MICROBIAL DEGRADATION OF SULFAMETHOXAZOLE AND EVALUATION OF BIOTOXICITY POTENTIAL IN FISH

By

MAIMOON ZAFAR

(Reg#00000170495)

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Submitted by

Maimoon Zafar

Has been found satisfactory for the requirements of the degree of

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Supervisor: _____

Dr. Imran Hashmi

Professor

IESE, SCEE, NUST

Member: _____

Dr. Muhammad Arshad

Associate Professor

IESE, SCEE, NUST

Member: _____

Dr. Habib Nasir

Professor

SNS, NUST

Member: _____

Dr. Asma Jamil

Senior Assistant Professor

Bahria University Islamabad

This Thesis is dedicated to my Parents and Family whose continuous support and prayers are always with me whenever and wherever required

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Maimoon Zafar

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LIST OF ABBREVIATIONS

API	Analytical Profile Index
BLAST	Basic Local Alignment Search Tool
CFU	Colony Forming Unit
COD	Chemical Oxygen Demand
DNA	Deoxyribo Nucleic Acid
DO	Dissolved Oxygen
EC	Electrical Conductivity
HCT	Hematocrit
HB	Hemoglobin
mg/L	Milligram per Liter
MCV	Mean Corpuscular Volume
MCH	Mean Corpuscular Hemoglobin
MCHC	Mean Corpuscular Hemoglobin Concentration
MSM	Mineral Salt Medium
NB	Nutrient Broth
NCBI	National Center for Biotechnology Information
PCR	Polymerase Chain Reaction
rRNA	Ribosomal Ribonucleic Acid
TDS	Total Dissolved Solids
µS/cm	Microsiemens per centimeter
SMX	Sulfamethoxazole
RBC	Red Blood Cells
WBC	White Blood Cells

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ABSTRACT

Ongoing industrial development, urbanization and agricultural trends have given rise to a series of environmental problems amongst which degradation of water bodies is an area of great concern. Various types of pharmaceuticals are detected which probably enter the water bodies through runoff water from poultry sheds, sewage and aquaculture use of antibiotics. Sulfamethoxazole (SMX) is amongst those antibiotics which are extensively used as human and veterinary medicine. This study aims to isolate and identify potential environmental bacteria to degrade SMX and to assess the effects of the antibiotic towards non target organisms such as fish. Dominant strains of bacteria were isolated from five tributaries (Ratahutar, Nupur, Jinnah, Shahdara stream and Korang River) that converge to form Rawal Lake. Water Samples were collected from upstream and downstream points. All samples values were under the permissible limits for TDS and turbidity. Degradation analysis revealed potential strains *Pseudomonas aeruginosa* was able to degrade SMX and indicated more than 90% removal in MSM and nutrient broth. Later the strain was used to evaluate degradation potential in the presence of fish along with SMX to evaluate toxicity. The results indicated removal 88.52 % of SMX from water. Fish exposed to SMX presented different effect on behavior and body length, weight, hematology and biochemistry which indicated behavioral effects of SMX on fish. During exposure, blood values such as Red blood cell count, Hemoglobin, Hematocrit were found to decrease, where as White blood cell count was found to increase 26.05 10^{-6}/µL. Likewise, other blood parameters such as mean corpuscular volume, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration also indicate decreasing trend, when fish is exposed to SMX. A significant decline was also noted in glucose and protein levels with time ($p<0.05$). The result from the above study suggests that bacterial strains isolated form stream samples may be used to biodegrade SMX. Bacterial strains used to degrade SMX have no significant effect on fish and may thus have potential for bioremediation for sulfonamide polluted water, whereas SMX has significant effect on fish population and affects them adversely. The alterations of these parameters led to the conclusion that these parameters may be used as biomarkers in monitoring SMX toxicity in aquatic organisms.

1. INTRODUCTION

1.1 BACKGROUND

One of the basic and essential building blocks for survival is water without which no livings organisms can survive and are mostly dependable on its availability. Only about 2.5% of all water on earth is freshwater, the rest 97.5% is salt water. Most of the freshwater is in ice form and snow covers in Antarctic and Arctic. About 30% of freshwater is present beneath the ground and only 0.3% is available on ground which can be easily used for human needs and other living organism .This small amount of groundwater and surface water is under stress due to uncontrolled pollution and the increasing demand. Among others, one major growing concern is the effect these polluted water has on all aquatic bodies and how they inevitably affects humans (Ghumman, 2011).

Addition of unwanted contaminants to water bodies resulting from anthropogenic means, which deterioration the water quality results in water pollution, these contaminants are harmful to living organism and are a cause of wide range of diseases throughout the world. Due to rapid increase in population, urbanization and the unsustainable use of water resources there is an immense stress on water availability (Azizullah *et al*., 2011).

Rapid industrial development and growing agricultural trends in the 21st century are giving rise to contaminates that are threating not only the environment but are also a concern for public health, a range of different chemicals such as pharmaceuticals and other personal hygiene and care product are among these contaminants and due to extensive usage these contaminants are present in the environment than ever before and are becoming more difficult to remove due to

their non-biodegradable nature (Gavrilescu *et al.*, 2015). Changes in environmental health account for up to 33% of total disease (David, 2003). Studies have shown that the living organisms exposed to the ever increasing non-biodegradable toxic contaminants are a major source (Khan and Nicell, 2015).

Any substance that is used to diagnose treat or prevent a disease or symptom is termed as a pharmaceutical (Enick and Moore, 2007). Pharmaceuticals have a wide range of structures functions and mode of action (Zwiener, 2007). Most of the pharmaceuticals used today are making their way back to environment and are a cause for pollution (Jones *et al*., 2005).

With rapid growth and development pharmaceutical industry has increased with time reflecting the increasing demand for drugs. These drugs are ultimately excreted or disposed off and end up in the environment, thus accumulation of these drugs is recognized as a huge threat (Gauthier, H, 2008). The presence of antibiotics in water bodies as a result the development of antibiotic resistant microorganisms have come to light as a major issue of the modern world and has raised potential concern (Fent *et al*., 2006 ; Segura *et al*., 2009)

The widespread overuse and misuse of antibiotics in humans, animals and agriculture is considered to be closely linked to the growing increase in antibiotic resistance (Levy and Marshall, 2004). Antibiotic burden may be also attributed to several other sources that include waste from hospitals, sewage treatment plants waste and inappropriate disposal (Kemper, 2008). The presence of antibiotics in water bodies may lead to the development of antibiotic resistance genes, making the environment a reservoir for pathogens (Khan *et al*., 2013). Antibiotics in water bodies also effects non target organism such as fish leading to suppressed immune systems and abnormal growth and related defects (Gaffney *et al*., 2016; Bai *et al*., 2017).

In aquaculture sector antibiotics prevent different kinds of infectious diseases. The use of antibiotics has increased since the last few years. These compounds have been known to persist in the environment due to their polar nature and low volatility (Brausch *et al*., 2012). The processes adopted in WWTP are not designed for the purpose of antibiotic removal (Chu *et al*., 2015). Removal of these antimicrobial agents form water bodies is a challenging task. Many conventional treatment processes fail to remove these micro pollutants because the treatment plants are not designed for the said purpose. Processes such as oxidation, adsorption or MBR are efficient for removing antimicrobials but are not feasible in most urban WWT plants. The removal of xenobiotic by the means of microorganisms via biological processes provides several advantages, the most important being regarded as a green technology with few impacts on the environment and low cost (Reis *et al*., 2014). Biodegradation of compounds such as antibiotics is a complex process and involves different biochemical reactions. Many enzymes are involves in the process, and is carried out by the use of microorganisms which may be exogenous or indigenous. A few studies have identified some bacterial strains that are capable of degrading antibiotics. The most reported genera being *Rhodococcus, Bacillus* and *Pseudomonas* reported by Gauthier and Larcher in 2010 and 2012 respectively. The studies indicate that the genera were able to co-metabolize SMX in the presence of glucose. Other organisms were also reported later on isolated form enriched activated sludge. The genera's include *Archomobacter, Ralstonia, Brevundimonas, Variovorax, and Microbacterium* (Bouju *et al*., 2012; Herzoz *et al*., 2013; Jiang *et al*., 2014). A wide range of bacteria are capable to degrade antibiotics may be attributed to the evolution of the bacteria of the degradation pathway or development of resistant mechanisms which may help in developing practical applications. These bacteria or their enzymes may be

used to develop low cost effective technologies and membranes that may help reduce the spread of antibiotic resistance (Reis *et al*., 2014).

1.2 Present Study

To achieve sustainable development, environmental preservation is a key factor. Currently the development of cheaper green technologies is considered to play a vital role to achieve the goals for a sustainable environment and provides means to conserve the existing one. Studies indicate that microbial degradation may be a useful technique for the removal of contaminants form the environment. Mostly bacteria works naturally in environment but bacteria may also be be encouraged via modifications to degrade contaminants like antibiotics. The presence of sulfamethoxazole has been reported throughout the world and their toxic effect towards aquatic life and humans. The present study was aimed at investigating the ability of isolated bacterial strains to degrade SMX in nutrient broth and minimal salt media, using SMX as the sole carbon source. Also keeping in view the harmful effects these antibiotics have on aquatic life such as fish the eco-toxicological effects of SMX was also conducted and health impacts of SMX were observed in Fish

1.3 Objectives

Following are the objectives of the research work for the study

1. Isolation, Identification and characterization of SMX degrading microorganism.
2. Determination of biodegradation potential of isolate through COD.
3. Degradation of SMX and bio toxicity evaluation in fish.

2. LITERATURE REVIEW

Water is an essential component of life. Two third proportion of earth surface is covered by marine water that is unfit for human use. The available form that may be used for drinking purpose is fresh water. It can cater human needs only if it has a high quality as it supports physiological activities of biological cell. Total fresh water present on earth is only 3%, out of which only 0.01% is available for use (Hinrichsen and Tacio, 2002). This fresh water is under stress due to high growth in population, and increased demand. In developing countries the condition is aggravated due to lack of management and professionals (PCRWR, 2005). Rivers, oceans, lakes and ground water are affected by water pollution. This is not only damaging for an individual specie or population but it also adversely affects biological communities. Adequate treatment of pollutants is necessary in order to remove harmful constituents. Untreated water when discharged into water bodies, causes pollution. It is a matter of concern as it leads to outset of diseases claiming deaths of over 14,000 people every day. In developing countries the condition is worse than developed countries. The occurrence of pharmaceuticals compounds in water bodies has increased in the last decade. These compounds are considered emerging pollutants due to their persistent nature and sub lethal and chronic effects on non-target species (Aissaoui *et al*., 2016).

2.1 PHARMACEUTICALS IN WATER

Pharmaceutical are defined as any over the counter prescription that can be used for the treatment of human and animal diseases. Over the past few years these compounds have been detected in water bodies, sediments and soil in concentrations that may be harmful for the environment and aquatic organisms, after use these pharmaceuticals reach back the environment

through different sources. The main sources as depicted in Fig 2.1 includes sewage form domestic hospital and industrial facilities, WWTPs and leachate and are detected in ng/L to µ/L range in surface water. Pharmaceuticals are often not completely removed by conventional WWT processes and thus make their way back to the environment. One major source of therapeutic drugs is when they are excreted by the body post use and released into sewage. The water is then used for irrigation or the treated solids are used as fertilizers where the liquid effluent is released into environment. Groundwater contamination can also occur through leaching form soil and contaminates drinking water and inevitably becomes a part of the food chain. Fig 2.1 illustrates sources of environmental contamination with pharmaceuticals (Ebele *et al*., 2017). Hospital wastewater; a major source also releases pharmaceuticals into the environment.

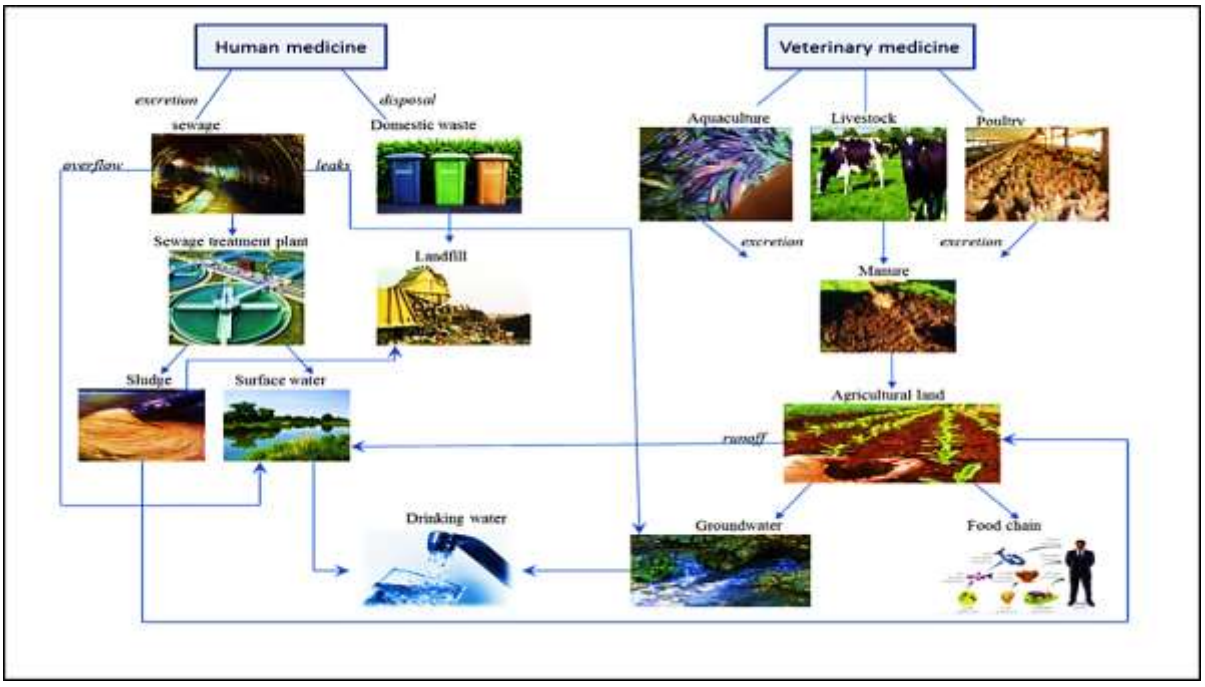


Fig 2.1: Common Sources of pharmaceuticals and how they end up in water (Ebele et al., 2017)

HWW is a major contributor to surface water contamination in developing countries like Pakistan. Most of the HWW released into sewage lines with no prior treatment (Ebele *et al*., 2017).

Antibiotics is a class of pharmaceuticals used to inhibit growth of bacteria. They are widely used for humans and veterinary disease, and due to their extensive use are released into the environment at an alarming rate and have been found in varying concentrations .Due to the uncontrolled use, antibiotics have been detected throughout the world and the situation is worse in the developing countries. Antibiotics detected in surface water in the UK include lefopramina tramadol, paracetamol coltrimazole, trimethoprim, sulfamethoxazole diclofenac in concentration of ng/L (Ebele *et al*., 2017).

In China about 19 antibiotics were detected in surface and ground water sample belonging to sulfonamide, tetracycline, macrolides and fluoroquniolones in ng/L concentrations. Some antibiotics were in higher concentrations on groundwater than in surface water. These highest concentration detected was erythromycin (381.5 ng/L) (Tong *et al*., 2014). In Vietnam antibiotics like sulfamethoxazole, sulfadiazine, trimethoprim, and enrofloxacin were detected in surface and ground water in concentration ranging from 4-21 ng/L. Although these concentration are not high but with time may cause harm to aquatic organism and damage the environment and pose risk to humans (Giang *et a*l., 2015).

2.2 SITUATION IN PAKISTAN

The presence of pharmaceuticals in water is considered to be a threat to not only the environment but also indirectly to human health. A wide range of antibiotics and other pharmaceutical drugs have been detected throughout the world in surface and groundwater which gives rise to new and

emerging problems such as eco-toxicity and new antibiotic resistant bacterial pathogens. Pakistan is among the largest producers and consumers of pharmaceutical products and the use of antibiotics has increased. Water bodies in Pakistan are contaminated by pharmaceuticals as reported by many researchers. The problem has increased and become more serious because of its high consumption rate. Tetracycline and fluoroquniolones are amongst the most consumed throughout the country. Antibiotic contamination of ofloxacin, ciprofloxacin, levofloxacin, ox tetracycline, and doxycycline was detected in underground water and soil near pharmaceuticals industry in Lahore ranging in concentration 0-0.80 ng/L (Hussain *et al.*, 2017).

Wastewater sludge from hospitals in Lahore was analyzed which showed the presence of ofloxacin, ciprofloxacin, sparfloxacin, moxifloxacin and gemifloxacin with concentration ranging from 0-224 µg/L in waste water and 0-219 µg/L in sludge (Ashfaq *et al*., 2016). Wastewater analysis of indicated the presence of six pharmaceuticals in Gujrat, Pakistan. Highest concentration was recorded of paracetamol 696 ng/ml (Ashfaq *et al*., 2017).

Samples collected from Ravi River that receive municipal wastewater and hospital wastewater indicated the presence of ofloxacin before mixing ranging from 7.31-39.13 µg/L in HWW and concentration of ofloxacin ranged from 0.26-0.43 µg/L in MWW and the concentration increased to 0.54 -1.29 µg/L after mixing (Ahmad *et al*., 2012). Hospital effluent and soil examined in Peshawar indicated the presence of cephtazidime and cephradine in wastewater effluent, soil and drinking water in concentrations of 0.47 µg/ml of cephtazidime and o.64 µg/ml of cephradine (Hassan *et al*., 2015). These concentrations in water bodies are an evidence of high rate of antibiotic consumption and improper disposal of wastewater activities in nearby areas which may have detrimental effects in other life forms.

2.3 PHARMACEUTICALS IN RAWAL LAKE

Rawal Lake the main drinking water reservoir for Islamabad and Rawalpindi; located near Margalla Hills National Park. A number of large and small streams contribute to its total storage.

Analysis of water samples from major rivers in Lahore and Rawal dam indicated high concentration of sulfamethoxazole, a member of sulfonamide group, in them depicted in Fig 2.2. Rawal Lake was also found to have relatively high concentration of this antibiotic. Frequent use, high stability and hydrophobicity of these compounds are the possible reason for their abundance in water. These substances interact with the target molecules in an organism that's why they have a potential to negatively impact the animal biota. The presence of antimicrobials into the environment also leads to the development of multidrug resistant strains thus there is a dire need to remove such contaminants from the environment (Khan *et al*., 2013).

