Toxicity Assessment of Selected Antibiotic with Titanium Dioxide Nanoparticles and Evaluation of Quorum Quenching Potential Using Fish as Model Organism

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Institute of Environmental Sciences and Engineering (IESE)

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بسم الله الرحمن الرحيم

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LIST OF ABBREVIATIONS

AO	Acridine orange
ANOVA	Analysis of variance
ALT	Alanine transaminase
BOD	Biological oxygen demand
COD	Chemical oxygen demand
DCF	Dichlorofluorescein
EDS	Energy dispersive x-ray spectroscopy
ERM	Embryonic rearing media
ENPs	Engineered nano particles
Hb	Haemoglobin
HPLC	High performance liquid chromatography
ICP-MS	Inductive coupled plasma mass spectrometry
K_{ow}	Octanol - water partition coefficient
MD	Molecular dynamics
NBT	Nitro blue tetrazolium
NMs	Nanomaterials
OECD	Organization for economic co-operation and development
PCR	Polymerase chain reaction
PE	Pericardial edema
PLT	Platelets
QQ	Quorum quenching
QS	Quorum sensing
RBCs	Red blood cells
SEM	Scanning electron microscopy
SMX	Sulfamethoxazole
TiO_2	Titanium dioxide
TG	Triglyceride
VMR	Visual motor response
WBCs	White blood cells
XRD	X-ray diffraction
YSE	Yolk sac edema

ABSTRACT

Growing trend of nano technology and excessive use of antibiotics in past few decades have given rise to global concerns regarding fate and toxicological impacts of these pollutants on ecosystem. Sulfamethoxazole (SMX) is a broad range bacteriostatic antibiotic widely used in animal and fish farming and also employed in human medicine. These antibiotics may ultimately end up in aquatic ecosystem and affect non-target organisms like fish. The overview of experimental work in this research followed three main phases linked to each other. In phase 1a, local fish *Cyprinus carpio* was used as bioindicator for toxicity assessment of SMX. Effects were determined by chronic exposure to environmentally relevant dosages of 25, 50,100 and 200 µg/L of SMX for 28 days. Cytotoxicity assessed through hematological and biochemical profiling showed a dose-response relationship. Hemoglobin, platelets, and erythrocytes levels were significantly reduced in exposed fish. Leukocytes count was considerably enhanced with values varying from 131-303 (x 10^3 /µL). Changes in biochemical indices showed biphasic trend with time and dosages tested in study. An inverse relation between concentrations applied and bioaccumulation in targeted fish muscles was discovered by HPLC analysis. The highest concentrations quantified in fish muscles exposed to 25, 50, 100 and 200 µg/L were 124, 202,104.5, and 123.2 ng/g, respectively at several sampling times. Moreover, exposure to SMX enabled ROS production and various histopathological lesions in various organs of SMX exposed fish. Organ pathological index showed that the intensity of tissue lesions increased as SMX dosage was increased. Upon completion of exposure time (28^{th} day), quantitative analysis of gill morphology revealed that the severity of histopathology increased over time for all exposure groups, suggesting physio-metabolic turmoil brought on by the biological and molecular action of SMX. The current study also determined the exposure effects of a broad range of SMX concentrations to developing zebrafish, an ideal indexical organism for ecotoxicological studies. To discern the effect of SMX on developing zebrafish embryos and larvae, Phase 1 b of studies investigated a broad range of toxicity endpoints including survivability, hatchability, malformations, oxidative stress, behavioral changes, mitochondrial bioenergetics, apoptosis, and immune-related transcripts. Results showed that higher concentrations of SMX affect survivability, cause

hatch delay and induce malformations including edema of yolk sac, pericardial effusion, bent tail and curved spine in developing embryos. Lower levels of SMX provoked an inflammatory response in larvae at 7 dpf as noted by up-regulation of *ifn* and IL-1β transcripts. SMX also increased transcript expression of genes related to apoptosis including *bad* and *bax* at 50 µg/L and decreased *casp3* expression in a dose-responsive manner. SMX induced hyperactivity at 500 and 2500 µg/L based upon light/dark preference test.

Titanium dioxide nanoparticles are most widely used nano materials employed in various industries. It has been reported that presence of nanoparticles alters the toxicity and bioavailability of organic toxicants. Still, combined toxic effects of nano titanium dioxide and SMX antibiotic that are used world-widely remains unclear. In Phase 2 of study, *Cyprinus carpio* was utilized as bioindicator for toxicity assessment of nano titanium and SMX. Juvenile fish were exposed to selected dosages of 25-100 µg/L of SMX alone or co-exposed with 1.5 mg/L of nano titanium for 96 h period. Results revealed that nano titanium bioaccumulates in fish and it may also adsorb SMX. Nitroblue-tetrazolium (NBT) reduction assay for determination of immunological changes provided clear evidence for increase in respiratory burst activity triggered by nanoparticles. Nano titanium accelerated the uptake of SMX, suggesting that it may increase the bioaccumulation of antibiotics in fish muscles. Fish biochemical characteristics including glucose, alanine transaminase, and total protein were also changed as a result of co-exposure to both contaminants. Current study demonstrated that nano titanium increased SMX bioaccumulation and enhanced SMX-induced toxicity in *Cyprinus carpio*.

Phase 3 of thesis is focused on the *in silico* identification of promising phytochemical agent as drug candidate (and an alternative to conventional antibiotics) against vibriosis infections by considering LuxR protein as a primary target involved in quorum sensing (QS). Process of quorum sensing in *Vibrio anguillarum* (fish pathogen) depends on interaction of (AHL) autoinducer molecule with a receptor protein LuxR that is a positive transcriptional activator and results in vibriosis. In aquaculture vibriosis is responsible for severe economic losses worldwide. Phase 3 of current study was designed to investigate phytochemicals as an effective natural inhibitor (quorum quenching agent) of LuxR

protein. The compounds with PubChem IDs; 99091, 443028, 44587196 and 42607999 showed successful binding with LuxR protein with minimum binding energy in the range of -10.1753 Kcal/mol to -8.79 Kcal/mol. Lipinski rule of five and ADMET analysis were further used to evaluate the drug like properties of selected compounds. Lead compound with best drug like properties was additionally evaluated by molecular dynamics simulations to evaluate the stability of the protein-ligand complex during the simulation period. The study's findings indicate that compound with PubChem ID_42607999 might serve as an effective quorum quenching agent for *Vibrio anguillarum.* Finally current study may possibly facilitate the development of cost-effective and natural drug against vibriosis in aquaculture.

Chapter 1

INTRODUCTION

1.1 Background

Industrialization, urbanization, and rapid growth in human population have resulted in proportionate increase in environmental pollution with numerous organic and inorganic contaminants. Among these contaminants, pharmaceutical products are considered as evolving pollutant due to their numerous uses, occurrence in different environmental compartments at high levels and possible risk to the living organisms (Khan & Nicell, 2015). Pharmaceuticals are complex, primarily carbon-based compounds having a variety of physical and therapeutic properties. These pharmaceutical compounds get into the environment through various point and non-point sources, including factories, hospitals, agricultural runoff, residential use, and inappropriate waste disposal (Dorival-García et al., 2013). Pharmaceuticals are chemical substances with biological activity that, depending on their chemical characteristics, may interact with certain biological systems or function generically all over the body (Patel et al., 2019).

Because of the high demand of food security aquaculture sector has grown very rapidly all over the world. In recent decade, antibiotics attracted great public attention as a most prevalent and emerging contaminants. In aquaculture industry they play a key role in fighting against various infectious diseases. Since the last decade, it is estimated that 200,000 tons of antibiotics have been used all over the world (Gao et al., 2012). Due to their large consumption, an increasing detection of pharmaceuticals have been found largely all around the world including North America China, UK and Europe (Yang et al., 2017). In total antimicrobials utilized, sulfur drugs occupy 6%. Among these, sulfonamides are frequently utilized as a veterinary medicine (Perlovich et al., 2014).

SMX is predominantly used in aquaculture and rigorous livestock farming and is considered as heavily used antibiotics in the European Union (Bielińska et al., 2014). In the early twenty-first century, 16000 tonnes of antibiotics were used annually in the USA,

with sulfonamides accounting for 2.3% of all antibiotics used in veterinary medicine. Throughout Europe, this percentage ranged from 11 to 23% (Mahmoud et al., 2013).

Adverse effects of SMX on marine fish species have been the subject of several studies (Yildiz & Altunay, 2011; Anskjaer et al., 2013). However, studies on the toxicity of SMX in freshwater fish particularly on Pakistani cultivable local fish are limited. And data about its long-term exposure at environmentally relevant concentrations is still scarce and further research is needed.

Self-cleaning exterior coating systems, light-emitting diodes, solar cells, antiseptic sprays, sports equipments, water treatment technologies, and topical sunscreens are just a few applications for nano titanium (Frenzilli et al., 2014; Irshad et al.,2021). Due to its applicability in a wide range of consumer products, nano titanium are currently produced in significant quantities, with an estimated 10,000 tonnes produced per year worldwide(Piccinno et al., 2012). Annual output of nano titanium is expected to reach 2.5 million tonnes by 2025, owing to rising market demand (Zhang et al., 2012).Such widespread use of nano-sized titanium dioxide could result in large nano-titanium release into the environment, potentially increasing titanium nanoparticle exposure in the environment (Menard et al., 2018). Nano titanium has been reported to cause DNA damage and decreased leukocyte viability, reduction of hatching time, genotoxicity in erythrocytes and altered swimming activity in aquatic organisms (Minetto et al., 2014).Apart from the toxicity of nano titanium themselves, they have a tendency to interact with other environmental pollutants. The interaction of nano titanium with other contaminants may have an impact on bioaccumulation and harmful effects, raising implications for ecological risk assessments. Nano titanium and other pollutants have been found to have an adsorptive interaction in several investigations. In *Daphnia*, increased copper metal retention and ecotoxicity were documented in the presence of nano titanium (Fan et al., 2011). In addition, some studies indicate that nano titanium may interact with organic pollutants. Previous research has found that the co-exposure with nano titanium enhanced the toxicity of cypermethrin pesticide in zebrafish (Li et al., 2018). They have been reported to enhance the bioaccumulation and bioconcentration factor of PCB77 and pentachlorophenol leading

to impaired development and endocrine disruption in zebrafish (Fang et al., 2015; Lammel et al., 2019).

As previously mentioned, aquaculture operations have grown extremely quickly in past few decades, establishing themselves as a significant sector. Increased disease outbreaks and undesired diseases in local production have also been observed as a result of fast development in aquaculture sector. Bacterial diseases are indicated as a vital factor in reducing the productivity of aquaculture industry by causing growth retardation and a higher rate of mortality. This becomes a key problem in intensive fish production and aquaculture development (Perlovich et al., 2014). Bacteria have their own specific ways of communication. Quorum sensing, discovered in 1970's was defined as a mechanism that coordinates phenotypic expressions at the population level, like bioluminescence, pathogenicity and toxin production (Mutlu et al., 2019). This method is used by microbes to evaluate the local densities that govern the expression of specific genes. Production, secretion, and detection of signal molecules operate this process. An increase in gene expression occurs when the signal molecules aggregate to a threshold level (Xiao et al., 2022).

Quorum sensing (QS) in bacterial cells is classified into two types: AHL (acyl homoserine lactone) based QS in (gram-negative bacteria) and AIP (Autoinducer peptide) based QS in gram-positive bacteria. At a low population density or high diffusion rate, AHL are at low concentration and the LuxR receptor is not activated. The receptor is activated only when the AHL amount reaches a specific level by establishing AHL- LuxR complex. To control the phenotypic expressions such as biofilm formation, virulence, motility, luminescence, competence that are regulated by QS, anti-QS techniques are being explored. Controlling QS is considered as a better option to avoid these phenotypic expressions as loss of QS activities are found to pose no threat to the cell activities (Siddiqui et al., 2015).

1.2 Present study and problem statement

Pollution of surface water channels has been well narrated all over the world and comprises a key issue at indigenous, national, and international levels. Various xenobiotics including antibiotics from different sources enter the water bodies and may get deposited at the soft-bottom and aquatic entities (Shi et al., 2022). Even though most antibiotics have a benefit

in aquaculture operations, misusing them can lead to some issues. The overuse of antibiotics in fish has been attributed to several hazards and adverse effects, including growth inhibition, immunosuppression, the emergence of bacterial strains that are resistant to the drugs, and ecosystem issues including drug residues. (Saglam and Yonar 2009).

Fish are among the many aquatic organisms that are important water biomonitors. Fish are the primary consumers and contribute significantly to the aquatic food chain and food webs by regulating pollution levels in the aquatic ecosystem. Common carp (*Cyprinus carpio*) and zebra fish (*Danio rerio*) were selected as model organisms for the current study. Common carp is one of the most widely consumed freshwater fish in Pakistan. It may tolerate adverse environmental conditions and stresses and has high sensitivity towards changing environment. Furthermore, it is a cool to temperate water fish being the main constituent of food chain in many areas of world. Additionally, zebrafish is considered as an excellent model for ecotoxicological studies as they have high fecundity rate, genetic similarities with humans, needs low maintenance cost, and rapid development from embryos to larvae stage.

Little information about the side effects of SMX exposure on a physio-metabolic system of fish is available. Studies on ecotoxicity of SMX in freshwater fish mainly on Pakistani cultivable fish under chronic exposure at environmentally relevant concentrations are still scarce and entail further research. The efficiency of using sulfonamides in aquaculture is still under discussion, So, pertinent information that contributes to an understanding of potential effects of SMX administration on fish physiology may aid in enhancing the knowledge on their efficacy. Keeping in view all the possible toxicological impacts, **phase 1 a** of the present study aims to illustrate effects of SMX in common carp (*Cyprinus carpio*) using biomarker approach. This study examined the possible bioaccumulation and toxicity of the targeted antibiotic (SMX) in *Cyprinus carpio* at environmentally relevant dosages (25, 50, 100, 200 µg/L) in terms of oxidative stress, histopathological, biochemical, hematological, and immunohematological effects over a prolonged period.

Based on a literature review, it has been found that there is a lack of data regarding SMX toxicity during the early developmental period of fish. Hence, in **phase 1 b** of this study investigated several toxicity assays over a range of antibiotic dosages found in environment

to comprehensively evaluate the effects of SMX to early stages of *Danio rerio* (zebrafish) embryos. Embryos were exposed to SMX dosages varying from 25-5000 µg/L. This study reports endpoints related to malformations, survival, hatchability, mitochondrial bioenergetics, apoptosis, reactive oxygen species (ROS), locomotor and behavioral (light/dark preference) assays. Based on the mechanism of action of SMX, it was expected that innate immunity of fish would be compromised, leading to apoptosis and oxidative stress. It was also predicted that immune system-related transcripts would change because antibiotics have immunosuppressive properties. Because of the distinct properties and potential damage to the ecology, nano titanium is gaining greater attention. Although antibiotics are frequently used in the environment, it is still unclear how coexisting nanoparticles might affect their impact on aquatic life. To comprehend the environmental fate and destiny of antibiotics in the presence of nanoparticles, data on bioaccumulation and ecotoxicity of common antibiotics in biota is needed. So, in **phase 2** of the study, the accumulation of SMX in edible muscle tissues of *Cyprinus carpio* after co-exposure with nano titanium was investigated. Furthermore, the cumulative cytotoxicity of nano titanium and SMX on fish biochemical markers (glucose, alanine transaminase, total protein, and triglycerides) were investigated. In addition, nitroblue tetrazolium reduction test has been run to evaluate the immunohematological changes in fish in the present study.

Last part of thesis, **phase 3** is focused on the *in silico* identification of promising phytochemical agent as drug candidate (and an alternative to conventional antibiotics) against vibriosis infections by considering LuxR protein as a primary target. Vibriosis is a lethal hemorrhagic septicemic disease caused by *Vibrio anguillarum* (gram-negative bacterium) that affects a variety of marine and freshwater fish, and crustaceans. Big economic losses in sector of aquaculture have been caused by this infectious disease globally. Because of the pathogen's high morbidity and mortality rates, literature reports extensive studies that have been conducted to comprehend its virulence processes and create quick detection methods and disease preventive techniques. Therefore, current study attempted to discover phytochemicals as a biological friendly and natural alternative to conventional antibiotics in aquaculture by using various bioinformatics tools including virtual screening, molecular docking, and molecular simulations techniques.

1.3 Scope and objectives

The current study was aimed to investigate the toxicological impacts of SMX antibiotic with nano titanium and evaluation of quorum quenching potential using fish as model organisms. The research work reported in this study was conducted in the laboratories of Institute of Environmental Science and Engineering (IESE), School of Civil and Environmental Engineering (SCEE), National University of Sciences and Technology (NUST), Pakistan, Super Computing Research and Education Centre (ScREC), School of Interdisciplinary Engineering and Sciences (SINES), NUST and Center for Environmental and Human Toxicology (CEHT) Department of Physiological Sciences, University of Florida (UF), USA.

The main objectives of the study were as follows:

1. To determine acute and sub-acute toxicity of SMX using fish as model organism
2. To investigate influence of titanium dioxide nanoparticles on uptake, bioavailability and biotoxicity of SMX
3. To identify *(in silico*) promising phytochemical as quorum quenching agent in controlling virulence of vibriosis in fish

1.4 Institutional Review Board and Ethical Statement

- The use of whole vertebrate organisms, such as zebrafish (*Danio rerio)* and common carp (*Cyprinus carpio*), in scientific research offers numerous ethical justifications and considerations:
- **Genetic Similarity and Insights**: Zebrafish and common carp share genetic, physiological, and anatomical similarities with higher vertebrates, including humans. This genetic proximity allows researchers to gain insights into complex biological processes, disease mechanisms, and potential treatments, benefiting both human and animal health.
- **Alternative to Mammals**: Using zebrafish and common carp as model organisms can reduce the need for higher vertebrates in research. Their rapid reproduction rates and transparent embryos make them suitable alternatives to mammals, helping to implement the principle of Replacement in animal research ethics.

- **Non-Invasive Imaging:** The transparency of zebrafish embryos enables researchers to observe internal processes and developmental stages without invasive procedures, reducing the impact on the animals and aligning with the principle of Refinement.
- **Environmental Monitoring:** Common carp are often used for environmental monitoring due to their sensitivity to pollutants and changes in water quality. Research involving these fish species can contribute to the conservation and protection of aquatic ecosystems.
- **Educational Value:** Zebrafish and common carp serve as excellent educational tools, facilitating the training of future scientists, veterinarians, and researchers who learn fundamental concepts through hands-on experience.
- **Understanding Behavioral and Neural Mechanisms:** Zebrafish are known for their complex behaviors and neural activities. Studying these traits can offer valuable insights into neurological disorders and behavioral patterns in vertebrates.

1.4.1 Relevant Committees and Ethical Frameworks:

All the experiments involving *Cyprinus carpio* were conducted after taking the approval of

Institutional Review Board (IRB) of Atta Ur Rahman School of Applied Biosciences (ASAB) and all the experimental procedures were according to the guidelines described by the National Institute of Health (NIH) Pakistan. All zebrafish experiments were approved by the Institutional Animal Care and Use Committee (UF IACUC#201708562) of the University of Florida ensuring compliance with federal animal welfare regulations.

1.5 Thesis organization

This dissertation has been organized into 5 chapters. A brief introduction of each chapter is given below.

Chapter 1 briefly introduces an overview of antibiotic SMX, nano titanium and quorum sensing/quenching techniques in general followed by scope and objectives of the current research.

Chapter 2 explains the comprehensive survey on literature review for the work done on toxicity assessment of antibiotics, nano toxicity and quorum sensing/quenching techniques. History, prevalence and background of antibiotics and nano materials has been presented in the first and second part of this chapter. The third part presented major application of *in silico* tools for virtual screening of phytochemicals for drug designing and providing a bio friendly alternative to antibiotics.

Chapter 3 is divided into three phases and each part corresponds to detailed experimental procedures for each objective including all chemicals and analytical techniques utilized.

Subsequently, in **Chapter 4** results and discussion are presented. An in-depth analysis of results is also stated in this chapter.

Finally, **Chapter 5** concludes research work and further recommendations are also provided at the end.

Chapter 2

LITERATURE REVIEW

2.1. Background

The rise in worldwide human affluence, along with widespread fear of a pandemic, has resulted in a steady growth in global drug use. Pharmaceuticals' durability in the environment, pace of dissemination, and capacity to accumulate in the biosphere are all varied. Their tremendous biological activity, on the other hand, shows that medicines, even in tiny doses, might induce substantial biosphere alterations. Pharmaceutical chemicals are environmental pollutants that are utilized by humans and animals. More than 200 medicines have been found in waters across the world. These chemical compounds can be found in the environment not just separately, but also in a complex combination, which might have unintended synergistic consequences (Breazeal et al., 2013).

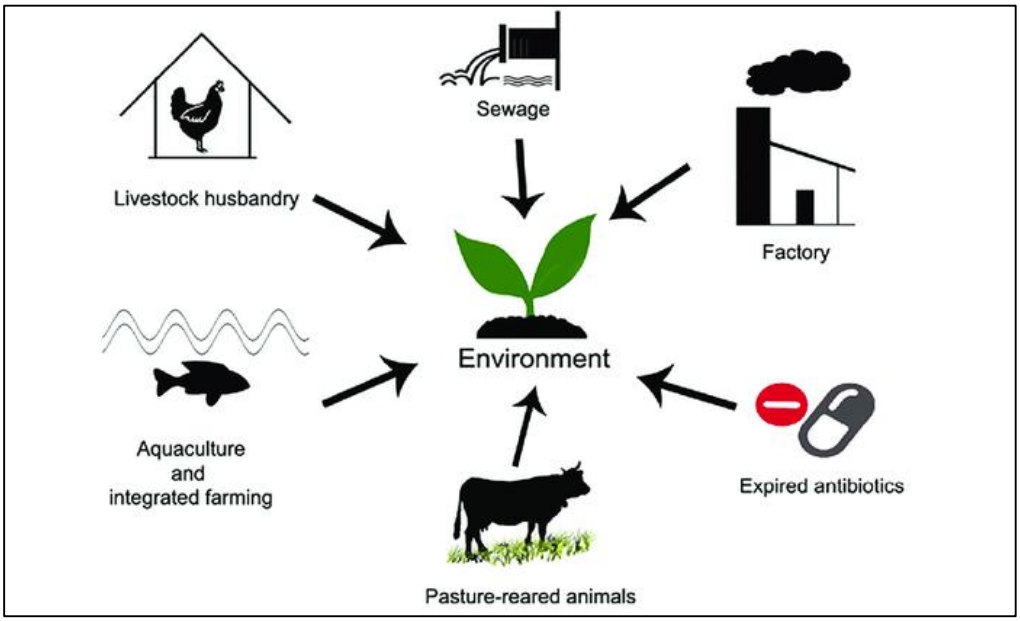


Figure 2. 1 Entry points of antibiotics into environment (Quaik et al., 2020)

2.2. Situation in Pakistan

The pharmaceutical has become one of Pakistan's most important industries, increasing at a pace of 10% each year. The pharmaceutical industry sells a variety of medicinal chemicals to over 27 nations across the world. In Pakistan, there are more than 386 pharmaceutical businesses with various capacities. These are positive economic indicators, however the pharmaceutical business at the community scale does not meet national environmental quality standards (NEQS) (Tauqeer et al., 2019). Compared to the G7 nations, Asia's pharmaceutical business is expanding at a rate of 10-15% annually. Every year, more than 4,000 pharmaceutical substances, weighing hundreds of tonnes, are commercially manufactured for animal and human care. Antibiotics account for roughly 200,000 tonnes of waste every year. Pharmaceutical chemicals are normally found in quantities ranging from nano to micrograms per liter in the environment (Kovalakova et al., 2020). At such low quantities, a single medicinal ingredient may not cause any harm. Total concentrations of all such pharmacological compounds with a common mode of action, on the other hand, might become a source of public concern.

2.3. Antibiotics in aquaculture and their infestation in aquatic environment

Aquaculture practices have intensified worldwide, leading to water pollution and waterborne infections that pose major risks to aquatic life. Aquaculture is considered a critical sector for meeting human demand for fish products. As a result of intense culture and climate change, disease outbreaks in local production have increased in frequency (Bashir et al., 2020). Bacterial diseases are now recognized as a significant factor resulting in the loss of productivity in the aquaculture industry by causing growth retardation and a higher rate of mortality in fish (Pepi & Focardi, 2021). As such, bacterial and viral diseases have emerged as a significant issue for intensive fish production and aquaculture development. To address this pressing issue, a number of antibiotics are applied in aquaculture to prevent or cure fish diseases (Perlovich et al., 2014)

Antibiotics have received increased attention in the last decade as an emerging contaminant. Antibiotics are used to treat infections, and boost up animal growth in both livestock management and aquaculture operations (Zhao et al., 2021). They are essential

in aquaculture for preventing devastating infectious illnesses and are found in a wide range of environmental matrices, such as surface and groundwater, as well as sediments (Mirzaei et al., 2018). Currently, significant amounts of antibiotics are entering the aquatic environment resulting in growing environmental concerns.

2.4. Fish as a bio-indicator of aquatic pollution

Fish occur at the top of food chain hierarchy in aquatic environment and is valuable bio-monitor of aquatic pollution. Since fish has the capacity to be directly exposed to many xenobiotics, it is a good indexical organism for assessing and documenting water pollution. When xenobiotics encounter fish, several responses between the body's biological and chemical systems are set in motion, ultimately leading to biochemical changes. Hence, it is important to identify the contaminant action mechanism and any relevant countermeasures. Fish can therefore be utilized as bioindicators of aquatic contamination for the evaluation of the aquatic system's health. (Bonomo et al.,2020) Several risks have been linked to the disproportionate usage of antibiotics in fish including immunosuppression and emergence of antibiotic resistance (Saglam & Yonar, 2009).

Chronic effects which may occur over a long period of time, are more likely to occur. Long-term exposure to antibiotics may result in anomalous physical processes and reproductive disorders and increased chances of cancer (Kolpin et al., 2002). Antibiotics have been found to bioaccumulate in aquatic species when exposed to extremely low quantities. For instance, bioaccumulation factors of chloramphenicol, roxithromycin, and enrofloxacin were 5376, 7410, and 4490 L/kg respectively, in marine fish (Na et al., 2013; Li et al., 2012). Likewise, antibiotics have been reported to cause negative impacts on the physiological functioning of non-target organisms, such as developmental and reproductive disorders, and neurological diseases; however, in most of the studies the concentrations applied were not environmentally relevant (Liu et al., 2014a).

2.5. Target antibiotic: Sulfamethoxazole

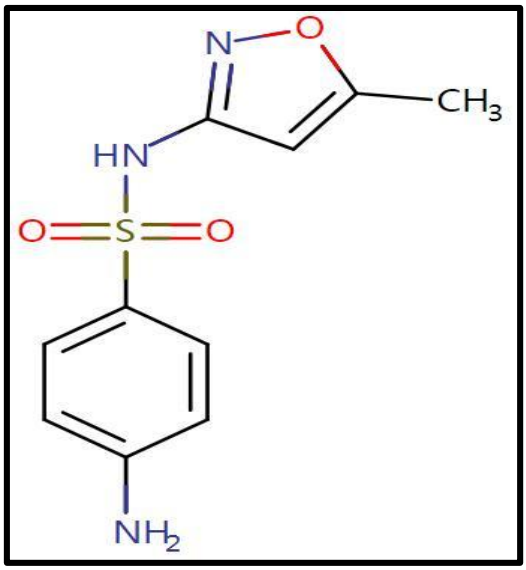


Figure 2. 2 Chemical structure of SMX

Sulfonamides, which include SMX, trimethoprim, and sulfadimethoxine, are present in surface waters all around the world (Shimizu et al., 2013). Sulfonamide consumption reached 7890 tons in 2013, with significant concentrations in rivers due to the failure of water treatment plants to remove various forms of sulfonamides (Zhang et al., 2016).

Sulfonamides are considered to be one of the concerning antimicrobial classes for the environment, first used in 1932 for medical purposes. These antibiotics are widely used in human and veterinary (including aquaculture) treatment because of their broad bactericidal spectrum and cheaper cost relative to other antibiotics (Xie et al., 2019).SMX , a prevalent member of the class, acts to inhibit the enzymatic pathway involved in bacterial folate production. By regulating the dihydrofolate synthetase enzyme, long-acting SMX prevents para-aminobenzoic acid from being converted to dihydrofolic acid, thus exerting its bactericidal effect (Fig. 2.3) (Romero et al., 2012).

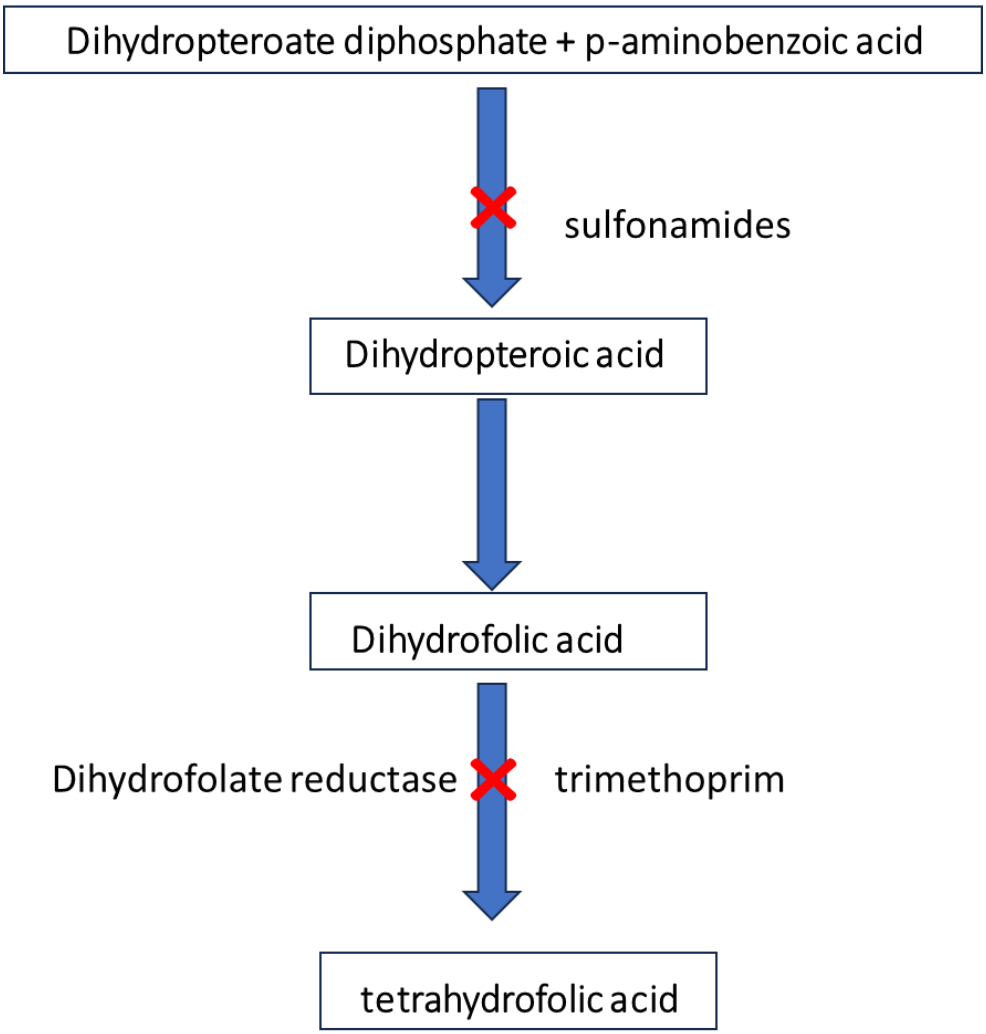


Figure 2. 3 SMX mode of action: inhibitor of folate synthesis in bacteria

It is one of the antibiotics that is eliminated in wastewater treatment plants with the least efficiency. Reported concentrations of SMX in hospital effluents are (0.4–2.1 mg/L) in New Mexico, USA (Brown et al., 2006) and (0.047–309 µg/L) in South Korea(Sim et al., 2011). Additionally, SMX was detected in ground water in the USA (0.015- 18 µg/L)(Baran et al., 2011), Pakistan (318 µg/L - 16000 µg /L) (Zafar et al.,2021) and China (8.4 ng /L to 211 µg/L) (Zhao et al., 2015). Taken together, SMX may be detected in several water systems on a global scale.

