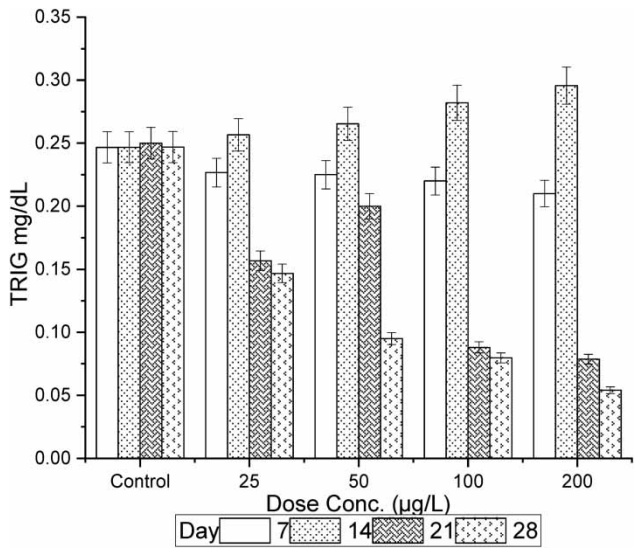


Figure 7 | Triglyceride level of control and SMX-exposed fish for 28 days (values are expressed as mean ± SE, n = 8).

intake or less absorption due to poor gut and liver functioning and initiation of membrane biogenesis (Van Meer *et al.* 2008).

The ALT level significantly ($p < 0.05$) increased with increasing dose concentration and time of exposure, as seen in Figure 8. ALT is principally present in the hepatocyte of the body and therefore their increased level reflects liver damage (Mikulikova *et al.* 2013). Rising ALT serum levels may be related to stress conditions in fish toxicological studies. Structural changes in the cell organelles could alter the level of ALT enzyme. Improper metabolism of protein and carbohydrate could be the reason for elevated levels of transaminase activities under exposure of antibiotics (Akrami *et al.* 2013). Sampaio *et al.* (2016) reported that SMX affects the liver and activates enzyme production at a higher rate. Elevated level of ALT is a symptom of hepatomegaly, which is triggered due to SMX toxicity.

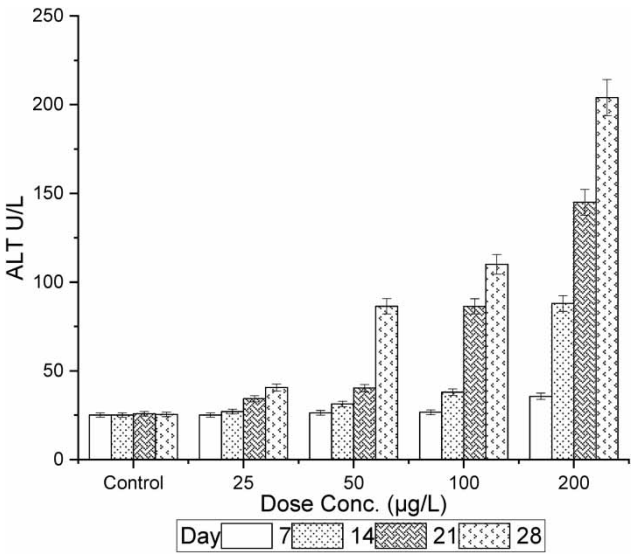


Figure 8 | ALT level of control and SMX-exposed fish for 28 days (values are expressed as mean ± SE, n = 8).

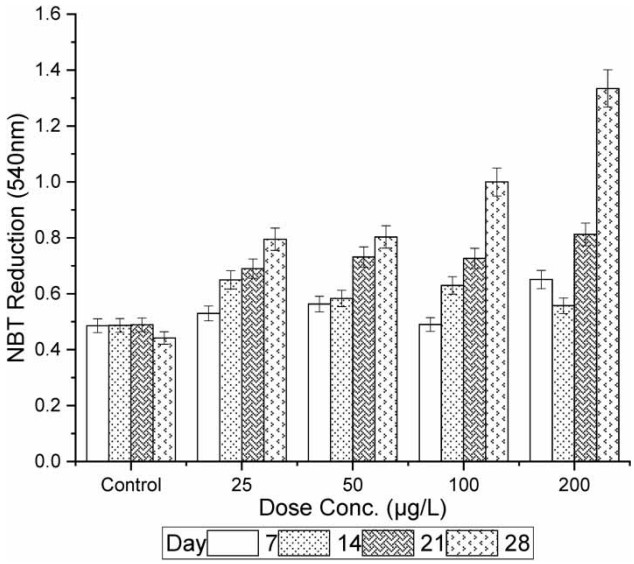


Figure 9 | Respiratory burst activity in control and SMX-exposed fish for 28 days (values are expressed as mean ± SE, n = 8).

Respiratory burst activity (NBT)

Figure 9 shows substantial increase in respiratory burst activity with increasing time of experiment, and the highest values of 0.79, 0.80, 1.0, and 1.33 were recorded for 25, 50, 100, and 200 µg/L groups, respectively, on day 28. Respiratory burst activity shows neutrophil and macrophage activation status that result in the generation of reactive oxygen species (ROS) such as hydrogen peroxide, hydroxyl radical and superoxide anions. Cytokines facilitate the phagocytosis of neutrophils and macrophages to remove bacteria by generating ROS during respiratory burst activity (Secombes *et al.* 2001). The current study revealed that SMX significantly ($p < 0.05$) enhanced the phagocytic activity in common carp. Limbu *et al.* (2018) reported that SMX cause lipid peroxidation and results in damage to fish-immune organs. Altunoglu *et al.* (2017) reported values of 0.8 and 2.5 for respiratory burst activity in rainbow trout after exposure to black cumin extract at the dosage concentration of 0.1 mg/kg and 0.5 mg/kg for the exposure period of 30 days.

CONCLUSION

The results of the present study show that SMX results in significant alterations in respiratory burst activity, hematological profile, and biochemical parameters of *Cyprinus carpio*, upon chronic exposure at nominal concentrations. Results of the current study could provide baseline data on the possible effects of antibiotics on non-target organisms, specifically on fish under prolonged exposure. Moreover,

this biomarker approach may be used to assess the risk associated with antibiotics in the aquatic environment. Further investigation of molecular toxicity could help in understanding of the SMX mode of action on organisms.

ACKNOWLEDGEMENTS

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DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

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