Fig: 2.2 SMX residues found in Lahore 1100-2700 ng/L and Rawal dam 14 ng/L (Khan et al., 2013)

2.3.1 Sulfonamides

Sulfonamide drugs are widely used as antibiotics against different diseases thus it is very likely they enter into the environment in large quantities. Sulfonamides are derived from sulfanilamide, these are synthetic antimicrobials are commonly used in aquaculture, agriculture, and humans for the treatment of diseases like respiratory and UTI's (Holm *et al*., 1995; Sukul and Spitteler, 2006). Sulfonamides are broad spectrum antibiotics used to inhibit pathogens by inhibiting DNA replication, protein and cell wall formation by inhibiting bacterial folic acid synthesis. Groups of antibiotics based on their mode of action are depicted in Fig 2.3.

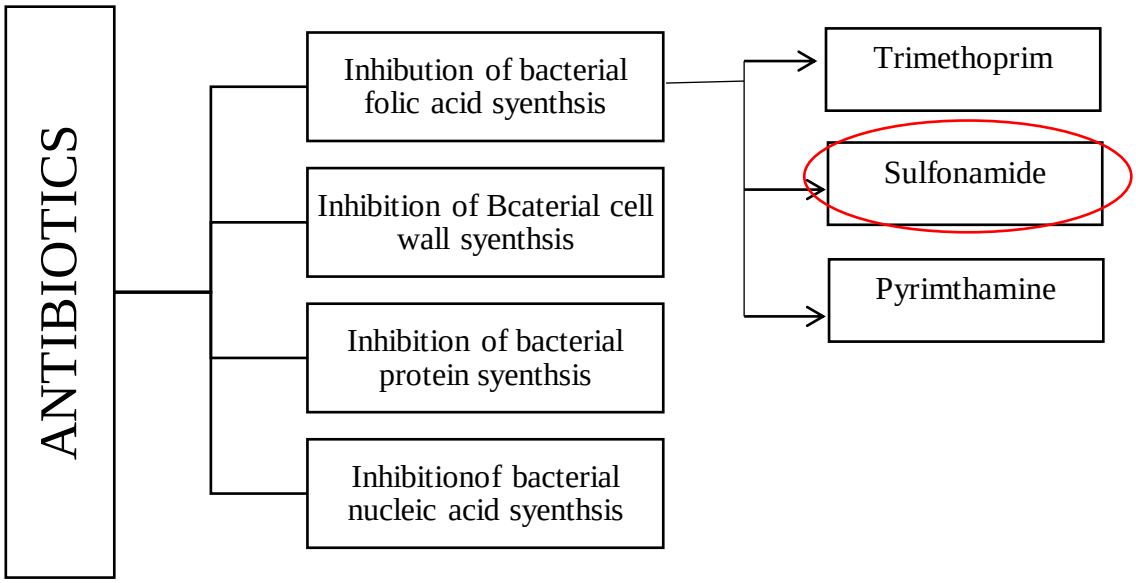


Fig 2.3: Classes of Antibiotics

2.3.2 Mode of action

Sulfonamide has a bacteriostatic effect on microorganisms and prevents growth by reducing the synthesis of folic acid (Craig and Stitzel, 1994). These antibiotics are reported to inhibit the enzyme carbonic anhydrase which causes different diseases. These antibiotics may also be used to treat glaucoma, obesity, cancer and osteoporosis (Supuran 2008, 2011). When taken by humans or animals only some part of the antibiotic is taken up by the body, and the rest is expelled through feaces, or bodily fluids such as urine in the form of as metabolites or in its original form (Herber, 2002).

2.4 SELECTED ANTIBIOTIC-SULFAMETHOXAZOLE

Sulfamethoxazole, or 4-Amino-N-(5-methyl-3-isoxazolyl) benzene sulfonamide Fig 2.4 depicts the structure of a sulfonamide drug. Sulfonamides have been, and continue to be, extensively used because of their low cost of production, relatively low toxicity and their efficiency (Connor, 1998). Sulfamethoxazole used to be prescribed as a single antibiotic, but the development of antibiotic resistance has changed the way it is prescribed today.

SMX is a para-aminobenzoic acid (PABA). It is an isoxazole compound having a methyl substituent at the 5-position and a 4-aminobenzenesulfonamido group at the 3-position (Fig 2.4) (www.ebi.ac.uk)

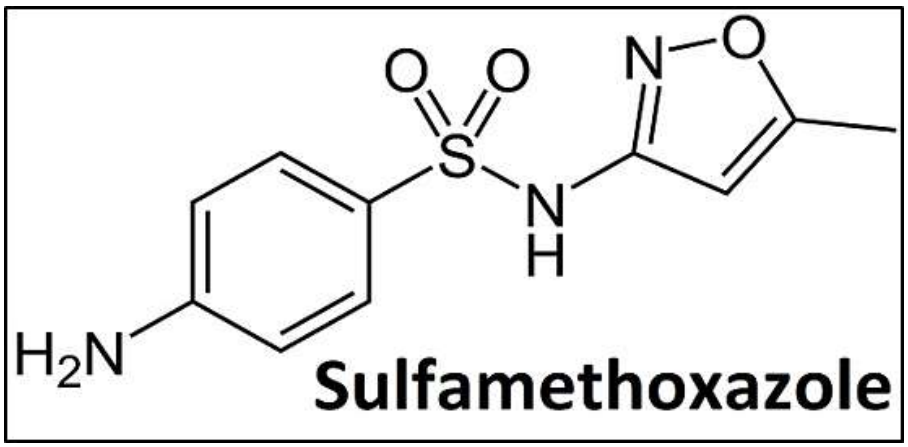


Fig 2.4: Structure of Sulfamethoxazole

Sulfamethoxazole is combined with trimethoprim, to enhance the antibacterial efficiency (Karpman and Kurzrock, 2004). Trimethoprim, is a derivative of pyrimidine indicated in Fig 2.4. The drugs separately are bacteriostatic, meaning that they hamper the growth of the bacteria, but together they have a synergic effect and they become bactericidal (Connor, 1998). Sulfamethoxazole, which is less active than trimethoprim, enhances the activity of the latter. The combination of the drugs (SMX+TMP), called co-trimazole, is fixed in a ratio of 5 sulfamethoxazole to 1 trimethoprim (Zhou and Moore, 1994).

Co-trimazole is particularly active against gram negative bacilli and certain Staphylococcus species (Karpman and Kurzrock, 2004). It inhibits two sequential two steps of the bacterial folate synthesis. The folate synthesis is essential to the formation of purines, which are precursors of deoxyribonucleic acid (Connor, 1998). The first step of the sequential mechanism is the inhibition by SMX of the synthesis of dihydro folic acid from para-amino benzoic acid. TMP acts as an inhibitor to the subsequent transformation to tetra hydro folic acid, which is it's active form (Connor, 1998). The properties of SMX are listed in Table 2.1.

Table 2.1: Properties of Sulfamethoxazole

Properties	Description
Chemical formula	($C_{10}H_{11}N_3O_3S$)
Color	White to slightly pale crystal powder
Solubility in water	Les soluble in water(0.5g/L) soluble in ethanol and methanol
Molar mass	253.279 g/mol
Melting point	167 °C (336 °F)
Drug indication	Bacterial infection(bronchitis, prostatitis) and UTI
Half-life in serum	8-10 hours
Septran Composition	160 mg trimethoprim and 800 mg Sulfamethoxazole

2.5 TOXICITY OF SMX IN AQUATIC ORGANISMS

With the rapid development of new analytical techniques, pharmaceuticals detection has become a main focus and has been found to contaminate the environment very rapidly. Their presence have been detected in surface, ground and drinking water and are becoming a major threat as these are inadequately removed by conventional treatment processes (Li *et al*., 2015). Norfloxacin and SMX have been detected in aquatic environment in µg/L (Zhao *et al*., 2010). In few cases, the presence of SMX and NOR in drinking water in trace amounts have been detected. Due to their wide spread presence in the ecosystem NOR and SMX are potentially toxic and effect biological conditions in non-target organisms most probably fish and fish cells (Liu *et al*., 2014). Uncontrolled use and overdose of pharmaceuticals like SMX have led to the formation of sulfonamide residues in animal tissues such as fish, shrimp and milk where they bio accumulate and in turn end up in humans through the food chain. Sulfonamides have been found to

accumulate in aquatic organism up to 1150 µg/kg form the surrounding contaminated waters (Zhao *et al*., 2015).

Research on the sub-lethal effect of SMX due to the biochemical activity in ongoing. Chronic effects which occur over a long durations of exposure are more often detected. Chronic toxicity refers to long term exposure with low doses of the chemical. Possible exposure of aquatic organisms to antibiotics for a longer time may lead to abnormal development, phenotypic retardation and loss of reproduction (Jobling *et al*., 1998), high risk of cancer (Davis and Bradlow, 1995; Kolpin *et al*., 2002).

2.5.1 Long term toxicity

Environmental problems that arise from the uncontrolled use and misuse of organic contaminants can be resolved using eco-toxicological tests. Long term/chronic tests using animal model such as fish help in estimating the requirements for drinking water and the possible risk assessment. Fish responses (health and survival) towards theses contaminates serves as an early warning tool and may be used as reliable biomarkers for environmental. In eco-toxicological studies fish biomarkers used for the purpose of risk assessment includes the study of internal mechanisms. Changes in the blood and other related parameters may be used as a tool to assess the health of the aquatic organisms. The changes in these blood parameters may unveil the presence of disease, and a compromised immune system due the presence of any toxic material in the environment (Poopal *et al*., 2017).

2.5.2 Hematological parameters

Blood the basic tool that maybe used to assess the internal conditions of an organism. Any alteration in the blood framework shows that the organisms might be not in good health condition. Hematological variables such as cell counts are widely used in clinical studies. Alteration in blood cell count and other parameters signifies weak immune system and a stressful environment

Alternations in hematological parameters was observed in *Arius Thalassinus* by Saleh and Marie in 2016, the results indicated a clear response of stress in fish towards the pollutants present in the environment. In another study Nile Tilapia (*Oreochromis niloticus*) was exposed to sulfamethazine 422mg/kg body weight for 11 days and the results indicated increase in the hematological parameters of the fish (Sampaio *et al*., 2016). Sanglam and Yonar in 2009 studied the hematological and biochemical effect of Sulfamerazine on Rainbow trout for 21 days and the results indicated a decrease in the hematological parameters (RBC, Hb, HCT, MCH, MCHC, MCV whereas an increase WBC count was noted and indicated the fish in stress conditions. The protein and glucose level also decreased in rainbow trout after application of sulfamerazine.

2.5.3 Biochemical parameters

Protein and Glucose are important molecules; these indicate the internal health of aquatic life. Increase in values for glucose may indicate the lack of carbohydrate metabolism. Low glucose may also indicate environmental stress in fish and other aquatic organisms. Similarly, a change level of protein depicts that the organism any have its internal functions in strain due the presence of any toxicant present in the environment. (Lavanya *et al*., 2011)

2.6 TREATMENT TECHNIQUES

Conventional methods of treatment for contaminated site and wastewater have been based on physical and chemical methods, but these processes may cause the formation of potentially harmful degradative byproducts (Dirany *et al*., 2011). Sulfonamides have been reported to undergo rapid photo degradation, but the process may indicate the formation of byproducts mixtures that are even more toxic then the parent compound at minimal concentrations (Matamoros *et al*., 2009). Removal of these compounds from the environment is a challenging task. Convention processes are not designed for proper removal or are technologically and economically not feasible for large scale wastewater treatment (Reis *et al*., 2014).

Due to these reasons biodegradation is preferred. Biodegradation is a new, cheap technique and may covert a harmful compound into a less toxic form. When biological process is properly designed and operated, it may result in complete oxidation of organic matter moreover the removal of xenobiotic via biological process has several advantages, regarded as a green technology it has minimal impact on the environment and is inexpensive (Müller *et al*., 2013).

Biodegradation of antibiotic uses oxidation of original toxic complex and produce CO_2 and H_2O, which provides sustenance for microbes to grow. Enzymes are involved in degradative process. If a potential degrading microorganisms is absent in the environment or if the biodegrading capacity of microbes is reduced due to high toxicity of the compound, specific microorganisms may be added to speedup up the process of biodegradation (Diez, 2010)

2.7 SMX DEGRADATION

Sulfonamides such as SMX are becoming very troublesome to remove from aquatic environment due to their highly stable nature and high rate of use. Theses have been reportedly found in

different water bodies. SMX plasma half-life ranges from 8-12 hours in body fluids (Grose *et al*., 1979)

The percentage removal of SMX through Biological removal observed in a number of processes ranged from negative removal due to SMX being re-formed from its metabolites to 99 % removal (Larcher and Yargeau 2012). SMX has been reported to undergo rapid photo degradation indicating that other biotic factors influence SMX degradation in aqueous mediums.

Although the degradation of SMX is thought to primarily occur by microbial activity, little is known about the microbes that carry out the process and the mechanism by which this occurs.
In previous studies *Geobacillus thermoleovorans* has been known to degrade 95% sulfamethazine in 24 h at 70 °C, pH 6.0, 50 rpm rotation speed and 50 mL of culture volume (Pan *et al*., 2017). Bacterial cultures, *Ochrobactrum* sp., *Labrys* sp. and *Gordonia* sp. were selected for their particular ability to resist SMX at of 5 mg/L. Results showed up to 45.2, 62.2 and 51.4 % degradation, respectively within 288 h (Mulla *et al*., 2018).

Seven individual strains of bacteria were able to degrade SMX in the presence and absence of glucose: *Bacillus subtilis, Pseudomonas aeruginosa, Pseudomonas putida, Rhodococcus equi, Rhodococcus erythropolis, Rhodococcus rhodochrous,* and *Rhodococcus zopfii*. The results revealed that SMX was successfully degraded by *R. equi* (15 % up to 29 % removal with glucose) while the other six bacterial strains including *Bacillus firmus, Bacillus cereus* have also been isolated from SMX contaminated river water and sediments which have indicated SMX degradation capacity of high levels up to (1 to 20 mg/L) at various temperatures (4 to 25 °C). The SMX removal achieved ranged from 40-90 % (Larcher and Yargeau 2012)

In various experimental studies SMX degradation carried out in the presence of different bacterial strains and the formations of the some metabolites have been summarized in Fig 2.5. During SMX degradation two most common degradation pathways are observed i.e. The formation of A.3-amino-5-methyl-isoxazole and 4-hydroxy-sulfamethoxazole, in which the p-amino group has been replaced by a hydroxyl group, when SMX is the only source of carbon and nitrogen; and the additional formation of N-acetyl-sulfamethoxazole and sulfanilic acid, when SMX is supplied with a growth substrate. The formation of the latter together with 3-amino-5-methyl-isoxazole may be seen as indication for hydrolytic cleavage of the sulfonamide group.