2.6. Antibiotics: A threat to aquatic biota

Despite the benefits of most antibiotics in aquaculture operations, there are significant risks linked with their use. Excessive use of antibiotics has been related to several adverse effects in fish, including developmental delays, immunodeficiency, antibacterial resistance, and ecosystem disturbances such as antibiotic residues in water and sediments (Lin et al., 2014 ; Yang et al., 2020; Zhou et al., 2022). Moreover, several studies demonstrate SMX can impact the physiology of freshwater fish. For example, hematological and biochemical disturbances following SMX exposure at environmentally relevant concentrations have been reported under chronic exposure (Iftikhar & Hashmi, 2021).Long term exposure to SMX (200 µg/L) also decrease body weight of zebrafish, indicating growth related effects (Yan et al., 2016). There is also rising indications that the immune system of fish responds to low-level antibiotic exposure in the environment. In adult Nile Tilapia (*Oreochromis niloticus*), exposure to 0.26 µg/L SMX promotes the expression of inflammatory cytokines (Limbu et al., 2018). SMX has been shown to impact a wide range of endpoints in fish, including oxidative stress, body development, bioaccumulation, histopathology and inflammatory changes(Lin et al., 2014; Elzagallaai et al., 2020; Iftikhar & Hashmi, 2021) . Even though antibiotics at mg/L have been demonstrated to produce minor inflammation and changes in healthy zebrafish, there are still concerns about environmentally relevant levels of exposure. Immunological toxicity is reported to be more prevalent in the embryo-to-larval period than in the adult phases (Mazurais et al., 2015), raising concern of a cascade of toxic events in fish. In this sense, although there is evidence that SMX exposure induces behavioral and histopathological abnormalities in adult fish, there are lack of toxicity data for the early developmental stages of fish.

There are significant human health issues raised by scientists regarding antibiotics of the aquatic environment. For example, exposure of fish to antibiotics can lead to deposits of sulfonamides in eatable animal tissues. Studies have quantified sulfonamides in fish muscles and liver(Zhao et al., 2015; Liu et al., 2018) . Therefore, residues may culminate in human tissue, leading to bacterial resistance (Gao et al., 2012) . Moreover, some members of the sulfonamide group of antibiotics are confirmed to be oncogenic (Van der

Oost et al., 2003). In terms of human safety, the maximum residue limit (100 µg/ Kg) in edible tissues of sulfonamides has been set by European Union and the USA (American Public Health Association). However, a recent study on *Cyprinus carpio*, a freshwater fish, has reported bioaccumulation of SMX at exposure concentrations as low as 25 µg/L. Therefore, continued diligence is warranted regarding the accumulation and toxicity of antibiotics in aquatic organisms.

2.7. Biomarker approach for toxicity assessment

The WHO defines a biomarker as "nearly any measurement reflecting an interaction between a biological system and a possible risk factor, which may be chemical, physical, or biological"(Athira et al., 2018).

The relationship between aquatic pollution and its adverse impacts on aquatic life forms can serve as a valuable tool for environmental preservation and biodiversity conservation. In this way, adverse impacts on the aquatic organism, specifically in fish health may be controlled (Rodrigues et al., 2019). Multiple biomarkers can be used to determine the sensitivity of different biological matrices to a given pollutant, as well as the mechanism causing that change. Survivability, malformations, and hatchability are among many biomarkers that can be applied in the field of toxicology. Biochemical biomarkers are the final outcome of physiological and biochemical changes. Consequently, the application of a multibiomarker approach could offer a more comprehensive understanding of the main effects of SMX in fish.

Histopathology is considered one of the most valuable tools for ecotoxicological investigations as it offers an effective and rapid detection of contaminant exposure results; it also enables the location and definition of lesions that take place on particular organs of targeted fish .The key advantage of using a histopathological technique in environmental impact assessments is that it allows for the examination of several organs at once, thus serving as a symptomatic indicator of a fish's overall health (Nero et al., 2006).

Numerous laboratory investigations have been performed to calculate the bioaccumulative potential of sulfonamides in aquatic ecosystems. Anskjaer et al. (2013) studied the influence of pH on uptake of sulfadiazine using *Daphnia Magna* as a model organism and

reported an inverse correlation between bioaccumulation and pH levels. Sulfadimethoxine was reported to bioaccumulate in brine shrimp, and notably reduced the hatching rate (Migliore et al.,1993; Saglam & Yonar, 2009).

Moreover, oxidative stress results in the production of ROS that can result in oxidation of various other molecules including lipids present in a cell and could result in change of mitochondrial bioenergetics in fish. Exposure to SMX for a long time may trigger cellular metabolism leading to the production of ROS in fish bodies. Increase in ROS is linked to the final pathway for the toxicity caused by several contaminants. Furthermore, xenobiotics and biological components may interact at a molecular level, resulting in tissue damage that can be measured using histological techniques and altered gene expressions (Rodrigues et al., 2017). Tissue modifications in different targeted organs of fish caused by both chronic and acute effects of pollutants are useful tools for toxicological studies. Most of the studies used qualitative data to investigate tissue damage, whereas quantitative analysis of histopathological changes allows for a more accurate understanding of the toxicological process at the molecular level (Bernet et al., 1999).

2.8. Engineered nanoparticles (ENPs)

Nanoparticles (NPs) are an important example of an emerging class of environmental pollutants. Increased rate of manufacture and consumption of NPs has led to increased human and environmental exposure. Engineered nanoparticles (ENPs) refer to a subset of nanomaterials with at least one dimension ranging from 1-100 nm in size. In addition to volume of industrial production, the possibility of anthropogenic agents ending up in aquatic environments also depends on how they are used. Concerns about the harmful impacts of nanoparticles on human health and ecosystems have increased due to their widespread application in industrial and consumer products (Rudramurthy et al., 2018).

2.9. Titanium dioxide (TiO_2)

Earth's soils contain naturally occurring titanium dioxide. It appears to be opaque and whitish. According to history, this mineral is as old as the planet itself. It is also among the top 50 chemicals produced globally, according to statistics. According to literature, there are eleven different polymorphs of TiO_2. Three polymorphs that are common in nature are anatase, rutile, and brookite. TiO_2 must go through several chemical procedures in order to

be purified (Rodríguez et al., 2019). It has a variety of uses because of its inherent qualities. This naturally occurring mineral has significant advantages for the paint and cosmetics industries. Among the nanomaterials, TiO_2-NPs have the highest concentration in surface water. Numerous applications of nano titanium at excessive rates inevitably results in their release into aquatic ecosystems (Wang et al., 2011). Estimated concentrations of nano titanium in water bodies are reported as 0.7 to 24.5 µg/L whereas predicted environmental concentration (PEC) for surface water is 0.7-16 µg/L (Troester et al., 2016). Moreover, nano titanium concentration as high as 900 µg/L has been recently reported in the surface water closer to beaches (Labille et al., 2020) Once released into an aquatic environment, significant impact of nano materials is subjected to their stability, mobility, and ultimately on the morphology in water resources used.

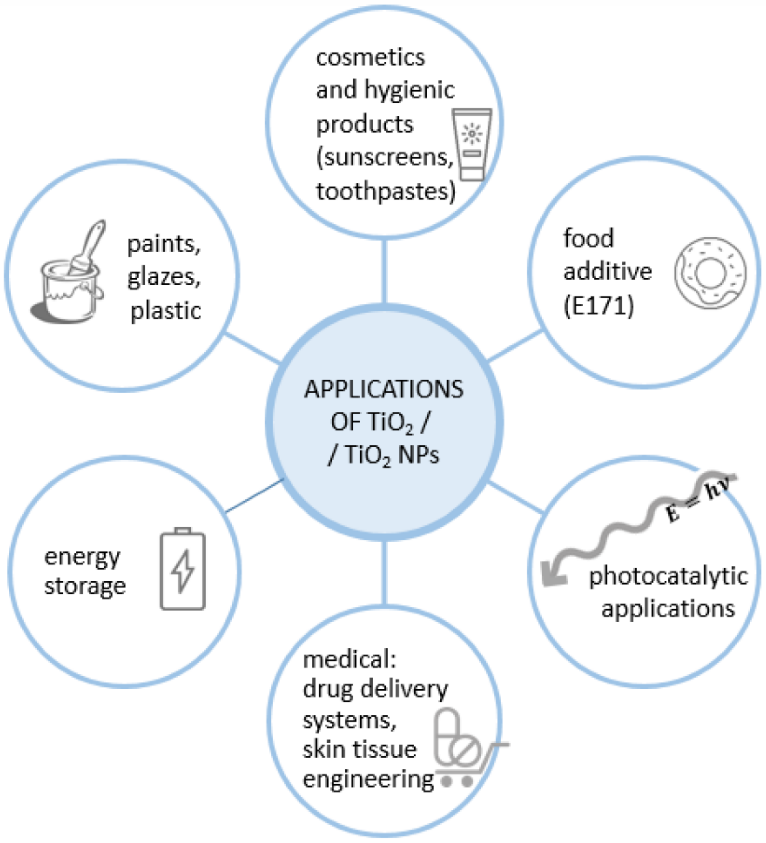


Figure 2. 4 Multiple applications of TiO_2 in daily life and industries (Musial et al., 2020)

2.10. Nano titanium toxicity in aquatic organisms

TiO_2 toxicity in aquatic animals in freshwater fish, mollusks, crustaceans, and algae have been reported in terms of immunotoxicity, cytotoxicity, oxidative stress, physiological and reproductive alterations. The sub-acute exposure of nano titanium to *Paralichthys olivaceus* resulted in oxidative stress at 100 µg/L concentration (Huang et al., 2018). In addition, negative impacts on production of embryos and oxidative stress during 1 mgL^{-1} exposures in zebrafish have also been reported (Ramsden et al., 2013). Study on genotoxicity and cytotoxicity due to titanium dioxide (TiO_2) nanoparticles exposure have resulted in genotoxicity in erythrocytes of marine fish, *Trachinotus carolinus* (Vignardi et al., 2015). Body growth rate of *Xenopus laevis* (amphibian) was reduced and their development was delayed when exposed to high concentrations of TiO_2 (>31 mg/L) (Zhang et al., 2012). TiO_2 nanoparticles have been known to generate reactive oxygen species (ROS), with •OH radicals being major radical species produced in the fish cells. These radicals are primarily responsible for the genotoxic consequences, specifically oxidative DNA damage (Reeves et al., 2008).

NPs might undergo active endocytosis. However, phagocytosis causes the activation of NADPH and ROS in phagocytic cells. NPs enter the cell through passive diffusion and reach the mitochondria and interfere with electron transport chain, which will cause excessive rate of oxidative stress. By interacting with the genetic material inside the nucleus, they may result in mutagenesis (Wang et al., 2022).

2.11. Combined toxicity of nanoparticles and organic pollutants

Several studies have been reported on combined toxicity of nano particles and other organic pollutants on several animal models. Du et al., 2021 studied the impact of ZnO-NPs on *Platymonas subcordiformis* swimming behavior. Results showed reduced motility due to lower energy availability, reflected by decreased ATP and pigments, and altered enzyme activity. Oxidative stress, ROS accumulation, and antioxidant responses further contributed to the observed reduction in swimming ability. Another study conducted by Zhang et al., 2023 explored the combined toxicity of nano-TiO_2 and organochlorines (OCs) on *Chlorella pyrenoidosa* in karst natural waters. nano-TiO_2 interact with OCs, resulting in varying toxicities. Synergistic and antagonistic effects were observed for different OC

pairs. TiO_2 enhanced pollutant accumulation in algae, with distinct effects from PeCB, atrazine, and PCB-77. Another study investigated the effects of nano-TiO_2 on 2,3,7,8-tetrachlorodibenzo-p-dioxin (2,3,7,8-TCDD) bioconcentration and toxicity in European sea bass over a 7-day exposure. Multi biomarkers were assessed in various organs. Nano-TiO_2 influenced 2,3,7,8-TCDD-induced immune response in spleen and reduced DNA damage in erythrocytes. However, it did not affect detoxification or bioconcentration of 2,3,7,8-TCDD. Interaction between nano-TiO_2 and organic pollutants in artificial sea water was minimal, indicating its independent effects on toxicity pathways (Della et al., 2015).

2.12. Bacterial quorum sensing in fish pathogenic bacteria

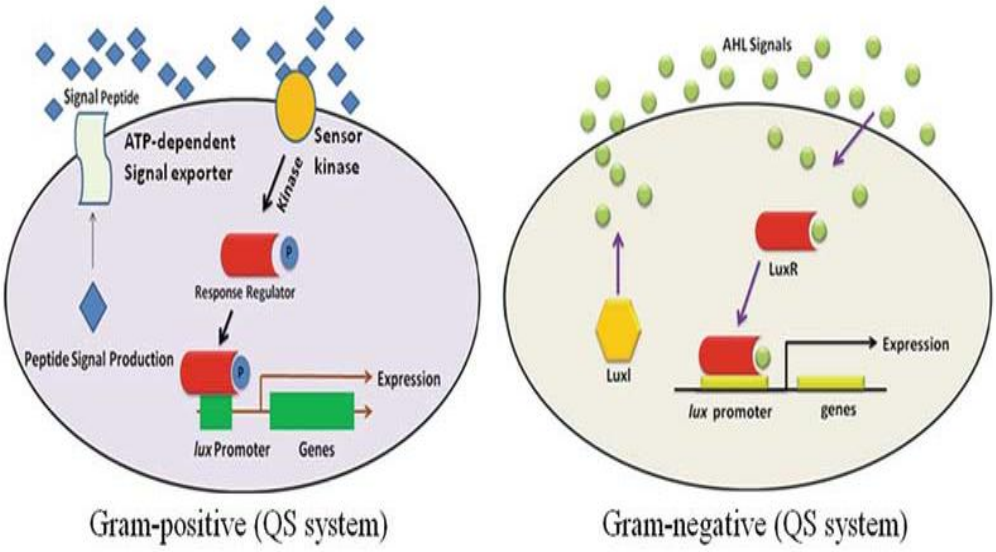


Figure 2.5 Quorum sensing types in bacteria: Autoinducer peptide-based QS in gram-positive bacteria and AHL based QS in gram-negative bacteria (Sankar Ganesh et al., 2018).

Quorum sensing, discovered in 1970's was defined as a mechanism that coordinates phenotypic expressions at the population level, like bioluminescence, pathogenicity, and toxin production (Nealson,1977). Microbial cells use QS as a cell-to-cell communication system to assess their local concentrations, which ultimately controls the expression of certain types of genes (Shao & Bassler, 2012). The mechanism is driven by signal molecules production, secretion and sensing. When the signal molecules are accumulated

to a certain threshold level, a shift in gene expression is activated in the population (Waters & Bassler, 2005). Number of studies have been conducted to elucidate quorum sensing mechanism in fish pathogenic bacteria (Table 2.1).

Table 2. 1: Effect of QS genetic mutations on the virulence of aquaculture related bacteria

S/No	Pathogen	Mutation of Gene	Virulence	Host	Reference
1.	*Vibrio harveyi*	luxO luxS luxP	Eliminated	*Artemia*	Defoirdt et al.,2005
2.	*Vibrio alginolyticus*	luxT	Slightly repressed	*Danio rerio*	Liu et al., 2012
3.	*Vibrio alginolyticus*	luxS	Repressed	*Pagrus major*	Ye et al., 2008
4.	*Vibrio vulnificus*	smcR	Repressed	INT-407 (human intestinal cells)	Lee et al., 2007
5.	*Aeromonas hydrophila*	ahyI, ahyR	Repressed	*Lota lota L.*	Natrah et al., 2012
6.	*Vibrio alginolyticus*	luxT	Slightly Repressed	*Danio rerio*	Liu et al., 2012
7.	*Aeromonas hydrophila*	ahyI	Repressesd	*Carassius auratus*	Chu et al., 2011

2.13. QS controlling strategies

Different mechanisms have been studied for the inhibition of QS activities, including:

1. QS signal production control (Chen et al., 2011). This strategy involves techniques to disrupt the signal molecules production. For instance, the *LuxI* genes in gram-negative bacterial species produces AHLs. The target in this mechanism is therefore the *LuxI* genes. It aims at complete disruption of signal molecules production.
2. QS signal (AHL) degradation (Sio et al., 2006). The targets in this strategy are the signal molecules. The production is not stopped while after production the density is controlled by degradation of signal molecules.
3. QS signal activity (AHL cognate receptor protein or AHL synthase) control (Parveen & Cornell, 2011). This method involves the control at expression site, that is the signal molecule interaction with genes that are expressed is targeted. In case of AHL based QS, the *LuxR*-AHL complex triggers pathogenicity, hence this the technique avoids the complex from forming and inhibits quorum sensing.
4. QS signal mimicking by synthetic compounds as signal molecule's analogues (Chen et al., 2011). This is a rare technique in which such compounds are introduced in the system, which have more affinity towards the group of genes otherwise expressed by signal molecules attachment.

2.14 Molecular Docking

Molecular modelling is a field in which methods like docking are used to calculate the ideal orientation of one molecule to another, establishing a stable complex. Calculating the strength of the bond between two molecules, for example using "scoring functions," can be done using knowledge of the preferred orientation. It is basically used for two purposes: one is signal transduction, and the other is drug design. The interaction of molecules with biological importance, such as lipids, proteins, nucleic acids, and carbohydrates, is known as signal transduction. Also, the type of signal generated may vary depending on the relative direction of the two working partners (agonism, antagonism). Hence, docking is useful tool for calculation of type and strength of the signals produced. In the case of drug discovery and design docking is needed to calculate the orientation of small molecular candidates of drugs, to their protein targets so that predictions on the affinity and action of

the small molecule can be made. Therefore, docking is vital for rational drug design and discovery (Meng et al., 2011).

2.15. Molecular Dynamics Simulations

It is a molecular mechanics program intended to imitate the movement of atoms within a molecule. Molecular Dynamics (MD) can be done on a molecule to produce altered conformation which upon energy minimization, give a range of steady conformations. The MD method is used for calculation of the molecular motion of several particles that are interacting in classical manner. In MD, motions of molecules are within some specific timeframe i.e., one time step (fs); atoms have some velocities and are subjected to some forces. The MD simulation is used to observe the conformational changes in molecules by assimilating Newton's second law of motion. Motion of a single molecule or of large number of molecules can be studied by this method (Sidorenkov et al., 2016)

Chapter 3

METHODOLOGY

This chapter illustrates the experimental procedures, biomarkers for the toxicity assessment of target pollutants - SMX and TiO_2 nanoparticles - and the in-silico techniques used for identifying a suitable phytochemical as a quorum quenching agent against vibriosis.

3.1 Phase 1a: Toxicity assessment of SMX on cultivable fish *Cyprinus carpio* using multiple endpoints.

3.1.1. Purchase and maintenance of experimental fish (*Cyprinus carpio*)

Healthy Common carp were purchased from Punjab Hatchery (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.

3.1.2 Acclimatization of fish

Fish were accustomed to laboratory conditions for a period of fourteen days and fed with commercial food pellets on daily basis. To avoid tank fouling, dead fish were immediately removed during the acclimatization period, and tank water was renewed on alternate days. Morphometric parameters of experimental fish were determined immediately after shifting to laboratory (Table 3.1).

Table 3. 1: Morphological characteristics of experimental fish

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

3.1.3. Water parameters of fish tanks

The physicochemical parameters of the experimental tank and lake water were assessed by following the standard OECD guideline method 203 (1992).

3.1.4 Experimental design

Experimental tanks were filled with approximately fifty liters of water and changed on daily basis during exposure period of 28 days. To prepare the experimental stock solution, 1 mg of SMX was dissolved in 1000 ml of water. To obtain the required dosages of toxicity solution, further dilutions of this stock solution were made. Air stones were placed in all the experimental tanks and the concentration of SMX was renewed on daily basis. Using random selection method healthy fish with desired morphometric characters (Table 3.1) were divided into experimental and control groups. A set of 8 fish in each tank was exposed to SMX for 28 days at following environmentally relevant concentration: 25, 50, 100 and 200 μg/L. All parameters were assessed on 7, 14, 21 and 28 days, respectively. Residual SMX concentrations in experimental and control tanks were analyzed after 1h and 24h of renewing the test solutions through (Agilent 1260 Infinity II LC System) HPLC. Toxicity in fish was assessed by different type of analysis following OECD guidelines 204 (1984). No fish death was noticed over the 28-day test period.

3.1.5. Chemicals

In the current study, only analytical-grade compounds were utilised. SMX > 98% purity was purchased from Sigma -Aldrich, USA. Nitrotetrazolium blue chloride (Bioworld, USA) and N, N dimethylformamide (Sigma-Aldrich, USA) were used for Nitrotetrazolium blue chloride (NBT) reduction assay. Biochemical parameters were measured using reagents kits purchased from AMP Diagnostic, Austria. 2,7-dichlorofluorescein diacetate (CAS: D6883 Sigma Aldrich) was used for measuring ROS in the brain and gills. For HPLC (Agilent-1260 Infinity II LC-System, USA) analysis all chemicals used including HPLC grade water and Acetonitrile (CAS-No-75-05-8) were purchased from Merck Germany.

3.1.6. Biochemical analysis

Fish blood samples were biochemically analyzed to assess toxic impacts of SMX using the Perveen et al. (2019) method. Biochemical parameters: total glucose level, triglyceride, total protein content, and alanine transaminase level in blood were selected for the present study. In biochemical analysis the blood samples were withdrawn with the help of syringe in gel activators for the preparation of blood serum. Samples were centrifuged at 4000 rpm for 10–20 minutes to create serum, which was then run through an AMP Piccos II chemistry analyzer.

3.1.7. Respiratory burst activity (Nitroblue tetrazolium (NBT) assay)

Immuno-hematological changes were assessed by using NBT reduction assay by following the procedure reported by Zanuzzo et al. (2015). 0.1 ml of both heparinized blood and 0.2% of NBT (in phosphate buffered saline solution) were co-incubated with at ambient temperature for period of 45 min. 50 µl from the resultant suspension was added to 1 ml of N,N-dimethylformamide and centrifuged to get supernatant. The optical density of resultant supernatant was than assessed using UV-Visible spectrophotometer at wavelength of 540 nm. The blank consisted of similar steps and components, excluding blood that was replaced with distilled water.

3.1.8. Hematological parameters

To determine the effect of applied dosages of SMX on hematological parameters of fish, experiments were conducted 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 dorsal fins using a 5ml syringe. The blood was collected in sterile purple topped EDTA vials containing anticoagulant. After collecting blood, 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, white blood cells, hemoglobin and platelets were measured using Sysmex blood analyzer XP-100.

3.1.9. Sample analysis for bioaccumulation

Fish muscle tissues were extracted by the procedure reported by Tang et al. (2012) with some modifications. Briefly, a precisely weighed amount (1 g) of muscle tissue samples from each fish with n=9 was mixed with acetonitrile within a falcon tube and then homogenized using WiseTis (HG-150) homogenizer, Korea at 9000 g. The resultant slurry was successively centrifuged (Centurion Scientific-K3 series, UK) at 5000 g for ten minutes. The supernatant was then collected and placed into a 50-mL glass flask. Entire procedure was repeated, and supernatants were pooled and placed into the incubator at 37 ºC temperature to evaporate near to dryness. Afterward, n-hexane was added to the residues for the removal of lipid content from fish tissues. The samples were finally filtered using syringe filters (0.45 µm) for HPLC analysis. Target antibiotic (SMX) was analysed using High-Performance Liquid Chromatography (Agilent-1260 InfinityII LC-System, USA) having quaternary pump, diode array detector, autosampler, Eclipse (4.6x250 mm) C18, 5 µm column. Flow rate of 1 mL/ min and sample injection volume (20 µL) was maintained for the analysis. The mobile phase used was composed of 100% acetonitrile. Equations were used to derive the detection and quantification limits:

$$LOD = 3.3\ x\frac{\sigma}{S} \qquad (1)$$

$$LOQ = 10\ x\ \sigma/\mathrm{S} \qquad (2)$$

where σ denotes the standard deviation and S shows the slope of the curve (Zafar et al., 2021). Determination of SMX in real samples was performed through standard curve that was constructed with SMX standard > 98% purity (Sigma-Aldrich) within the concentrations range of 10 to 75 µg/mL. Response factor (3.19) was calculated from the calibration curve with an R^2 value of 0.99 and retention time of SMX as 2.48 min. The procedural blanks were also determined simultaneously by following the same sample preparation method as for the exposed fish samples.

3.1.10. Bioconcentration factor and data analysis for quantification of SMX

Quantification of SMX in targeted muscles tissues was calculated using equation 3 and 4.

$$Amount\ of\ analyte\ in\ real\ sample = (Peak\ area\ of\ the\ sample)/ (Response\ Factor) \quad (3)$$

$$Response\ factor = Peak\ area\ of\ standard / Amount\ of\ standard \quad (4)$$

The bioconcentration-factor was evaluated as a ratio between SMX quantity in fish muscle tissue to the quantities measured in experimental water tanks. The bioconcentration factor was calculated using the actual concentrations of SMX (23.0, 47.75, 92.35, and 188.4 g/L) determined using HPLC in water tanks, which corresponded to the applied concentrations of 25, 50, 100, and 200 µg/L, respectively. The following equation was used to calculate the bioconcentration factor (BCF kg):

$$BCF = k1/k2 \quad (5)$$

where k1 is SMX concentration in water and k2 shows the concentration in fish muscles (Zafar et al., 2021; Rome et al., 2012). Moreover, the estimated bioconcentration factor was calculated from the regression equation:

$$\log BCF = a\ logKow + b \quad (6)$$

3.1.11. Oxidative stress

Determination of oxidative stress in brain and gills was based on the methods of Zhang et al. (2008) where DCFH-DA probe was applied to measure ROS. Brain and gill tissues were homogenized using a homogenizer (WiseTIS HG- 15D, Korea) in ice-cold Locke's buffer (100 mg tissues/ml buffer), the homogenate was left at ambient room temperature for period of 5 min. After that,5 µL of DCFH-DA was added to the homogenate followed by incubation of 45 min at 37 °C. A spectrofluorometer (F4000 Hitachi, Japan) with an excitation/emission wavelength of 485/525 nm was used to monitor the conversion of DCFH to DCF. Parallel blanks were used to correct background fluorescence.

3.1.12. Histopathological procedure and gills morphological analysis

Histopathological analysis and gills morphometric characteristics were performed following the protocol of Rodrigues et al. (2019). Briefly, after completion of the exposure period fish in the control and experimental groups were dissected and targeted organs were removed carefully. For histopathology dissected organs were then immersed in Bouin's liquid and the fish's tissues were fixed for 24 to 30 h, dehydration was performed in gradients with different solvents. Small sections of 4-6 mm were prepared using a rotary microtome and prepared slides were examined under a light microscope (Zeiss primo star-

415500-1811-000, USA) with 10 to 100 x magnification. (Rodrigues et al., 2019; Bonomo *et al.*,2020). Quantitative evaluation of histopathological lesions was based on the Scoring (Sc) and importance factor (Fi). Consequently, by using values of scoring and importance factor for each lesion, the tissue-specific alteration-index and the organ pathological-alteration index was calculated using equation 6 and 7 (Bernet et al., 1999).

$$\text{IAlt} = \text{Fi x Sc} \quad (6)$$

$$Iorg = \Sigma Alt \quad (7)$$

A light microscope (Zeiss primo star-415500-1811-000, USA) was used to examine photomicrographs of gill tissues. For secondary-lamellar-width, interlamellar-distance, and basal- epithelium-thickness, three measurements at different points were made for each secondary lamella and primary lamellae respectively. The proportion of secondary-lamellae available for gaseous exchange (PAGE) by fish was calculated on the 28th day using the following formula:

$$PAGE\ (\%age) = 100\ x[SLL/(BET + SLL)]$$

Whereas BET is (Basal-epithelium-thickness), ID (interlamellar-distance), SLL (secondary- lamellar-length) and SLW is (secondary- lamellar -width)

Data analysis

ANOVA was run to test the differences among experimental and control groups to determine the effect of different dosages of SMX on *Cyprinus carpio.* Post-hoc Tuckey test was performed to compare each treatment with the control and with selected dosages. Statistical analysis was performed by using Microsoft Excel and Origin software 8 (Origin Lab Corporation, USA) using a significance level of 0.05.

3.2. Phase 1 b: Toxicity assessment of SMX in early staged zebrafish (*Danio rerio*) using multiple biomarkers.

Zebrafish (*Danio rerio*) stand out as a significant model in toxicological research due to their genetic resemblance to humans, transparent embryos aiding in non-invasive imaging, rapid development allowing for efficient assessments, and ethical considerations. Their sensitivity to toxicants, behaviour observation capabilities, and conservation of pathways

enhance their value. Zebrafish serve as a versatile tool, facilitating high-throughput screening and offering insights into both developmental and environmental toxicology, making them pivotal in advancing toxicological understanding and chemical safety assessment.

3.2.1. Chemical Preparation

SMX (Cat :723-46-6) purity > 99% was provided by Sigma Aldrich. Stock solution (400 µg/mL) was prepared in embryo rearing media (ERM) and stored in refrigerator. Before the setup of all experiments, test solutions were freshly made to create final concentrations of 0, 25, 50, 100, 200, 500, 1000, 2500, and 5000 µg/L of SMX depending on the endpoint measured.

3.2.2. Zebrafish husbandry

The Cancer-Genetics Research Center (UF) reared adult zebrafish *Danio rerio* (AB x Tübingen) Zebrafish staging recommendations followed established protocols (Kimmel et al.,1995). Adults were maintained at ambient temperature (28±1 ºC), 14:10 (light-dark period), a dissolved-oxygen level greater than 6.0 ppm, and pH level of 7.2±1. The night before embryo collection, zebrafish (3-6 months of age), two pairs of adult zebrafish (males and females) were transferred to a breeding tank. Male and female zebrafish were separated overnight with a divider. Dividers were removed in the morning to initiate spawning. All experiments were performed at the Aquatic Toxicity Center using the collected eggs after getting approval by (UF IACUC#201708562) University of Florida.