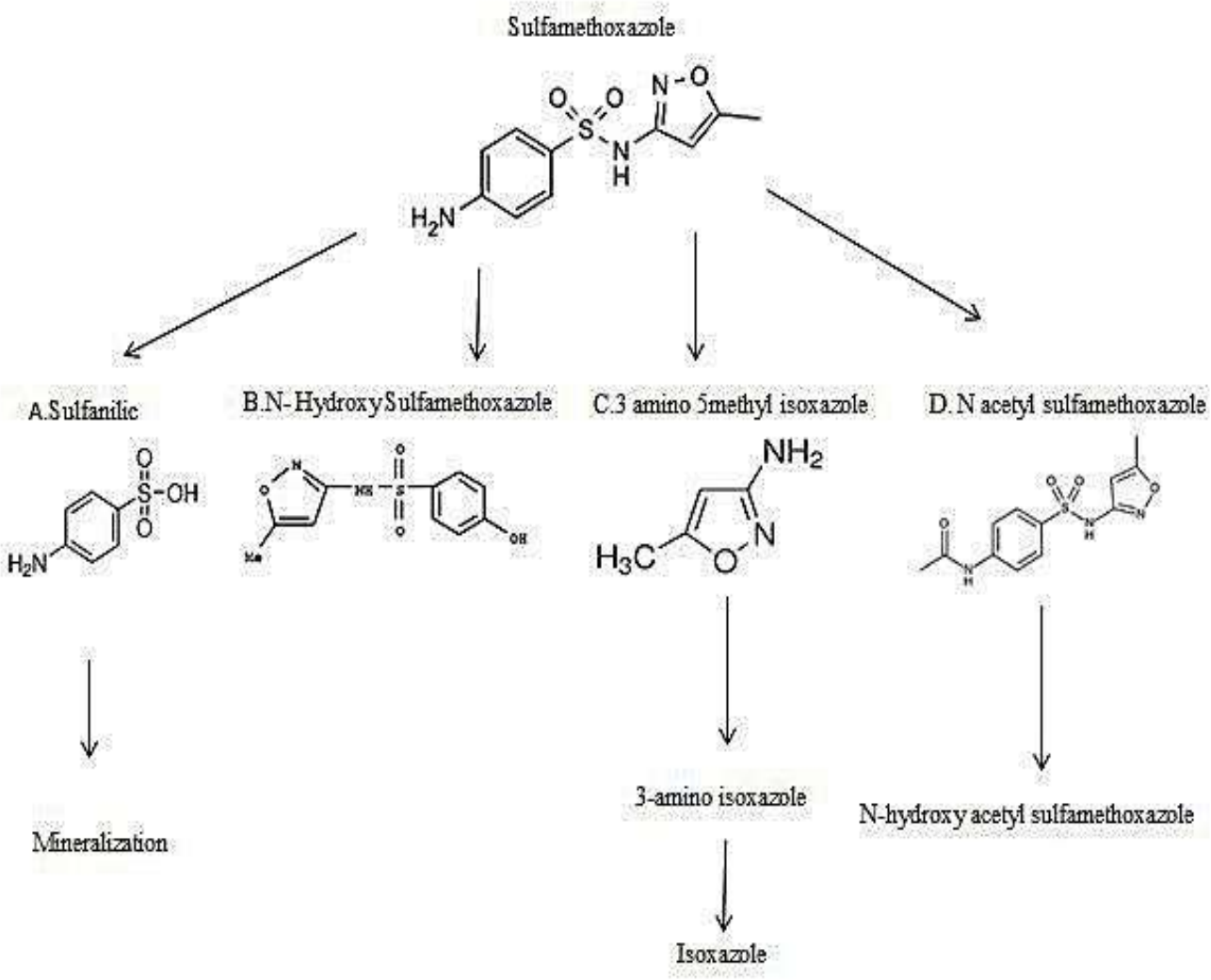
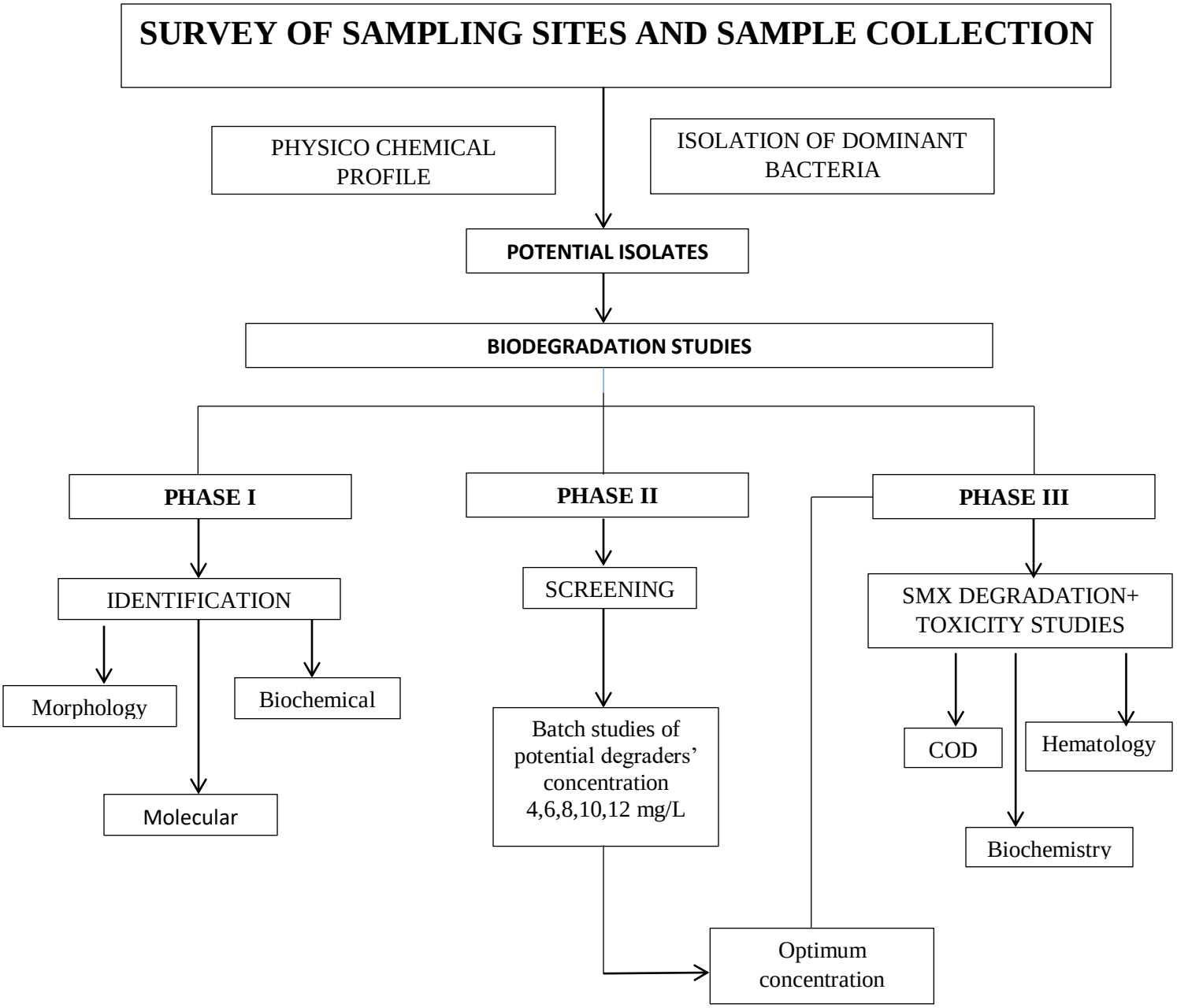


Fig 2.5: SMX biodegradation pathway and selected metabolites (Gauthier *et al*., 2010; Larcher and Yargeau 2012; Müller *et al*., 2013)

Some of the modified by-products of sulfamethoxazole that maybe formed by means of metabolic processes of microbes through co-metabolism and chemical reactions yield different transformations products some of which are completely mineralized by other microorganisms.

The bacteria that are able to degrade sulfa drugs most sulfonamides are mostly found in activated sludge in consortium which may also be supplemented by providing SMX which may act as a sole substrate. The bacteriostatic nature of sulfamethoxazole may cause a slight change in the microbial growth which may be distinguished from co-metabolism activities brought about by adaptation process. Further research in degradation process carried out by microorganisms that degrade sulfonamides and other pharmaceutical products my provide more insight (Fischer and Majewsky, 2014).

2.6 EXPERIMENTAL DESIGN (flow chart)



3. MATERIALS AND METHODS

3.1.1 Reagents and Chemicals

Commercial grade Sulfamethoxazole tablet (septran DS) was purchased from local pharmacy. The drug is a mixture of SMX and TMP at a ratio of 5:1. Methanol for standard stock preparation was acquired from Merck (Germany). All COD chemicals used were purchased from sigma Aldrich. For fish toxicity testing Glucose (100 ml) and Protein (125 ml) kits for analysis in Chemistry Analyzer (AMP Piccos II) were purchased from AMP, Austria.

3.1.2 Concentration of SMX

To conclude which is the preferred media for growth of selected strains, nutrient broth and mineral salt media were used to perform comparative studies. SMX was the carbon source in mineral salt media. Various concentration of SMX 4, 6, 8, 10 and 12 mg/L were used.

3.1.3 Washing and sterilization of glassware

All glassware used in the experiments was washed thoroughly and then sterilized by autoclaving for 15 mins at 121°C and 15 psi pressure. All glassware was oven dried 1 hour at 150 °C

Preparation of Media

For degradation studies nutrient broth and minimal salt medium was used both.

- **Minimal Salt Medium (MSM)**

MSM was prepared for the isolation and experimental purpose. The composition of minimal salt media is shown in Table 3.1

Table 3.1: MSM Composition

Chemicals	Quantity (gms/L)
$(NH_4)_2HPO_4$	0.5
$MgSO_4.7H_2O$	0.2
K_2HPO_4	0.1
$FeSO_4.7H_2O$	0.001
$Ca(NO_3)_2$	0.01

The above mentioned chemicals were mixed in 1L distilled autoclaved water. The pH of the media was 6.2 (Hashmi and Kim, 2003). Sulfamethoxazole was added to MSM to acquire different concentrations of 4, 6, 8, 10 and 12 mg/L.

- **Nutrient Broth**

1L Nutrient broth was made by adding 8g of media in distilled water. Broth was autoclaved for 15 minutes at 121°C and 15 psi pressure. For sterility test the broth was placed in incubator at 37°C for 24 hours.

3.2 PHASE-I

3.2.1 Selection of sampling sites and collection of water samples

Rawal Lake located in Islamabad is the main drinking water supply for Islamabad and Rawalpindi. Rawal lake and its neighboring streams receives water from local springs discharge

and divert untreated wastewater from houses nearby industries, poultry farms, agricultural effluent and polluted runoff during the rainy season.

Fia 3.1: Rawal lake tributaries

Sampling sites from the upstream tributaries that converge to form the Rawal Lake were identified and selected as represented in Fig 3.1. The sampling locations with its GPS coordinates are given in table 3.2. Water sampling was done according to standard APHA protocols from the upstream and downstream locations of the 4 tributaries and Korang River just before they join the Rawal Lake. Water samples were collected from August to November 2016. The water samples collected were analyzed for physicochemical properties onsite using multimeter and were then transferred to laboratory for further analysis.

Table 3.2: Sampling locations with its GPS Coordinates

Sampling locations	Upstream GPS coordinates	Downstream GPS coordinates
Ratahutar stream	33°450‘06.8”N 73°050‘56.7”E	33°44’12.3”N 73°06’30.5”E

Nurpur stream	33°44’48.5”N 73°06’34.2”E	33°44’32.4”N 73°06’56.3”E
Jinnah stream	Inaccessible	33°744867”N 73°116639”E
Shahdara stream	33°746738 ”N 73°168261”E	33°45’35.0 ”N 73°10’25.2 ”E
Korang river	33° 41.631”N 73° 8.416” E	33° 42.707”N 73° 8.245” E

For the physicochemical parameters, water samples were collected in autoclaved glass sampling bottles of 500ml. All samples were collected in triplicate from upstream and downstream locations of each tributary and korang river. TDS, pH, EC were analyzed onsite and turbidity was checked after samples were transported to laboratory. Table 3.3 characterizes the parameters along with the instrument and method used for analysis.

Table 3.3: Parameters and instrument along with method used for

Parameters	Units	Instruments used	Method of analysis
Ph	-	pH meter (HACH 156)	Potentiometric method
Temp	˚C	HACH session 1	Laboratory method
Total dissolved solids	(mg/L)	TDS meter (Ino Lab 720)	Potentiometric method
EC	(µS/cm	Conductivity meter (720 WTW probe)	Potentiometric method
Turbidity	(NTU)	Turbidity meter (HACH 2100 P)	Laboratory method

3.2.2 Collection of Water Samples

Triplicate water samples were collected in in sterile glass bottles from tributaries carefully to avoid sediment collection. After collection the samples were kept in refrigerated cooler and transferred to laboratory to be further analyzed.

3.3 ISOLATION OF MICROORGANISMS FROM WATER SAMPLES

Pure cultures of microorganisms were isolated from water samples following standard protocols. Petri plates were washed and put in autoclave for sterilization at 121°C, 15psi for 15 minutes and put in oven for drying at 150 °C for at least 1 hour. Nutrient agar was prepared following manufactures directives. Autoclaved cooled; media was poured into sterilized petri plates inside a laminar flow chamber to reduce contamination. Plates containing media were left for 15 minutes to solidify were incubated overnight at 37°C overnight for sterility testing .

The water samples were spread on agar plates after serially diluting the samples (10^{-1} - 10^{-5}). From each dilution, 0.1 ml of water samples was placed on the center of the prepared nutrient agar media, the sample suspension was spread on media plate using a glass spreader. Plates were incubated at 37°C overnight. Plates that showed a countable range of colonies (30-300) were selected. The colonies were counted using a colony counter.

Dominant colonies based on morphology were selected and isolated with a sterilized wire loop and subculture onto agar plates to obtain pure colonies. The procedure was followed for all samples collected including replicates. Each colony was subjected to repeatedly (4-5 cycles) of streaking to get different colonies separated. Each colony was stored in the refrigerator at 4°C for further use.

3.4 IDENTIFICATION OF MICROORGANISMS

Dominant isolated bacterial species were then distinguished using morphology, biochemical test and molecular techniques.

3.4.1 Morphology

Morphological identification of the dominant bacteria isolated form water samples was performed by noting their form, elevation, margin, surface, odor, color pigmentation and opacity.

3.4.2 Biochemical

- Gram staining

Gram staining differentiates bacteria into two major groups: gram positive and negative based on their cell walls.

A smear of the isolated fresh culture was prepared on slides and dried then heat fixed. Slide was then flooded with crystal violet for 1 minute and rinsed with distilled water. Then slide was flooded with iodine solution for 1 minute and then washed with decolorizer (70% ethanol) for 15-20 seconds. Lastly the smear was stained with safranin for 1 minute was and washed with distilled water. The smears were were observed under oil immersion using 100X resolution (Hucker and Corn, 1923).

- Catalase

A loop full of 24-hours fresh culture of isolated bacterial strains was smeared on to a grease free glass slide using a sterilized inoculating loop, about 2 drops of 3% hydrogen peroxide solution was poured on smear. Gas produces white bubbles, indicating presence of catalase enzyme in the bacteria (Cheesbrough, 2006).

- Oxidase

Oxidase test helps in differentiating bacterial species that produces enzyme cytochrome c oxidase. N, N, N', N'-tetra methyl-p-Phenylenediamine I (TMPD) is used which is an electron acceptor for the enzyme oxidase. Colonies from 24 hour fresh culture were smeared onto a filter paper using a sterilized inoculating loop. 2 -3 drops of TMPD reagents already prepared was dropped on the colony. Change in colony color to deep purple color indicates positive oxidase test (Cheesbrough, 2006).

- Motility test

Bacteria was grown in liquid media (nutrient broth), the fresh 24 hour liquid cultures were then used to observe motility of bacteria using hanging drop technique. A drop of nutrient broth that contains grown bacteria is placed onto the hollow depression of slide and covered with a coverslip sealing the edges of the depression, the slide was then observed under 100X resolution (Tittsler and Sandholzer, 1936).

- Lactose fermenting test

Already prepared McConkey agar plates and were used to streak 24 hour fresh bacteria. The plates were then incubated. Colonies that turned pink were considered as lactose fermenters

- Analytical Profile Index 20-E

API 20E is a test kit used for the identification of non-fastidious and enteric bacteria. It consists of a plastic strip that has 20 mini cupules. Each cupule contains a specific medium for the biochemical characterization. For performing the tests saline suspension (0.85%NaCl) was

prepared and autoclaved. Fresh colonies of 24 hours were added to suspension and mixed well. The cupules of the API strip were completely filled with the suspension containing bacteria except for the capsules of citrate utilization (CIT), voges-proskauer (VP) and gelation liquefaction (GEL). They were filled half and were filled with a drop of mineral oil to create anaerobic conditions. The strip was covered with lid provided and placed in incubator at 37 °C for 24 hours. Color changes were noted and results were recorded in table after incubation reagents were added to few cupules, TDA was added to TDA cupule. James/Kovacs reagent was added to IND while VP1 and VP2 were added to VP. The test was allowed to develop for a few minutes and results were recorded. An additional oxidase test was performed to develop a 7 digit code required for identifying the bacteria. List of tests performed in API 20 E kit are given in Table 3.4.

Table 3.4: list of test in API 20E kit along with positive and negative results

Cupule Medium	Positive Results	Negative Results
O-Nitrophenyle-B-D galactoside	Light yellow to yellow	Colorless
Arginine Dehyrolase (ADH)	Light to dark red	Yellow
Lysine Decarboxylase (LDC)	Light to dark red	Yellow
Onthonine Decarbolyase (ODC)	Light to dark red	Yellow

Citrate Utilization(CIT)	Blue green to blue	Pale green to yellow
Hydrogen Sulfide	Black	Grey to colorless
Tryptophan Deaminase (TDA)	Deep red	Brown
Indole (IND)	Pink	Colorless/Pale/Green/Yellow
Voges –Proskauer (VP)	Pink/Red	Colorless/Slight pink
Gelatin Liquefaction (GEL)	Goes black (digested)	No change
Glucose (GLU)	Amber	Greenish Yellow, Indigo
Mannitol	Yellow	Greenish Yellow, Green, Indigo
Inositol	Amber	Greenish Yellow, Indigo
Sorbitol	Yellow	Greenish Yellow, Green, Blue
Rhamnos	Amber	Greenish Yellow, Green, Indigo
Sucrose	Yellow	Greenish Yellow, Green, Indigo
Melibiose	Amber	Greenish Yellow, Indigo
Amygdaline	Yellow	Greenish Yellow, Green, Indigo
Arabinose	Yellow	Greenish Yellow, Green, Blue

3.4.3 Molecular Identification

Molecular method included isolation of DNA from bacterial strains isolated from water samples collected form Rawal lake tributaries. DNA sequence analysis was carried out after extraction of pure DNA from the pure bacterial samples.

3.4.3.1 Primer Sequences

PCR primer and sequences used in the study are mentioned in Table 3.5. The primers used are universal primes and are widely used.

Table 3.5: Selected primers for PCR amplifications

Primers	Sequence (5'-3')	Targeted Genes	References
27F	5' AGAGTTTGATCMTGGCTCAG 3'	16SrRNA	Erguven *et al*., 2016
1492R	5' TACGGYTACCTTGTTACGACTT 3'	16SrRNA	

3.4.3.2 DNA Extraction

Genomic bacterial DNA was extracted using commercial kit Norgen Biotek Corporation by following manufactures instructions.

- Lysate preparation

  Cell pellet were prepared by harvesting up to 2×10^9 cells. Cell pellet was suspended in 180 µl genomic digestion buffer and 20 µL of proteinase K was added followed by brief vortexing. Tubes were incubated at 55° C for one hour with occasional vortexing. 20 µl

of RNAs was added followed by brief vortexing. 200µl of lysis buffer was added to yield a homogenous solution. 200 µl of pure ethanol was added and vortexed well for 5 seconds to get the homogenous solution.

- Binding of DNA to the column

  Approximately 650 µl of prepared lysate was added in spin column supplied with the kit and was centrifuged at 10,000 rpm for 1 minute. Collection tubes were discarded and spin column was placed into the pure collection tube supplied with the kit.

- Column wash

  500 µl of wash buffer I was added in the column and was centrifuged at 10,000 rpm for one minute at room temperature. Collection tube was discarded and was replaced with pure collection tube from the column. 500 µl of wash buffer II was added in the spin column and was centrifuged at maximum speed for 3 minutes at 14,000 rpm. Collection tube containing supernant was discarded.

- Elution DNA

  Collection tube was placed in 1.5 mL micro centrifuge tube. 50 µl genomic elution buffers were added in the column and were centrifuged at 10,000 rpm for 1 minute. Spin column was discarded and the tube contained pure DNA

- Storing of DNA

  Purified DNA was stored at -20° C for use in PCR process.

3.4.3.3 Gel Electrophoresis for DNA band visualization

Agrose gel electrophoresis was carried out to visualize the extracted DNA. 1% (w/v) agarose gel was prepared by adding 0.6g of agarose gel in 60ml of 1X TBE buffer. Ethidium bromide

solution (5µg/ml) was added as a staining agent. Electrophoresis was done at 100 volts for 30 minutes. After that gel was observed by placing it under UV trans-illuminator.

3.4.3.4 PCR Amplification

PCR was performed to amplify the extracted DNA. The reaction mixture was prepared (25 µl) having composition mentioned in Table 3.6.