3.2.3. Exposure experiments with SMX

Embryos (6 hpf) were collected and washed 3 times using ERM in petri dishes to remove any contamination from breeding tanks. Unfertilized embryos were identified using a microscope and removed to select only viable ones for the exposure. A total number of 15 or 20 embryos were transferred in a random fashion into Pyrex beakers with 10 mL ERM to comprise the experimental treatments (ERM, 25 - 5000 µg/L SMX). Four independent experiments were conducted with five to eight replicates to assess the survival, deformities, and hatchability of embryos using EVOS™ Auto Imaging System. Deformities measured included edema of yolk sac, pericardial effusion, and kinked tails.

Zebrafish were constantly exposed for seven days, with the exposure medium being prepared and refreshed every day to maintain the standard.

3.2.4. Mitochondrial bioenergetics

For the assessment of mitochondrial bioenergetics, 6hpf zebrafish embryos were transferred to experimental beakers with 10 embryos each. Exposure treatments included ERM, 2, 20, 200, and 2000 µg/L SMX. Four replicate beakers were maintained for each exposure condition and control group. Following a 48-hour exposure to SMX, embryos from exposed beakers (n=4/treatment as biological replicate) were selected to assess the rate of oxygen consumption (OCR) using Seahorse XFe-24 Extracellular-Flux-Analyzer (Bioscience, USA)(Souders II et al., 2019). Wave Desktop v 2.6 (Agilent Technologies, USA) software was used to export data to GraphPad PRISM v9.4 (La Jolla, CA, USA). Mitochondrial bioenergetics included: basal, non-mitochondrial, maximal and ATP associated respiration (User Guide: 103015-100, Agilent).

3.2.5. Reactive oxygen species

ROS was measured following published protocol (Souders II et al.,2019). Briefly, embryos were exposed to ERM (control), 25, 100 and 500 µg/L SMX (n=5 beakers per experimental group), each containing ~ 20. Experiment was conducted for 7 days with daily media change. At 7 dpf, ROS levels in zebrafish larvae were measured using H2-DCFDA probe and multi-detection microplate reader (New-Synergy 4, Bio-Tek).

3.2.6. Visual motor response test (VMR)

Dark photo kinesis response in zebrafish larvae being exposed to various concentrations of SMX (0-5000 µg/L) and ERM was assessed using VMR test. Five replicate beakers (15 embryos /beaker) were prepared for all treatments. Fish were exposed continuously for 7 days with 90% daily exposure media changes along with daily chemical renewal in incubator and photoperiod pf 14:10 h. Eight independent trials were performed to assess the potential effects of SMX on developing larvae behavior. In mid-afternoon (~ 2:00- 3:00 pm), normally developed zebrafish larvae (n=17-33 individuals/treatment) were placed into a 96-well plate. Activities of larvae were tracked using Danio-Vision™

(Leesburg, VA) (Liang et al., 2019). A single graph was created from all the trial data and the total distance covered represented the level of locomotor activity.

3.2.7. Anxiety test

Embryo and larval exposures proceeded as other experiments. 7dpf larvae were shifted into a company manufactured 12 -well plate with ERM. Twenty trials were conducted with n= 13-36 fish per treatment. A 12-well plate with a cover over the plate's bottom created a dark and light zone in plate for the dark/light preference test (LDPT). Poorly tracked larvae were excluded from analysis and data from twenty distinct runs were blended into a single graph to reflect all runs (Huang et al., 2021).

3.2.8. Gene expression analysis

Zebrafish larvae were exposed to ERM or one of several concentrations of SMX (25, 50, 100, 200, 500 µg/L) for gene expression analysis over 7 days. Eleven to twelve fish from a single beaker were pooled in a tube to make one biological replicate. Prior to RNA extraction, samples were immediately immersed in liquid nitrogen and kept in an ultra-freezer to prevent RNA degradation. The TRIzol® Reagent was used to extract nucleic acids, and pellets were then reconstituted in water devoid of DNase and RNase. RNA integrity was evaluated using the RNA-6000 nano kit on the 2100- Bioanalyzer (Agilent Technologies, CA, USA). The samples' average RIN value was 8.8 ±0.66. TURBO DNA free™ Kit was used to remove genomic DNA (ThermoFisher Scientific). The iScript^{TM} cDNA (Bio-Rad, CA, USA) was used to create the cDNA using 750 ng of RNA. The no reverse transcriptase (NRT) controls were created using 5 randomly chosen RNA samples in the same manner as above, but without the enzyme. One no template control (NTC) without RNA template was also included in the plate. qPCR plates were prepared following the protocol:forward and reverse primers (0.8 µL : approx. 100-200 nM), 3.33 µL (cDNA), and 5.025 µL (SsoFast™- EvaGreen® -Supermix solution). The CFX Connect™ RT- PCR Detection System (Bio-Rad) was used to collect data as per methods by Wang et al. (2018). Two technical replicates were used to measure each biological replicate. To standardize target expression; rps18, and β-actin, housekeeping genes with (M-value = 0.82, CV = 0.27) were used. CFX Manager^{TM} software (v3.1) was

used to obtain normalized expression values for each target gene, and the relative ΔΔCq technique based on BioRad software was applied.

3.2.9. Acridine orange staining/Apoptosis assay

The effect of SMX treatment on zebrafish apoptosis was assessed using the nucleic acid-selective staining technique known as acridine orange dye. 7 dpf larvae from each experimental group were chosen (n=15) and washed with ERM after exposure to various dosages of SMX (ERM, 25, 100, and 500 µg/L. Apoptotic cells were detected using an EVOS^{TM} FL equipped with a GFP filter after being washed with ERM five times for 30 seconds. Apoptotic cells were identified by bright green fluorescence patches. The histogram tool of the Image J programme (http://rsbweb.nih.gov/ij/) was used to quantify the fluorescence intensity.

Data analysis

GraphPad PRISM v9.4 was used to statistically analyze and visualize all data sets, and the results are reported as mean standard deviation (SD) (La Jolla, CA, USA). Kruskal-Wallis followed by Dunn's multiple comparison test was used to assess deformities, survival, and hatch rate data. Data related to ROS, apoptosis, and transcript levels were assessed for normality by applying a Shapiro-Wilk test. Differences in group means were compared to the ERM and were analyzed using a one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test. For locomotor activity and endpoints of anxiety, ANOVA (one-way) followed by Holm-Sídak post hoc test was used. The significance difference limit for each analysis was set at $p< 0.05$.

3.3. Phase 2: To investigate influence of titanium dioxide nanoparticles on uptake, bioavailability and biotoxicity of SMX

3.3.1. Chemicals

Sigma Aldrich provided SMX (CAS: S7507) with a purity of 98 percent. All compounds used in the HPLC analysis (Agilent-1260, USA) were purchased from Merck, Germany. For the manufacture of pure titanium nanoparticles, titanium dioxide powder (Daejung, Korea) was acquired. The NBT reduction experiment was performed using Nitroblue Tetrazolium (Bioworld, USA) and N, N-dimethylformamide (Daejung, Korea). Total

proteins, alanine transaminase, glucose, and triglycerides were measured using reagents from AMP Diagnostic in Austria.

3.3.2. Experimental design and fish maintenance

A total number of 200, healthy specimen (12-15 cm, length; 40-60 g weight) of common carp (*Cyprinus carpio*) were purchased from Rawal lake hatchery, Rawal Town, Islamabad and were transported to the lab in oxygenated polyethylene bags, avoiding mechanical injuries. They were transferred and kept in aerated glass tanks. Specimen were divided into different batches for each test. Triplicates were retained for every dosage and control. For the first two weeks, the fish were acclimatized under laboratory conditions. They were given dry commercial food pellets to eat (YDJ004, Zhejiang, China). Throughout the experiment, the physicochemical characteristics of water were determined on a regular basis using the standard protocols outlined in APHA (2017) (American Public Health Association). The fish were sorted into 12 fish per tank using a random selection process (containing 50 L water). 1 mg/L SMX stock solution was prepared. Test solutions of concentration: 25, 50, and 100 µg/L of SMX, equivalent to nominal amounts of SMX occurring in natural environments were freshly prepared before starting the test, both alone and in combination with 1.5 mg/L (non- lethal dosage) of titanium dioxide nanoparticles (Khan et al., 2013a; Van Doorslaer et al., 2014).To reduce tank fouling and maintain SMX and nano titanium concentrations in exposure tanks, the exposure media were replaced daily. Fish were exposed for period of four days and research work was performed after obtaining approval from Internal Review Board of ASAB (NUST). After the exposure of four days, the fish were sampled for subsequent biochemical profiling, NBT reduction assay and bioaccumulation of both contaminants.

3.3.2. Synthesis of TiO_2-NPs

Liquid Impregnation method was performed for the synthesis of pure titanium dioxide nanoparticles (Khan et al., 2013a). In a beaker, 50 g of TiO_2 was combined with 300 mL of distilled water to create a slurry. On a magnetic stirrer hot plate, this suspension was continuously mixed for 24 hours before the solution was given another 24 hours to settle. It was oven dried for 12 hours at 105 ^{0}C. After drying, the material was crushed properly

using a pestle and mortar. To produce pure TiO_2-NPs, the ground powder was transferred to a china dish and heated in a muffle furnace for six hours at 550°C.

3.3.3. Characterization of nanoparticles

SEM (Scanning Electron Microscope JEOL JSM-6460) at 10,000x magnification was used to examine the topography, morphology, and direct determination of particle size of TiO_2-NPs. The crystalline phase of prepared nanoparticles was identified by X-ray Diffraction (JEOL JDX-II) (Khan et al., 2013b).

3.3.3. Biochemical analysis

To investigate the harmful effects of SMX, biochemical analysis of fish blood samples was performed according to a previously mentioned procedure (Perveen et al., 2018). The following biochemical indices were used for the current study: glucose, triglyceride, total protein, and ALT (alanine transaminase). Blood samples were extracted using a syringe in gel activators to produce blood serum for biochemical examination, and then analysed with an AMP Piccos II Chemistry analyzer.

3.3.4. Nitroblue tetrazolium (NBT) reduction assay

Stimulated ROS (reactive oxygen species) were evaluated using the blood/Nitroblue tetrazolium (NBT) method with some minor modifications (Smith et al., 2013). This method works by converting NBT into the colourful chemical formazan. For this test, 0.1 ml blood was co-incubated 0.1 ml of (0.1 percent) NBT. Following the incubation period, 1 ml of DMF was added to the blood/NBT mixture and centrifuged for 10 minutes to collect supernatant. A spectrophotometer set to 540 nm was used to measure formazan in the final supernatant, which was used to assess ROS.

3.3.5. Adsorption experiment of SMX on nano titanium

To evaluate the adsorption of SMX on nano titanium, a mixed solution containing 1.5 mg/L of nano titanium and 25 µg/L of SMX was utilized. Experiment was set for 24 h. To separate the nano titanium particles, 100 mL of the combined solution was collected at 0, 2, 4, 8, 16, and 24 hours and centrifugated for 5 min at 14000 *g*. HPLC was used to determine the concentration of SMX in the supernatant (Agilent-1260, USA). The amount

of SMX adsorbed was determined by calculating the reduction in SMX concentration over time (Fang et al., 2015).

3.3.6. Bioaccumulation of SMX in fish muscles

The method used by Tang et al. (2012) to extract fish muscle tissues was adopted with some modifications. Briefly, muscle tissues (1 g) from each of the six fish (n = 6) was mixed with 10 mL acetonitrile and homogenized at 9000 g using a WiseTis (HG-150) homogenizer from Korea. Using a centrifuge (Centurion Scientific-K3 series, UK), the slurry was centrifugated at speed of 5000 g for 10 min. The supernatant was placed in an incubator at 37 ^{0}C to evaporate. The residues were then combined with n-hexane to remove the lipid content from muscle tissues (Tang et al.,2012). For HPLC analysis, prepared samples were filtered using 0.45 m syringe filters. The following HPLC settings were used: Eclipse (4.6 250 mm) C18 column, sample injection volume (20 µL), flow rate of 1 mL/ min, and acetonitrile as the mobile phase (100 percent). Equations were used to derive the detection and quantification limits:

$$(1)\ Limit\ of\ detection = 3.3x\sigma S$$

$$(2)\ Limit\ of\ quantification = 10x\sigma/S$$

where σ is the standard deviation and S demonstrates the slope of curve (Zafar et al., 2021). Quantification of SMX in real fish muscles was done through standard curve which was constructed with SMX standard (> 98% purity) within the concentrations range of (10–75 µg/mL). Response factor (3.19) was determined from the calibration curve (R^2 value =0.99). The procedural blanks were also included simultaneously by adopting the same sample preparation method as for the exposed fish sample. Response factor and amount of antibiotic in real samples were done by using the following formula:

$$RF\ (Response\ factor)\ = Peak\ area\ of\ standard/Amount\ of\ standard$$

$$Amount\ of\ analyte\ in\ real\ sample = (Peak\ area\ of\ sample)/(Response\ Factor)$$

3.3.7. Nano titanium analysis in fish muscles

Quantification of nano titanium in muscle tissues was performed by acid digestion method. In a digestion flask, concentrated nitric acid (10 ml) and perchloric acid (2 ml) were combined with freeze-dried muscle samples (1 g; n=6). The mixture was then heated in a fume hood on a hot plate at 100°C until the yellow tint faded, and then a few drops of H_2O_2 were added. Each digested sample's volume was evaporated down to 2 ml. The liquid was cooled, reconstituted with 50 mL of distilled water, and then filtered using filter paper (pore size 0.45 µm). Inductively coupled plasma - optical emission spectrometry was used to evaluate these samples (ICP-OES iCAP 6500, Thermo Scientific, UK) (Ashraf et al., 2021).

Statistical analysis

The results of the current investigation were presented as mean and standard deviation (SD). The results were assessed through one-way (ANOVA) using the Tukey's test (post-hoc) to see whether there were any significant differences between the groups. The statistical analysis was performed using Origin software 8 (by Origin Lab Corporation, United States), with a significance level of $p<0.05$.

3.4. Phase 3: To identify (*in silico*) promising phytochemical as quorum quenching agent in controlling virulence of vibriosis in fish

3.4.1. Preparation of ligands library

The chemical structures of 2000 bioactive phytochemicals in SDF format were downloaded from MPD3 (medicinal plants database) and their energy minimization and 3D protonation were done using MoE software (v. 2015) to prepare ready to dock library in MDB format.

3.4.2. Protein structure preparation

Fasta sequence was retrieved from Uniprot database and tertiary structure of LuxR protein was prepared by swiss model, intFold, i-tasser and phyre-2 (Table3.2). All resultant structures were further validated through SAVES analysis (v.2016) (https://saves.mbi.ucla.edu/).

Table 3. 2 Modelling approaches used for protein structure preparation

Protein structure preparation	
Homology Modelling	Swiss Model
Homology Modelling	i-tasser
Ab-initio Modelling	Phyre-2
Threading	Int-FOLD

3.4.3. Analysis of protein active binding sites and molecular docking

The active sites in the target LuxR protein were found using a Dogsite scorer online tool (https://proteins.plus/help/dogsite), and docking analysis was done employing Molecular Operating Environment (MOE) software (v. 2015). Protonation and ionic effects were addressed when the target protein structure was uploaded to the MOE program. The software was used to upload the ligand library and estimate the energy of phytochemical interactions with the target protein LuxR using the software's default parameters. For each iteration, 10 conformations were produced, and the minimum optimal conformational binding was chosen.

3.4.4. Evaluation of drug like properties

The concept of drug likeness emerged from the identification of characteristics of compounds that are more likely to exhibit satisfactory ADMET qualities. Molecular properties including pharmacokinetics and drug likeliness attributes obeying Lipinski rule of five were predicted utilizing online tools such as molsoft, swissADME, and ADMETsar (Table 3.3).These characteristics serve as a filter, screening out additional leads.

Table 3. 3: Parameters and range values of Lipinski rule of five

Rule	**Parameters**	**Range**
Lipinski rule of five	Molecular Weight(MW)	<500 Da
	H-bond Donors	< 5
	H- Bond Acceptor	<10
	Lipophilicity of ClogP	<5

3.4.5. Molecular Dynamics Simulation

A molecular dynamics simulation was run on the best protein ligand complex geometry generated by the molecular docking studies.MD simulations were performed using Groningen Machine for Chemical Simulations (GROMACS v. 5.1.0), following GROMACS tutorial-5 (Protein-ligand Complex) while using the parameter files provided within the tutorial, with the CHARMM36 force field for all simulations. The force field parameters for the ligands were acquired using ParamChem with Chemistry at Harvard Macromolecular Mechanics (CHARMM) general Force Field (CGenFF). The protein topology file was created using the pdb2gmx. Periodic boundary conditions were used. Newton's equations of motion were incorporated by means of leap-frog algorithm. The temperature was sustained at 310K (kelvin). Using a semi-isotropic Parrinello-Rahman barostat, the system box was allowed to vary under 1atm (atmospheric pressure). Using the Berendsen weak coupling technique, all systems, including NVT (constant number, volume, and temperature) and NPT (constant number, volume, and pressure), were optimized and then equilibrated for a total of 10 ns. The GROMACS tutorial-5 (Protein-ligand Complex) was the basis for the use of all of these parameters, making them broadly applicable to all MD simulations. The GUI used to manipulate input files were PyMol v2.0 and Avogadro v1.2.0, similarly in order to visualize the output files VMD v1.9.3 was used. The simulations were carried out on supercomputing facility of research and education centre , ScREC, SINES, NUST.

Chapter 4

RESULTS AND DISCUSSION

In this chapter, results and discussion of experimental work is presented, followed in three main phases as discussed in Chapter 3.

4.1. Phase 1a: Toxicity assessment of SMX on cultivable fish *Cyprinus carpio* using multiple endpoints.

4.1.1. 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 experiment and compared with physicochemical values of Rawal lake hatchery water from where fish samples were procured.

Table 4. 1: Physicochemical parameters of the experimental tanks and lake water

Parameters **Mean values (minimum- maximum)**				
	Temperature (^oC)	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

The physicochemical analysis of water parameters is presented in Table 4.1. The results showed that mean values of temperature, dissolved oxygen, and hardness (28.5 ^{o}C, 6.9 and 230.7 mg/L) increased in lake water in comparison to experimental tanks (23.4 ^{o}C, 5.5 and 220 mg/L). The probable reason for significant increase in temperature and hardness may be due to the entry of pollutant load from the nearby areas as reported by Malik and Nadeem in 2011.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 .They also reported that the quality of Rawal lake water was 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 from nearby settlements. Ayaz et al. (2016) conducted a study to evaluate water quality of Rawal lake and quantified that most of the physicochemical parameters including total hardness and temperature exceeded the permissible limits prescribed by WHO.

4.1.2. Effects of SMX on hematological profile

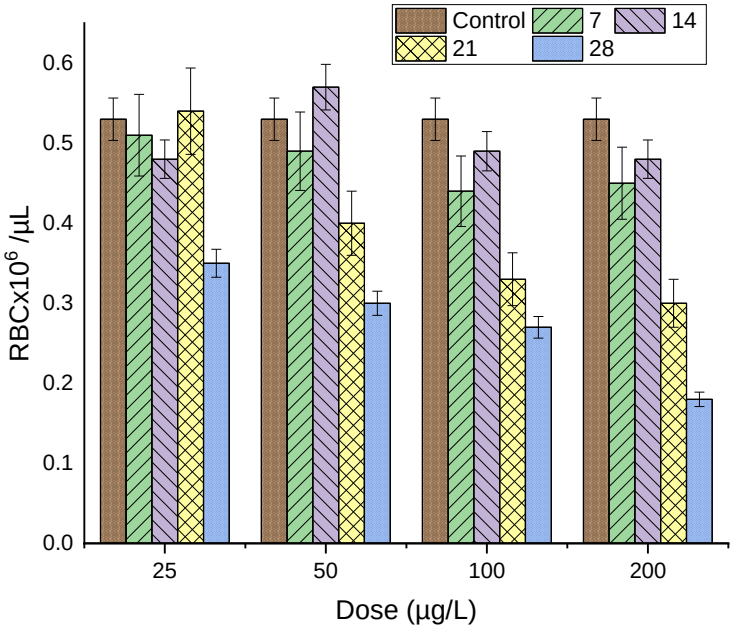


Figure 4. 1 Red blood cells (RBCs) level of control and SMX exposed fish for 28 days (values are expressed as mean ± SE, n = 8)

Alterations in hematological count have been extensively used as powerful tool for the determination of health and physiological status of fish (Gabriel et al., 2011). This allows fast and rapid evaluation of sub-acute toxicity of xenobiotics on target organs. It results in assessing the pathophysiological status of fish and parameters help in diagnosing structural and functional changes in fish due to chemical exposure. Figures 4.1, 4.2, 4.3 and 4.4 shows variation in RBCs, Hb, WBCs and PLT count after SMX exposure. The levels of RBCs, Hb and PLT count were decreased with time in all the exposure groups whereas number of WBCs were found to be increased. The effect of SMX on RBCs of the fish is illustrated in Figure 4.1. RBCs count of (0.53,0.52,0.51,0.53 $x10^6$ /µL) was observed in control group for 7^{th},14^{th},21^{st} and 28^{th} days respectively. Initially at 7^{th} day the RBCs count did not change significantly (one-way ANOVA followed by post hoc Tukey's test) for all the exposure groups but as time passed there was significant ($p < 0.05$) decrease in the RBCs count on the 14^{th}, 21^{st} and 28^{th} day, where the values were found to be ranging from 0.54-0.78 $x10^6$ /µL. The maximum decline (0.18 $x10^6$ /µL) was detected for 200 µg/L group at the end of the 28^{th} day. RBCs are a very important component of the blood and helps 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. RBCs' membranes can be penetrated by SMX because of its lipophilic qualities, which also make RBCs brittle and susceptible to damage causing anemic conditions in fish. A low red blood cell count, or anemia, can have significant effects on fish histopathology and immunity. Anemia can lead to tissue hypoxia, where tissues and organs receive insufficient oxygen. In fish, this can result in histopathological changes, including cellular damage and tissue degeneration. Organs like the liver and gills may show signs of hypoxic injury as manifested by histopathological results of current study. Additionally, anemia-induced stress on tissues can lead to inflammation and alterations in cellular structure. Red blood cells play a vital role in supporting the immune system of fish. With reduced oxygen-carrying capacity, immune cells may struggle to reach infection sites, hampering the fish's ability to fight off pathogens effectively. As a result, anemic fish may become more susceptible to infections, parasitic infestations, and diseases. Their overall immune response may also be weakened, prolonging recovery from illnesses. In summary, anemia in fish can have a cascading effect on their health, affecting both histopathology and

immunity. Proper identification, diagnosis, and treatment of anemia are essential to ensure the well-being and disease resistance of fish populations. Additionally, the membrane of RBCs may be impacted by the ion and gas exchange during high energy demands. The fish in the current study may have reacted strongly to the toxicity of SMX as manifested through changes in the quantity of RBCs (Cicha et al.,2013)

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

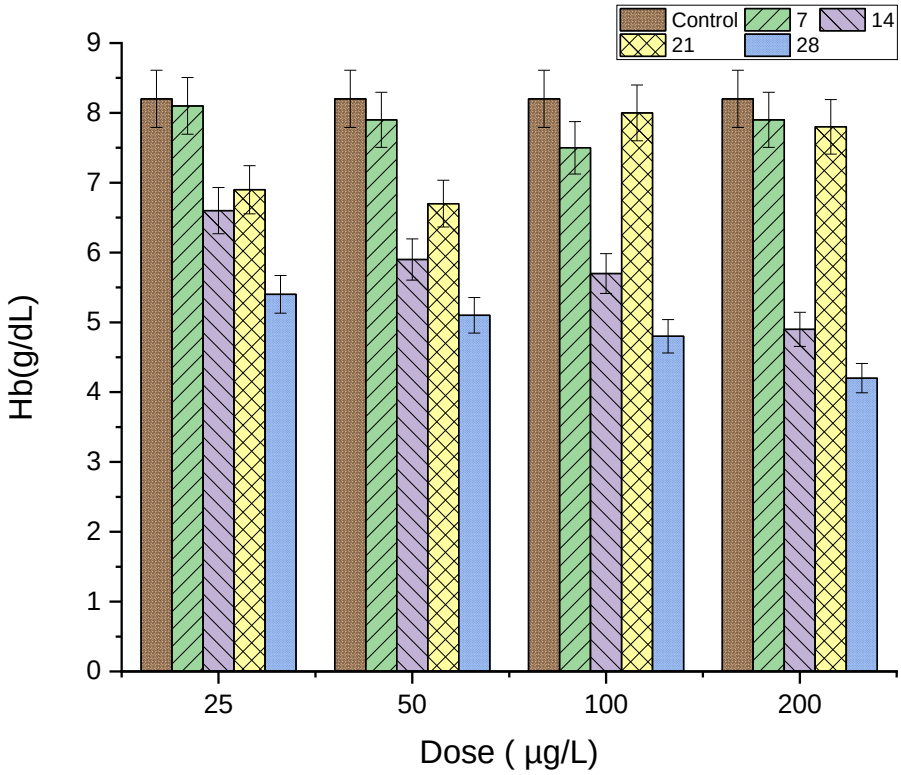


Figure 4. 2 Hemoglobin (Hb) level of control and SMX exposed fish for 28 days (values are expressed as mean ± SE, n = 8)

WBCs play a major role in providing defense to the body and any alteration in WBCs count may indicate an infection in the organism. These infections may be caused by stress or damage to tissues. The effect of SMX on WBCs of the fish is illustrated in Figure 4.3. As compared to control group, the exposed fish shows sharp increase in WBCs count. WBCs count started to increase with passage of time and continued till 28th day with values ranging from 131-303 (x 10^3 /µL) for all the exposure groups. Maximum increase was seen for the highest concentration exposure group (200µg/L) on the 28th day with a value of 303 x 10^3 /µL. Primarily, mononuclear phagocytes and white blood cells carry out the process of phagocytosis in fish by producing huge amounts of superoxide anion (O^{-2}) following stimulation with a variety of xenobiotic substances. 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 into the accumulation of H_2O_2. That ultimately increases number of white blood cells in fish body. Due to the likely rise in H_2O_2 levels caused by SMX, this immunostimulatory effect may have occurred (Saglam *et al.*,2009). According to Lunden and Bylund (2002) study, the number of WBCs correlates with the body's immunological response. As a result, whenever the body is under stress from an outside source, the spleen creates new WBCs. Increased WBC counts are a sign that the body is producing antibodies. The immune system is essential for maintaining healthy biological functions and providing defence against illnesses.

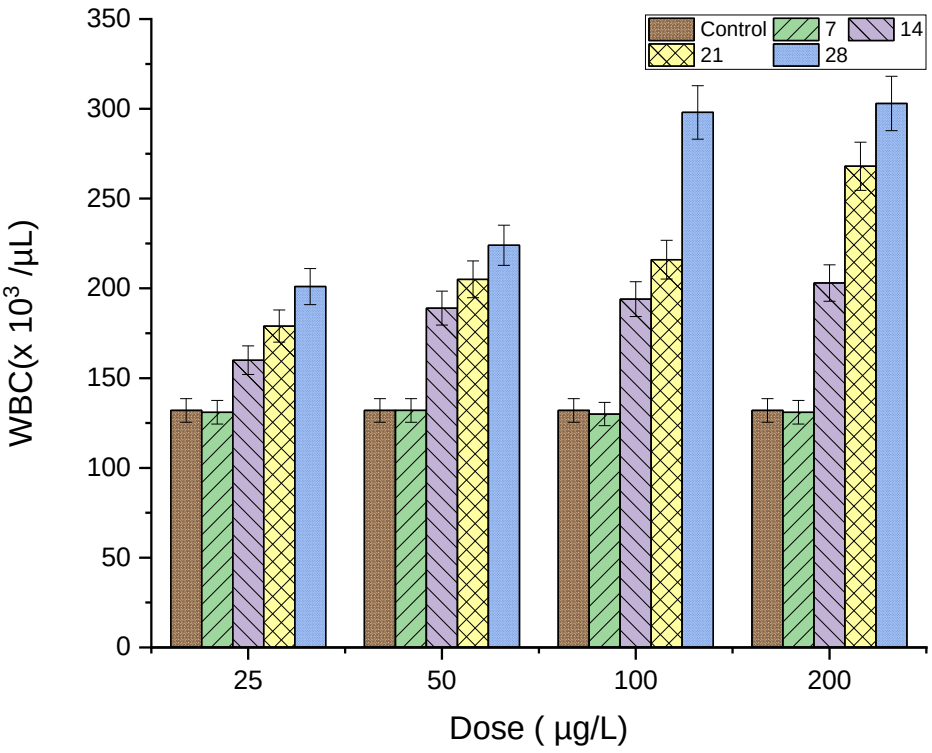


Figure 4. 3 White blood cells (WBCs) level of control and SMX exposed fish for 28 days (values are expressed as mean ± SE, n = 8)

Figure 4.4 shows number of PLT count of control as 330, 331.2, 329.5,330.75 x(10^3/µL) for 7, 14, 21 and 28 days respectively. When compared to control, the exposed group shows

gradual decrease in PLT count with values ranging from 330-150 x(10^3/µL) indicating onset of thrombocytopenia; a type of disorder in which there is a lower number of PLT. Drug-induced thrombocytopenia occurs when certain drugs rescind PLT or inhibit the body's ability to make enough of them. Some drugs result in the production of antibodies in the body, which destroy PLT, and process is known as drug-induced immune thrombocytopenia (Bemt et al.,2004). Sulfonamides have been proven for the cause of thrombocytopenia (Warkentin et al.,2018). Numerous infective mechanisms have been correlated with drug induced thrombocytopenia. Out 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 antibiotic and specifically target two genes; GPIIb/IIIa or GPIb/IX leading to the drastic decrease in number of PLT count in the blood of target organism (Bakchoul et al., 2018).