Table 3.6: Recipe of PCR reaction mixture

Reagents	**Volume µL**
Taq PCR master mix	25
DNA template	1
Primer F (10µM)	2
Primer R (10µM)	2
Nuclease free water dd H_20	20
Total volume	**50**

For the 16SrRNA gene detection, the PCR program includes 5 minute at 95°C for template denaturation, and 40 cycles for template amplification consisting of three steps: 95°C for 1 minute for DNA denaturation into single strand, 61°C for 1 minute for primer to anneal to their complementary sequences on either side of the target sequence, 72°C for 1 minute for extension of complementary DNA strand from each primer and final elongation at 72°C for 10 minute for Taq DNA polymerase to synthesize any unextended strand left.

Fig 3.2: Thermal Cycler (Company: Extra Gene 9600)

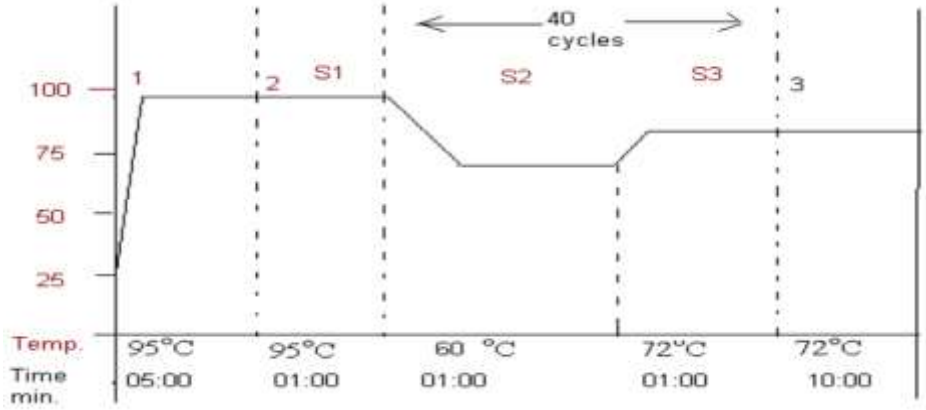


Fig 3.3: PCR program for 16SrRNA gene amplification

3.4.3.5 16S rRNA sequencing

PCR products were kept in ice box and the preserved isolates were sent to Genome analysis department Macrogen Inc, Seoul, South Korea for 16S rRNA sequencing.

- Phylogenetic analysis

  Once the sequences were obtained, they were trimmed through Bioedit software and junk data was removed. Properly trimmed sequnces were analyzed through BLAST tool of National center of biotechnologcal information (NCBI). After proper detection of the obtained species, accession numbers were obtained from NCBI gene bank library. FASTA sequences were run in MEGA 7 software to get the phylogenetic tree which showed linkages between the isolated strains and those at GENBANK of NCBI.

3.5 PHASE –II

3.5.1 Biodegradation studies

Degradation studies were performed in order to screen out and isolate potential strains for bioremediation of SMX and to find out optimum concentration of SMX that may indicate significant degradation. Experiments were conducted in triplicates. The results obtained were further used in toxicity studies on fish.

3.5.2 Screening for SMX degrades

Screening for potential isolates was done in nutrient broth containing SMX 4 mg/L (sole carbon and energy source) incubated at 37°C. Growth was monitored regularly by single beam spectrophotometer at 600 nm. Strains that possessed the highest degrading capability were selected for further SMX degradation studies. The bacterial strains that indicated capacity in the presence of SMX were further acclimatized under increasing SMX concentrations

3.5.3 Optimum concentration for degradation

Batch studies were conducted to find out the optimum concentration for SMX bioremediation. For this purpose various concentrations of SMX (4, 6, 8, 10, 12 mg/L) was added to NB and MSM in conical flasks. Acclimatized 24 hour fresh culture of bacteria which showed the capability of degrading SMX were added to the 5 selected concentrations of both medias in equal amounts. The flasks were incubated at 37°C and were analyzed for SMX degradation for 72 hours. Degradation was monitored using COD titration closed reflux method and optical density was measured by means of spectrophotometer. Each group was run in duplicate. Optical density was also noted for both Media at 600 nm using UV-visible spectrophotometer. Each group was run in duplicate. A control was also used which contained SMX but no bacterial culture for all concentrations.

3.5.4 COD titration closed reflux method

Both media were monitored for a period of 72 hours for COD levels and optical density. Aliquots were withdrawn and diluted to 1000 times. COD was measured by standard 5220C closed reflux titrimetric method (APHA, 2017). According to this method, 1.5 ml of standard potassium dichromate digestion solution ($K_2Cr_2O_7$) and 3.5 ml of 0.0176M Ag_2SO_4 solution were added to a 2.5 ml sample. The samples were refluxed in a thermo reactor for 2 hours at 150°C. The samples were then cooled, and transferred to erlenmeyer flasks and 3 drops of ferroin indicator ($FeSO_4.7H_2O$) were added to the samples. Then, samples were titrated with 0.1M standard ferrous ammonium sulfate (FAS) until color change from yellow to green and endpoint of brown. The initial and final volume of FAS used was noted and COD results were calculated.

3.5.5 COD analysis

After titration samples were calculated with the following equation:

$$\textbf{COD } O_2\textbf{/ L = (A-B) × M×8000 / Volume of Sample (ml)}$$

Where:

A = Volume of FAS (ml) used for blank

B = Volume of FAS (ml) used for sample

M = Molarity of FAS

8000 = Mill equivalent weight of oxygen × 1000ml/L

3.6 PHASE –III

To determine whether SMX if present in water may be degraded using bacteria in the presence of living organisms. Fish being a good environmental biomarker and shows prominent signs of environmental contamination was used in the following study. An optimum SMX concentration was added to fish tank along with a bacterial inoculum that was capable of degrading the antibiotic. The effect of SMX on fish was also noted and SMX degradation was monitored. The detailed methodology of phase III is as shown in Fig 3.4

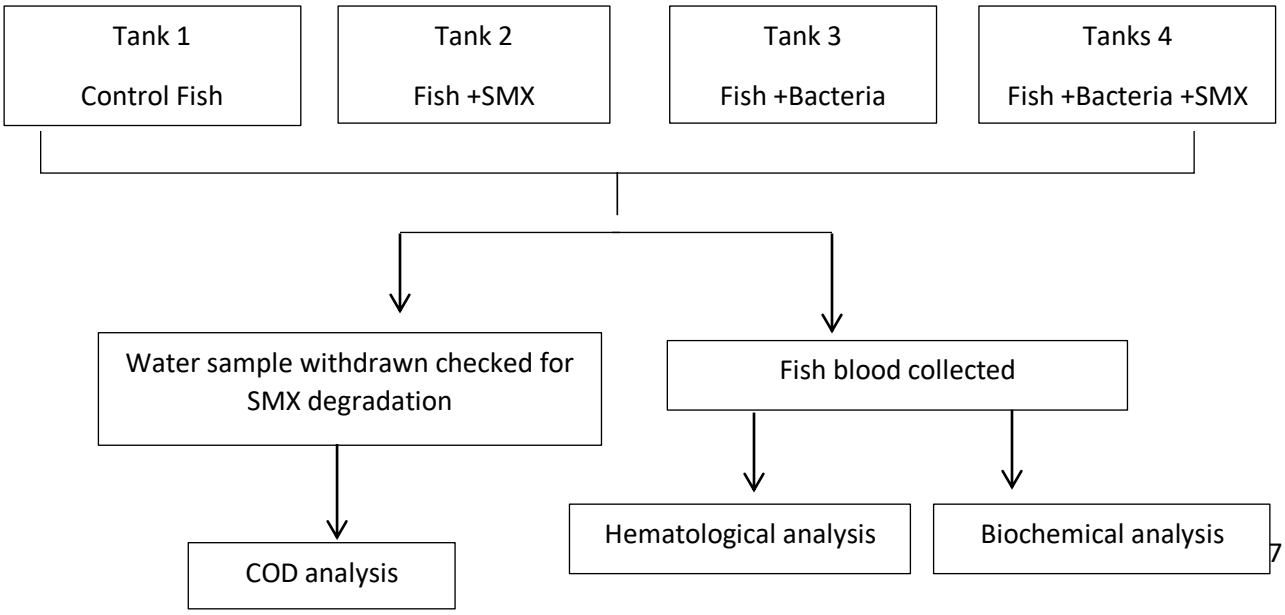


Fig 3.4: Methodology Phase III (In Detail)

3.6.1 Purchase and maintaince of experimental fish

Toxicological studies were carried out in environmental toxicology laboratory. White carp was selected as bio-indicator for the current study as it was available in the season and because it may bear stress and extreme conditions. During exposure period fish were evaluated for different conditions and health status. Semi static tanks were used following OECD 204 guidelines for chronic toxicity tests. Healthy fish specimens of white carp were purchased from Punjab hatchery Rawal town. Number of fish per batch depended on the nature of test to be conducted. Fish were brought to the laboratory avoiding mechanical injuries and kept in experimental tanks.

3.6.2 Acclimatization of fish

Before starting the experiment, fish were acclimatized to laboratory conditions for a period of 1 week. They were fed commercial dry food pellets, leftover food in tanks was removed daily and the water in tank was changed weekly during this period. The tanks were provided with air diffusers for proper oxygen supply and filter was installed for removal of waste produced by fish. Dead fish were removed immediately to avoid biofouling.

3.6.3 Water parameters

Water was changed weekly followed by the addition of SMX solution. Temperature, pH, dissolved oxygen were determined according to standard methods regularly (APHA, 2017). The

physico-chemical parameters of the water may influence the toxicity of SMX. The physico-chemical parameters of water presented in Table 3.7 were estimated on regular basis

Table 3.7: Physicochemical parameters of tap water

Parameters	Units	Instrument used	Values/Results
pH		Multimeter, 156 Hach sension, Germany	7.8±0.2
Temperature	(^{o}C)	Thermometer (720 WTW probe)	25.15±1.5
Dissolved Oxygen (DO)	(mg/L)	Winkler Method	8±0.6
Electrical Conductivity (EC)	(µS/cm)	Conductivity meter (720 WTW probe)	744±80
Total Hardness	(mg/L)	Titration Method	139±25
Chlorine		34)	*BDL

**BDL = Below Detection Limit*

3.6.4 Stock preparation

Sulfamethoxazole (SMX) sold under the commercial name of Co-trimazole Spetran DS was purchased from local pharmacy. Commercial product SMX tablets was purchased from local pharmacy and crushed, it was dissolved in 1 ml ethanol and mixed with 1L distilled water to make a primary stock solution. From this stock solution further working solution were prepared.

3.6.5 Exposure Period

Healthy fish were exposed to an optimum SMX concentration. Fish were exposed for a period of 28 days to find out the chronic toxicity at environmentally relevant concentrations. Toxicity test was performed using 10 fish in experimental tanks containing 50L water. Two replicates were also maintained along with treatment and a control group. The study was conducted in two batches. To find out chronic toxicity, fish were exposed to an optimum dose of SMX. The debris

was removed before renewal of toxicant in water. Sampling was done at 7, 14, 21, and 28 days of exposure (Ramesh *et al*., 2018).

3.6.6 SMX degradation in fish tank

To monitor SMX degradation in fish tank, an optimum concentration of SMX was applied to fish and a bacterial inoculum of potential SMX degrading strain was added to the experimental tank. Degradation of SMX was monitored using COD. Samples were withdrawn from tank and were centrifuged to removes any debris from the water samples and were diluted up to 1000 fold and COD was performed using closed reflux titrimetric method.

3.6.7 Bio toxicity Evaluation

Fish blood was collected from the caudal vein puncture using medical grade disposable syringe after exposure and collected in EDTA (ethylene diamine tetra acetic acid) tubes which serves as an anticoagulant. Fresh blood was used for hematological analysis and the remaining was put in heparinized tubes containing gel and was used for biochemical testing. Samples were taken on 7, 14, 21 and 28 days after exposure to SMX.

Study Hematological Parameters

Chronic toxicity was determined according to OECD guidelines 204 (1984). A 30 day of exposure duration was given. Fish were divided into four experimental tanks as shown in Fig 3.6 containing 10 fish each. One group served as control. At the end of stipulated time, blood samples were collected for hematological analysis in clean vials containing anti-coagulant EDTA. Red blood cell count (RBC), white blood cell count (WBC), Hemoglobin (Hb), Hematocrit (HCT), Mean Corpuscular Volume (MCV), Mean hemoglobin concentration, (MHC)

and mean corpuscular hemoglobin concentration (MCHC) was measured using complete blood count using sysmex blood analyzer XP-100.

Study of Biochemical parameters

Whole blood form caudal vein puncture was collected in heparinized tubes continuing gel and centrifuged at 12,000 rpm for 2-3 mins to separate the plasma from the cells. After centrifugation a clear yellow liquid separates above the gel and cells settle at the bottom. Protein content was determined using AMP Piccos II chemistry analyzer the blood serum was processed according to manufacturer's instruction. For glucose 10µL separated serum sample was transferred in clean eppendorf tube and mixed with about 1 mL of glucose reagent and incubated at room temperature for 5 minutes and was then aspirated by the analyzer. The results were expressed in mg/dL. For protein analysis separated 20µL serum was taken in eppendorf and mixed with 1mL of protein reagent and aspirated immediately. The results were expressed in g/dL.

3.7 Statistical Analysis

Standard deviation and mean calculation

Mean was calculated of replicate values and standard deviation applied.

ANOVA

Two- way ANOVA was to estimate the significance of the differences found among the groups. $P<0.05$ was considered statistically significant. Correlation was also used to represent the association between of target compounds and toxicity.

4. RESULTS AND DISCUSSIONS

The current study aimed to evaluate water quality and to investigate the degradative capacity of indigenous microorganisms to degrade an antibiotic that has been detected previously; furthermore toxicity of the compound was also assessed using biological tests associated with fish as a biomarker.

4.1 PHASE I

4.1.1 Sampling site survey and water sample collection

The Rawal Lake is the main source of water supply for Rawalpindi city and cantonments and for parts of Islamabad. The lake has a catchment area of 106 square miles, which generates 84,000 acre-feet of water in an average rainfall year. There are four major streams and 43 small streams which contribute to its storage. It has a total storage capacity of 47,500 acre-feet (12,994 million gallons (Ayaz *et al*., 2016). The four major streams and Korang River were selected as sampling sites. Locations along with coordinates are presented in Table 3.2

R1-RATAHUTAR Up Stream 33°450‘06.8”N 73°050‘56.7”E

Upstream location was about 7 km away Rawal Lake. Water apparently was visibly clear and clean owing to fact that not many houses and other settlements were seen in the area. The stream was originating at the base of the mountains. No water discharge was visible from the residences. The physical conditions of the upstream location of Ratahutar stream is as shown in Fig 4.1a and 4.1b.

From left to right Fig: 4.1a and Fig: 4.1b Ratahutar Upstream

R2-RATAHUTAR Down Stream 33°44'12.3"N 73°06'30.5"E

The Ratahutar Downstream location as shown in fig 4.2a and 4.2b were completely opposite in conditions to the upstream location. The stream is passing through slums. Heaps of garbage, animal waste and discharge from houses were seen in close vicinity to the stream. The water did not look clean and was visibly contaminated and not fit for use. It was evident that as the water was moving from downstream it was facing contamination from nearby settlements.

From left to right Fig: 4.2a and Fig: 4.2b Ratahutar Downstream

N1-NURPUR Up Stream 33°44’48.5”N 73°06’34.2”E

The selected upstream location of the Nurpur stream is shown in Fig 4.3a and 4.3b.The stream was passing through a village settlement. The stream itself was vastly contaminated with garbage, plastic bottles, and plastic bags. Along the sides of the stream huge heaps of animal manure (dung) was also seen which was aesthetically unpleasant and produced a bad odor.

From left to right Fig 4.3a and Fig 4.3b Nurpur upstream

N2-NURPUR Down Stream 33°44’48.5”N 73°06’34.2”E

As shown in Fig 4.4a and 4.4b the downstream location of sample collection was also passing through different settlements and was in almost the same conditions as the upstream location. Visibly the stream was contaminated and direct discharge from houses was observed during sample collection. Massive algal growth and sediments was also observed in the stream water as well.

From left to right Fig: 4.4a and Fig 4.4b Nurpur Downstream

S1-SHAHDARA Up Stream 33°746738”N 73°168261”E

The upstream location indicated in Fig 4.5a and 4.5b. The water was flowing through rock area and apparently looked clean and clear. Few residential settlements were seen nearby. The banks of the stream had vegetation.

From left to right Fig 4.5a and 4.5b Shahdara Upstream

S2-SHAHDARA Down Stream 33°746738”N 73°168261”E

From left to right Fig 4.6a and 4.6b Shahdara Downstream

The water of the downstream location of Shahdara stream Fig 4.6a and 4.6b had completely opposite conditions to upstream location. The water was filled with algal growth and wild vegetation along the banks. Wild plants were common and could be seen as far the naked eye could see. Garbage dumps and poultry farms were seen nearby causing bad odor.

J1-JINNAH Up Stream

The upstream location of Jinnah stream was inaccessible which required additional support and was not performed.

J2-JINNAH Down Stream 33°744867”N 73°116639”E

The downstream location was close to Bari Imam Shrine Fig 4.7a and 4.7b. The stream was passing through a village and was highly crowded with a lot of traffic seen. The stream was

contaminated with garbage and construction material was also seen nearby. The Ratahutar, Nurpur and Jinnah stream eventually merge together before falling into the Rawal Lake

From left to right Fig 4.7a and 4.7b Jinnah Downstream

K1-KORANG RIVER Up Stream 33° 41.631”N 73° 8.416” E

The Korang River serves as both an inlet and an outlet from the Rawal Lake. The upstream water sampling site Fig 4.8a and 4.8b appeared static. The water appeared murky and muddy which may be due to large number of brick and tile factories located nearby. The water was being used for vehicle washing by the nearby residences.

From left to right Fig 4.8a and 4.8b Korang River Upstream

K2-KORANG RIVER Down Stream 33° 42.707”N 73° 8.245” E

Almost similar situation were observed at the downstream location of the stream Fig 4.9a and 4.9b the water appeared muddy murky and static. Cattle were seen nearby in the river water and many people were washing cars in the river water as well.

From left to right Fig 4.9a and 4.9b Korang River downstream

4.1.2 Physicochemical analysis of rawal lake tributaries

For the study of physicochemical parameters, water samples were collected in clean and sterilized glass sample bottles in triplicate. The physicochemical analysis of water samples showed that mean values of pH ranged from 7.97-8.7 at various locations of the tributaries. The values were within the World Health Organization (WHO) permissible limits of 6-8.5. The highest pH value of 8.63 was observed at sampling location S2 as show in Fig 4.10.