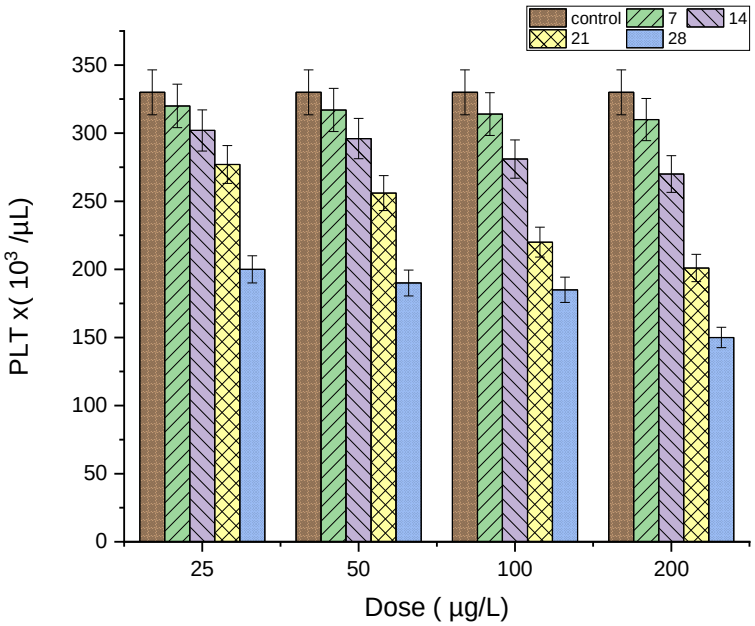


Figure 4. 4 Platelets count of control and SMX exposed fish for 28 days (values are expressed as mean ± SE, n = 8)

4.1.3. Biochemical parameters

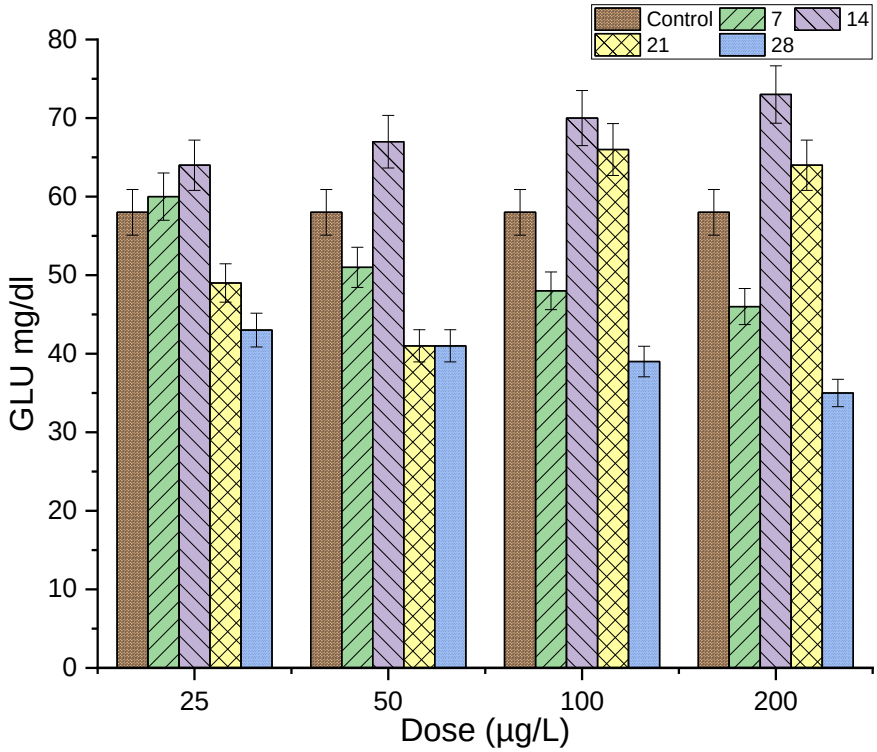


Figure 4. 5 Glucose level of control and SMX exposed fish for 28 day (values are expressed as mean ± SE, n = 8)

In a regular examination, biochemical parameters are assessed to evaluate an organism's physiological systems. Glucose and total protein level are important parameters used to determine the normal bodily function in toxicology studies (Vutukuru, 2003). The effect of SMX on glucose levels of the fish is illustrated in Figure 4.5. When compared with control (57.5,58.0,59.0,58.5 mg/dL) values for exposed group shows 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 73-35 mg/dL with a lowest value recorded on 28th day for 200 µg/L exposure group. The breakdown of glucose in the body is a major process for energy production, and the 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, caused by antibiotics, leading to a natural stress response. This response results in the secretion of hormones such as corticosteroids, epinephrine, and dopamine, primarily to reactivate glycogenesis and overcome the high energy demand. Fluctuations in glucose levels due to the exposure of fish to antibiotic may be due to the influence on carbohydrate metabolism which may cause a rise or fall in the cortisol production which is a primary stress response. In the current study, an initial increase in glucose level was observed. This was then followed by a decrease which may be due to the high metabolic demand caused by SMX. Similar biphasic trend was also observed by for Rohu (*Labeo rohita*) exposed to 80 mg/ L of oxytetracycline where serum biochemical profile showed increasing values for first ten days and then subsequent decrease was observed at the completion of exposure (day 25) (Ambili et al., 2013)

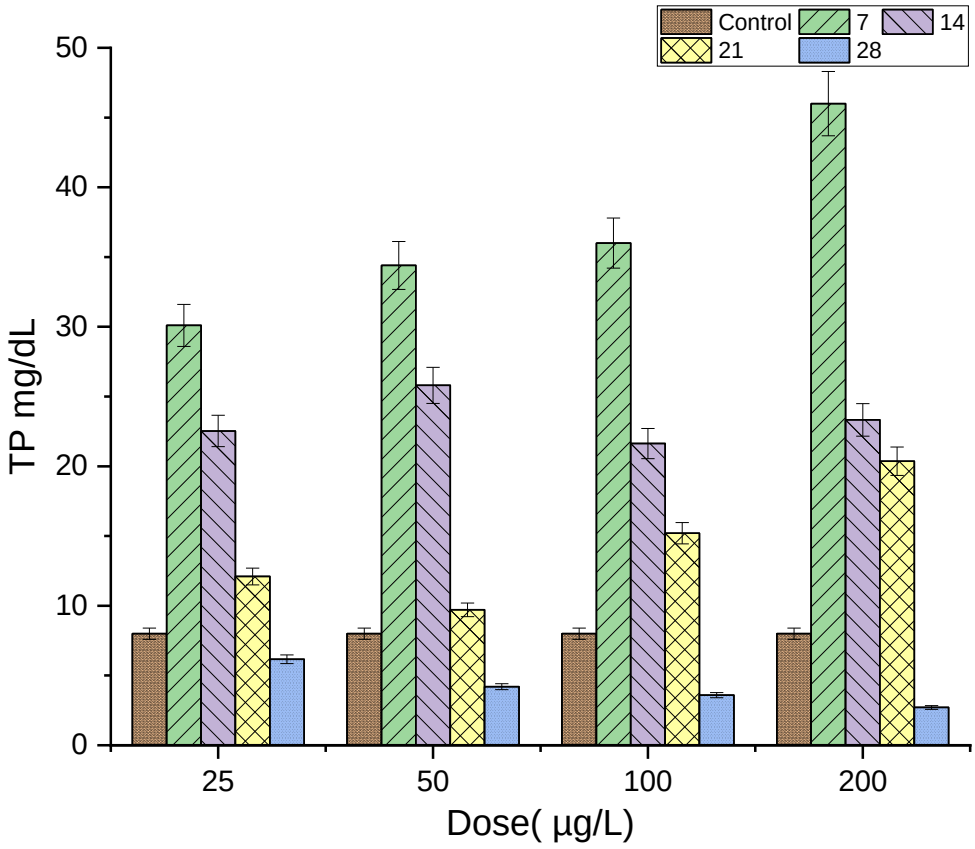


Figure 4. 6 Total protein level of control and SMX exposed fish for 28 days (values are expressed as mean ± SE, n = 8)

A fluctuating trend was also observed in the protein levels as indicated in Figure 4.6. An overall decrease ranging from 20-34 mg/dL was observed in the protein level in comparison 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 7th day, maximum increase (46 mg/dL) was observed on the 7th day of exposure for 200 µg/L, afterward it started to decrease till last 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).

A decrease in protein levels can indicate that protein production has been inhibited, while an increase in protein levels could indicate that fish have become acclimated to the pollutant. In current study the fish exposed to SMX shows 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 200mg/kg SMTZ showed a decrease in protein content 29 mg/dL on day 7 and 28 mg/dL on day 21which 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 characteristics of fish with decrease level of glucose and protein under high toxicity effect. According to Ejraei et al. (2015), fish hematological and blood plasma indices saw several changes due to ageing and hormonal therapy.

TG also showed biphasic fluctuations with maximum level at 0.25, 0.26, 0.28, 0.29mg/dl for 25, 50, 100 and 200 µg/L groups respectively on day 14 as shown in Figure 4.7. Whereas it decreased after the 21st day as compared to control group.

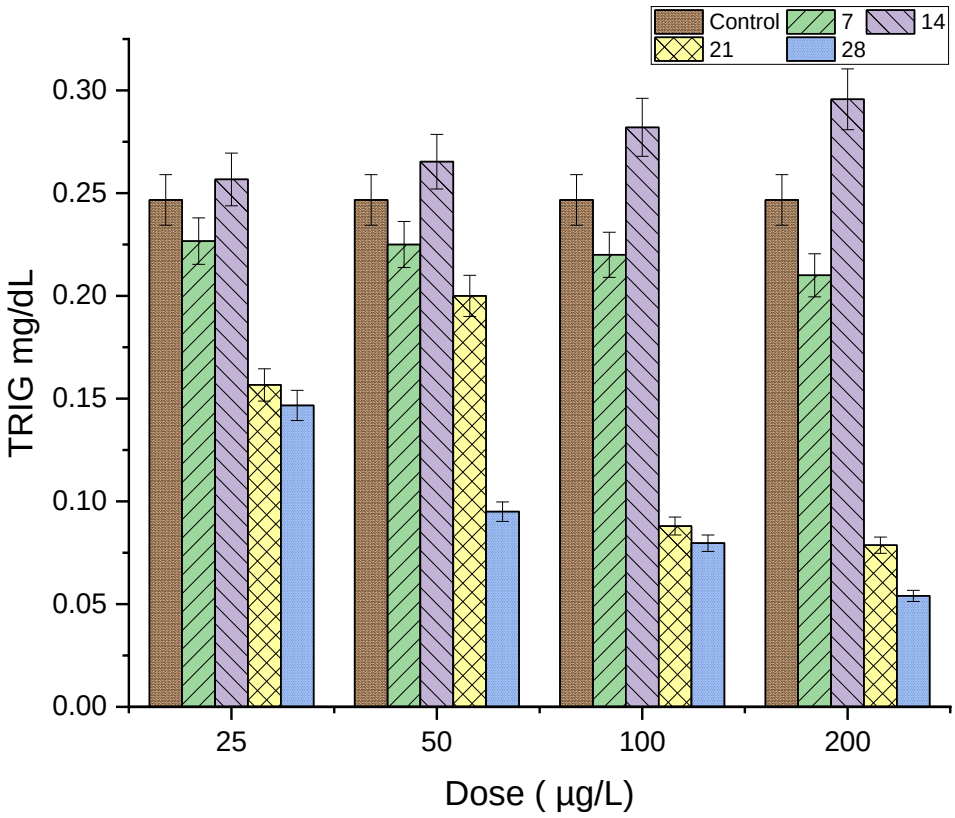


Figure 4. 7 Triglyceride level of control and SMX exposed fish for 28 days (values are expressed as mean ± SE, n = 8)

When under stress, TG is a crucial source of energy; a rise in TG levels may be caused by lipid mobilization to meet the increased energy requirement (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 promotes increase in their level (Gaber et al., 2013) Nonpolar lipophilic nature of SMX can degenerate lipid containing cell membrane leading to elevated level of TG in fish body. Decreased triglyceride at completion of exposure may be due to low feed intake or less absorption due to poor gut and liver functioning and initiation of membrane biogenesis (Van et al., 2008). The ALT level significantly ($p <$

0.05) increased with increasing dose concentration and time of exposure as seen in Figure 4.8. The ALT is principally present in the hepatocyte of the body and therefore their increase level reflects liver damage (Mikulikova et al., 2013). Rising ALT serum levels may be related to stress conditions in fish toxicological studies. The amount of the ALT may change because of structural changes in the cell organelles. A higher amount of transaminase activity could be caused by improper protein- and carbohydrate-metabolizing processes when antibiotics are present in the environment (Akrami et al., 2013). Sampaio et al. (2016) specified that SMX affects the liver and activates the enzymatic activity of liver. Elevated level of ALT is the symptom of hepatomegaly, which is triggered due to SMX toxicity

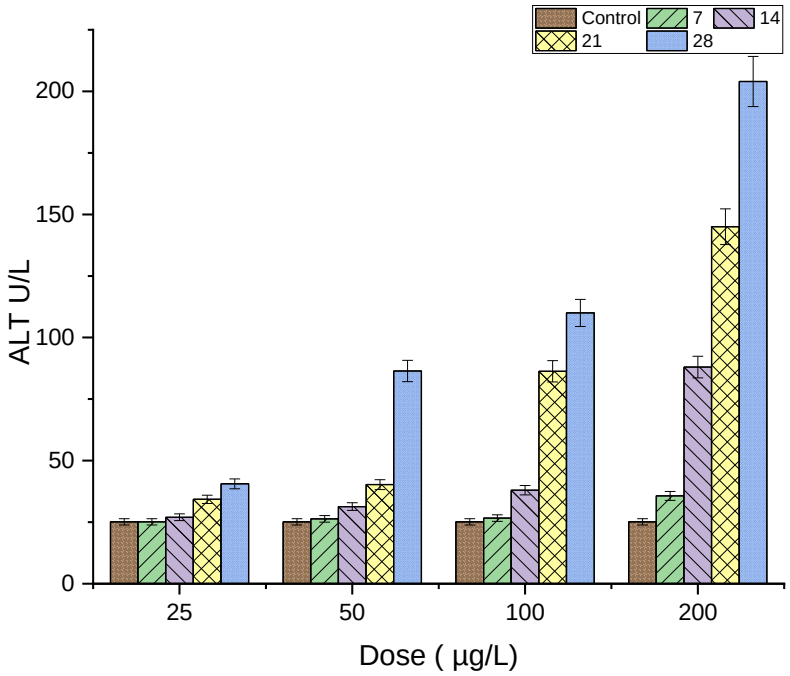


Figure 4. 8 Alanine transaminase level of control and SMX exposed fish for 28 days (values are expressed as mean ± SE, n = 8

4.1.4. Respiratory burst activity (NBT)

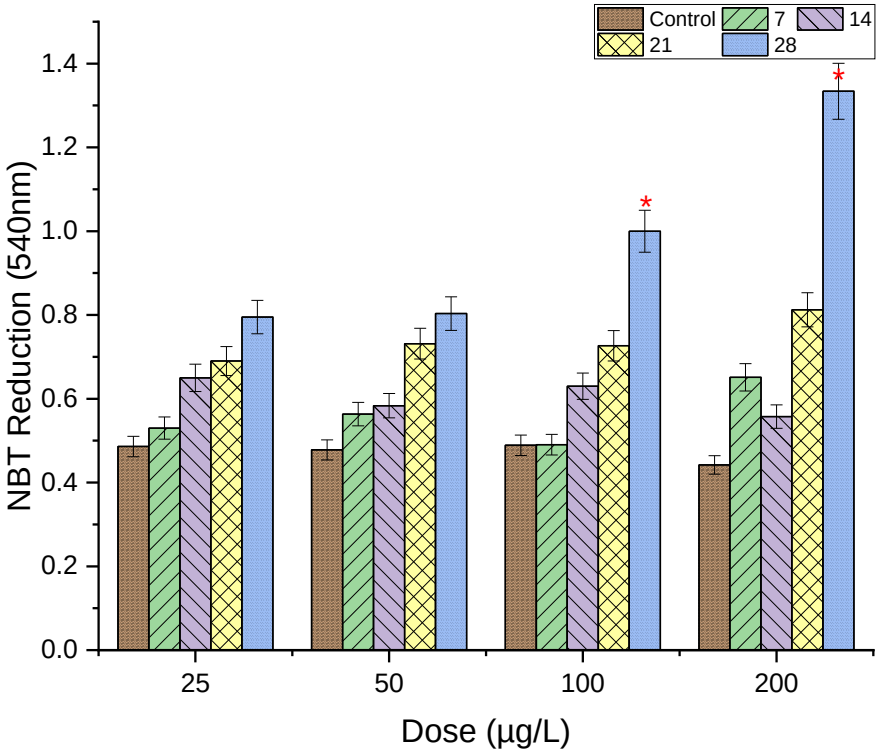


Figure 4. 9 Respiratory burst activity in control and SMX exposed fish for 28 days (values are expressed as mean ± SE, n = 8. Asterisks indicate values that are significantly different from the control values ($p < 0.05$).

The effect of SMX on respiratory burst activity of the fish is illustrated in Figure 4.9. Results show substantial increase in respiratory burst activity with increasing time of experiment, highest values of 0.79 ,0.80,1.0,1.33 were recorded for 25, 50, 100 and 200µg/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 reactive oxygen species (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 organ. Akrami et al. (2013) 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 (0.1mg/kg and 0.5mg/kg) for the exposure period of 30days.

4.1.5. Bioaccumulation and bioconcentration of SMX in fish muscles

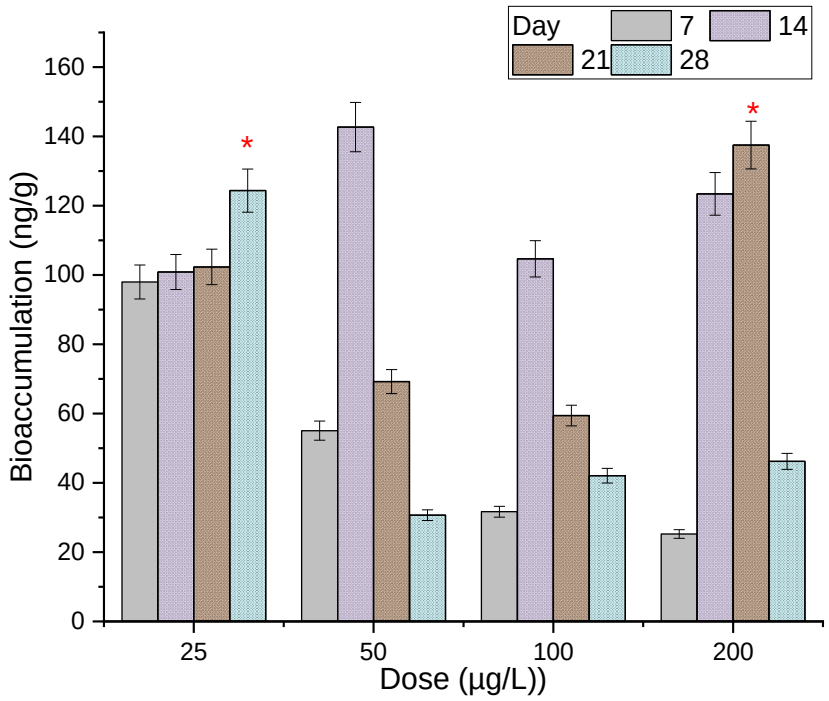


Figure 4. 10 Concentrations of SMX in muscles of common carp subjected to SMX (25-200 µg/ L) dosages measured at 7, 14, 21, and 28 days, respectively (n = 9).

In control fish, SMX peaks were not detected in examined muscle tissues. The quantified concentrations of SMX in fish muscle tissues during different exposure periods (7, 14, 21, and 28 days) are presented in Figure 4.10. Antibiotics revealed no distinct dosage-related accumulation in the muscle tissues. The SMX concentrations in the muscle tissues were higher at 25µg/L than other dosage groups after the 7th day of exposure. SMX concentration increased gradually with respect to time and reached the maximum concentration of

142.69, 104.68, and 123.41 ng/g at day 14 of exposure for the 50 and 100 and 200µg/L concentration groups. After reaching the maximum level at day 14 bioaccumulation of SMX started to decrease in muscles with the calculated values of 30.668, 42.06, and 46.18 ng/g for 50, 100 and 200 µg/L dosage groups respectively at day 28 of the exposure. However, bioaccumulation of SMX was gradually increasing for 25 µg/L with the maximum measured concentration of 104.141 ng/g on the final day of exposure. Furthermore, depending on the different SMX dosages applied to the water, bioconcentration factor values ranged from 0.13 to 4.52 in muscles for 28 days exposure. However, BCF values were considerably below the USEPA regulatory standards (USEPA, 2003).

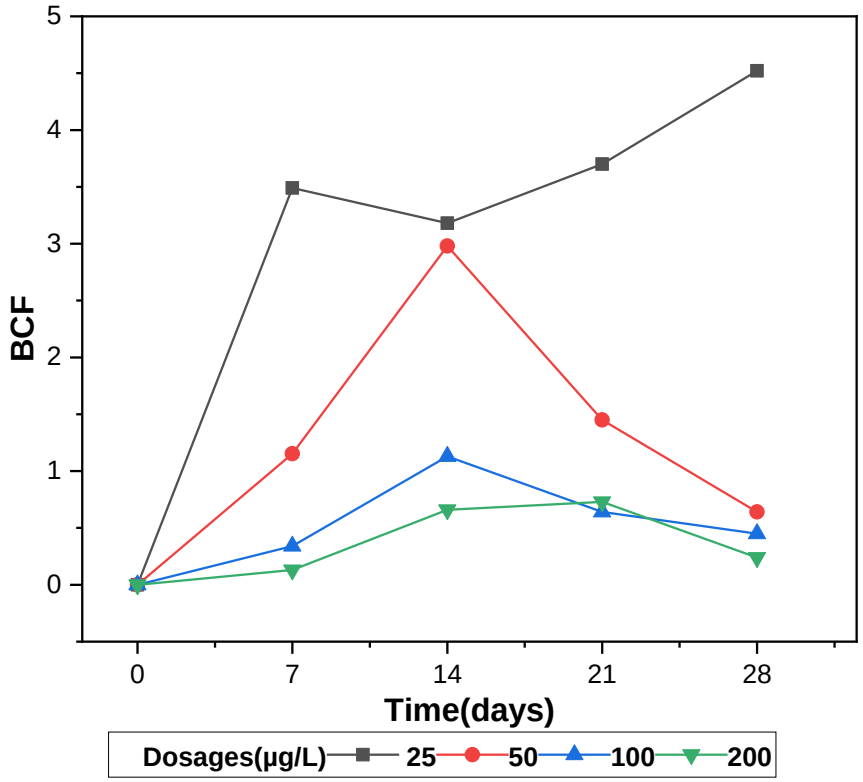


Figure 4. 11 Bioconcentration factor (BCF) of SMX for all exposure groups on days 7,14, 21, and 28.

Maintaining a healthy aquatic environment is critical for maintaining fish harvest levels in the fishing industry. Thus, effective water quality monitoring and accurate assessments of the state of aquaculture ecosystems are critical. Chemical analysis is a popular viewpoint in ecotoxicology and environmental risk assessment. However, it is unable to provide a reliable indication of contaminants' deleterious impacts on the biota. Aquatic organisms can easily uptake anthropogenic contaminants, which can cause major changes at the molecular, cellular, physiological, and behavioral levels. As a result, biomarkers, which are defined as quantifiable assessments of alterations at various biological levels, have been proposed as effective and appropriate indicators of pollutant exposure to supplement the information provided by chemical tests. The bioaccumulation and bioconcentration factors of antibiotics in different tissues are often examined to describe their possible impacts on exposed organisms. Knowledge of bioaccumulation allows one to assess the risk associated with the presence of various chemicals in the environment, food, and ecosystem as well as to present quantitatively the ability to control the use and emissions of chemicals. In current study, the highest concentrations were detected in the lower exposure group (25 µg/L), followed by the other exposure groups in descending order. The drop-off in the SMX concentrations in muscle tissues after 14 days of exposure might be the result of slower uptake due to fast metabolism and excretion of antibiotics out of the fish body. Furthermore, the existing phenomena are further supported by the discovery of increased oxidative stress in the brains of the exposed fish. Lower bioaccumulation of compound in higher dosage groups (50, 100, and 200 µg/L) as compared to 25 µg/L group is the result of nearly complete saturation of the muscle tissues by the antibiotic in the higher-concentration groups, consequently activating metabolism in the body and excretion of antibiotic out of the body (Schwaiger et al., 2004). These results were consistent with the former studies that confirmed an increasing trend in bioaccumulation of drugs from specific therapeutic groups in fish muscle tissues concerning exposure time (Togunde et al., 2012). This process of complete saturation happened faster in higher exposure concentrations of SMX. The same trend of bioaccumulation of carbamazepine has been previously reported in which the concentration still increased until the end of the exposure period of 7 days at the lowest concentration, but at higher exposure concentrations the equilibrium was attained on the first day of exposure experiment (Contardo-Jara et al.,

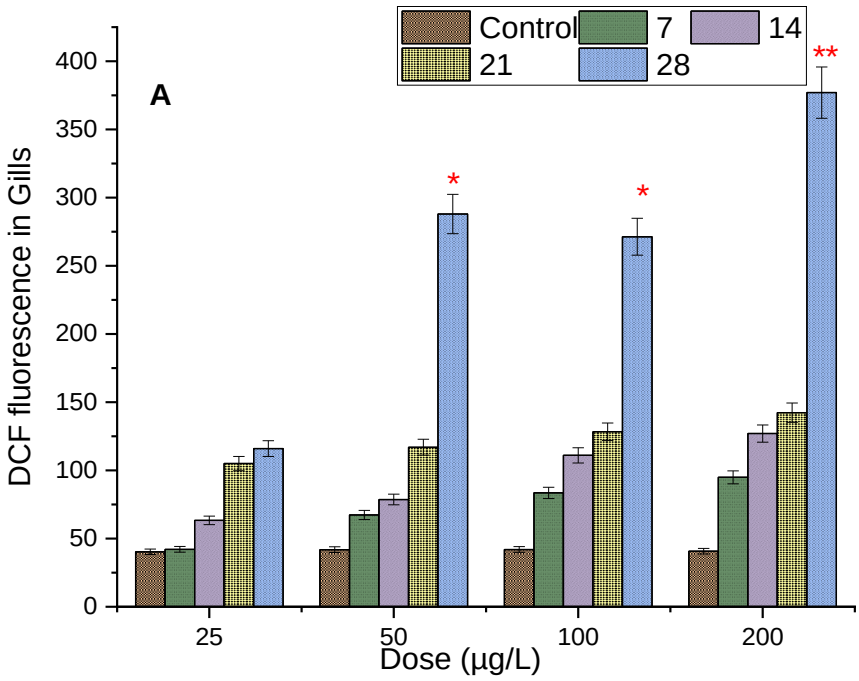
2011). It was also apparent that the SMX concentrations in the muscle tissues increased quickly at the early period of exposure for all the dosage groups and acquired a maximum level on day 14 for 50,100 and 200 µg/L and then decreased along exposure time except for the lowest dosage (25 µg/L) group. It can be attributed to the fact that a certain threshold level for pollutant concentration and exposure time exists; and above that level, the fish responsive biomarker signals are activated as compared to the normal range in the stress-free environment (Van der Oost et al., 2003). Fig. 4.11 shows that the measured bioconcentration factor (BCF) values, recorded at all sampling times, were inversely proportional to the exposure concentrations in the fish. This is due to the tissue's self-elimination function, which was almost completely saturated with SMX within the applied dosage range. So, our results suggest that SMX residues in water bodies at lower levels may possess higher bioconcentration potential for aquatic biota, which will attract more attention for assessing the risk caused by SMX on ecological security.

According to the model developed by Meylan et al. (1991) ($r2 = 0.73$), where a and b are empirically calculated constants and using log Kow of 0.89 for SMX, the theoretically estimated bioconcentration factor of SMX is approximately 3.162. that is very much closer to the calculated values in the current study confirming the fair approximation of the BCF for our targeted antibiotic in laboratory conditions. A significant increase ($p<0.05$) in the reactive oxygen species during the 28 days of exposure indicated that SMX has induced oxidative stress in the common carps. For the lower concentration group (25 µg/L), the continuous bioconcentration of SMX may cause a significant decrease in superoxide dismutase activity and increased ROS over 28 days of exposure as observed in our study. Studies have shown that SMX might lower the basal inflammation caused by bacteria and, subsequently, the standard levels of antioxidant enzymes in the body. After 14 days of exposure, with the biotransformation reinforcement, the concentration level of SMX was decreased, and a large amount of metabolic and reactive oxygen species were produced. When exposed to 100 and 200 µg/L of SMX, the BCF values in muscles were only 0.45 and 0.24 at the end of the exposure period, suggesting that the metabolic and reactive oxygen species were continuously generated in fish subjected to higher concentrations during all exposure periods. Whilst the process of detoxification occurs mainly in the liver,

muscle tissues are also involved in these processes. Muscle cells have been demonstrated to secrete different kinds of xenobiotic-metabolizing enzymes, including Glutathione S-transferases and cytochrome P450 (Hussey et al., 1991). Even though the relative number of enzymes in muscle tissues is lower than in the liver, its significance in the detoxification processes should be considered since it denotes a great body mass percentage. Thus, our findings suggest that the stimulation of antioxidant mechanisms in muscle was effective against oxidative damage.

4.1.6. Oxidative stress

Reactive oxygen species (ROS) concentration in the brain and gills of common carp at different exposure concentrations and time points is presented in Figure 4.12.ROS was significantly increased (p<0.05) by higher concentrations of SMX (≥ 50 µg/L) after 14 days of exposure in gill tissues (Fig. 3A), Similarly, statistically significant ROS values were observed for the 200 µg/L dosage group after 21 days of exposure (p<0.05) for brain tissues (Fig. 3B), and the increasing rate of ROS production matches the dosage concentrations applied in the studies.



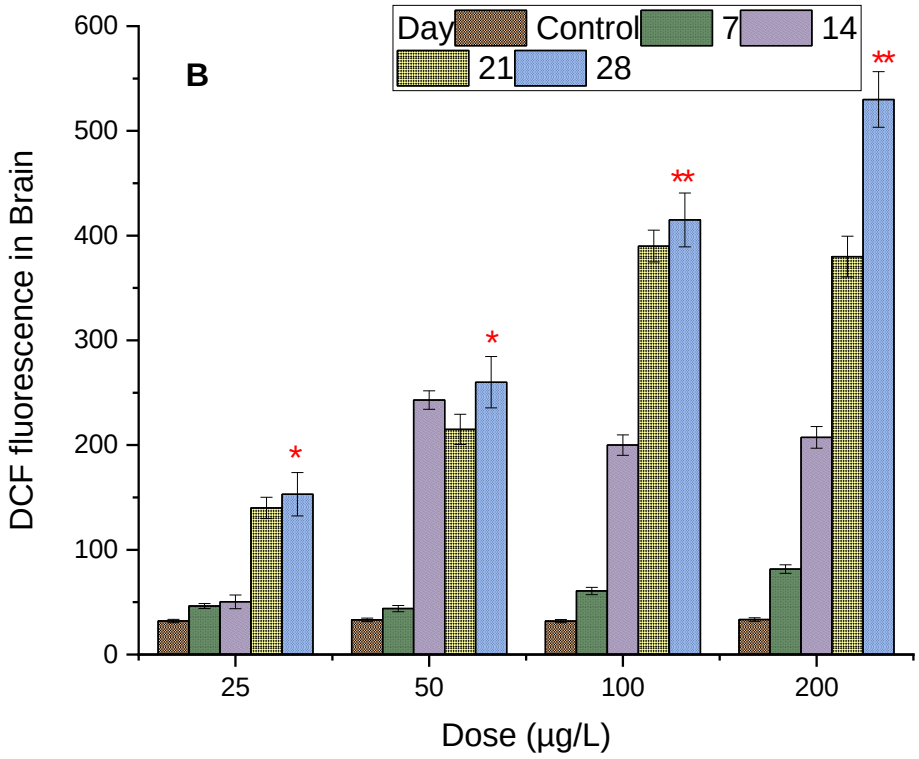


Figure 4. 12 Oxidative stress in gills (A) and brain (B)of control and SMX (25, 50, 100 200 µg/L) exposed common carp for 7, 14, 21, and 28 days (n=9)

Biomarkers of oxidative stress, representing a distinctive subclass of effects biomarkers, have been inconsistently employed in aquatic studies. It is part of aging process and happens in all living organisms when reactive O_2 species or their byproducts cause cell and tissue injury. As response of the living organism can be xenobiotic or tissue-specific, it is important to investigate various endpoints associated to oxidative stress in different tissues.

Oxidative stress results in the production of reactive oxygen species - oxygen-centered electrophilic compounds capable of oxidising other molecules, most commonly cellular lipids. Exposure to SMX for a long time may trigger cellular metabolism leading to the production of ROS in fish bodies. The generation of ROS is reported to be linked with the final pathway for the toxicity caused by several chemical toxicants (Saies &Jones, 2020).