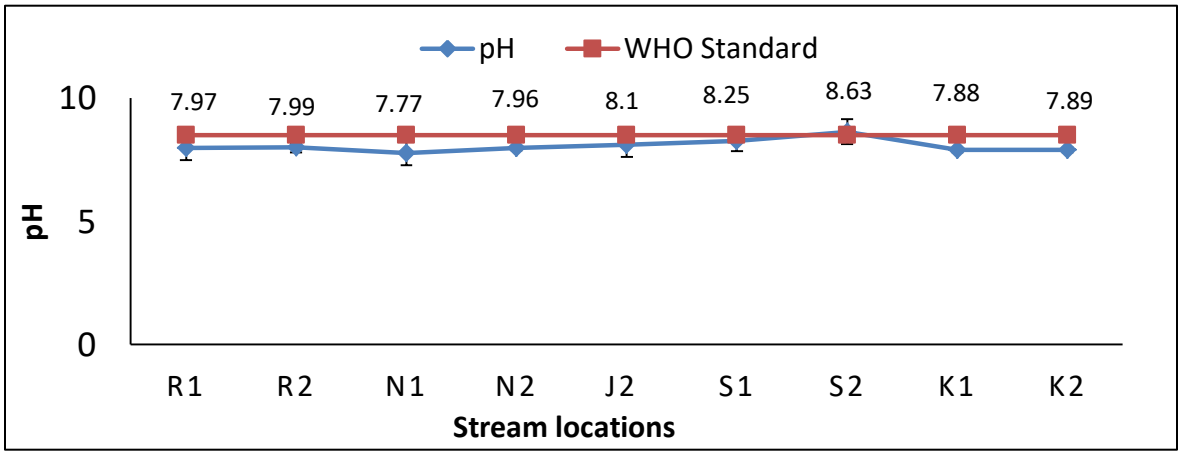


Fig 4.10: pH of water samples at upstream and downstream locations

The value of turbidity ranged from 1.0 to 10 NTU at various locations of Rawal Lake tributaries. The highest value of turbidity was observed at sampling point location R2, K1 and K2 and is shown in Fig 4.1. High values of turbidity may be due to contamination of the tributary water flowing from houses as well as sedimentation and algal growth. Suspended solid particles may also cause high levels of turbidity (Ghumman, 2011).

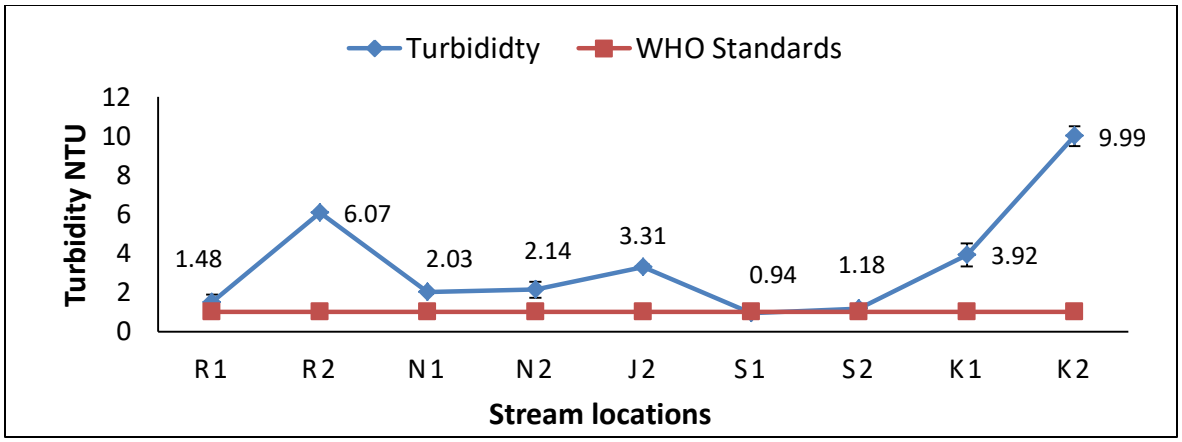


Fig 4.11: Turbidity of water samples upstream and downstream locations

Fig 4.12 shows the values of electrical conductivity ranging from 200-350 µS/cm, the values found were to be slightly higher at some points such as R1 N1 and K2 but were under the permissible limits. The WHO standard limit for EC lies within 400-600 µS/cm. High EC values which may be attributed to the physical condition of the stream and the presence of high ionic species which may contribute to contamination (Ayaz *et al*., 2016).

TDS values were higher at site R2, N1 and K2 (Fig 4.13). TDS values showed the highest fluctuation among various sampling locations and the values ranged from 500 to 800 mg/L with highest values recorded at site R2 which is above the WHO permissible limit for drinking water.

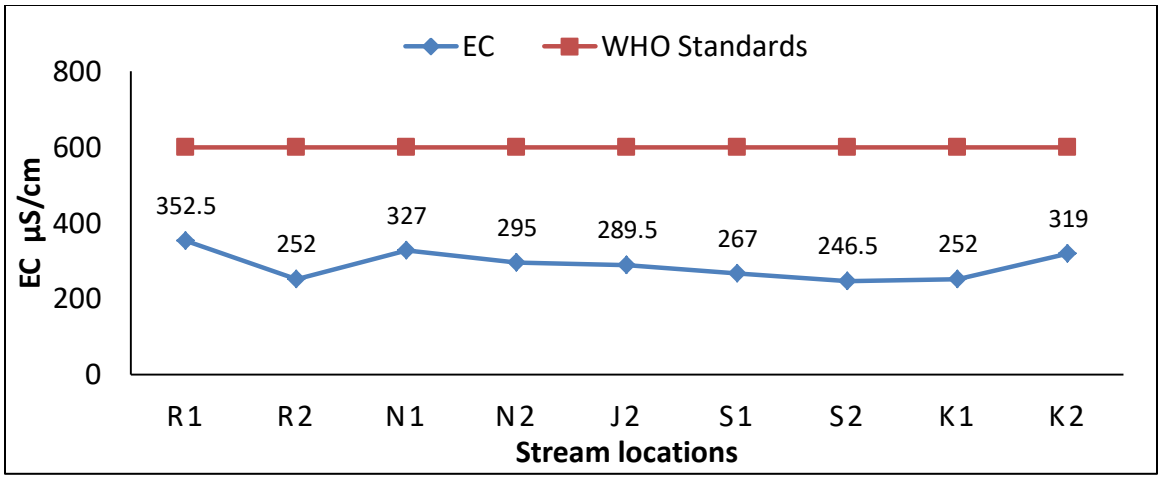


Fig 4.12: Electrical Conductivity at upstream and downstream locations

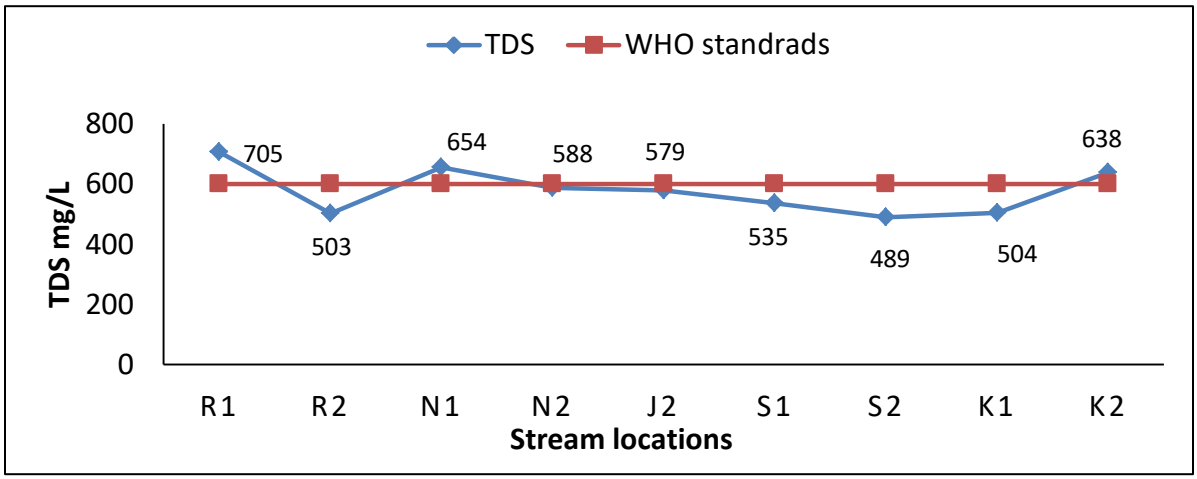


Fig 4.13: TDS of water samples at upstream and downstream locations

The physicochemical analysis and the physical conditions of the rawal lake tributary suggest that the pollution load to the lake is coming from the residential areas and uncontrolled settlements near the tributaries. Untreated household waste is directly dumped into the water which ultimately reaches the lake and causes further deterioration. Ayaz *et al*., 2016 reported that the water quality of various location of rawal dam could be good for agricultural purpose but may be rarely used for drinking moreover water found was mostly contaminated with animal waste and

poor drainage system. Ali *et al* in 2015 studied the water quality of Korang River and reported that contamination of the Korang River was mostly attributed to household waste discharge, poultry waste, agricultural runoff and solid waste dumping. Saeed and Hashmi in 2014 reported similar findings of water quality in tributaries and various location of Rawal Lake. The values found were higher in tributaries as compared to the lake water as the contamination is lessened due to dilution factor. Malik and Nadeem in 2011 reported that water quality based on the physicochemical profile in areas with less anthropogenic activities and sites near residential areas near Korang River were found to be contaminated. Runoff from the nearby areas may contribute to the sediments formation during rainy season in Korang River (Ghumman, 2011).

4.1.3 Isolation and Identification of predominant microorganisms

Water samples collected form rawal lake tributaries was subjected to standard agar plating method for the isolation of various bacterial strains. Strains were differentiated based on colony morphology and biochemical tests followed by molecular identification. Total 15 dominant bacterial strains were isolated from different location of the rawal lake tributaries. The strains were given ID name from W1-W15 along with the location of isolation is presented in Table 4.1. Maximum of strains were isolated from Korang River location.

Table 4.1: Isolated bacterial strains from different locations

Strain ID	Locations
W1	Shahdara stream
W2	Nurpur stream
W3	Jinnah stream
W4	Ratahutar stream
W5	Jinnah stream
W6	Nurpur stream
W7	Korang river

W8	Korang river
W9	Korang river
W10	Korang river
W11	Shahdara stream
W12	Jinnah stream
W13	Korang river
W14	Shahdara stream
W15	Nurpur stream

4.1.3.1 Colony Morphology

Table 4.2 represents the colony morphology of isolated strains (W1-W15). Colony morphology was studied in terms of form, color, elevation, margin, surface texture and opacity. Maximum percentage of strains had circular shape, white color, raised elevation, smooth texture and was opaque.

Table 4.2: Colony morphology of bacterial strains from rawal lake tributaries

Strain ID	Form	Color	Elevation	Margin	Surface texture	Size
W1	Circular	Cream	Convex	Entire	Smooth	Small
W2	Circular	Cream	Flat	Entire	Slimy	Large
W3	Circular	Cream	Convex	Entire	Moist	Small
W4	Circular	Cream	Raised	Undulated	Moist	Small
W5	Circular	Cream	Raised	Entire	Smooth	Small
W6	Round	White	Convex	Entire	Smooth	Small
W7	Circular	Cream	Flat	Entire	Moist	Large
W8	Circular	White	Flat	Curled	Smooth	Small
W9	Round	Cream	Convex	Undulated	Dry	Large
W10	Circular	Cream	Convex	Entire	Moist	Small
W11	Circular	White	Flat	Entire	Smooth	Small

W12	Circular	Grey	Raised	Undulated	Smooth	Small
W13	Round	Off-white	Raised	Entire	Smooth	Small
W14	Puntiform	Off-white	Raised	Undulated	Smooth Moist	Small
W15	Circular	Blue green	Flat	Entire	Slimy	Large

4.1.3.2 Cell Morphology

Cell morphology of isolated bacterial strains in terms of gram reaction, shape, arrangement and motility is mentioned in Table 4.3. All of the isolated strains were identified as gram negative and maximum percentage of bacteria had rod shape. Cell motility of maximum strains were observed when examined under 100X resolution of light microscope.

Table 4.3: Cell morphology of bacterial strains isolated from Rawal Lake Tributaries

Strain ID	Gram Reaction	Shape	Arrangement	Motility
W1	Negative	Rod	Single Pair	Non-motile
W2	Negative	Rod	Single Pair	Fast
W3	Negative	Rod	Single Pair	Fast
W4	Negative	Rod	Short chain	Fast
W5	Negative	Rod	Cluster	Fast
W6	Negative	Rod	Single Pair	Fast
W7	Negative	Rod	Single	Fast
W8	Negative	Rod	Single Pair	Fast
W9	Negative	Rod	Single Pair	Fast
W10	Negative	Rod	Single Pair	Non-motile
W11	Negative	Rod	Single Pair/Short chain	Fast
W12	Negative	Rod	Short chain	Fast
W13	Negative	Rod	Single Pair	Fast

W14	Negative	Rod	Single Pair	Fast
W15	Negative	Rod	Single Pair/Short chain	Fast

4.1.3.3 Biochemical characterization of isolates

After morphological characterization, strains were subjected to biochemical characterization.

The details are mentioned in Table 4.4.

Table 4.4: Biochemical characteristics of bacterial strains isolated from Rawal Lake

Strain ID	Oxidase	Catalase	Citrate
W1	Negative	Negative	Positive
W2	Positive	Positive	Positive
W3	Positive	Positive	Positive
W4	Positive	Positive	Positive
W5	Negative	Positive	Positive
W6	Positive	Positive	Negative
W7	Negative	Negative	Positive
W8	Positive	Positive	Negative
W9	Positive	Positive	Negative
W10	Negative	Positive	Positive
W11	Negative	Positive	Positive
W12	Negative	Positive	Positive
W13	Positive	Positive	Positive
W14	Positive	Positive	Negative
W15	Positive	Positive	Positive

4.1.3.4 API 20E kit

Further biochemical identification was performed by API 20E kit. Bacterial genus were identified using the 7 digit code obtained. Strain ID along with the genus identified is listed in Table 4.5.

Table 4.5: Identified bacterial strains through API 20E kit

Strain ID	Bacteria Identified
W1	*Flav.multivorum*
W2	*Pseudomonas spp*
W3	*Tatumella spp*
W4	*Bacillus spp*
W5	*Ser.liquefican*
W6	*Salmonella spp*
W7	*Shigella spp*
W8	*Alcaifaciens*
W9	*Bacillus spp*
W10	*K.Pemuno spp*
W11	*Erwinia spp*
W12	*Proteus spp*
W13	*Aeromonas spp*
W14	*Escherichia spp*
W15	*Pseudomonas spp*

4.1.3.5 Molecular characterization

Strains were characterized molecularly at genus and specie level through PCR amplification and 16S rRNA sequencing process. The detail of which are mentioned below:

4.1.3.6 DNA extraction and PCR amplification

DNA of the isolated strains were extracted through kit method and were confirmed by running it on agarose gel. Extracted DNA of the isolated strains were further subjected to PCR amplification process for the genus identification 27F and 1429R primers were used to amplify the 500 bp fragment of 16S rRNA genes of isolated bacterial strains. PCR amplification products of 500 bp were obtained for all the isolates. Amplified genes of strains W1-W15 were visualized by 1% agrose gel, stained with loading dye and was observed under UV trans illuminator. Fig 4.14 is the gel picture of amplified genes of isolated strains.

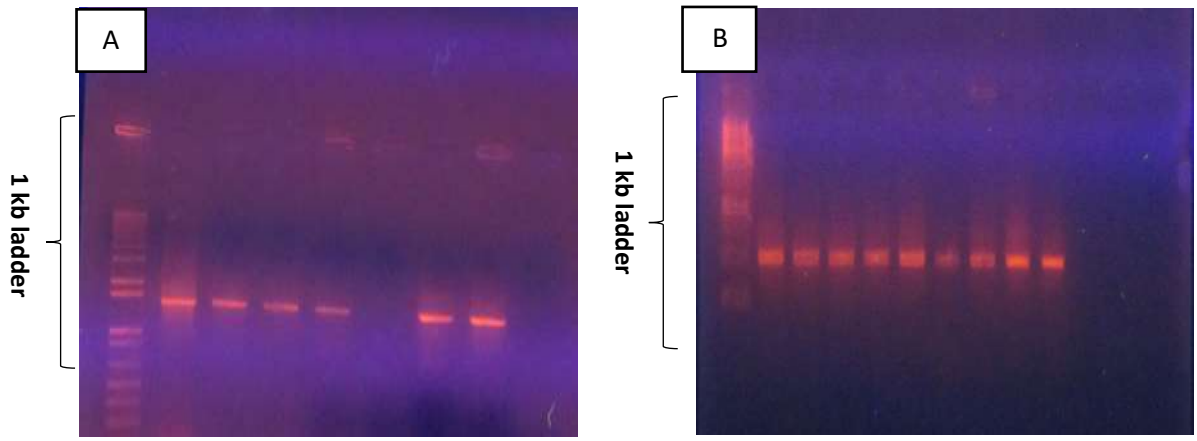


Fig 4.14 (a) From left to right, amplified genes of strains W1-W6 (b) from left to right, amplified genes of strains W7-W15

4.1.3.7 16S rRNA sequencing

For further degradation studies three strains were selected on the basis of growth studies mentioned in section (phaseII). Maximum sulfamethoxazole degrading potential was exhibited by isolates W13, W14 and W15. PCR products of the strains were sent to genome analysis department, Macrogen. Sequences that were obtained were trimmed through bio-edit software and were identified through BLAST tool of NCBI. After obtaining the accession number phylogenetic tree was constructed which demonstrate the relatedness and linkages of different bacterial strains identified. The phylogenetic tree is represented in Fig 4.15. The bacterial strains along with accession number are given in Table 4.6

Table 4.6: Bacterial strains along with location and accession number

STRAIN ID	LOCATION	SPECIE IDENTIFIED	ACCESSION NUMBER
W13	Korang River	*Aeromonas hydrophila*	MK280751
W14	Shahdara Stream	*Uncultured Escherichia sp.*	MK280752
W15	Nurpur Stream	*Pseudomonas aeruginosa*	MK280753

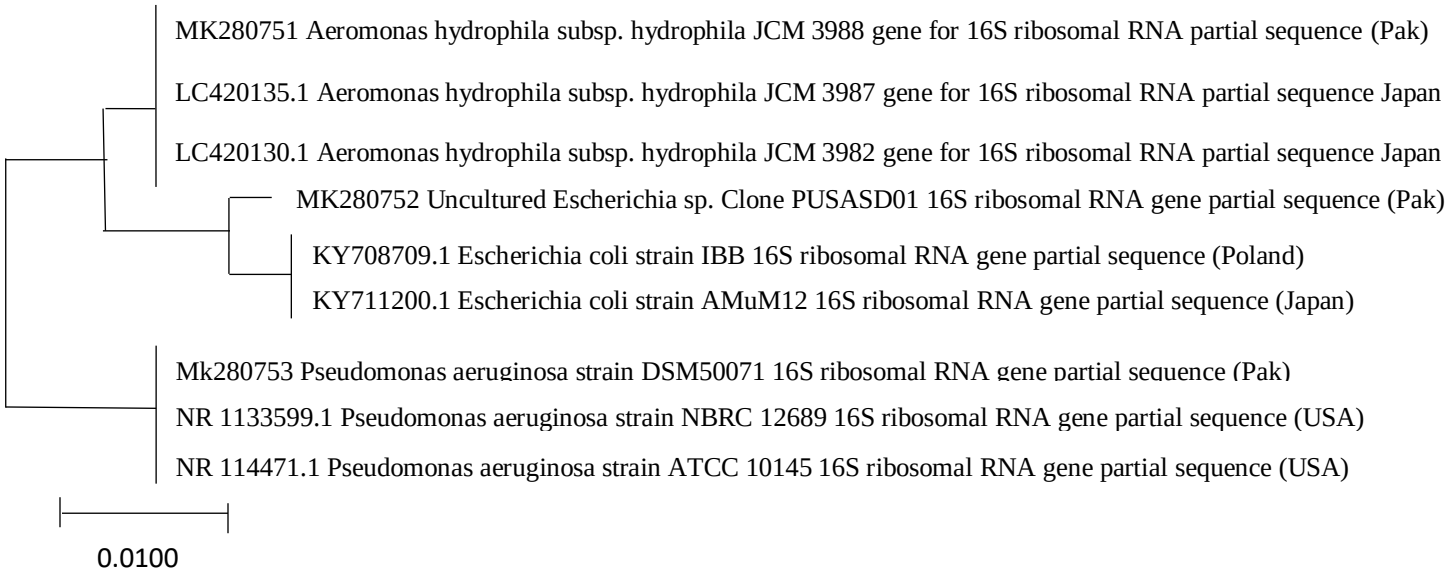


Fig 4.15: Phylogenetic tree demonstrating relatedness and linkage to different bacterial strains

4.2 PHASE II

4.2.1 Screening of potential isolate

Screening was done in order to determine the effect of SMX at initial concentration on the growth of bacteria. Strains that exhibited the maximum growth were then selected for further SMX degradation. The effect of initial SMX concentration of 4mg/L on the growth of potential degrader strains is presented in Fig 4.16, 4.17 and 4.18. Bacterial strains W13 identified as *Aeromonas hydrophila*, W14 identified as *Escherichia coli* and W15 identified as *Pseudomonas aeruginosa*. Growth in nutrient broth confirms that SMX is not posing any inhibitory effect on the particular bacterial strains.

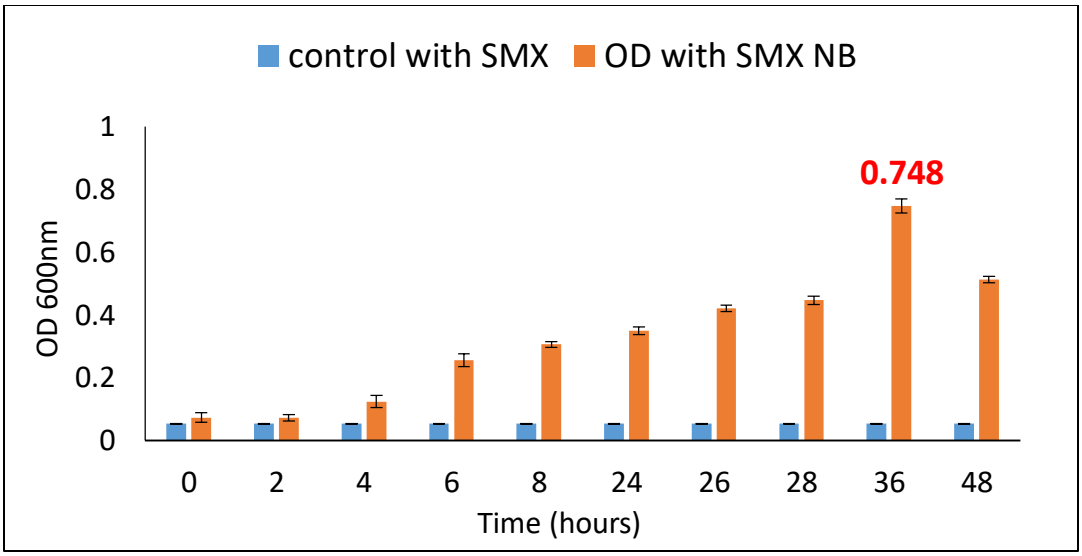


Fig 4.16: Growth curve bacterial strain Aeromonas hydrophila

Fig 4.16 represents the growth curves for bacteria strain *Aeromonas hydrophila* the bacteria was capable of growing in SMX at initial concentration and rate of bacteria was recorded highest at 36 hours and declined till 48 hours. Initially growth was detected from 2 hours of incubation

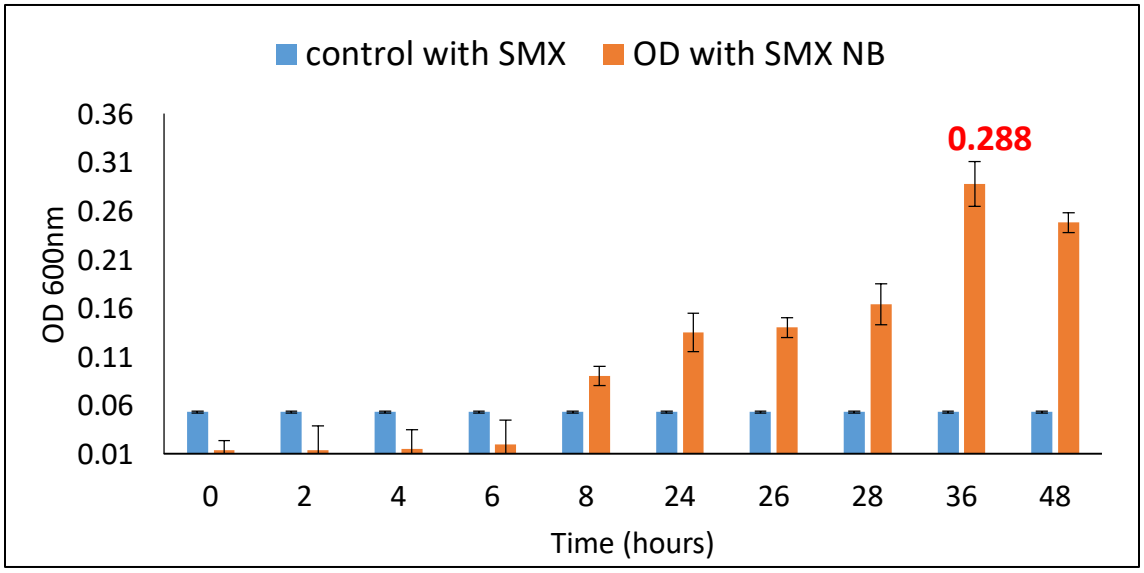


Fig 4.17: Growth curve bacterial strain Escherichia coli

Fig 4.17 shows growth curve of bacterial specie *Escherichia coli*. The growth curve indicates that the bacteria was able to grow under SMX and used it as a source of carbon and energy highest growth was observed at 36 hours and growth declined at 48 hours.

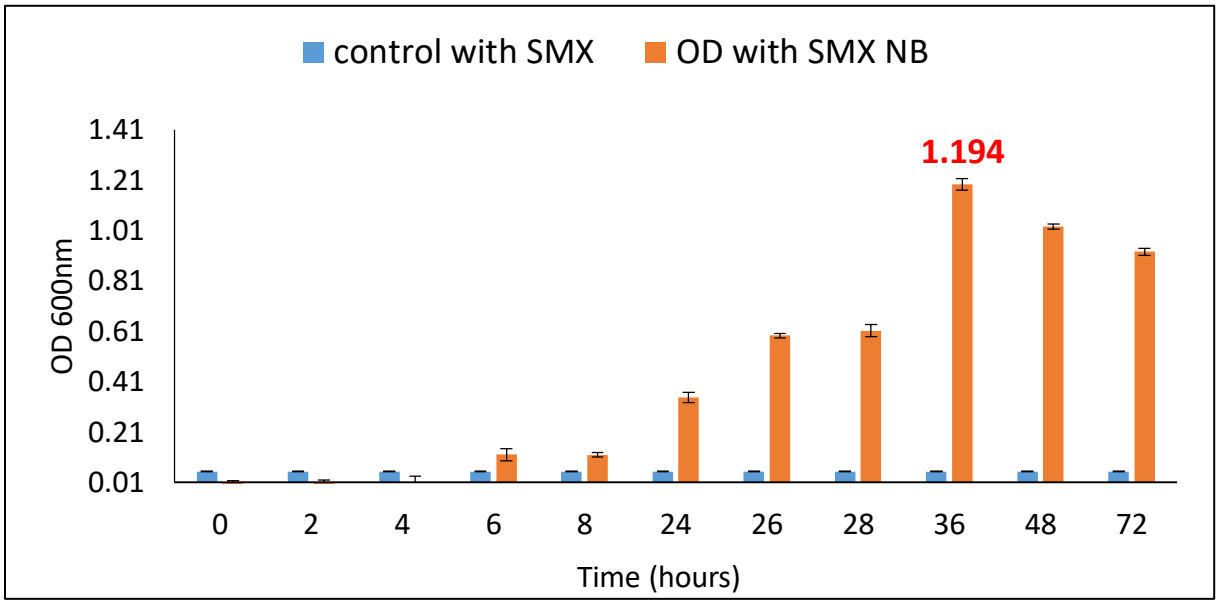


Fig 4.18: Growth curve bacterial strain Pseudomonas aeruginosa

Fig 4.18 illustrates the growth curves of bacteria strain *Pseudomonas aeruginosa*; this strain depicted highest growth capability under SMX exposure and was able to grow. Highest growth was observed at 36 hours and growth rate declined after 48 hours up till 72 hours.

4.2.2 Biodegradation of SMX

The effect of antibiotic concentration on bacterial strain and the its capability of SMX biodegradation was observed by adding different concentrations in NB and MSM which varied from 4-12 mg/L. SMX biodegradation was checked at regular intervals (0-72 hrs.) using chemical oxygen demand (COD) closed reflux titrimetric method. The bacteria that indicated the

highest capability of growth under initial concentration of SMX was then grown under increasing SMX concentrations to find out the optimum concentration. The result indicated that bacterial growth was inhibited as the concentration of the compound was increased. COD reduction rates indicated different results depending on differences in bacterial species in the liquid medium and the type of medium used for degradation experiment. The COD reduction efficiencies of *Aeromonas hydrophila, Escherichia* and *Pseudomonas aeruginosa* species are further explained.

4.2.2.1 Degradation of SMX by *Aeromonas hydrophila*

Fig 4.19a represents COD removal rate and OD measured at 600 nm for bacterial strain identified as *Aeromonas hydrophila***.** Degradation was monitored for 72 hours in both media. The bacteria showed inhibiting growth as the concentration was increased. In nutrient broth increased percentage removal of SMX was observed at 72 hours for all concentration but was decreased as the concentration of SMX increased. The degradation of SMX by *Aeromonas hydrophila in* nutrient broth was recorded to be 79.8, 75, 70.8, 71 and 50.4 % at 4, 6, 8, 10 and 12 mg/L respectively.

Maximum percentage removal (79.8) of SMX at 4 mg/L was observed with an optical density 1.309 nm at 72 hour of incubation. Almost similar trend was observed for 6 mg/L but as the concentration increased to 8 and 10 mg/L the % removal of SMX decreased considerably by 75 and 70.8% respectively after 72 hour. The lowest percentage removal was observed at 12 mg/L (50.4%) with a recorded optical density of 1.337 nm.

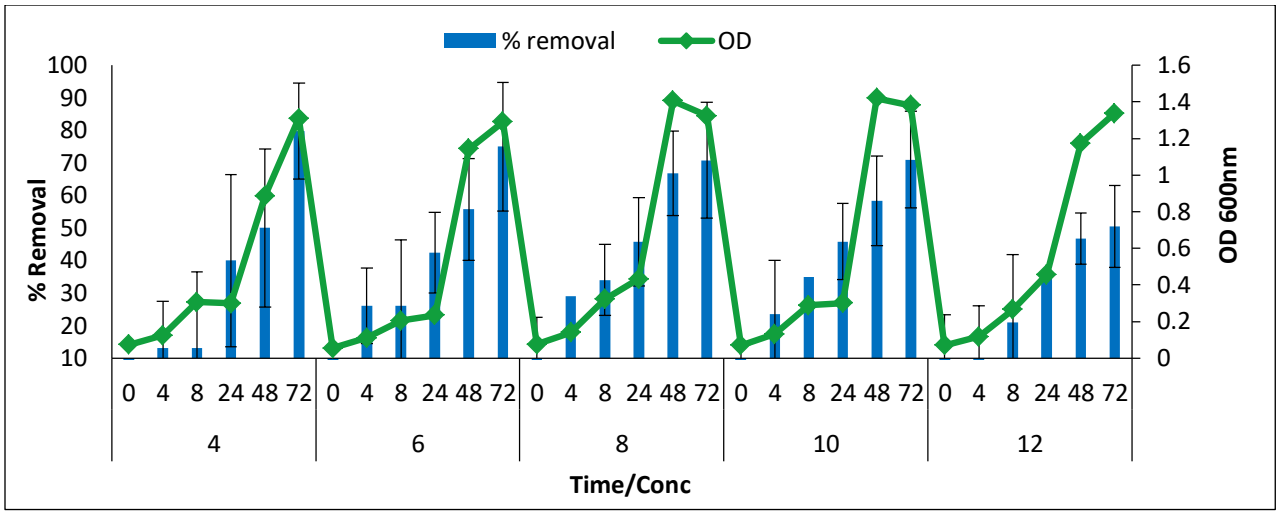


Fig 4.19 a: ***Percentage removal of SMX related with optical density for Aeromonas hydrophila measured in nutrient broth culture media***

In MSM (Fig: 4.19b*) Aeromonas hydrophila* a similar trend was observed and percentage removal of SMX was recorded to be highest for all concentration at 72 hours of degradation but as the concentration of the compound increased a decrease of SMX removal was observed. Highest percentage removal of SMX was recorded at 4 mg/L (75.5%) with an optical density of 0.026 nm at 72 hours of incubation. As the concentration increased percentage removal decreased and lowest removal rate was recorded at 12 mg/L, 57.9% with an OD of 0.033 nm at 72 hours. Experimental results on monitoring microbial activity in the both media medium with SMX showed an increase in OD at 4 hours of incubation. A distinct increase in OD was recorded at 72 hours for both media.

Degradation of Sulfamethoxazole in MSM indicates that the bacteria may be able to use the compound as a sole carbon and energy source. The resistance of *Aeromonas hydrophila* to SMX may be attributed to its dissimilatory sulfate reduction and resistance mechanisms against toxic compounds encountered in polluted waters. These enzymes may have bio-remediative as well as industrial potential (Seshadri *et al*., 2006). A study reported the presence of multi antibiotic

resistant *Aeromonas* species from aquatic sources, which indicated the indiscriminate use of antibiotics and poses public health problems to both humans and aquatic animals (Odeyemi & Ahmad, 2017).

Aeromonas hydrophila have been reported to degrade industrial dye (Reactive Black 5 RB5) at higher concentration of about 100 mg/L. Degradation by *A. hydrophila* was found to be 76% at 100 mg/L within 24 h. The optimum pH and temperature for the decolorization was 7 and 35 °C respectively (Bouraie *et al.*, 2016). In another study other xenobiotic such as benzyldimethylalkylammonium chloride was degraded by *A. hydrophila*. The results indicated that the bacterium may use the contaminant utilized as sole sources of carbon and nitrogen (Patrauchan *et al.*, 2003).

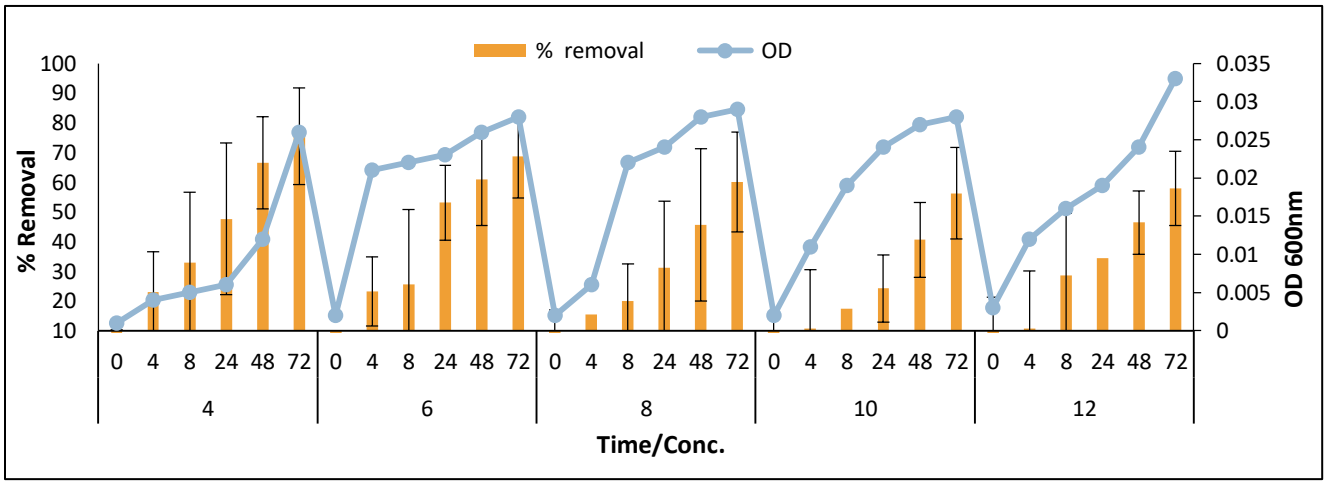


Fig 4.19b: Percentage removal of SMX related with optical density for Aeromonas hydrophila measured in MSM culture media

4.2.2.2 Degradation of SMX by *Escherichia sp.*

Similar results for SMX degradation were observed for *Escherichia sp*. results for SMX degradation is indicated in Fig 4.20a and 4.20b as the concentration of SMX increased a decrease

COD removal percentage was detected which may be attributed to the inhibitory effects of SMX towards the bacterial strain. In NB the maximum COD removal % (83.59) was observed at the lowest concentration of 4 mg/L concentration at 72 hour with OD of 0.954 nm. A gradual increase in removal rate and OD was observed for all concentrations. Significantly higher removal rate was recorded at 72 hours; lowest removal rate of 59.4% for *Escherichia sp* was recorded at 12 mg/L at 72 hour of incubation. The adherence of *Escherichia sp.* towards sulfonamide drugs may be attributed to presence of sulfonamide-resistant dihydro pteroate synthase enzyme which is determined by the R plasmid (Wise and Abou-Donia 1975).