It is a rapid and low-cost approach to measure effects of water contamination in vital organs of affected fish (Zhang et al., 2008). Oxidative stress refers to an imbalance between the production and removal of ROS within the body. The growth of ROS can be sparked by changes in the environment, and larger levels of membrane lipid peroxidation can destroy the antioxidant system's ability to protect cells from oxidative damage, which can harm internal organs. Fish brain and gill tissues exposed to SMX produced ROS; the amount of ROS increased dose- and time-dependently. The highest value of DCF fluorescence in gill tissues was observed to be 377 AU at a concentration of 200 μg/L of SMX after 28 days of exposure. The highest value of DCF fluorescence of brain tissue was 530 AU at the highest concentration after maximum exposure time (Fig.4.12). The brain is primarily subjected to oxidative stress caused by ROS production because it contains a high level of polyunsaturated lipids The metabolism of sulfonamides, such as SMX, by the cytochrome P450 isozyme, CYP 2C9, produces the reactive metabolite (SMX-HA), which is then spontaneously auto oxidized to form the highly lethal metabolite (SMX-NO). Indeed, cells exposed to these electrophilic metabolites experience considerable GSH depletion, resulting in cellular oxidative stress. SMX-HA can generate ROS during auto-oxidation by reducing a molecule of oxygen to form the reactive O2-•, which can then be spontaneously dismutated by SOD to generate H_2O_2. The latter, in turn, partially reduced to the highly cytotoxic OH-• via the Fenton reaction. As a result, SMX metabolism may result in oxidative stress (Hu et al., 2021). In the present study, ROS generation in the fish brain and gill homogenates was measured using probe 2,7-dichlorofluorescein (DCF), which is the fluorescent product of dichloro-dihydro fluorescin (DCFH). DCF, proven to be a useful marker of ROS, is used to measure the toxic effects of various neurotoxic chemicals. Rather than measuring various products of oxidative stress, this method measures the direct generation of ROS. When the perilous equilibrium of the body is disturbed due to depletion of antioxidants or surplus generation of ROS, oxidative stress occurs. An increase in ROS production is a common toxicologic impact triggered by many environmental pollutants (Yan et al., 2016). In the conditions of this experiment, there was a linear relationship between ROS generation, exposure time, and SMX concentrations. Previous studies by Xing et al., 2012 support these findings, where SMX and ciprofloxacin significantly induced oxidative stress in microalgae and fish. Comparison of brain and gills ROS

generation indicates that the brain is more sensitive to an antibiotic residue. Anti-oxidative dysfunction was observed in previous studies, for example, Alak et al. (2017) measured the reduction in antioxidant enzyme activity in the organs of *Oncorhynchus mykiss* exposed to eprinomectin at concentrations ranging from 0.001 to 0.05 μg/L for four days. Another study reported an increase in oxidative stress activity in the liver of Carassius auratus exposed to SMX with concentrations ranging from 3.2 μg/L to 0.4 μg/L for one week (Liu et al., 2014b). The results of the present study revealed that SMX is toxic to fish even at lower concentrations with the increase in exposure time. Our study reveals a new insight that antibiotic exposure, specifically SMX, can induce inflammation in common carp, characterized by high levels of reactive oxygen species at higher dosage levels. The process of inflammation is a dynamic one. When an organism is attacked by a pathogen or injures itself by a pollutant, neutrophils generate a number of cytokines to fight pathogens or external pollutants, as well as increased ROS, despite the surrounding tissues being affected (Campbell et al., 1994). The assessment of antioxidant mechanisms can be achieved by measuring the activity of certain enzyme intermediates, serving as oxidative stress biomarkers to understand the potential oxidative changes induced by xenobiotics, but antioxidant responses may not be enough to alleviate oxidative stress, and ROS can interact with other macromolecules including lipids, cellular proteins, and DNA, resulting in cell death (Van der Oost et al., 2003). Enzymatic oxidation can have negative implications in pathways that control important physiological functions including neurotransmission. Literature findings suggest that pro-oxidative substances can cause cholinesterases in specific organisms to be oxidized (and hence inactivated), demonstrating a relationship between oxidative stress, enzymatic inhibition, and probable behavioral, morphological, and histopathological repercussions, all of which are of undeniable ecological importance.

4.1.7. Histopathology in liver and gills

The photomicrographs of examined organs (liver and gills) in the control and SMX treated fish are displayed in Figure 4.13. Compared to control groups, fish treated with SMX showed some histological changes in targeted organs, with damage severity increasing over time and with antibiotic concentration. The liver of the control fish showed uniform

parenchyma, multidimensional hepatocytes, globular nucleus, and central nucleoli. The liver cells were organized like cords that were adjunct to sinusoid vessels. In some of the inspected regions, hepatocytes showed vacuolization (Fig. 4.13 B).

Many histopathological alterations in the liver of fish were observed after exposure to SMX including pycnotic nuclei, vacuolization, melanomacrophages, bile stagnation, and hypertrophy, some histopathological lesions were considerably higher in *Cyprinus carpio* exposed to higher concentrations (100 and 200 µg/L) of SMX such as pycnotic nuclei, cytoplasmic vacuolization, and blood congestion. Intracellular bile stagnation (yellowish granules) was present in the hepatocytes of fish from the groups exposed to 100 and 200 µg/L of SMX. Organ pathological index (Iorg) showed a greater extent of alterations in the liver of fish exposed to higher concentrations of SMX after 28 days of exposure (Fig 4.6).

Fish exposed to SMX showed significant changes in gill morphology after chronic exposures (Table 4.2). The interlamellar distance was observed to be significantly reduced ($p< 0.002$) in all concentration groups concerning time. An increasing trend in width of secondary lamella was noticed with the value of ($p< 0.02$) whereas no significant changes ($p=0.83$) were observed in basal epithelium thickness. Secondary lamellar length SLL was also significantly increased ($p=0.0002$) in individuals exposed to the highest dosages of SMX applied (100 and 200 µg/L). Moreover, the PAGE percentage (Figure 4.14) showed decreasing trend ranging from 65.18 for the control group and 45.97 for the highest concentration group (200 µg/L).

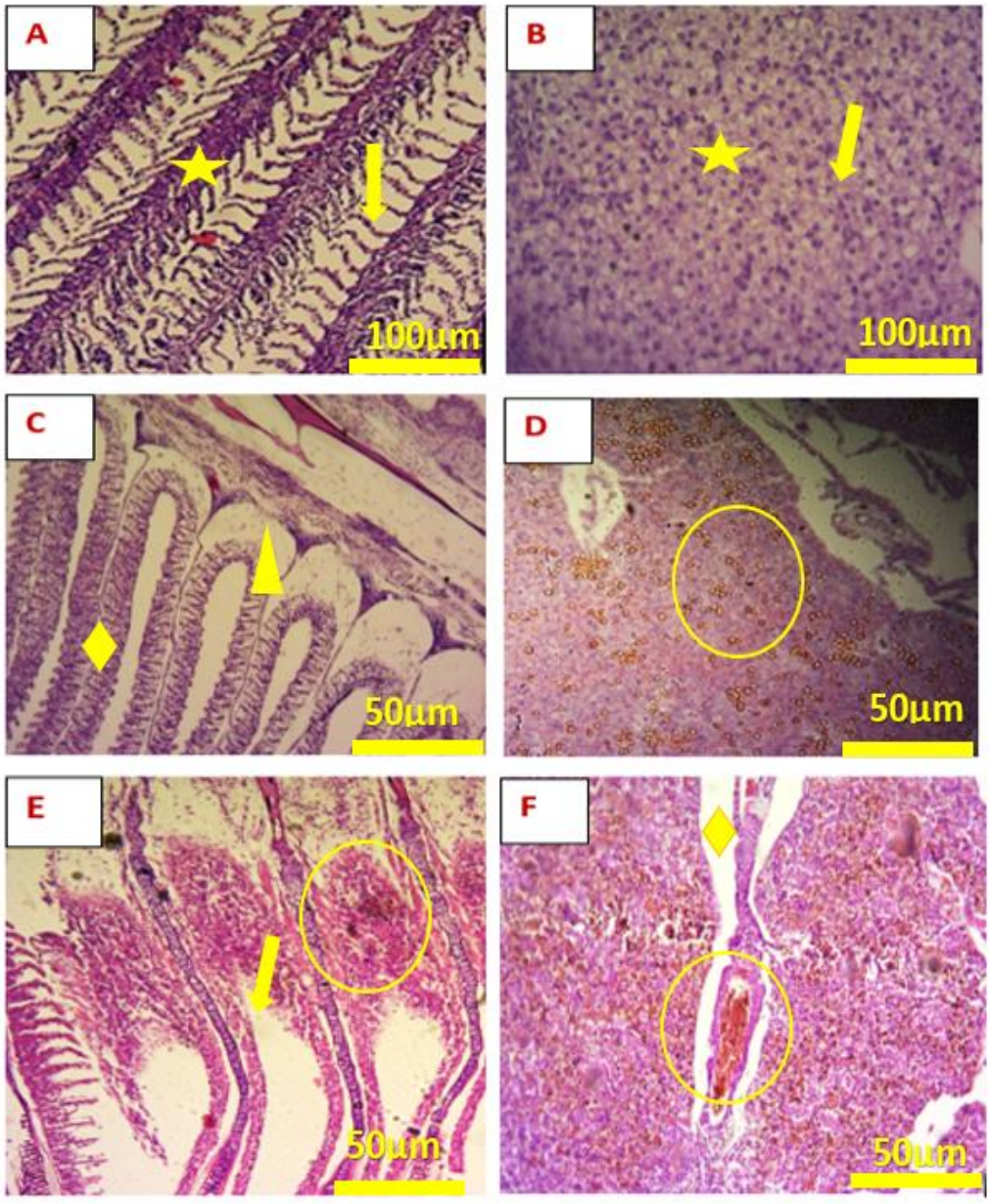


Figure 4. 13 Various histopathological changes observed in gills and liver of *Cyprinus carpio* (stained with H& E ×400). Control gills showing normal filament (star) and intact lamella (arrow); B control liver with normal hepatocytes (star) and blood sinus (arrow); C gills showing epithelial lifting(triangle) and partial fusion of lamella(diamond); E gills showing blood congestion (circle) and complete fusion of lamella (diamond); D liver with melanomacrophages (circle); F liver showing vacuolization (diamond) and blood congestion (circle).

Table 4. 2 Basal epithelium thickness (BET), secondary lamellar length (SLL), secondary lamellar width (SLW), and interlamellar distance (ID), of *Cyprinus carpio* gills, following chronic exposure to SMX. Values represent the mean of values from each treatment ± SE

SMX	**BET (**µm)				**ID (**µm)				**SLL (**µm)				**SLW (**µm)			
Days	**7**	**14**	**21**	**28**	**7**	**14**	**21**	**28**	**7**	**14**	**21**	**28**	**7**	**14**	**21**	**28**
0 µg/L	50.1 ±0.8	50.4 ±0.1	51.3 ±0.4	52.1 ±0.3	23.1 ±0.3	24.6 ±0.7	22.1 ±0.5	23.5 ±0.4	98.6 ±0.9	98.3 ±0.7	96.4 ±0.9	97.4 ±0.2	10.7 ±0.3	10.9 ±0.4	10.5 ±0.3	10.3 ±0.1
25 µg/L	52.0 ±1.1	53.1 ±1.6	56.3 ±0.8	57.0 ±2.1	19.8 ±1.3	17.3 ±0.3	17.1 ±0.2	14.4 ±0.3	94.8 ±1.2	91.2 ±0.6	90.1 ±1.6	88.3 ±0.2	11.6 ±0.2	11.9 ±0.1	12.1 ±0.3	13.3 ±1.6
50 µg/L	52.7 ±0.5	54.0 ±0.6	57.1 ±1.2	58.1 ±0.7	14.5 ±0.7	13.0 ±0.9	12.9 ±1.3	11.9 ±0.3	96.1 ±0.4	90.6 ±1.4	89.2 ±0.7	87.6 ±1.3	12.0 ±0.8	13.3 ±0.3	13.2 ±0.7	14.6 ±0.7
100 µg/L	53.7 ±0.6	55.0 ±1.2	58.7 ±2.1	63.1 ±1.1	13.2 ±0.3	12.1 ±0.6	10.3 ±0.9	8.63 ±2.3	97.3 ±0.2	88.7 ±2.1	88.8 ±1.2	86.1 ±0.8	13.1 ±0.2	12.5 ±0.4	12.1 ±0.7	15.5 ±0.7
200 µg/L	55.3 ±1.1	56.3 ±1.8	59.6 ±1.0	67.0 ±0.8	11.8 ±1.1	11.0 ±2.2	10.1 ±0.3	7.12 ±0.7	90.4 ±0.8	86.2 ±0.7	86.1 ±0.3	85.1 ±0.4	14.5 ±0.5	15.7 ±0.7	16.3 ±0.8	16.7 ±0.8

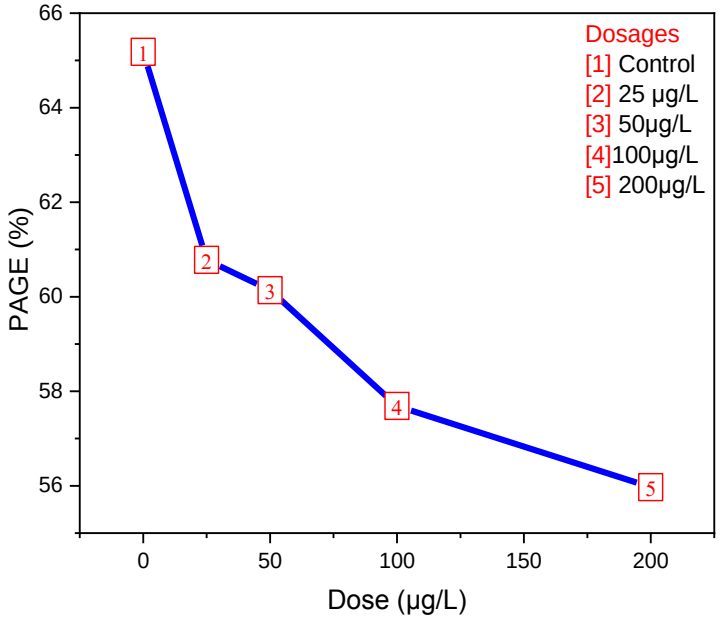


Figure 4. 14PAGE analysis of *Cyprinus carpio* exposed to SMX and control at (28thday).

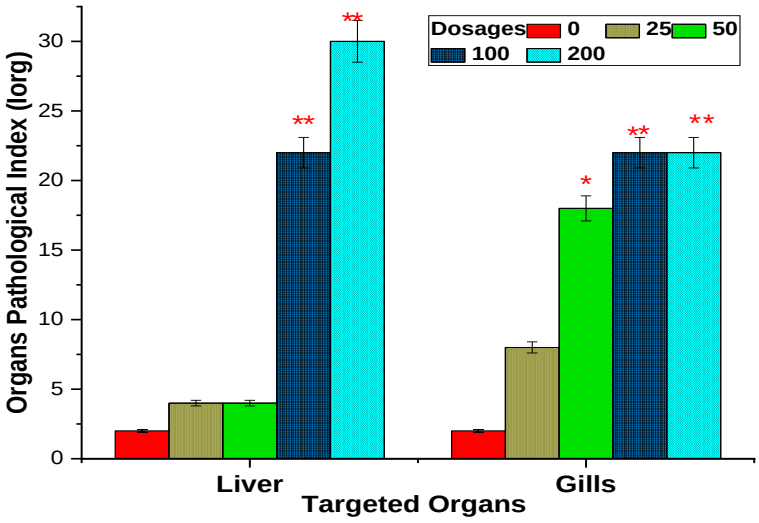


Figure 4. 15 Organ alteration index (Iorg) of liver and gills of control and SMX (25, 50, 100 200 µg/L) exposed common carp for 7, 14, 21, and 28 days (n=9).

Xenobiotics and biological components may interact at a molecular level, resulting in tissue damage that can be measured using histological techniques (Rodrigues et al., 2017). Many human or environmental stresses can alter an organism's histopathology, which is relevant to the field of ecotoxicology. Histopathology is an essential analytical link between whole-organism or population research and molecular, cellular, or biochemical assays. It is a well-established methodology that can provide data to support population-relevant endpoints. In order to further extrapolate to population/community effects, histopathological reactions can be used as biomarkers of contaminant exposure and repercussions that are connected to an individual's general health and fitness. Histopathological changes in the fish body are caused by antagonistic fluctuations in the organism's metabolic profile (such as glucose, protein, calcium levels, etc.), oxidative stress, and numerous physiological abnormalities. A key advantage of using a histopathological technique in environmental impact assessments is that it enables the examination of several organs at a time (Paulino et al., 2020). When a fish encounters any chemical pollutant, it stimulates the appearance of some different types of lesions in tissues of different organs of the body (Altinok and Capkin, 2007). Gills and liver respond easily to the xenobiotics making them more suitable for histopathological examinations. Since they come into close touch with toxins in the water and respond quickly to contaminants of any concentration, fish gills are important organs for assessing the toxicity of aquatic contaminants. The liver is regarded as a crucial location for the storage of substances, their biotransformation, and ultimately their elimination from the body. Moreover, the histology of the liver is a sign of the general health of fish (Nunes et al., 2014). Nonetheless, the liver of exposed fish showed various histopathology, the intensity of which enhanced as the antibiotic concentration was increased. These morphological alterations are the result of an indirect or direct action of SMX on the liver tissues. Indirect impacts were induced by physiological and biochemical changes (Iftikhar & Hashmi, 2020). According to studies, environmental pollutants can cause oxidative stress, which in turn triggers cell death. Sulfa medicines are known to activate cell apoptosis and necrosis by accumulating matching metabolites, which causes the formation of ROS (Elzagallaai et al., 2020). Some changes in the nucleus and cytoplasm were non-specific, however cytoplasmic vacuolization is the indication of cellular degeneration.

Furthermore, the presence of intracellular substances like bile stagnation and melanomacrophages, predominantly in fish exposed to higher concentrations of SMX were signs of degenerative process related to blockage of bile canaliculi. Similar characteristics were noted in *Oncorhynchus mykiss* exposed to erythromycin (Rodrigues et al., 2019). Changes in the nucleus of observed tissues in the current study were probably the outcome of genotoxicity caused by SMX. Another explanation for the rise in melanomacrophages when SMX concentrations increased is that these cells play a crucial role in clearing the fish's body of potentially hazardous substances such free radicals and byproducts of cellular degradation (Agius and Roberts,2003). By considering everything, the pathological index (Iorg) of the liver that has been calculated using Fi (importance factor) and Sc (pathological score) evidenced that this antibiotic induces histopathological changes exhibiting dosage dependence. A lack of oxygen as a result of gill tissue deterioration may be the cause of hepatocellular degeneration. Histological lesions in fish caused by pollution have been explained by a number of theories, including the buildup of oxidative stress. According to some research, excessive oxidation can harm cells in ATP and delay the process of apoptosis, which eventually results in cell necrosis (Rodrigues et al., 2019).

During the 28th day of the exposure period, SMX had notable detrimental effects on the gills in all exposure groups compared to controls (Fig. 4.13 C& E). The control group's histopathological analysis revealed normal-appearing gills, which were demonstrated by the presence of intact primary and secondary lamella and a prominent inter-lamellar gap. On the contrary, exposed groups depicted gills with various tissue abnormalities including partial and complete fusion of lamella, uplifting of the primary lamella, blood congestion, and shortening of the secondary lamella that showed prevalent damage to gills tissues. Concentrations of SMX in the water are directly correlated with the degree of histological abnormalities in fish gills. Antibiotic exposure may result in toxic and hypoxic circumstances that can lead to cellular damage. (Minski et al., 2020). Fish may be able to counteract the impacts of pollution due to histopathological changes such epithelial raising and lamellar fusion. Moreover, these alterations might potentially be an indication of systemic hypoxia caused by a lack of oxygen. (Rajeshkumar et al., 2017). In the 25 µg/L exposure group, the alterations were not very evident as compared to other exposure groups. Lesions were more noticeable at higher doses, and in the 200 µg/L group on day

28, damage frequency was widespread. Nunes et al. (2015) also noted abnormalities in *Gambusia holbrooki* subjected to another antibiotic tetracycline for four days at exposure dosages of 50-500 ng/L, including lamellar fusion, hyperplasia, shortening and curling of lamellae, and cellular necrosis.

Epithelial thickening noticed here may be the result of the emergence of special types of cells (like macrophages and leucocytes) belonging to the defense and tissue repair system of the fish body. Another protective system is the rise in the number of hypertrophic mucous cells as observed in the current study since mucus is a layer of glycol-lipids and glycol-proteins that acts as a defensive layer over gill epithelium. This seems to be a long-term adaptive behavior of the fish body (Nero et al., 2006). The loss of membrane structure (due to necrosis) may result in the discharge of cellular elements into the extracellular environment that initiates a pathological process that can stimulate a significant inflammatory reaction as indicated by our oxidative stress results. Moreover, gills possess a large surface area to provide an efficient gaseous exchange with surrounding water (Cinar et al., 2009). The delicate structure of gills usually counters the environmental stressors by structural changes depending on the dose and time of exposure, specifically for sublethal concentrations of water pollutants (Nero et al., 2006). Taken all together, organ pathological index, that was determined considering each I_{Alt} evidenced that SMX causes histopathological changes exhibiting dose-dependence. Thus, multi-biomarker approach reflected pathways involved in the regulation and maintenance of the general health status of *Cyprinus carpio*, by analyzing different biological samples (tissues/organs) after chronic exposures to SMX.

4.2. Phase 1 b: Toxicity assessment of SMX in early staged zebrafish (*Danio rerio*) using multiple biomarkers.

Antibiotics are considered emerging contaminants and are ubiquitously present in aquatic environments because of their widespread use in human and animal medicine. Several biomarkers have been used to quantify the toxicity of these chemicals in a range of aquatic animals. However, the toxicity of several antibiotics in aquatic organisms has not been fully assessed and continued surveillance is needed for risk assessment. SMX has been reported to be as high as 16 mg/L (16,000 µg/L) in some aquatic environments (Zafar et al., 2021) thus raising concerns as to its toxicity to exposed organisms. Exposure to SMX has been reported to induce immunosuppression, antibiotic resistance, biochemical responses, and histopathological changes in aquatic organisms (Iftikhar et al., 2022; Iftikhar and Hashmi, 2021; Yan et al., 2018; Saglam and Yonar, 2009). The current study determined the exposure effects of a broad range of SMX concentrations to developing zebrafish, an ideal indexical organism for ecotoxicological studies. Zebrafish stand out as a significant model in toxicological research due to their genetic resemblance to humans, transparent embryos aiding in non-invasive imaging, rapid development allowing for efficient assessments, and ethical considerations. Their sensitivity to toxicants, behavior observation capabilities, and conservation of pathways enhance their value. Zebrafish serve as a versatile tool, facilitating high-throughput screening and offering insights into both developmental and environmental toxicology, making them pivotal in advancing toxicological understanding and chemical safety assessment. The current study provides information regarding SMX's potential sublethal effects on zebrafish embryos, such as their survival, development, gene expression, oxidative stress, mitochondrial metabolism, and behavioral performance.

4.2.1. Survival, morphological malformations, and hatchability

In four different trials, the percentage of zebrafish larvae that survived each trial was noted, and daily images of malformations were obtained. Data from all trials were combined to create a single graph.

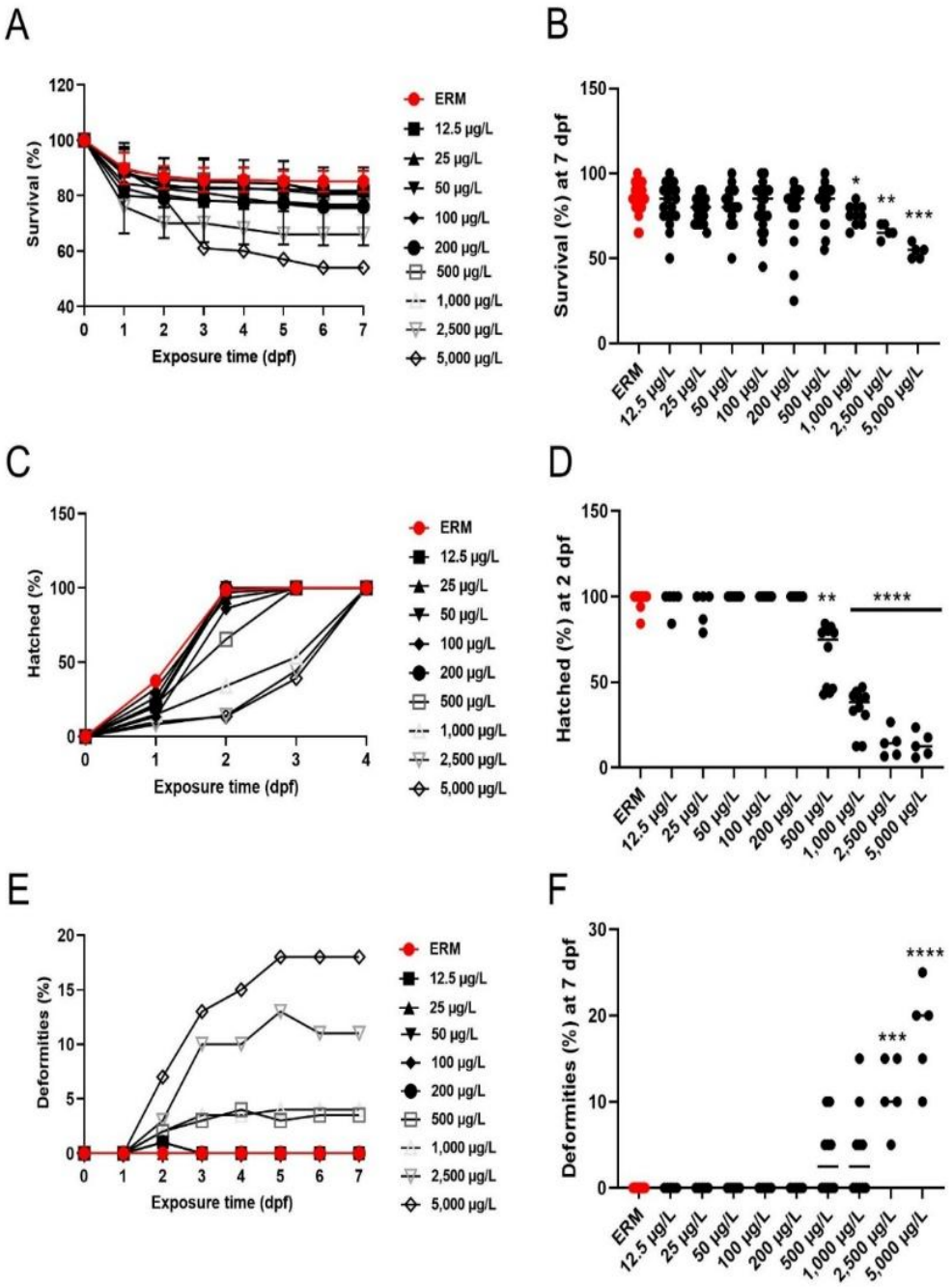


Figure 4. 16 Percent survival, deformities and hatch rate of zebrafish exposed to ERM (control), 12.5, 25, 50, 100, 200, 500, 1000, 2500 and 5000 µg/L dosages of SMX over time (A, C and E). Percent survival and deformities at 7dpf and hatch rate at 2dpf of zebrafish embryos (Dunn's test after Kruskal-Wallis test) (B, D and F respectively). For 7 days exposure all fish were hatched by day 4.

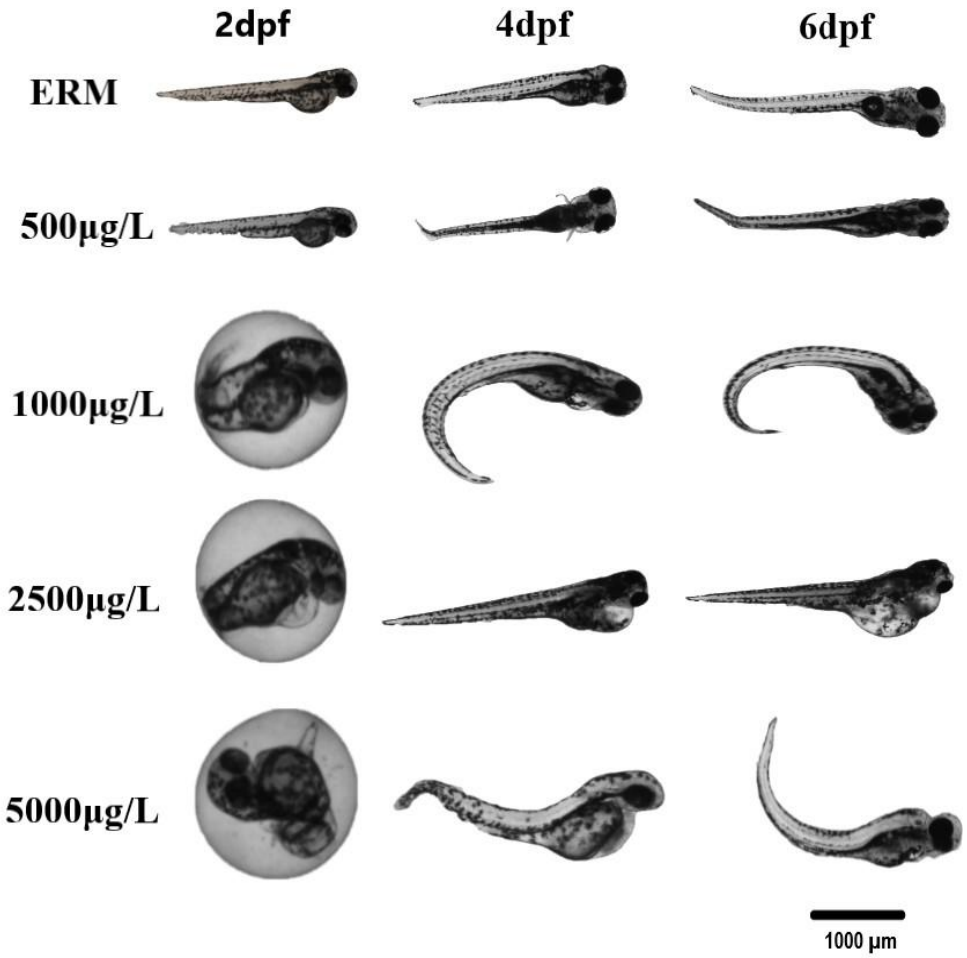


Figure 4. 17 Predominant morphological deformities observed in zebrafish embryos/larvae after being exposed 500, 1000, 2500 and 5000 µg/L SMX and ERM (control).

Exposure to SMX had a significant impact on the survival rate of larvae ([$F_{(DFn, DFd)}$, P value]; $F_{(9, 156)} = 5.268$, $p < 0.0001$) (Figure 4.16 A). At 7 dpf statistically significant increase in mortality at concentrations ranging 1000 to 5000 µg/L was observed (Figure 4.16 B). upon completion of the exposure, the mortality rate was around 30–40% at the highest concentration examined (5000 µg/L). The hatch rate was monitored over the first three days to determine if SMX exerted any influence on hatch rate of zebrafish. All embryos in all treatment groups and the ERM control hatched after three days. At 2 dpf, there was a statistically significant difference between the groups in the hatch rate. ($F_{(9, 61)} = 92.76$, $p < 0.0001$) (Figure 4.16 D). ERM (control) and environmentally relevant

concentrations of SMX showed no apparent deformities in larvae (<2%). However, there was a significant increase ($F_{(9, 61)} = 20.89$, $p < 0.0001$) in the occurrence of malformations at 7 dpf with SMX exposure (Figure 4.16 E, F). Most of the larvae were noted to be deformed and non-viable by 7 dpf after exposure to higher concentrations (2500 and 5000 µg/L). The most common deformities observed in higher dosages tested were yolk sac and pericardial effusion, deformed tail and arched spine (Figure 4.17).

SMX affected survival, and hatchability and caused malformations in developing zebrafish. Gross morphological abnormalities were brought on by SMX exposure at higher doses of 2500 and 5000 µg/L, and they typically manifested as early as 2 dpf. The pericardial effusion, arched tail, curved spine, and yolk sac edema were the most often seen malformations. Research performed to evaluate the effects of three types of sulfonamides: SMX, sulfadiazine, and sulfadimidine at (1-1000 µg/L) revealed that SMX induced deformities in zebrafish that included yolk sac edema, hemagglutination, and axial malformation (Lin et al., 2013).

Moreover, the hatchability overall decreased with higher exposure doses, according to the same study. No apparent differences were noted for lower concentrations tested (1 µg/L and 100 µg/L) of all three sulfonamides. A substantial decrease in hatching rate was observed at higher concentration of 1000 µg/L SMX and hatchability was reported for SMX exposure as 11.5% lower compared to control groups. Our data agree with those of these past investigations and acute toxicity is not observed until 500 µg/L exposure or more. However, decreased body length and delayed hatching was observed in zebrafish embryos exposed to 100 µg/L SMX (Liu et al., 2020). We observed effects on the hatch rate in zebrafish embryos exposed to 500 µg/L, but not 100 µg/L.

In another study, zebrafish embryos exposed to sulfamethazine (0.2- 2000 µg/L) presented a reduced embryo hatching rate at any given concentration between 58 and 96 hpf (Yan et al., 2018). Spine curvature and edema were also reported as the two primary types of malformations induced by the antibiotic. Thus, exposure to SMX is responsible for a delay in hatchability in zebrafish embryos.

The hatchability delay and malformations noted in the present study may be explained by the chemical actions of SMX (Lin et al., 2013) and several mechanisms may underlie developmental effects. Epiboly is observed in zebrafish embryos during the gastrula stage. Sulfonamides are an example of an exogenous antibiotic that can harm cells in the pre-epiboly stage as well as inhibit embryonic growth.

According to Isidori et al., 2005, the lack of epiboly function brought on by sulfonamides can impede the developmental process of the anterior-posterior body axis. The tail bending seen in the current study, for instance, could have been caused by a neural tube defect. According to Ralph et al. (2008), the emergence of yolk sac edema is also associated with an atypical decrease of epiboly activity, which the current study also found in some fish. Before reaching the nucleus, sulfonamides may interact with cytoplasmic receptors, impairing embryonic development (Kamata et al., 2009; Gunnarsson et al., 2008).Another mechanism for developmental effects may also involve the thyroid hormone system. As thyroglobulin (Tg) disruptors, sulfonamides cause dilatation and degranulation of rough endoplasmic reticulum (Lin et al., 2013); these modifications could result in low levels of Tg secretion. The development of organs and the central nervous system would be impacted by subsequent hypothyroidism. Lastly, the delayed hatchability and malformations of zebrafish embryos may be explained by the chemical actions of SMX specifically impairing cell division by regulating folate metabolism (Lin et al., 2013).

4.2.2. Mitochondrial bioenergetics

Following a 2-day treatment, the oxygen consumption rate of SMX-exposed embryos (at 54 hours of age) was measured (Figure 4.18 A-E). For all parameters examined, no significant change was seen.: basal respiration ($F_{(4, 15)} = 1.57$, $p = 0.23$), ATP linked respiration ($F_{(4, 15)} = 1.81$, $p = 0.18$), maximal respiration ($F_{(4, 15)} = 0.74$, $p = 0.68$), and non-mitochondrial respiration ($F_{(4, 15)} = 0.34$, $p = 0.85$).

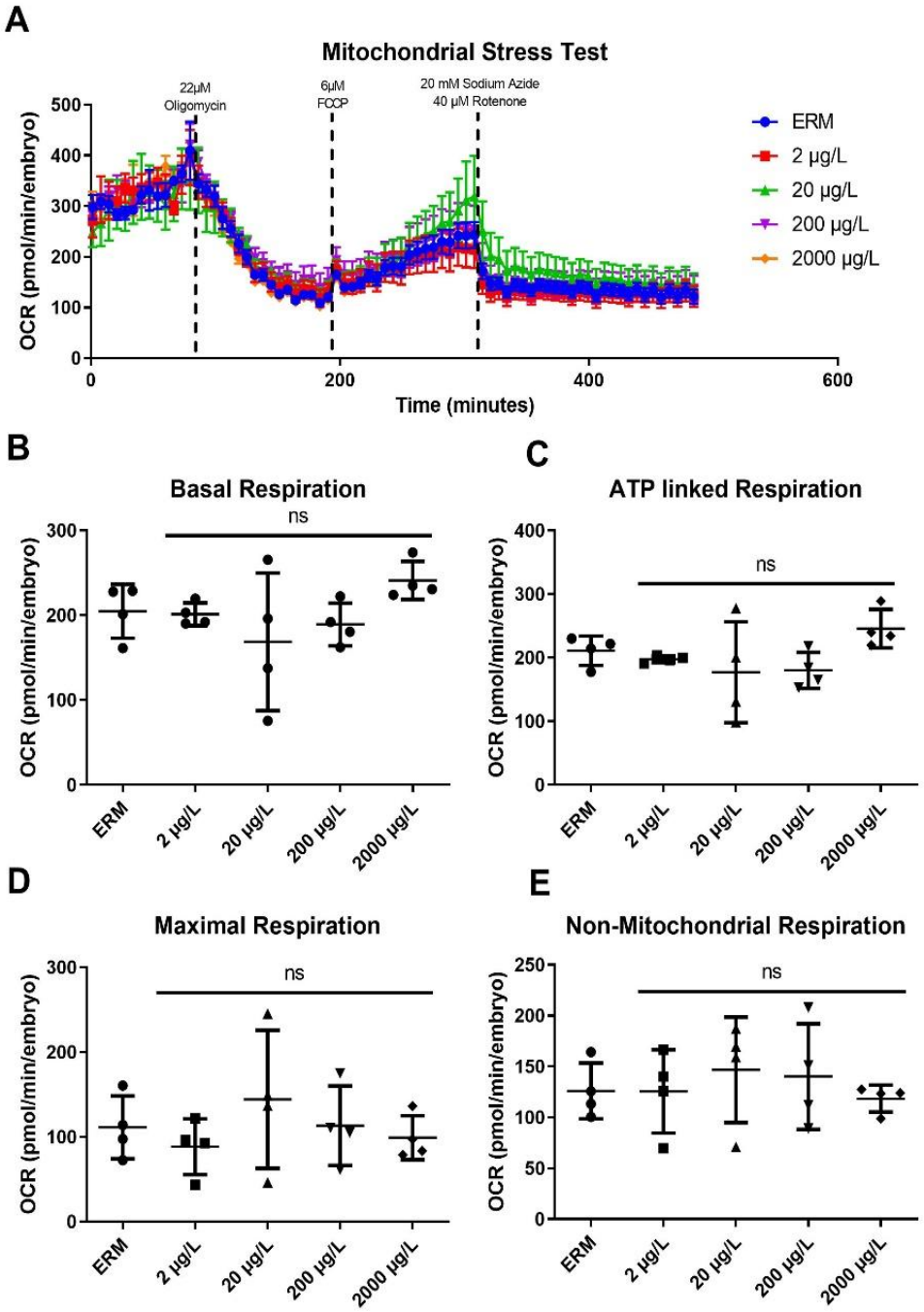


Figure 4. 18 Mitochondrial bioenergetics in 54 hpf zebrafish embryos. (A) OCR; (B) Basal respiration rate; (C) ATP-linked respiration rate; (D) Maximal respiration rate; (E) Non-mitochondrial respiration rate. Data are reported as mean ± SD (Tukey's test after one way ANOVA, n = 4/group).

4.2.3. Reactive oxygen species

Zebrafish embryos were given a 7-day exposure to 25, 100, and 500 µg/L of SMX and ERM. None of the treatment groups displayed any variations in the level of ROS ($F(3,16) = 0.79$, $p = 0.51$; Figure 4.19).

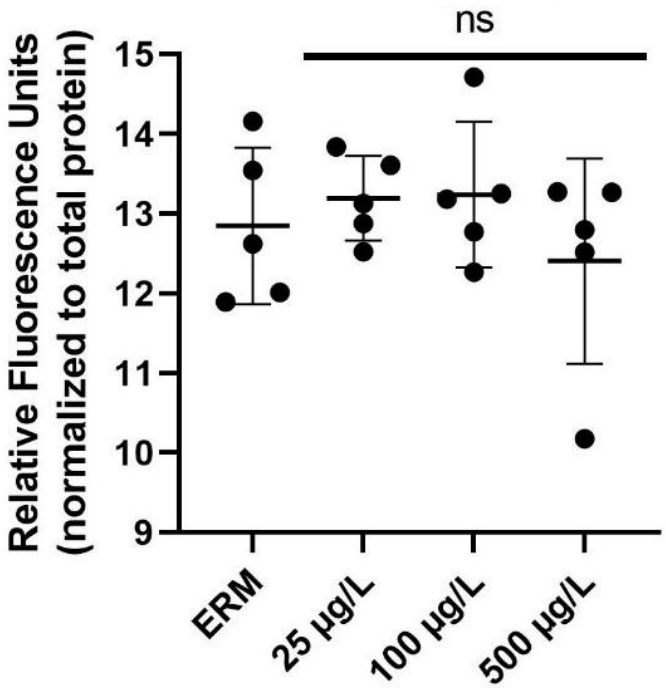


Figure 4. 19 Reactive oxygen species expressed as relative fluorescence units (µg/mL protein) (n=5).

Abnormal oxidative respiration is indicative of oxidative stress and mitochondrial dysfunction. In our study, SMX exposure did not affect oxygen consumption rates of embryos, nor did it induce ROS in larvae at the tested concentrations. Surprisingly, we also did not observe a significant change in the oxidative stress-related transcripts (*cat, gpx1, gst, hsp70, nrf2a, sod1* and *sod2*). No change in mitochondrial function was observed at 73 µg/L concentration as compared to 14.7 mg/L of erythromycin confirming dysfunction of mitochondria at higher dosages of antibiotics (Yang et al., 2022). Aquatic studies have utilized biomarkers of oxidative stress frequently.Investigating diverse endpoints

connected to oxidative stress is crucial as an organism's reaction to a xenobiotic or tissue-specific stressor can vary. To cope with ROS produced by oxygen-involved natural and external stresses, organisms often have a variety of enzymatic systems. Reactive oxygen species have a high intrinsic reactivity, which makes them potentially harmful to cells. As a result, antioxidant defense systems may not be sufficient to prevent the emergence of oxidative stress. For the exposure time and SMX concentrations investigated here, ROS production was not a significant mechanism as SMX did not induce oxidative stress nor mitochondrial dysfunction in early tagged embryos. There have been reports of zebrafish lacking antioxidant defence after exposure to antibiotics in the past. Iftikhar et al. (2022) reported onset of oxidative stress in the brain of *Cyprinus carpio e*xposed to SMX in a 28-day chronic exposure. However, exposure to SMX at 50- 500 µg/L for two weeks did not level of antioxidant enzymes (Tokanová et al., 2021). Accordingly, aquatic organisms exposed to sulfonamides show mixed evidence of oxidative stress. Different fish species, life stages, concentrations, exposure durations, and time points may explain these variations.

4.2.4. Analysis of genetic expression (RT-PCR)

Zebrafish larvae were tested for the potential effects of SMX on oxidative stress, apoptosis, and the immune system using mRNA levels of a wide range of transcripts. Transcript levels of immune-related gene *ifn* -γ ($F_{(6, 22)} = 4.8$, $p = 0.0029$) (Figure 4.20 B) in larvae showed increased expression with exposure to 500 µg/L SMX. In addition, *IL-1β* was upregulated ($F_{(6, 18)} = 6.07$, $p = 0.0013$) in zebrafish at 50 µg/L exposure group (Figure 4.20 C) Transcript levels for apoptosis-related gene *casp3* ($F_{(6, 20)} = 5.84$ $p = 0.0012$) (Figure 4.21 D-E) were reduced in larvae in a dose-response manner while transcript levels for *bad* ($F_{(5, 15)} = 4.78$, $p = 0.0082$) (Figure 4.21 A) and *bax* ($F_{(6, 23)} = 8.69$, $p < 0.0001$) (Figure 4.21 B) were upregulated in fish exposed to 50 µg/L treatment. SMX exerted no effect on oxidative stress-related transcripts tested in the study ($p > 0.05$).

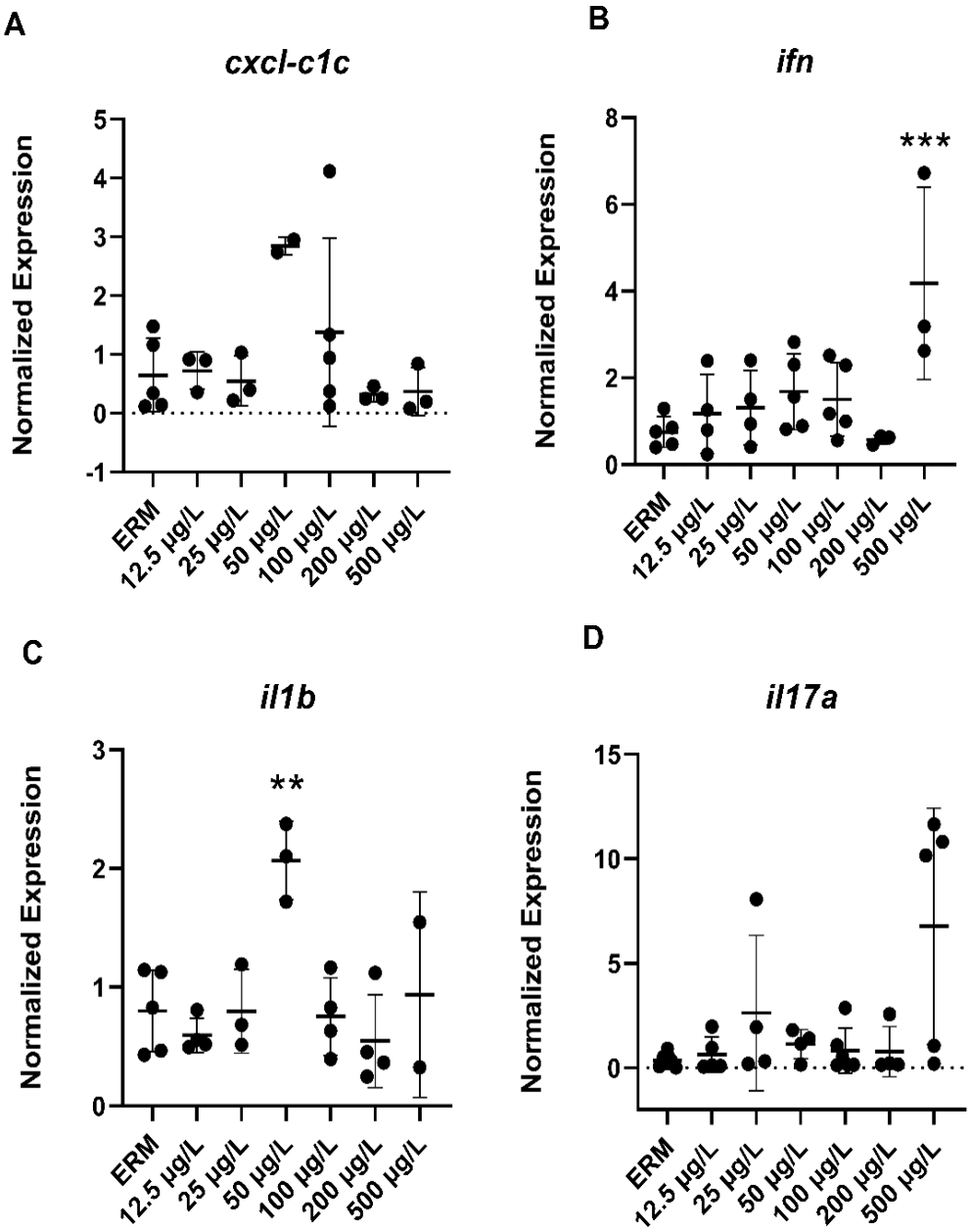


Figure 4. 20 Transcript levels of immune system (A) chemokine *cxcl-c1c*; (B) inter-feron-gamma, *ifn*; (C) Inter-leukin-beta, *il-1b*; (D) Interleukin-17A, *il-17* in 7 dpf zebrafish larvae exposed to SMX.

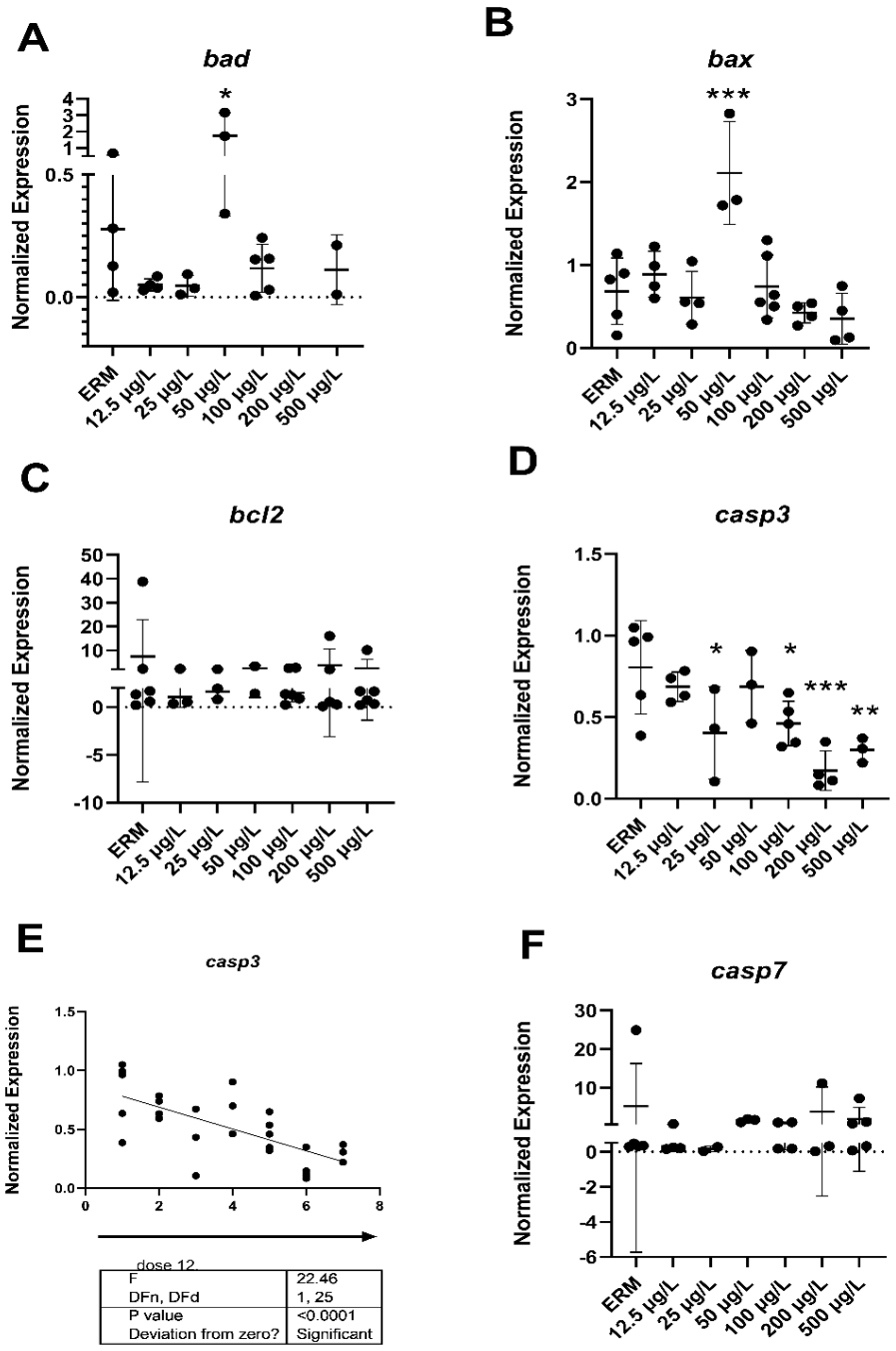


Figure 4. 21 Transcript levels of apoptosis (A) Bcl2 associated agonist of cell death, *bad*; (B) Bcl2 associated x, apoptosis regulator, *bax*; (C) Bcl2 apoptosis regulator, *bcl2*; (D) Caspase 3, *casp3* (E) Linear regression of caspase 3, *casp3* (F) Caspase 7, *casp7* in 7 dpf zebrafish larvae exposed to SMX.

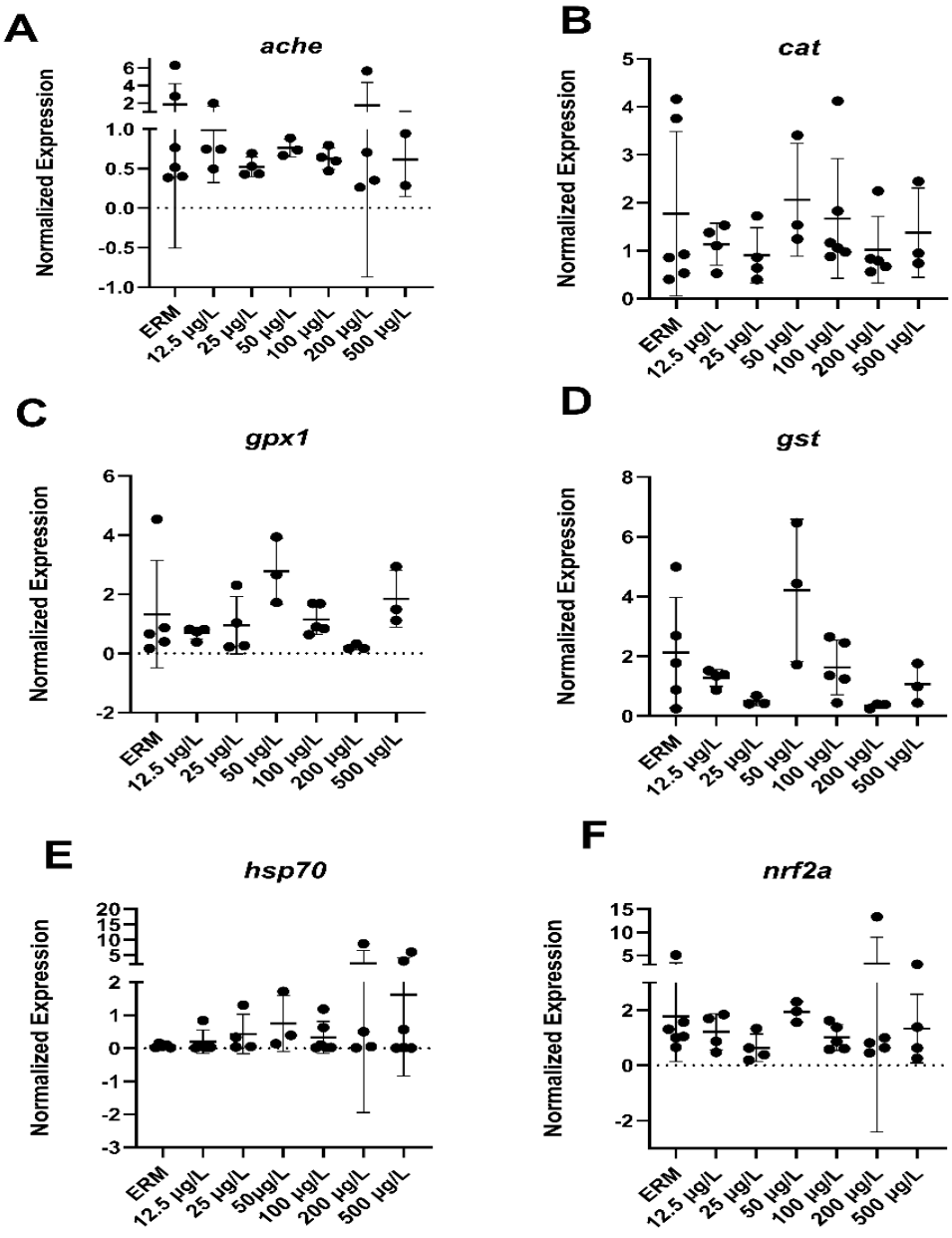


Figure 4. 22 Transcript levels of oxidative stress (A) acetylcholinesterase, *ache*; (B) catalase, *cat*; (C) glutathione peroxidase 1, *gpx1*; (D) glutathione S-transferase, *gst*; (E) heat shock protein 70, *hsp70*; (F) nuclear factor erythroid 2-related factor 2, *nrf2a* in 7 dpf zebrafish larvae exposed to SMX.

A series of immune-related transcripts were measured in zebrafish to assess the immune response of zebrafish embryos, comprising inflammatory cytokines such as IL-1β, ifn,

il17a and cxcl-c1c. These genes were chosen because antibiotic exposure has been reported to affect the cytokines of fish (Lin et al., 2014). Our results indicated up-regulation of proinflammatory cytokines ifn-γ and IL-1β, generating insight into host exposure to antibiotics. Healthy zebrafish embryos appear to be inflamed by SMX during early development, as shown in this study. Inflammation is a dynamic phenomenon. Neutrophils in the body's immune response generate several cytokines along with increased ROS when fish are infected by pathogens or harmed by pollutants (Kobayashi et al., 2003). Increases in IL-1β may result in the generation of lipid mediators, proteases and ROS and are regarded as a key proinflammatory cytokine. Moreover, ifn is known to facilitate inflammation by inducing macrophages to produce TNF-α and IL-1β (Cavaillon, 1993). We found that larvae exposed to 50 g/L SMX overexpressed proinflammatory cytokines, suggesting that environmental antibiotic levels may provoke inflammation in healthy zebrafish larvae. This is consistent with earlier research showing that fish, including zebrafish and Nile tilapia, are sensitive to low doses of SMX exposure in terms of proinflammatory cytokines production (Liu et al., 2020; Limbu et al., 2018). This research expands our knowledge of the immune system in the early life stages of fish and raises the possibility that environmental antibiotic exposure may result in immunological problems.

Apoptosis is a crucial cellular process for normal embryonic development. It is usually assessed to determine how antibiotics may affect the normal apoptosis rate in developing zebrafish larvae. Additionally, several transcripts related to apoptosis were evaluated in our study including bad, bax, bcl2, casp3, casp7, and casp9. There was a concentration-specific decrease in casp3 expression with SMX exposure in zebrafish. A 50 µg/L concentration of SMX that is environmentally relevant also led to the upregulation of the bad and bax transcripts. Among the members of Bcl-2 family are both anti-apoptotic proteins (Bcl2) and pro-apoptotic proteins (BAX, BAD). When exposed to pollutants, *p53* transcription directly targets the *bax* genes, which are located on the mitochondrial outer membrane and play a key role in the commencement of apoptotic damage. The *p53-Bax* cascade primarily activates the intrinsic apoptosis pathway, which subsequently causes cell death via the mitochondria-dependent pathway(Li et al., 2020). Higher levels of the genes bad and bax were expressed in exposed zebra fish, which was consistent with this

mechanism and suggested that fish apoptosis may be accelerated by SMX exposure. The Nile tilapia showed similar outcomes as well (Limbu et al., 2018).SMX exposure's detrimental effects on fish health were also shown by the increased inflammation at lower doses of SMX. The effector protein known as caspase 3 is essential for both endogenous (the mitochondrial) and exogenous (the death receptor) pathways of apoptosis. Additionally, it is the enzyme that controls synaptic activation and regulates the process of neurogenesis in developing larvae (Yabu et al., 2001). Transcripts for casp-3 were decreased in relative abundance after SMX exposure in a dose-dependent manner, revealing disturbances in the normal apoptotic process, which is crucial for development of embryos, repairing of damaged tissues. This may lead to developmental malformations in zebrafish embryos. In embryonic development, the apoptotic process mainly occurs during the eradication of redundant cellular material which is essential for correct morphogenesis of tissues and organs in addition to maintaining tissue homeostasis throughout the life of the cell (Porter et al., 1999). The expression of casp-3 is essential for development; knockout mice for caspase-3 were born infrequently and died in a short amount of time (Nguyen et al., 2021). During the developmental period of zebrafish, caspase-3-dependent apoptosis ensures normal development and also aids in stress tolerance. In a previous work, Yamashita et al. (2008) described the precise effects of microinjecting antisense MO (morpholino-oligonucleotide) into zebrafish embryos to suppress caspase-3. Zebrafish embryos displayed a little dorsalized morphology at lower doses of caspase-3-MO. In targeted zebrafish embryos, the phenomena of epiboly was arrested at a higher dose of caspase-3-MO at 8–12 hours after fertilisation, demonstrating that the absence of caspase impeded the process of embryogenesis (Yamashita et al., 2008).

Moreover, components of the apoptotic pathway, including caspases, are essential for apoptosis as well as being involved in other physiological functions in a wide range of cell types, including neurons. According to a study by Campbell et al. (2013) the central nervous system of zebrafish embryos undergoes axon reorganization as a result of caspase activation. Reduced Caspase-3 activity led to a limited amount of synaptogenesis and arbour growth (Campbell & Okamoto, 2013).

Our results are corroborated by other studies that found elevated expression of BAX and bcl-2 in response to SMX, such as in the brain of the *Cyprinus carpio* after exposure to cypermethrin antibiotics (Arslan et al., 2017). Furthermore, norfloxacin antibiotic caused apoptosis in the zebrafish embryos (Xi et al., 2019)

4.2.5. Apoptosis analysis

Exposure effect of SMX on zebrafish embryonic apoptosis were determined at 7 dpf using acridine orange staining (Figure 4.23 A) No significant difference was detected for apoptotic signal between experimental groups ($F_{(3, 56)} = 0.72$, $p = 0.55$) (Figure 4.23 B). We did not detect a change in apoptosis based upon AO staining; however, there may be a more subtle response of SMX and low-level effects on the transcriptome that is not fully captured with AO staining. Subsequently, a longer exposure may be required to induce apoptosis following up-regulation in the apoptosis-related transcripts at environmentally relevant concentrations. Considering that SMX can be persistent in the environment, apoptosis may be observed with sub-chronic exposures.