In MSM *Escherichia sp.* presented good growth capability for SMX removal from media, the bacteria was able to use the compound as energy and carbon source. The maximum removal rate of 82% was recorded at 4 mg/L with an OD of 0.032 mm at 72 hours and lowest removal rate 59.5 % was recorded at 12 mg/L with an OD of 0.049 mm as indicated in Fig 4.20b. COD removal rate recorded for *Escherichia sp.* were 82, 73.8, 68.8, 56 and 59.9 at 4, 6, 8, 10 and 12 mg/L respectively. Zhang and coworkers in 2012 used *Escherichia* and *Acinetobacte*r to degrade two sulfonamide drugs (sulfapyridine and sulfathiazole). About 66 or 72% of sulfapyridine and 45 or 67% of sulfathiazole contained in the media was degraded by *Escherichia* or *Acinetobacter* sp. respectively, after incubation for 2 days.

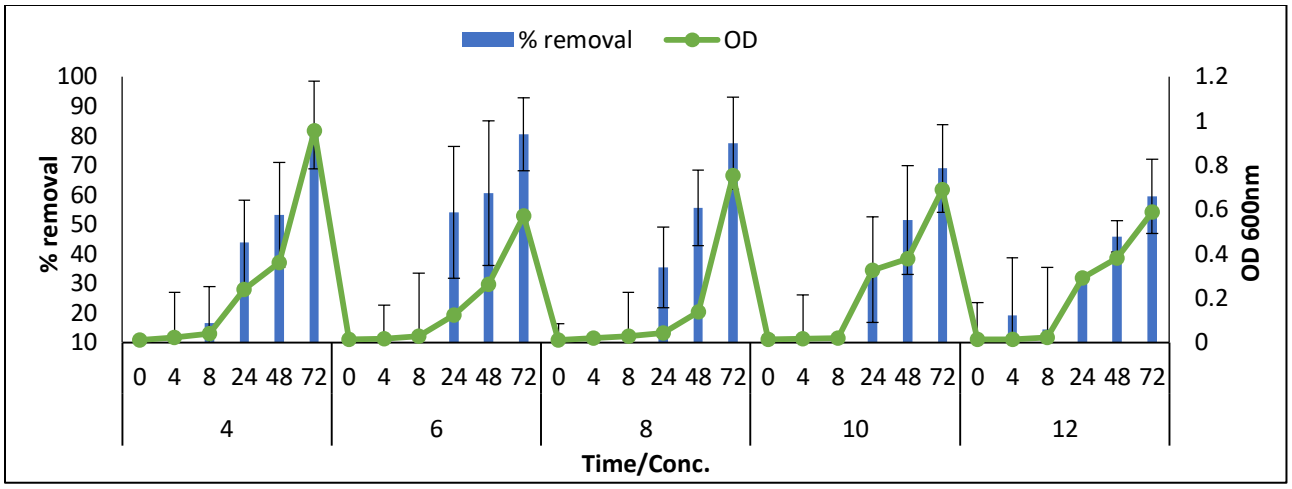


Fig 4.20 a: Percentage removal of SMX related with optical density for Escherichia sp measured in nutrient broth culture media

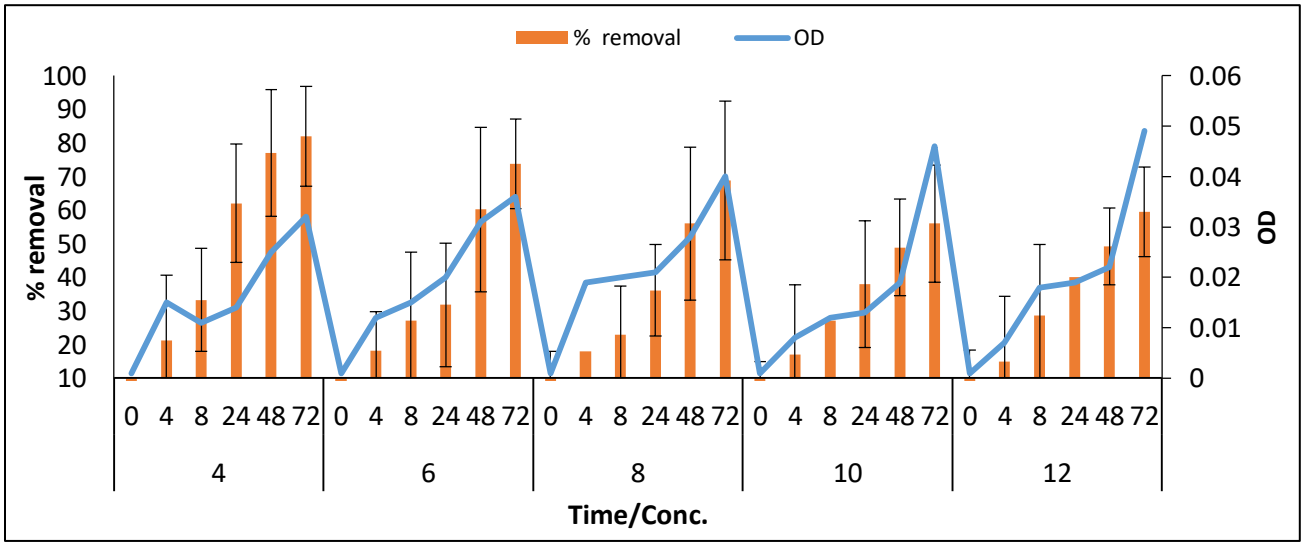


Fig 4.20 b: Percentage removal of SMX related with optical density for Escherichia sp measured in MSM culture media

4.2.2.3 Degradation of SMX by *Pseudomonas aeruginosa*

Degradation of SMX by *Pseudomonas aeruginosa* is sown in Fig 4.21a and 4.21b in NB and MSM respectively. A gradual increase in the removal rate was observed for all concentrations in both media from 0-72 hours. The maximum degradation with removal rate of 9.2% in NB is at 4 mg/L after 72 hours of incubation with an OD of 1.364 nm, slight change in OD was observed

after 8 hours of incubation and maximum increase in OD is at 72 hours. Increase in OD with respect to time indicates bacterial growth in the presence of compound. Similar trend of decreased removal rate with increase in concentration was observed. Lowest removal rate of 68.7% was recorded at 12 mg/L after 72 hours of incubation in NB. In MSM similar trend of degradation was observed for *P. aeruginosa* maximum removal rate of 93.75% was observed at 4 mg/L at 72 hours with an OD of 0.019 nm. The removal rate increased with time and decreased as the concentration of compound increased. Lowest degradation rate in MSM was recorded at 12 mg/L after 72 hours as indicated in Fig 4.21b. *Pseudomonas sp.* has been reported to remove many organic and inorganic compounds due to its multifactorial mechanism of responses and resistances (Morita *et al*., 2014).

Aissaoui and coworkers in 2016 used *Pseudomonas sp.* and tested its ability to grow in minimal mineral salt medium (MMSM) in presence of 6 mg/l of SMX as a sole source of carbon and energy. The results revealed that all the strain was able to grow and resist SMX. The removal rate achieved by the strain after 48 hours of incubation in MMSM at 26°C was 34.27%. Degradation of SMX by means of *Pseudomonas sp.* has also been reported by Herzog and co-workers in 2013 and confirmed that the specie plays an important role for the biodegradation of micro pollutants and reported an SMX biodegradation rate of 1.7 mg/L/day. A study reported the use of *Pseudomonas sp.* DX7 was inoculated into mineral salt media containing 10 mg/L of sulfonamide for 2 days at 30°C and achieved a removal rate of 20–30%. Jiang and co-workers in 2014 employed the use of a cold adapted strain *Pseudomonas psychrophila* HA-4. Strain HA-4 removed sulfamethoxazole at temperatures ranging from 5.0°C to 30°C, with the maximal removal rate at 10°C. The maximal removal rate of sulfamethoxazole by strain HA-4 was 34.30% after 192 hours at 10 °C.

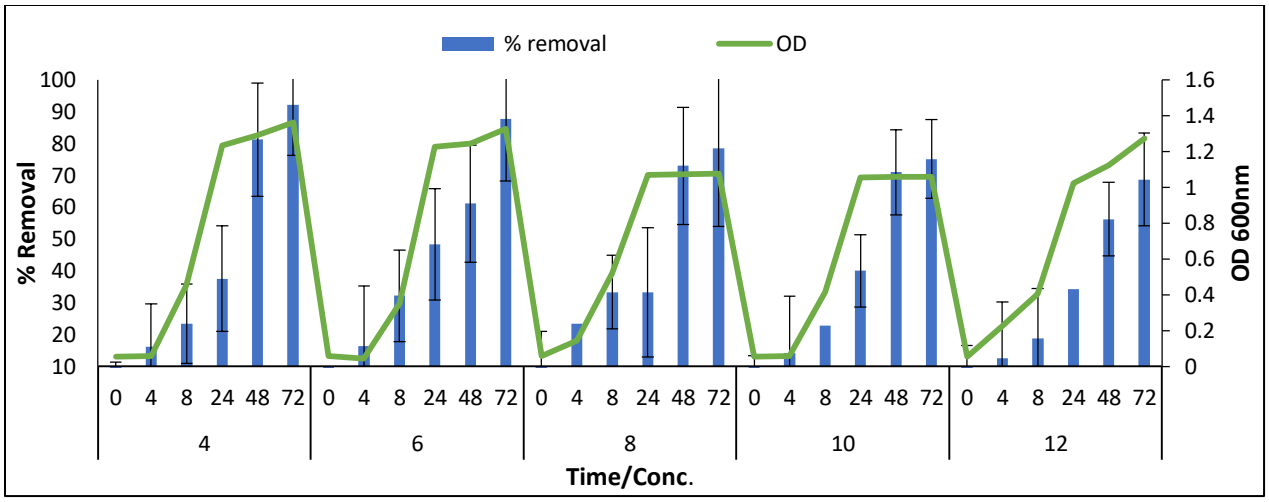


Fig 4.21 a: Percentage removal of SMX related with optical density measured for Pseudomonas aeruginosa in nutrient broth culture media

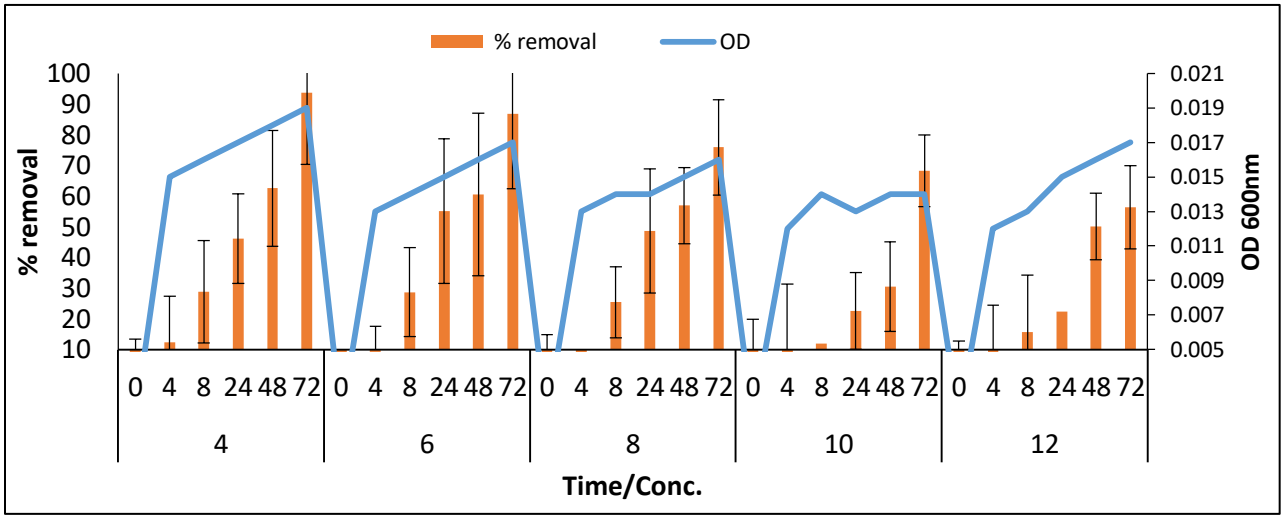


Fig 4.21 b: Percentage removal of SMX related with optical density measured for Pseudomonas aeruginosa in MSM culture media

4.3 PHASE III

4.3.1 SMX DEGRADATION IN FISH TANKS

To assess whether SMX degradation may occur in water that contained living organisms, 4 experimental tanks of 50L capacity each contained 10 fish were setup in Environmental Toxicology laboratory. Tank 1 served as control and only contained fish, tank 2 (Fish + SMX) was setup to evaluate SMX effect on fish, tank 3 contained inoculum of bacteria used for degrading SMX; the tank was setup to check whether the SMX degrading bacteria has any adverse health effects on fish, tank 4 (Fish + SMX + Bacteria) was used to assess the degradation of SMX in the presence of fish, the methodology is depicted in Fig 3.4. Water samples from each experimental tank were withdrawn to check degradation of SMX using COD analysis. An optimum SMX concentration derived from previous degradation experiment was assessed to be 4 mg/L and the potential SMX degrading strain was identified to be *P. aeruginosa.*

Following titration, COD was calculated and it revealed a clear indication of decrease in COD values for tank 2 and tank 3 in 96 hours (Fig 4.22). The tank containing Fish and SMX (tank2) indicated low COD after 24 hours. Tank 3 containing fish and bacteria also indicated slight variation in COD values with time but overall the fish in tank appeared in good condition. The lowest COD was recorded in tank 4 (SMX and bacteria along with fish) as compared to control and kept on decreasing with time, lowest COD values were recorded at 96 hours of sample inoculation. The results of the degradation experiments using *P. aeruginosa* indicated that the tank 4 containing SMX and potential SMX degrading strain had lowest COD as compared to control after 96 hours. A COD removal rate of about 88.52% was observed in 96 hours thus it maybe concluded that SMX was being removed from the water by the bacteria. The fish in tank

4 were least effected by SMX as compared to tank 2 were significant changes was observed. Tank 2 also indicated decreasing COD values with time indicating that SMX may be consumed by fish. The fish exposed to bacteria were not affected and did not show any signs of changed behavioral pattern toward the bacteria and were least effected by exposure.

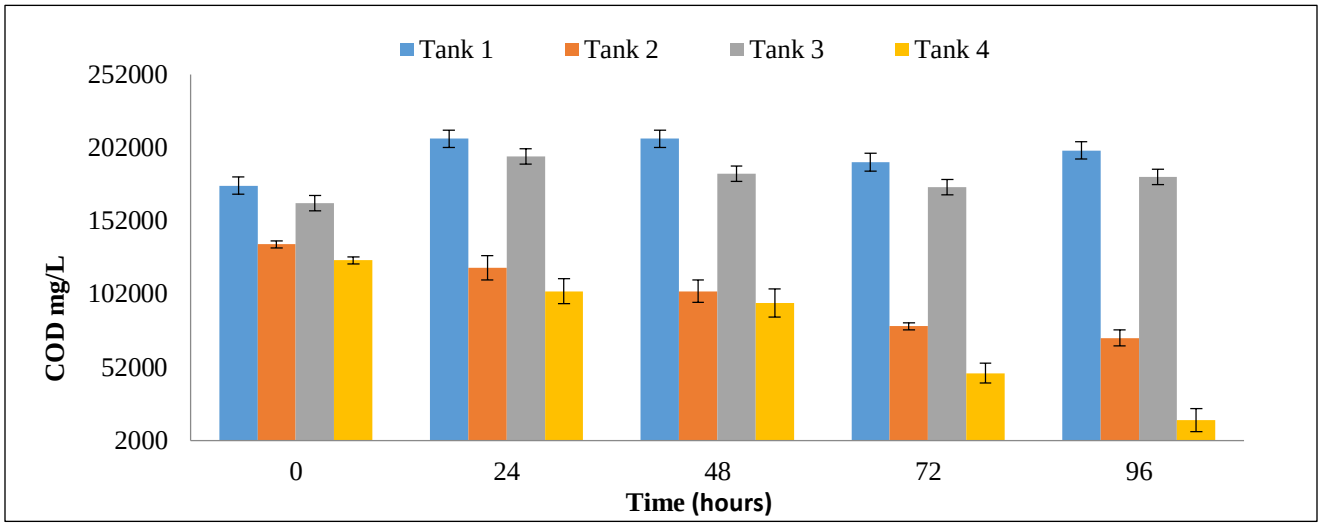


Fig 4.22: SMX degradation in Fish tanks using Pseudomonas aeruginosa and 4 mg/L SMX

4.3.2 BIOTOXICITY EVALUATION

In the present study fish were exposed to environmental relevant nominal concentration of 4 mg/L, of SMX for a period of 28 days. Fish showed a common and concentration based behavioral signs. As a common sign, fast schooling movement towards the opposite end was observed when any object moved closer to the tank. Mild behavioral signs such as schooling behavior disruption, swimming pattern, opercula movement, mucus secretion were observed and finally they settled at the bottom of the experimental tanks. The observed behavioral changes may be due to the toxicity of SMX. A similar trend of concentration based behavior was observed in *Cirrhinus mrigala* when exposed to Sulfamethazine (Ramesh *et al*., 2018).