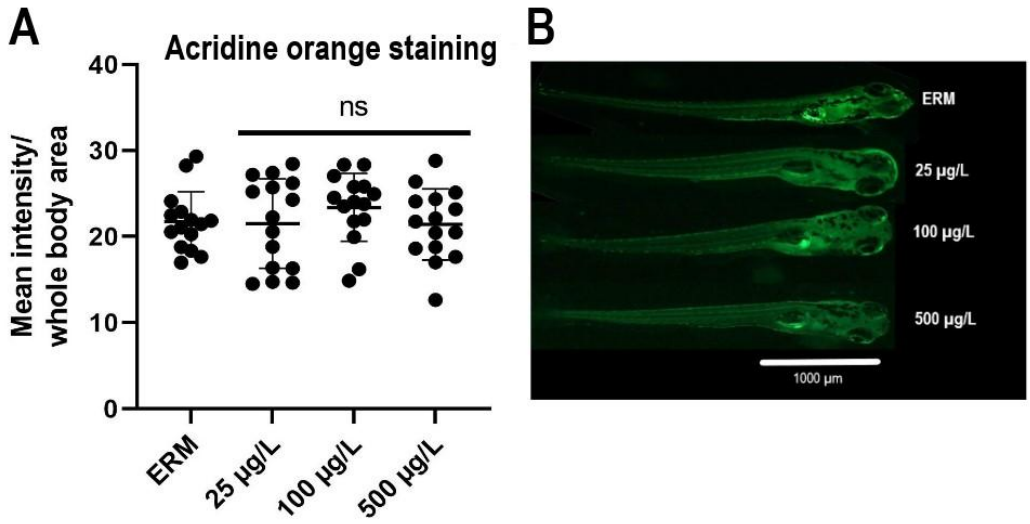


Figure 4. 23 (A)Fluorescence intensity in zebrafish larvae exposed to ERM (control), 25, 100 and 500 µg/L SMX at 7dpf. Data represented as mean (±SD) with (n=15). (B) Representative photomicrographs of zebrafish larvae stained with acridine orange (AO) being exposed to (0, 25,100 and 500 µg/L) SMX. The scale bar is 1000 µm.

4.2.5. Visual motor response test (VMR) and light-dark preference test (LDPT)

The VMR test was run eight times independently, and the data from all eight runs was combined to create a single graph (Figure 4.24) In both light and dark zones, the combined distance travelled by experimental groups was different from the control ($F_{(49, 450)} = 6.8$, $p < 0.0001$). For zebrafish treated with the 5000 µg/L group, analysis of the individual light and dark cycles revealed an increase in locomotor activity during the first light cycle. However, in further dark and light cycles, none of the tested treatments had a discernible impact on locomotor activity. Zebrafish larvae are often used to study the neurotoxicity of aquatic pollutants. Several antibiotics have a negative impact on the locomotor activity of fish (Gonçalves et al., 2020; Wang et al., 2014). Here, the visual motor response test revealed hyperactivity in the first light period at 5000 µg/L. However, that concentration is considerably higher than environmentally relevant levels of SMX reported globally. According to previous reports, SMX raises plasmatic bilirubin levels, causing kernicterus and related brain damage (Thyagarajan & Deshpande, 2014).Moreover SMX has been reported to induce neurotoxicity in grass carp *by* changing the permeability of blood brain barrier and down-regulating tight junction proteins (occluding, claudins) thus causing abnormal behavior, histopathological changes, and ultrastructural damage (nerve cell damage and synapse reduction) (Zhao et al., 2021).

Wang et al. (2014) studied the effect of six selected antibiotics on zebrafish behavior and the results showed that at 6.26 mg/L concentration, zebrafish larvae showed increased movement and signs of neurotoxicity showing that antibiotics would seem to affect behavior only at high concentrations. In another recent study performed by Gonçalves et al. (2020), zebrafish exposed to amoxicillin at (10 mg/L) showed locomotory changes and reduced social interaction manner.

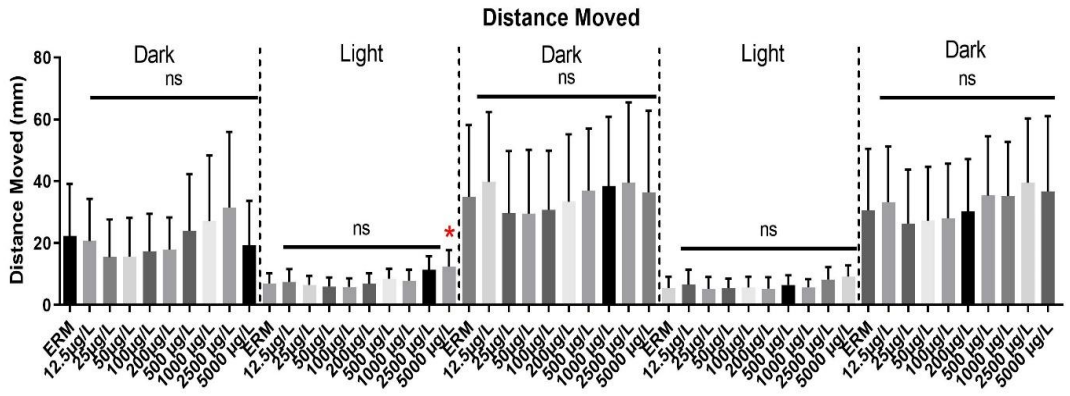


Figure 4. 24 VMR for zebrafish larvae exposed to ERM (control), 12.5, 25, 50, 100, 200, 500, 1000, 2500 and 5000 µg/L dosages of SMX at 7 dpf. Each light and dark cycle shows 10-minute interval.

We also used a light-dark preference test to evaluate the anxiolytic characteristics of larval zebrafish. A significant increase in total distance traveled in larval fish ($F_{(10,213)}$ =8.44, p <0.0001) (Figure 4.25 A) was observed with 500 and 2500 µg/L SMX. Frequency (dark zone) **(**$F_{(10, 213)}$ = 1.42, p =0.17) (Figure 4.25 C) did not differ between experimental groups.Average time spent in the dark zone total ($F_{(10,200)}$ = 3.81, p <0.0001) (Figure 4.25 B) showed a decreasing trend with respect to concentration. Cumulative time spent in the dark zone ($F_{(10,213)}$ = 2.98, p =0.0015) (Figure 4.25 D) revealed that only the positive control buspirone enhanced stay time of zebrafish in dark zone and there was no effect of SMX. Taken together, there was a subtle effect of SMX in larval fish, and a slight increase in locomotor activity with the highest concentrations tested. In our study, some hyperactivity was also observed in fish undergoing the light-dark preference test. In this test, we found that responses in zebrafish larvae were affected by SMX in terms of total distance moved, but there was no evidence of anxiety in the larvae with SMX exposure. Almeida et al. (2019) noted anxiolytic behaviors in zebrafish larvae following exposure to the antibiotic oxytetracycline. Their results reported that 10,000 µg/L exposure induced hyperactivity, changed feeding patterns, and reduced antioxidant enzymes in zebrafish larvae after long-term exposure. According to Burgess and Granato (2007), the startle reaction in zebrafish larvae is characterised by an abrupt increase in movement (hyperactivity), while adults may suffer freezing, hyperactivity, and unexpected

movements in response to environmental stress .This phenomenon may also underlie antibiotic exposures SMX has been reported to induce changes in intestinal microbial community of zebrafish at environmentally relevant dosages that may also affect brain- gut communication leading to changes in behaviour of zebrafish (Zheng et al., 2022). Taken together, SMX may pose a minimal risk to zebrafish for neurotoxicity at environmental concentrations (as no change in acetylcholinesterase expression was observed). However, further investigations into the hyperactivity response should be conducted in other fish species following exposure to antibiotics.

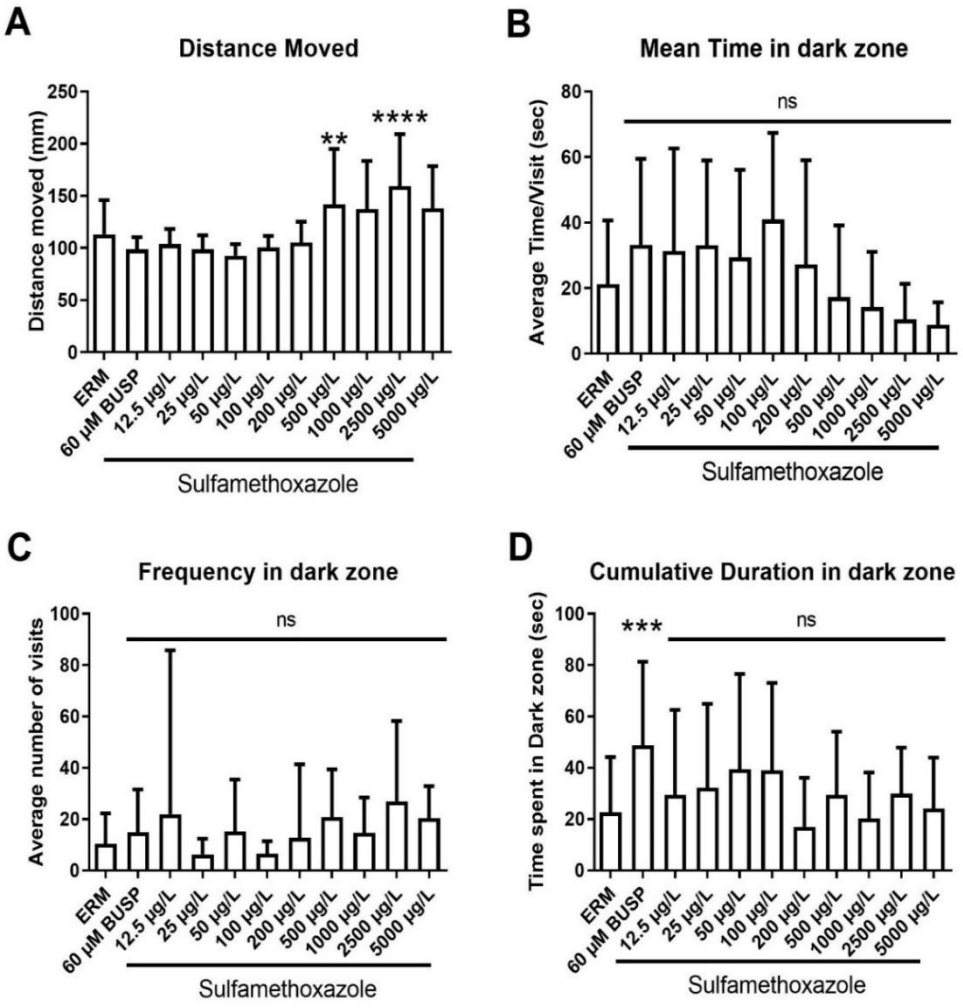


Figure 4. 25 LDPT showing anxiolytic behavior of zebrafish at 7dpf , (0-5000µg/L) SMX

4.3. Phase 2: To investigate influence of titanium dioxide nanoparticles on uptake, bioavailability and biotoxicity of SMX

By co-exposing *Cyprinus* carpio with nano titanium, the current study showed that SMX uptake increased. Moreover, the presence of nano titanium triggered more pronounced biochemical alterations that disrupted fish's physiological function. To understand immunohematological impacts, reactive oxygen species were measured by NBT reduction assay.

4.3.1. Characterization of nano titanium

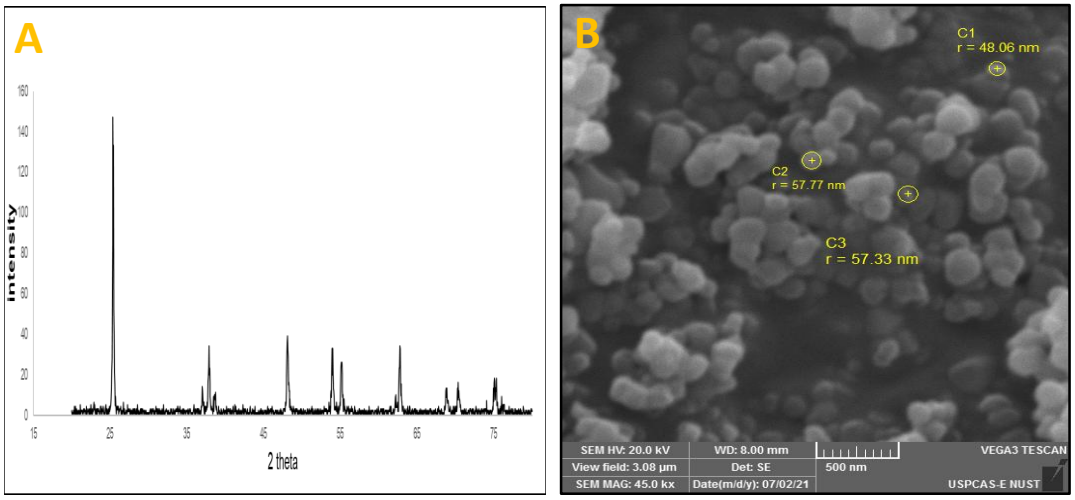


Figure 4. 26 XRD pattern and SEM image of prepared TiO_2 nanoparticles.

X-ray diffraction (XRD) in the 2-theta range of 20º-80º at ambient temperature was used to examine the synthesized titanium dioxide nanoparticles. XRD results are illustrated in Figure 4.26 A. Peaks of XRD at 25º revealed that nano titanium had anatase crystalline structure (Sirdeshmukh, et al., 2006). The Scanning Electron Microscopy images show surface morphology of nano titanium. The average particle size of nano particles, at 50,000 x magnification, was found to be 54.36 (Figure 4.26 B). The crystalline size of nanoparticles has been confirmed to be smaller than 100 nanometers.

4.3.2. Effects on biochemical biomarkers and respiratory burst activity

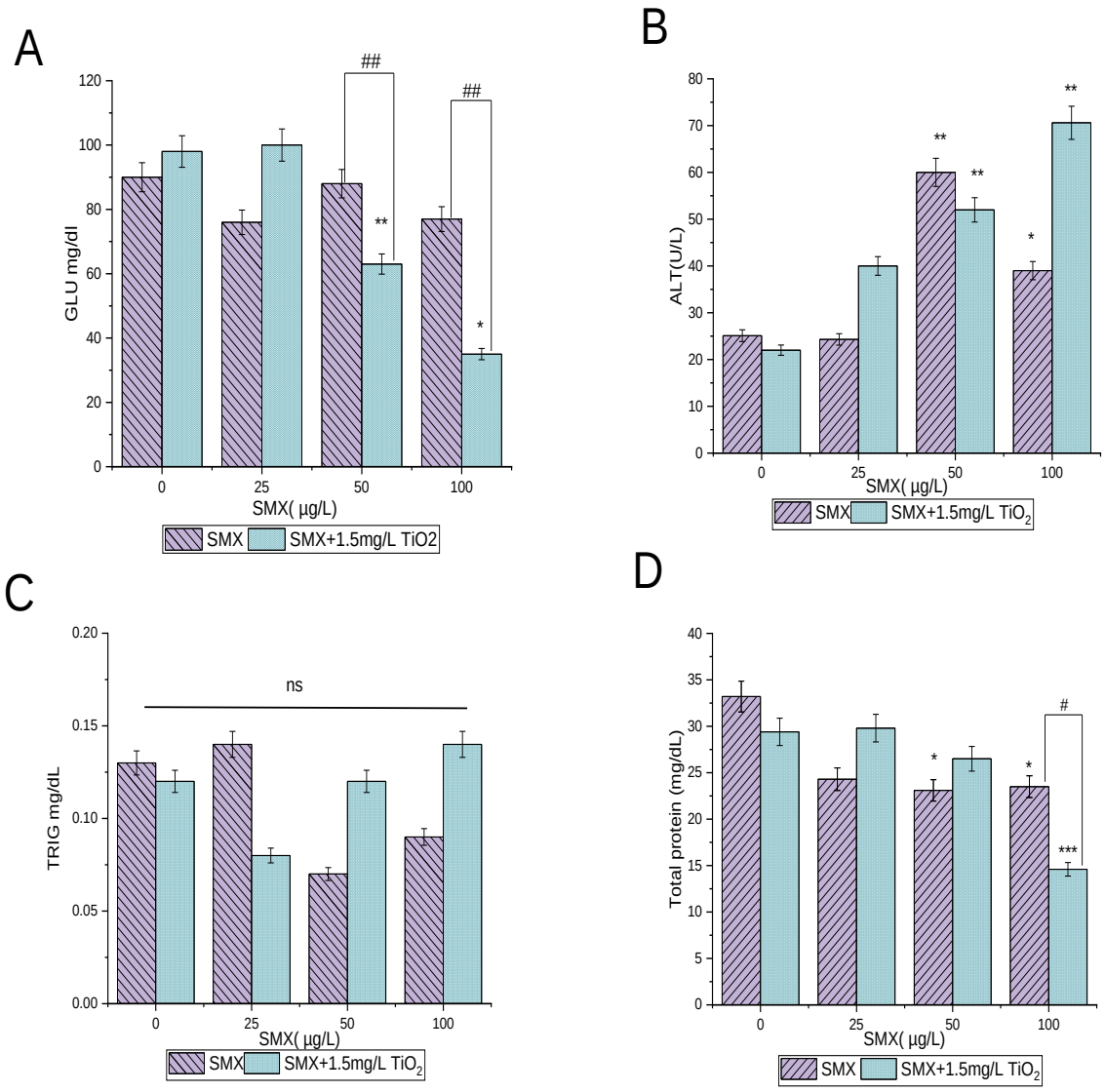


Figure 4. 27 Biochemical changes (A) Glucose level; (B) Alanine transaminase; (C) Triglycerides; and (D) Total Protein content in *Cyprinus carpio* being exposed to SMX alone (0, 25, 50, and 100 µg/L) and in the presence of nano titanium (1.5 mg/L) for 96 h. ($p < 0.05$) are shown by asterisk (*) vs. control group; and hash (#)vs. the corresponding SMX groups without nano titanium; ($p < 0.01$) are shown by (**)and (##). (one-way ANOVA followed by post hoc Tukey's test) (n = 6).

With respect to the dosages used in the experiment, a downtrend in glucose level was found. At higher dosage groups, co-exposure of SMX and nano titanium resulted in a considerably reduced ($p<0.01$) glucose level compared to SMX exposure alone. Lowest value of 35.1 mg/dL glucose as compared to control (90 mg/dL) was recorded at 100 µg/L SMX group in co-exposure with nano titanium (Figure 4.27 A).

The 50 and 100 µg/L SMX groups experienced a considerable rise in ALT (alanine transaminase) levels both alone and in combination with nano titanium ($p<0.01$) (Figure 4.27 B). Although there was a modest rise in ALT in the treatment group receiving SMX (100 µg/L) with nano titanium, this rise was not statistically different from the group receiving SMX alone. Triglycerides content didn't exhibit any significant change in stipulated time of exposure and dosages tested in the experiment as compared to control and with titanium co-exposure groups. The total protein contents were also measured in the fish at completion of exposure. Total protein levels were significantly reduced in the 50 and 100 µg/L ($p<0.05$) SMX group (Figure 4.27 D). In the combined SMX and nano titanium groups, a markedly decreased content of total protein was measured in higher dosage group (100 µg/L, $p<0.01$). When analyzing total protein content, no significant difference was recorded for the 25 µg/L SMX alone group. Likewise, In the combined SMX and nano titanium groups, the total protein content was not altered in the 25 µg/L group. The quantity of glucose and total protein significantly decreased, while the level of a liver-functioning indicator called alanine transaminase increased. The breakdown of glucose molecules in the fish body is a critical step for energy generation; glucose levels can fluctuate when the fish is stressed, requiring a lot of energy. Antibiotics can cause external or internal stress in the fish, which can lead to these conditions. This triggers a normal stress response in which the body secretes hormones such as dopamine, corticosteroids, and adrenaline to reactivate the glycogenesis process in order to meet the increased energy requirement. The current study's observed decrease in glucose levels is due to the fish being exposed to SMX, which initiates carbohydrate metabolism and may result in decreased cortisol production, which is a common stress response.

Likewise, protein is considered as a major constituent of various of cells in the body. It helps the body to form new tissues and repair the damaged ones. It helps to produce

enzymes and hormones and constitutes bones, skin, muscles, and blood. Under stress condition fish body may face oxygen deficiency which may alter the protein content of the body(Iftikhar and Hashmi, 2021).Decreased protein level at higher dosages in co-exposure treatments indicate the inhibition of protein synthesis under stress. Similar results have been observed for rainbow trout exposed to 200 mg/kg sulfamethazine, with total protein level considerably lower than control at the end of the exposure(Saglam and Yonar, 2009). Consistently, variations in biochemical parameters of freshwater fish with decreased amounts of glucose and total protein have been reported under substantial toxicity effect(Ejraei et al., 2015).

Moreover, Alanine transaminase content is measured as a biomarker of liver function as it is mainly present in hepatocytes and their elevated level shows liver damage (Mikulikova, et al., 2013). ALT level was recorded to increase significantly ($p<0.05$) with increasing dosage concentration showing that fish is under stress condition. Lipophilic nature of SMX could interfere with cell membranes and lead to elevated level of ALT enzyme. Furthermore, as evidenced by our findings, inefficient total protein and carbohydrate metabolism could be the cause of elevated alanine transaminase levels in the presence of antibiotics and nano titanium. The same results have been reported, indicating that SMX impacts hepatocytes and increases enzyme synthesis (Akrami et al., 2013). Hepatomegaly is provoked by SMX and nanotoxicity, and a raised level of ALT is an indicator of it in current study. Antibiotics alone, as well as co-exposure with nano titanium, were found to cause biotoxicity in *Cyprinus carpio* in present study.

In both single and co-exposure with nano titanium, respiratory burst activity was considerably altered in the higher dosage groups (50 and 100 g/L SMX). Only the 100 µg/L SMX group had substantially lower ROS levels than the control group ($p < 0.01$). While co-exposure with nano titanium resulted in substantially higher ($p< 0.05$) ROS values of 1.45 and 0.96 in 50 and 100 g/L treatments, compared to the values of 0.49 and 0.22 in SMX alone treatments (Figure 4.28).

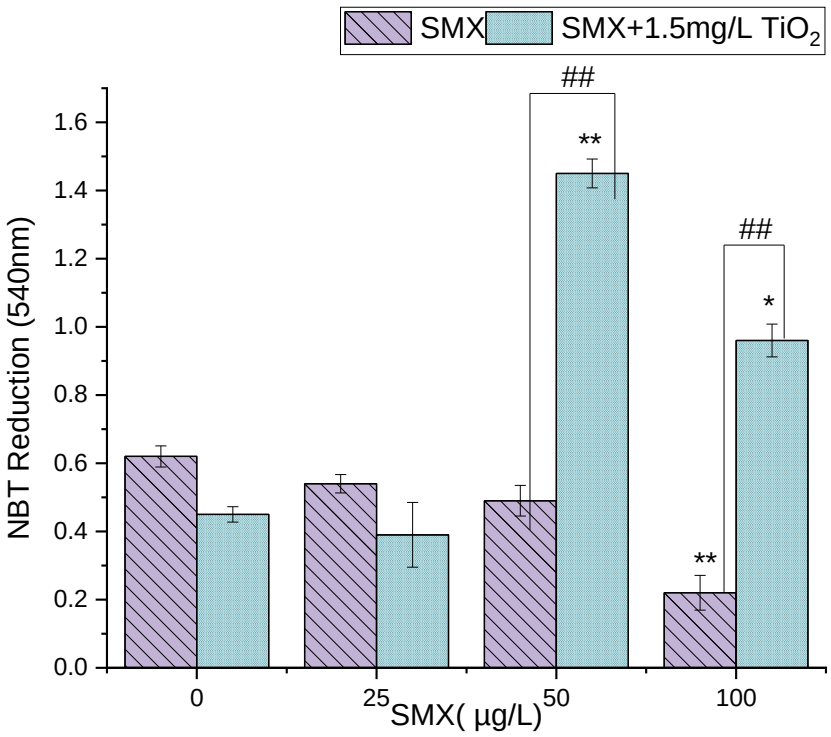


Figure 4. 28 Respiratory burst activity *Cyprinus carpio* being exposed to SMX alone (0, 25, 50, and 100 µg/L) and in the presence of nano titanium (1.5 mg/L) for 96 h. Significance differences ($p < 0.05$) are shown by asterisk (*) vs. control group; and hash (#)vs. the corresponding SMX groups without nano titanium (n = 6).

In fish and other animal models, antibiotics have been proven to cause hematological and biochemical toxicity. However, there is currently little information regarding how co-exposure to SMX and nano titanium affects freshwater fish's immunohematology and metabolic processes. Studies have suggested increased rate of oxidative stress is the key mechanism underlying the bio-toxicity of various pollutants.

Hence, current study investigated the effects of SMX on ROS generation. Results of nitro blue tetrazolium assay showed that SMX triggered ROS generation in fish only at highest dosage tested with reference to control, on the other hand, in the co-exposure groups, there was a significant increase in ROS generation, demonstrating that enhanced SMX uptake

by fish in the presence of nano titanium may be the cause of the elevated ROS formation. The reactive metabolite (SMX-HA) is produced during the metabolism of SMX by the cytochrome P450 isozyme and CYP 2C9. This metabolite is subsequently spontaneously auto-oxidized to produce the extremely lethal metabolite (SMX-NO). Cells exposed to these electrophilic compounds exhibit significant GSH depletion, leading to oxidative stress. SMX-HA can produce ROS during auto-oxidation by reducing an oxygen molecule to form the reactive O2-•, which can subsequently be dismutated spontaneously by SOD to produce H_2O_2. Through the Fenton reaction, the latter is largely converted to the highly cytotoxic OH-•. As a result, SMX metabolism might contribute to oxidative stress. The induced generation of ROS may result in biochemical disruptions in fish because oxidative stress-induced (ROS) has been connected to a variety of abnormalities, including developmental and histopathological alterations. These changes could attribute to the greater toxicity in the co-exposure treatments. The generation of ROS might influence the antioxidant system and if the oxidative stress overcomes the innate immunity and natural defense system, physio metabolic turmoil occurs, which could be demonstrated by changes in biochemical behavior of the fish.

4.3.3. Quantification of nano titanium in fish muscles

The concentration of nano titanium in fish muscles for SMX co-exposure treatments (0, 25, 50 and 100 µg/L SMX and 1.5mg/L nano titanium) was quantified at day 4. The detected concentrations of nano titanium in muscle tissues in co-exposure groups were 0.73 ± 0.01, 0.41 ± 0.06, 0.18 ± 0.02, 0.17 ± 0.03 µg/g in 0, 25, 50 and 100 µg/L groups respectively. No significant difference was observed for the bioaccumulation of nano titanium regarding SMX doses applied in co- exposure treatments.

4.3.4. Quantification of SMX in fish muscles

The results showed that the measured SMX amounts in water at 0 and 24 hours were close to the nominal values. The amount of SMX in exposure media was tested at 0 and 24 hours. The bioaccumulation of SMX alone and co-exposed with nano titanium in fish muscles was assessed at 24, 48, 72, and 96 hours, respectively. The control groups exhibited no SMX peak.

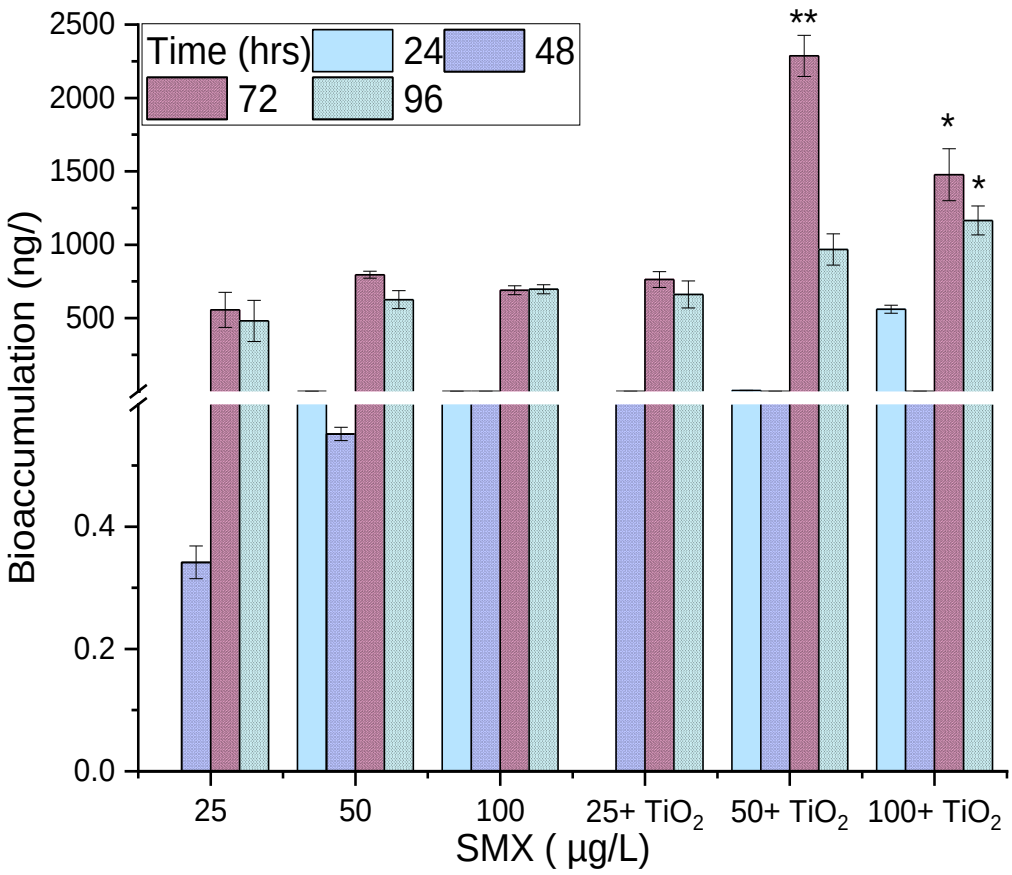


Figure 4. 29 Bioaccumulation of SMX in *Cyprinus carpio* being exposed to SMX alone (0, 25, 50, and 100 µg/L) and co-exposed to nano titanium (1.5 mg/L) at 24, 48, 72 and 96 hrs. Significance differences ($p < 0.05$) and ($p < 0.01$) and are indicated by * and ** respectively. The data is presented as a mean with standard deviation (n = 6)

Figure 4.29 shows the measured amounts of SMX in muscle tissues after different exposure times (24-96 hrs). For 25, 50, and 100 g/L SMX doses alone, SMX concentrations in muscle tissues showed an increasing trend. SMX concentrations at 50 and 100 µg/L dosage groups increased gradually over time in co-exposure with nano titanium, reaching a

maximum of 2286.54 and 1477.2 ng/g respectively at 72 hours. After peaking at 72 hours, SMX absorption in muscle tissues began to decline, with quantifiable values of 968.47 and 1165.41 ng/g at 96 hours for the 50 and 100g/L SMX co-exposure groups, respectively.

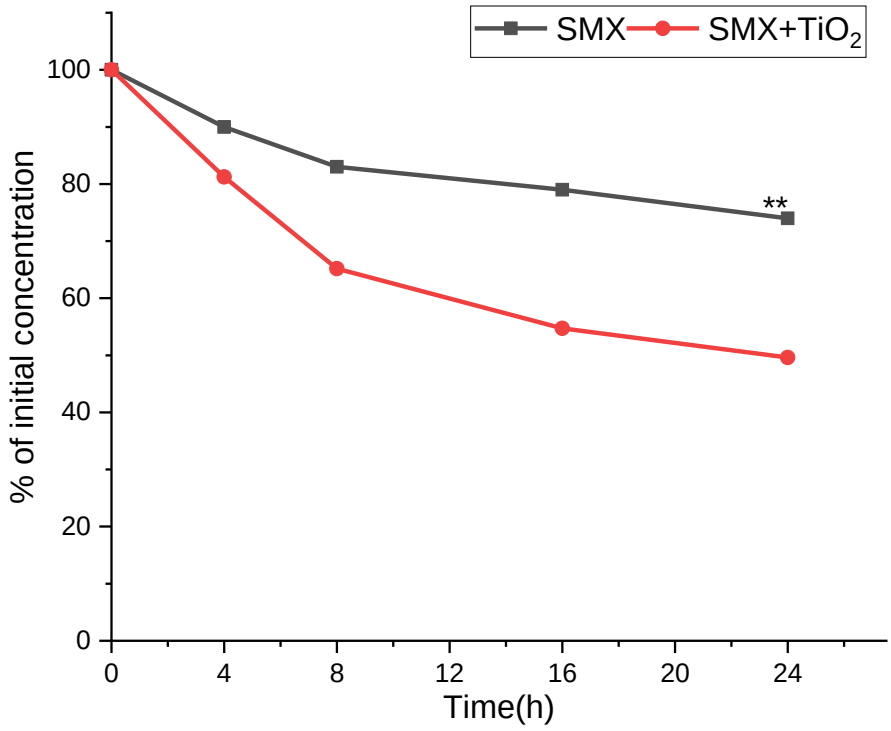


Figure 4. 30 Sorption kinetics of SMX onto nano titanium (1.5 mg/L) with an initial concentration of 25 µg/L. Significant difference is shown as double asterisk (**)

Figure 4.30 depicts the adsorption kinetics of SMX on nano titanium over time. The observed concentration of SMX in the SMX alone groups did not change appreciably over time. SMX concentration was reduced to 49.6 percent of original concentration at 24 h in the groups co-exposed to SMX and nano titanium. The co-exposure group had a significantly reduced SMX concentration compared to the SMX alone group at 24 hour (p<0.001).

In current work, the bioaccumulation of SMX in muscle tissues of *Cyprinus carpio* for four days was recorded ranging from 1.4 to 2286 μg/g in different treatments. Results revealed that SMX could bioaccumulate in the fresh water fish, which is in line with past studies showing that fish exposed to antibiotics in both wild and laboratory environments may accumulate them (Contardo-Jara et al., 2011; Togunde et al., 2012). In co-exposure treatments, enhanced bioaccumulation of SMX in fish muscles was found. For the adsorption reaction, nano titanium has a higher surface area to volume ratio, and our adsorption kinetics experiment confirmed that SMX could be adsorbed by nano titanium. Furthermore, previous research has shown that fish may uptake nano titanium upon water exposure (Zhu et al., 2010 ; Fang et al., 2015). ICP-OES findings showed the presence of nano titanium in muscle tissues of fish. As a result of SMX adsorption on the nanoparticles, *Cyprinus carpio* ingested more SMX. Nano titanium has already been documented in the literature as a carrier of organic contaminants in freshwater organisms. Earlier studies reported that co-exposure to nano titanium increased the bioaccumulation of cypermethrin pesticides, pentachlorophenol herbicides, and BDE-209 polybrominated diphenyl ethers in freshwater fish, resulting in negative effects (Wang et al., 2011; Fang et al., 2015; Li et al., 2018;). As a result, we might speculate that increased pollutant uptake by fish in the presence of nano titanium may have a major impact on the negative effects of certain contaminants.

4.4. Phase 3: To identify (*in silico*) promising phytochemical as as quorum quenching agent in controlling virulence of vibriosis in fish

4.4.1. Protein Structure

3D structures of LuxR protein prepared through swiss model, intFold, i-tasser and phyre-2 were further validated through SAVES analysis based upon Ramchandran plot, ERRAT quality, Whatcheck and Procheck quality factors. IntFOLD model with maximum number of amino acid residues (90.4%) in favorable region, ERRAT quality (82.926) and least number of errors in Whatcheck and Procheck was selected as final protein structure (Figure 4.31).

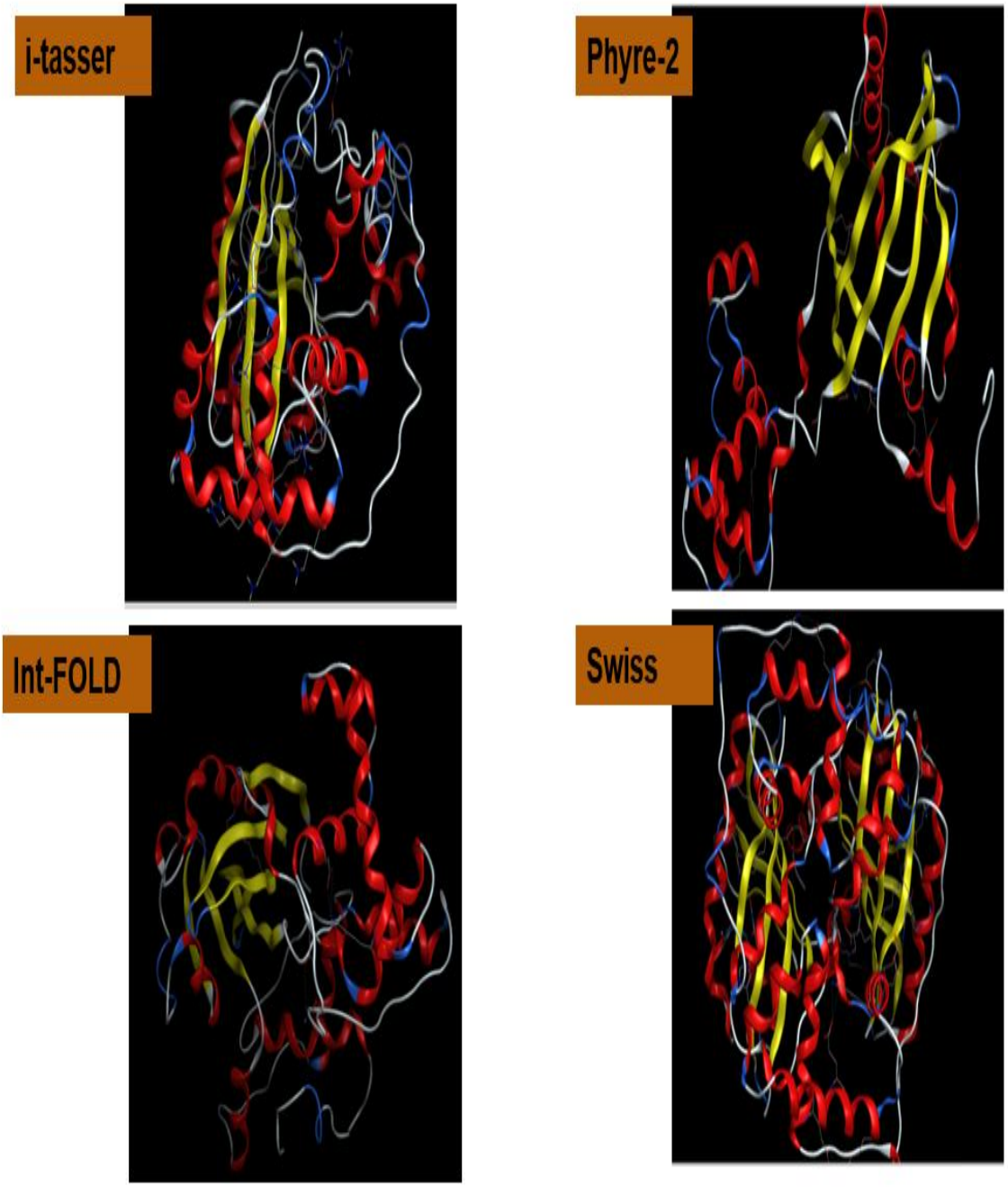


Figure 4. 31 3D structure of LuxR protein prepared by i-tasser, Phyre-2, int-fold and swiss modelling approaches.

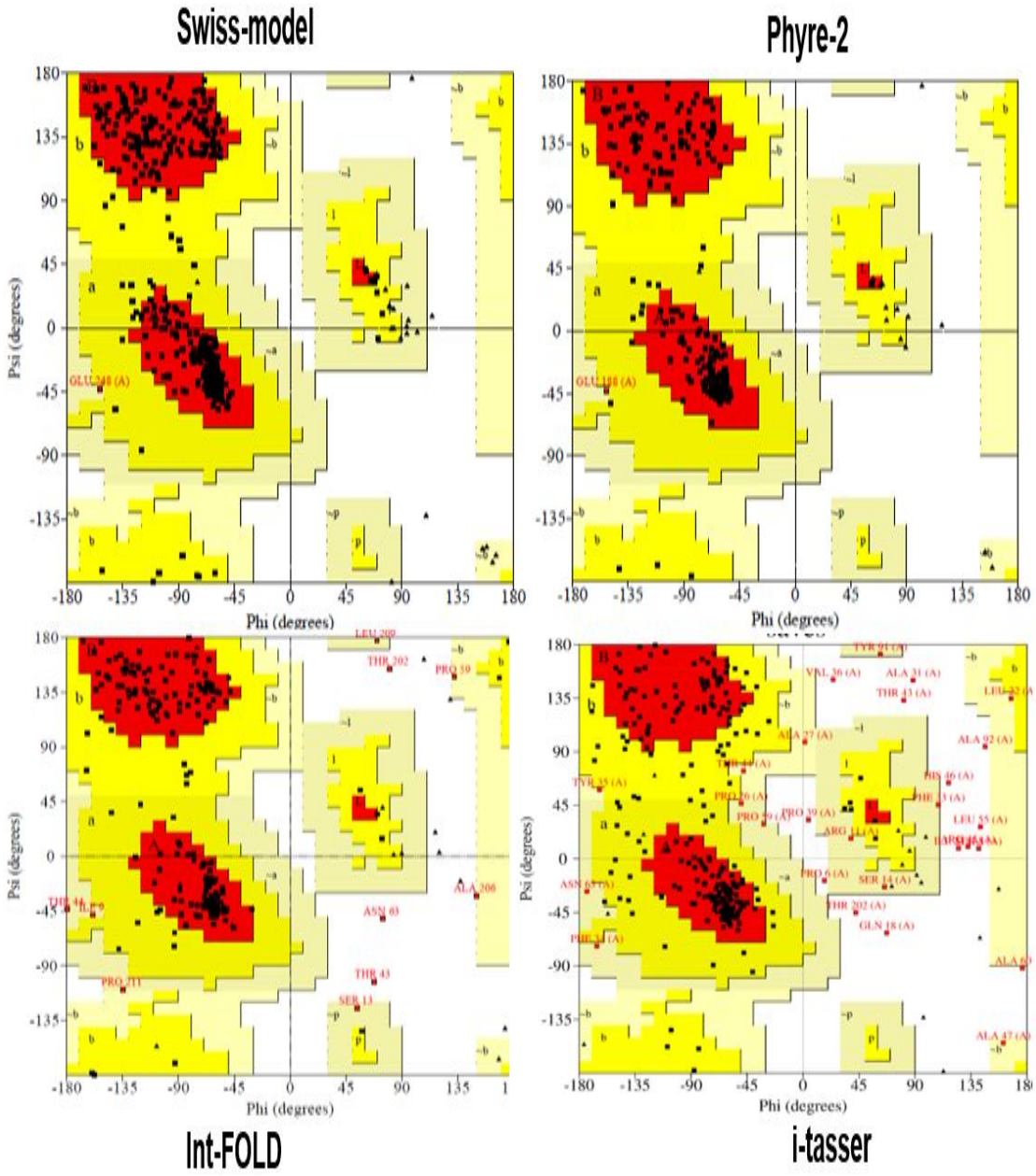


Figure 4. 32 Ramachandran plots showing amino acids in favourable (yellow) regions and non-favourable (red) regions.

4.4.2. Molecular Docking Analysis

For many years, molecular docking has played a significant role in drug discovery. Here,2000 phytochemicals were docked with the target protein LuxR. Table 4.3 summarizes the docking

information for the top ranked poses in top 10 compounds. For each ligand, docking produced ten distinct conformations. For further investigation, only the results for the top-ranked docked complex were retrieved from the table browser. Binding energies for the top ten compounds ranged from -10 kcal/mol to -8.64 kcal/mol. The main conditions for efficient activity are the free binding energy and RMSD refine values as shown in Table 4.3.

Table 4. 3 Results of molecular docking analysis

PubChem ID	**Docking score (Kcal/mol)**	**RMSD refines**
10327115	-9.4132	1.8695
10416673	-9.6317	1.4248
10723258	-9.6712	1.2310
42607999	-9.0869	2.0955
643463	-9.0210	2.3520
5281771	-9.1950	2.1313
5319292	-9.9008	1.2141
73122	-10.1753	1.4680
443028	-8.6423	0.8906
44487196	-8.7923	2.6577
99091	-8.1048	0.9689
53319974	-9.1723	1.7148

4.4.3. Drug Likeliness

The Lipinski rule is employed to examine whether a chemical molecule has properties that would make it likely to act orally in the target body and have a particular pharmacological or biological activity. This rule is employed as a first step filter in virtual screening of

compound libraries to swiftly reject lead candidates with poor physicochemical qualities (Rajamanikandan et al., 2017). According to Lipinski's proposal, potential drug candidate must have the following features (Ro5): (1) less than 5 hydrogen bond donor; (2) less than 10 hydrogen bond acceptors; (3) molecular weight less than 500 Da; and (4) logP value less than 5. Here, out of ten lead compounds identified from docking results only 5 compounds with PubChem IDs: 53319974, 42607999, 99091, 443028, 44587196 passed the Lipinski's rule of 5 test, implying the fact that they have a better chance of becoming a successful medicine.

Table 4.4 Lead compounds on basis of Lipinski rule of 5

Pubchem ID	**Violations**
53319974	Yes; 0 violation
42607999	Yes; 0 violation
99091	Yes; 0 violation
443028	Yes; 0 violation
44587196	Yes; 0 violation

4.4.4. ADME/T Selection

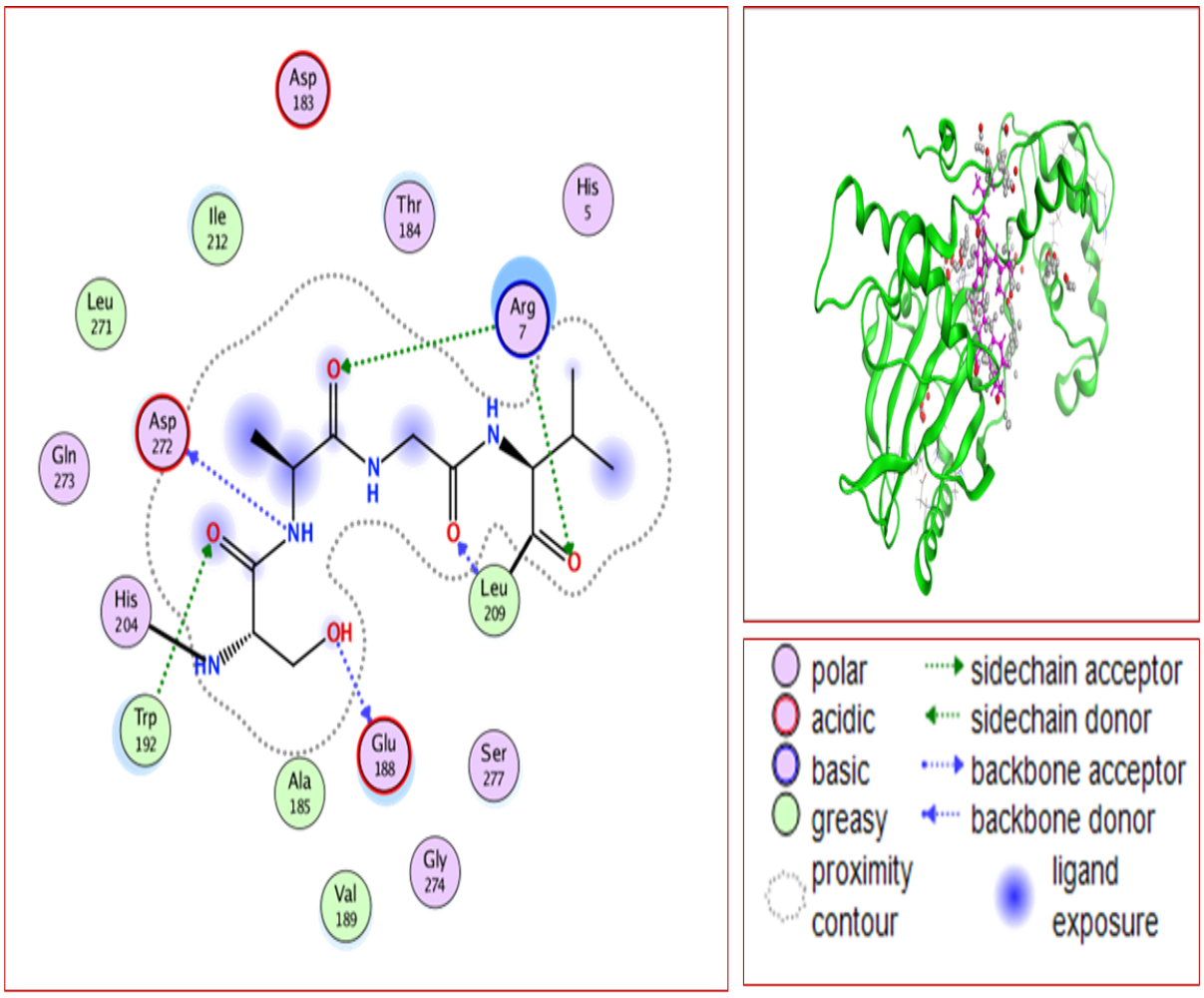
ADME/T qualities play a significant part in drug screening when drug-likeness is determined by analysing the physicochemical properties (Lipinski Ro5) and structural aspects of current drug candidates (Reddy et al., 2013). So, we used the ADMET selection after evaluating other drug-likeness features. During the design of a pharmacological molecule, it is vital to forecast the movement of the medication in the body. The ADMET characteristics of a medicine are assessed throughout the early phases of drug development. The process of a medicine being absorbed into the circulatory system is known as absorption. The diffusion of the medicine across the cell membrane barrier into different tissues, body fluids or organs, is referred to as distribution. The initial (parent) substance is transformed into new chemicals termed metabolites during metabolism. Redox enzymes,

like CYP3A4, are responsible for the drug metabolism in the liver. The removal of the initial form and metamorphosis is referred to as excretion (Lipinski et al., 1997). Based on ADMET analysis final compound PubChem ID 42607999 showed zero violation appearing to be the best drug candidate for vibriosis with appropriate pharmacokinetic characteristics and low toxicity in terms of absorption, distribution, metabolism and excretion to successfully pass the clinical trials.

Table 4. 5 ADMET profiling of drug like parameters of candidate compounds

Models	**Ideal Case**	**53319974**	**42607999**	**99091**	**443028**	**44587196**
Blood-Brain Barrier	-ive	+ve 0.78	**-ive 0.23**	+ive 0.95	+ive 0.95	+ive 0.90
Human Intestinal Absorption	+ive	+ive 0.99	**+ive 0.98**	+ive 0.99	+ive 0.99	+ive 0.94
CYP3A4 substrate	+ve	+ve 0.72	**+ve 0.65**	-ive 0.56	-ive 0.66	+ve 0.80
CYP3A4 inhibition	-ive	-ive 0.70	**-ive 0.86**	+ive 0.70	+ive 0.70	-ive 0.60
AMES Toxicity	-ive	-ive 0.73	**-ive 0.62**	+ive 0.66	+ive 0.66	-ive 0.81
Carcinogenicity	-ive	-ive 0.90	**-ive 0.88**	-ive 0.88	-ive 0.88	-ive 0.87
Total Violations		01	**00**	04	04	01

4.4.5. Molecular dynamic simulation of lead compound with LuxR compound



(5,7-dihydroxy-2-(8-hydroxy-2,2-dimethylchromen-6-yl)-6,8-bis(3-methylbut-2-enyl)-2,3-dihydrochromen-4-one (Pub chem ID 42607999)

Figure 4. 33: 2-dimensional (left) and 3-dimensional (right) interaction of lead compound PubChem I D 42607999 with LuxR protein showing different types of bonding between amino acid residues

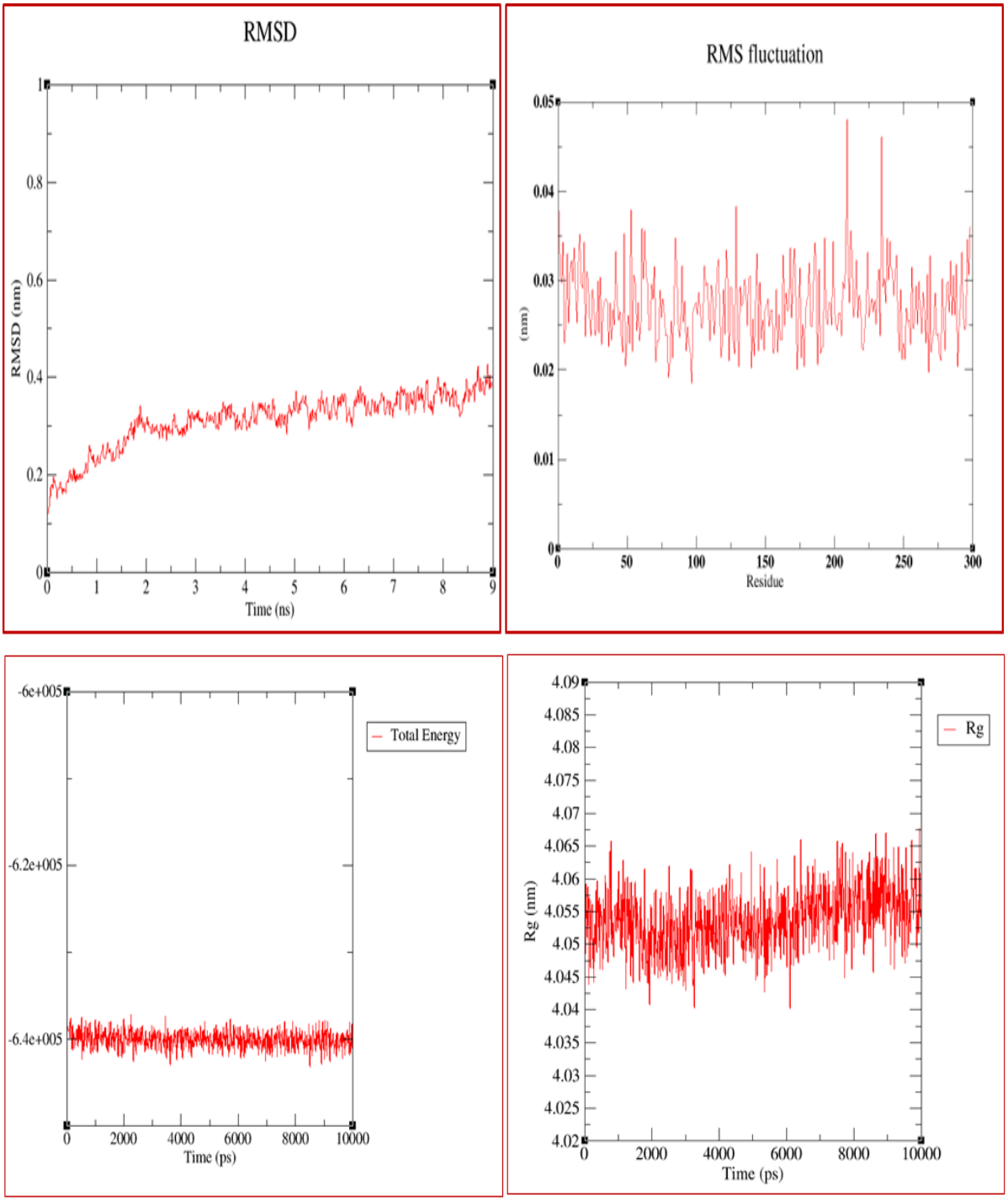


Figure 4. 34 The root mean square deviation (RMSD), root mean square fluctuation (RMSF), total energy and radius of gyration (Rg) of the LuxR protein-42607999ligand complex over the simulation time.

2D and 3D interactions of lead compound with LuxR protein are shown in Figure 4.33. Molecular docking results showed the involvement of different amino acid residues including Arg 7, Asp272, Leu209, Glu188, Trp192 and His204 in different types of bonding interactions with ligand. MD simulations were further performed for lead compound 42607999-LuxR protein docked complex. To check the stability of the structure, root-mean-square deviation (RMSD), root-mean-square fluctuations (RMSF), radius of gyration (Rg) and total energy plots were generated throughout a course of 10 ns. The backbone RMSD from the beginning structure (Figure 4.34) was determined over the course of the trajectory to assess the conformational changes in the LuxRprotein-42607999ligand complex. The RMSD values of the protein-ligand complex are shown in Figure 4.34. The figure shows that the RMSD of targeted complex initially increased until 2 ns, which could be attributed to the lack of restraint throughout the MD simulation generation phase. Average RMSD change was found to be 0.2-0.4 Å with an SD of 0.05. It was discovered that 2 nanoseconds were enough to reach an equilibrium state. The minor drifts in the RMSD plot revealed the protein structural flexibility during the simulation procedure. However, after 2 ns of simulation, the RMSD fluctuations converged, indicating that the protein structure conferred stability. Another crucial factor for determining the rigid and flexible parts of the protein complexes is the RMSF evaluation. Amino acids involved in RMSF fluctuations were identified as Ala60, Ala196, Arg238 and Leu266. Radius of gyration (Rg) refers to the compactness of the complex during simulation thus reflecting its stability. The steadier Rg value is, more stable is the structure. Our results showed an average Rg value of 4.05 nm with no distinct fluctuations during simulation period thus indicating the compactness of protein-ligand complex. Moreover, total energy of the system was calculated as -6.4e+005. Higher (in negative) shows stronger interaction. It must be lower to make a stable interaction. Total energy in negative values indicated that internal interactions surpassed systems internal strains and repulsions referring to a stable system.

Chapter 5

CONCLUSIONS AND RECOMMENDATIONS

5.1.1. Toxicity assessment of SMX on cultivable fish *Cyprinus carpio* using multiple endpoints

Cyprinus carpio, when chronically exposed to SMX at nominal concentrations, exhibits significant changes in respiratory burst activity, hematological profile, and biochemical parameters. This study also reports bioaccumulation and bioconcentration potential of commonly used antibiotic, SMX in edible fish tissue at environmentally realistic concentrations. These results indicate that exposure concentrations have a significant impact on their toxicokinetic processes and could substantially affect antibiotic bioaccumulation and bioconcentration in common carp. Furthermore, an increasing trend in oxidative stress and higher organ pathological index with respect to time revealed ecotoxicological effects of SMX upon chronic exposure. Based on the results of this study, we may be able to provide baseline data on how antibiotics might affect non-target organisms, particularly fish, when they are exposed for a prolonged period. Therefore, more attention is required to prevent the excessive use and release of antibiotics into aquatic biomes. Further, this biomarker approach may be used to assess antibiotic risks in aquatic environments. The molecular toxicity of SMX could aid in understanding its mechanism of action.

5.1.2. Toxicity assessment of SMX in early staged zebrafish (*Danio rerio*) using multiple biomarkers.

According to a recent study, SMX is acutely toxic to zebrafish larvae and developing embryos at higher concentrations. SMX caused hatching delay, affected survivability, and induced deformities at higher concentrations tested. On the completion of exposure, at the maximum dosage tested (5000 µg/L), about half of the population had died. The pericardial and yolk sac edema, bent tail, and spinal curvature were the most often seen malformations. No change was observed for oxidative stress, mitochondrial bioenergetics, and reactive oxygen species. The expression of genes linked to inflammation and apoptosis was changed by environmentally relevant amounts of SMX. Observed delay in hatchability

and various malformations observed in zebrafish embryos are most likely the result of disturbed apoptosis rate, inflammation, and reduction in innate immunity of healthy zebrafish larvae. Therefore, additional effort is needed to stop the overuse and discharge of antibiotics into aquatic ecosystems. The current study provided baseline information for the safety and risk evaluation of antibiotics and highlighted potential ecological concerns of antibiotics in the aquatic biome.

5.1.3. Combined toxicity of SMX and titanium dioxide nanoparticles on immunohematology, bioavailability, and biochemical status of *Cyprinus carpio*

To better understand the environmental fate of antibiotics in the presence of nanoparticles, data on bioaccumulation and ecotoxicity of SMX in fish has been investigated. In the current work, nano titanium increased SMX bioaccumulation, resulting in increased biotoxicity and metabolic disruption in *Cyprinus carpio*. According to the findings, the presence of nano titanium in the environment may alter the behaviour and bioavailability of co-existing antibiotics. Current research emphasises the potential threat of nanoparticles in the aquatic environment, as well as their risk of interacting with other contaminants, which must be considered during environmental risk assessments.

5.1.4. To identify (*in silico*) promising phytochemical as as quorum quenching agent in controlling virulence of vibriosis in fish

A high throughput in silico technique reduces the time and money required on drug production and testing prior to clinical trials. The current study identified PubChem ID 42607999 as a potential phytochemical with significant binding capability to the LuxR protein and drug-like properties. This discovery could facilitate the development of new, natural drugs that could effectively combat vibriosis, a common and destructive fish disease that significantly affects the aquaculture industry. To corroborate the study's efficacy, in vitro and in vivo research are strongly advised.

5.2. Recommendations

1. Employing a multi-omics approach (including transcriptomics, lipidomics, metabolomics, meta-transcriptomics, and epigenetics), in tandem with traditional biomarkers, could develop a more comprehensive understanding of contaminants' molecular mechanisms. This enhanced understanding can provide critical insights

into how contaminants affect the aquaculture ecosystem and how their impacts can be mitigated, contributing to more effective management of aquaculture.

2. The current study highlights the importance of considering the role of prevalent nano materials in the aquatic environment as carriers of other pollutants for environmental risk assessment and hazard mitigation. Given the growing use of nano materials in various sectors and their potential interactions with different pollutants, more in-depth experiments are needed to explain the toxicokinetic and toxicodynamic linkages between nanoparticles and organic pollutants.
3. This study's findings could guide the discovery and development of new natural drugs with improved inhibitory activity against vibriosis. To verify the effectiveness and safety of the identified phytochemical, more in vitro and in vivo studies are strongly advised. Furthermore, commercial production of phytochemicals as an alternative to conventionally used drugs is an important consideration in tackling the problems faced by the aquaculture industry.

5.3. Contribution to Knowledge

This research elucidates the environmental impacts and ecotoxicology of SMX and nano titanium on aquatic life, presenting innovative approaches to assess these effects. Additionally, it identifies a promising phytochemical for treating vibriosis in fish, offering potential advancements in aquaculture and environmental risk mitigation.

5.4 Impact Statement

The findings provide regulators and governments with important data on the environmental risks posed by SMX and nano titanium in aquatic environments. This information is crucial for formulating appropriate regulations on antibiotic and nanoparticle usage and disposal. The identified phytochemical also presents a potential alternative for antibiotic usage in aquaculture, potentially reducing antibiotic pollution and improving industry sustainability.

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