4.3.2.1 Hematological Parameters

Fig 4.23 to 4.26 shows variations in RBC, Hb, HCT, and WBC after SMX exposure. Except for WBC the levels of all three parameters were found decreasing with time.

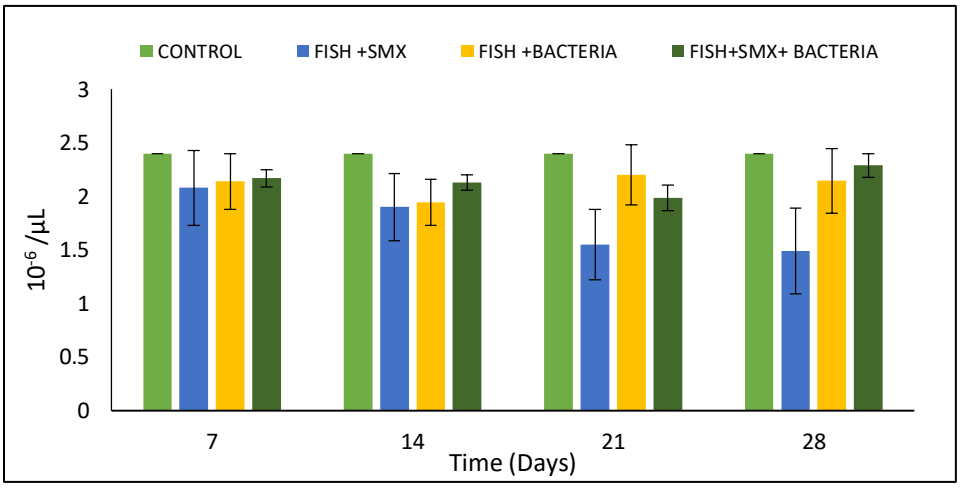


Fig 4.23 Effect of SMX on RBC of Fish

Fig 4.23 indicates the number of RBC of control and exposed groups to SMX for a period of 28 days. Fish in tank 2 indicated prominent alteration in RBC levels. An RBC Count of 2.4×10^{-6} /µL was observed in control group however, it reduced with the passage of time. Initially at 7 days the RBC count was not significant however significant decrease in RBC count on the 14^{th}, 21^{st} and 28^{th} day, where the values ranged from 1.9, 1.55 and 1.49×10^{-6} /µL respectively. The maximum decline was observed at the end of the 28^{th} day with value of 1.49×10^{-6} /µL RBCs are a major component of the blood and helps in the circulation of components important for normal bodily functions, hence there measurement is vital and any slight deviation may provide information about the health status of an organism. SMX has lipophilic property that may affect the membrane of the RBCs and make it fragile and prone to disruption (Cicha *et al.*, 2003). The data obtained from CBC test was statistically analyzed using two-way ANOVA. The time

dependent changes included that SMX has major effect on red blood cells (RBC) and hemoglobin (Hb) ($p<0.05$).The fish present in tank 3 and tank 4 only presented slight variation and were not affected by exposure.

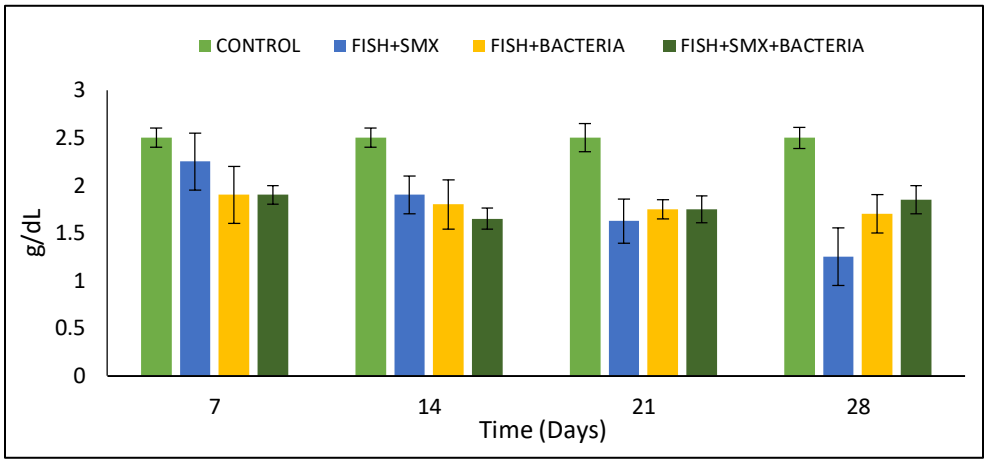


Fig 4.24: Effect of SMX on Hemoglobin of Fish

A similar pattern to RBC was observed in the Hemoglobin and Hematocrit ($p<0.05$) content as it showed decrease in value over the passage of time as presented in Fig 4.24 and 4.25. The normal value of hemoglobin in control group (2.5 g/L) exhibited slight variation after the 7th day but remained almost similar till the 28th day. HB content when compared with control was found to decrease from the 21st day with a value of 1.625 g/dL. A notable reduction in Hb content was observed on the 28th day displaying a value of 1.2 g/dL. Hb content in blood is a good indication of oxygen level in blood, during internal and external stress the body goes into anoxic conditions which disturbs the body's alternative energy synthesis process in the organism. The decline in Hb content might indicate that the fish is in stress conditions. Fig 4 shows the HCT values indicated in percentage also decreased with time and indicated a decline on the 28th day with a value of 2.45%. HCT indicated the amount of RBC in plasma and indicates blood viscosity. The

deviation in HCT values may be caused due to stress (internal or external). The decrease in RBC count, Hb and HCT levels indicates the anemic conditions due to exposure of SMX for a longer duration (Vazquez and Guerrero, 2007). Fish exposed to bacterial inoculum and those present along with SMX and bacterial strain presented only slight alteration in hematology (Samuel *et a*l., 2018).

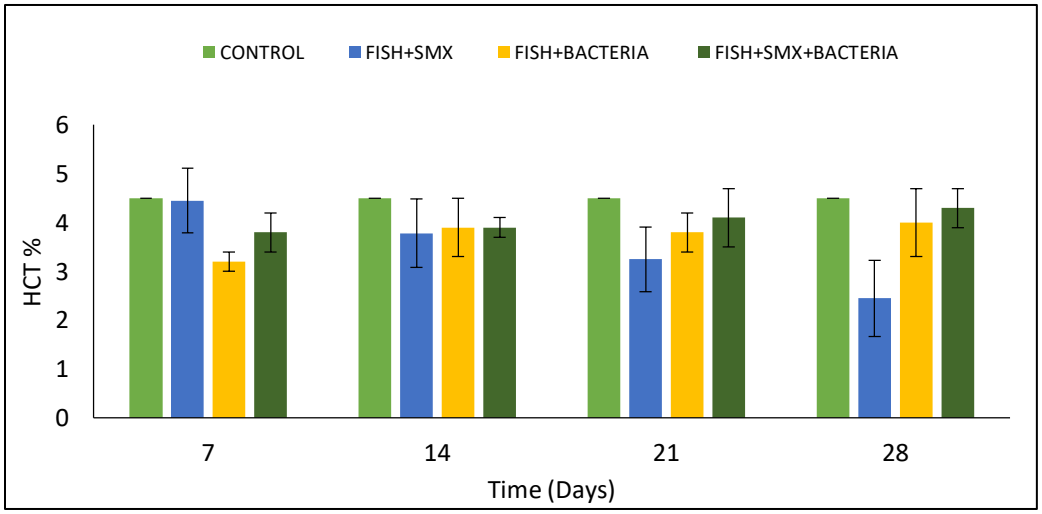


Fig 4.25: Effect of SMX on Hematocrit of Fish

White Blood Cells play a major role in providing defense to the body and any alteration in the WBC count may indicate an infection in the organism. These infections may be caused by stress or damage to tissues. Fig 4.26 shows WBC count of control and exposed fish to SMX for 28 days. Fish that were exposed to SMX when compared to control (14.6×10^{-6} /µL) indicated increase in the WBC count. The WBC count started to increase on the 14th, 21st and continued till the 28th day with values ranging from 16.3, 22.4 and 26.05×10^{-6} /µL. Maximum increase was observed on the 28th day with a value of 26.05×10^{-6} /µL. Lunden and Bylund (2002), verified that WBC count is related to the immune response of the body, thus in the presence of any foreign stress the spleen produces new WBC's. Proliferation in WCB count indicates production

of antibodies in the organism. A decrease in the WBC count indicates leucopenia in the fish due to SMX toxicity. Only slight change in WBC count was observed in Fish exposed to bacterial inoculum and those present along with SMX and bacterial strain (Oliveira *et al*., 2016).

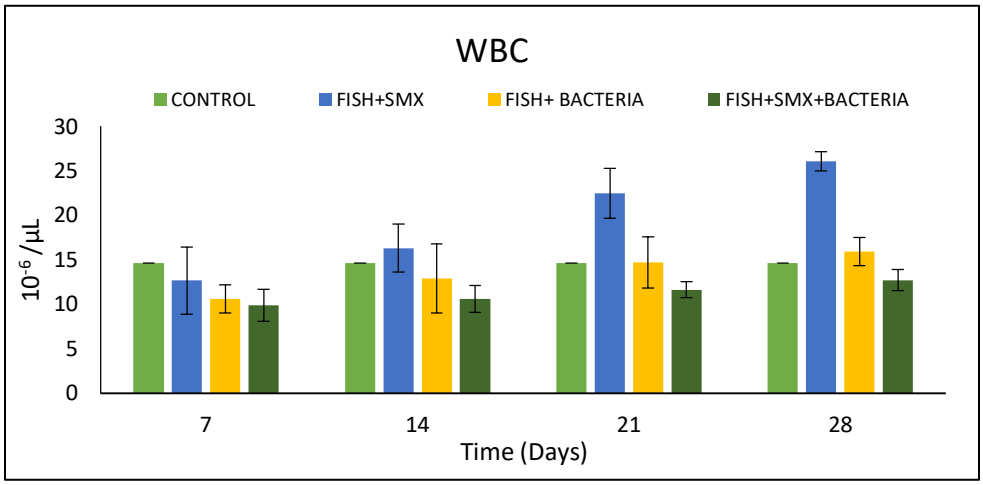


Fig 4.26: Effect of SMX on WBC of Fish

Fig 4.27, 4.28, and 4.29 shows blood indices values for MCV, MCH and MCHC, indicating considerable decrease in values with the passage of time signifying that the fish is under stress from the contaminant. This decreasing trend may indicate that the fish is under anemic conditions (Rodrigues et al., 2015). MCV levels of control (192.5fL) and fish exposed to MSX. Fig 4.27, the values of the fish exposed to a concentration of SMX shows a decreasing trend with values ranging from 178-148.85 fL. A decrease in the MCV values was witnessed on the 21st day of exposure (153.15 fL.). In Fig 4.28 MCH values also indicates a decreasing trend as compared to the control value 67.1 pg. The values of the treated groups range from 55.3-44.7 pg. with a lowest value noted on the 28th day. Both MCH and MCV levels were significantly lowered with time ($p<0.05$).

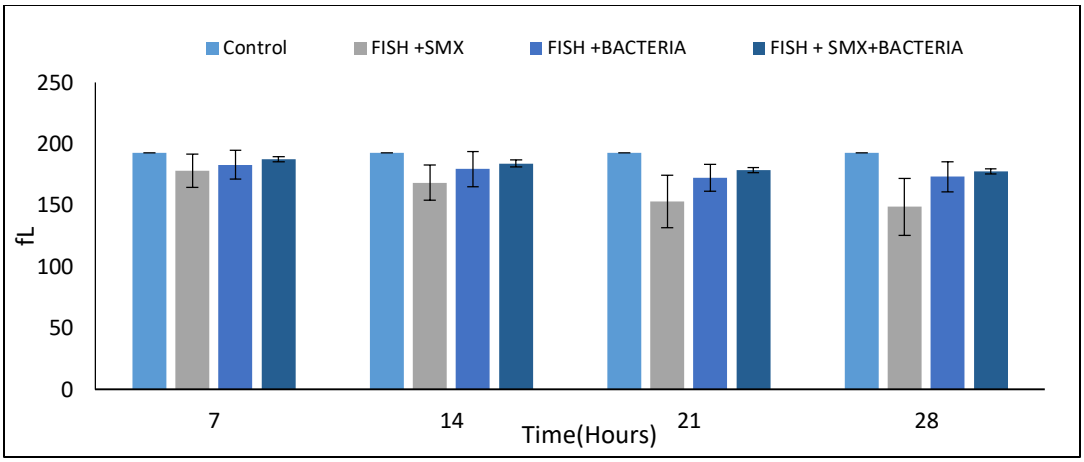


Fig 4.27: Effect of SMX on MCV of Fish

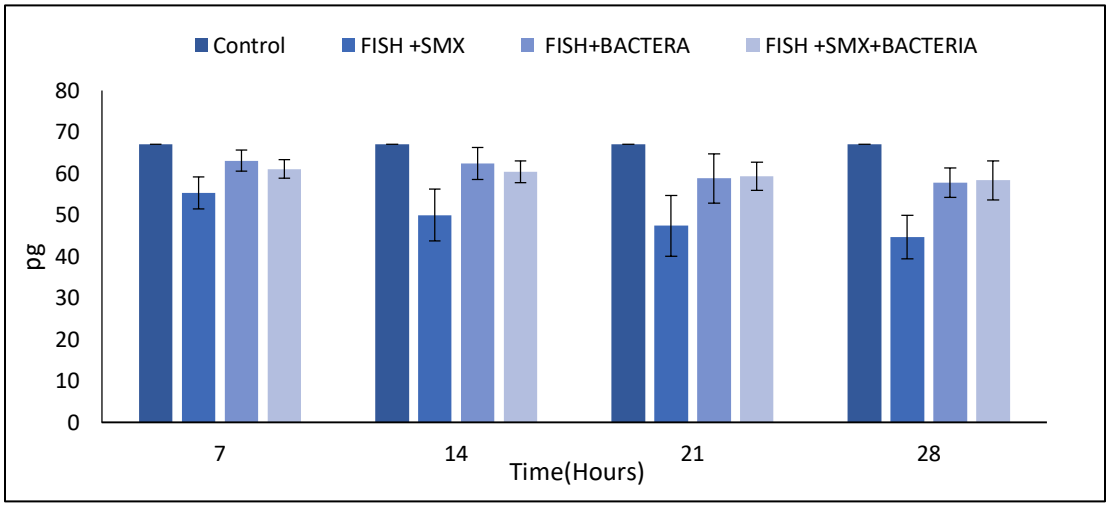


Fig 4.28: Effect of SMX on MCH of Fish

The values of MCHC are indicated in Fig 4.29, where a linear decrease in value was observed as compared to the control group (67.5 g/dL), the maximum decline in the MCHC values was observed at the 28th day (39.9 g/dL). When the fish was exposed to stressful conditions it creates oxygen deprivation in tissues resulting in carbon dioxide increase making the blood acidic which leads to RBC rupture, which may cause alteration in the values of MCHC (Vazquez and Guerrero, 2007).

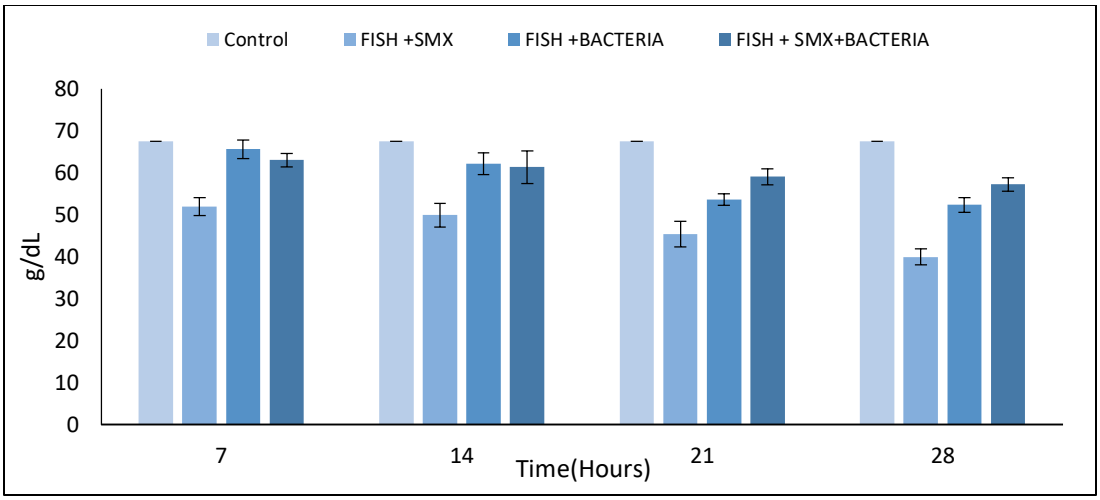


Fig 4.29: Effect of SMX on MCHC of Fish

The decrease in all blood indices may indicate formation of reticulocytes, shrunken and swollen blood cells may also cause the variation in MCHC levels (Sobecka, 2001). SMX only had a minor effect on MCHC ($p>0.05$). Sampaio and coworkers in (2016) studied the effects of SMTZ on *Oreochromis niloticus* and reported similar results of decrease in RBC indices with time when exposed to the drug. SMTZ was administered to Nile tilapia (*Oreochromis niloticus*) at a dose level of 422 mg kg/body weight, for a period of 11 days, via medicated feed. The results indicated a decrease in concentration of RBC, HCT, Hb, MCH and MCHC. Saglam and Yonar in 2009 reported the effects of a sulfonamide (Sulfamerazine) on rainbow trout the fish was exposed to a concentration of 100, 200, 400 mg/kg for 21 days. Blood parameters indicated decrease in values as compared to controls indicating anemic conditions in fish. Ramesh *et al.* in 2018 studied the response of *Cirrhinus mrigala* to 1 and 10 mg/L of sulfamerazine and reported a decrease in RBC, Hb and HCT values indicating anemic conditions, whereas an increase in WBC count was observed indicating fish under stress from infection and a biphasic trend was

observed in the values of MCV, MCH and MCHC which may indicate the inhibition of RBC formation due to lack of oxygen (Spadoto *et al*., 2018).

4.3.2.2 Biochemical Parameters

Biochemical parameters are studied to assess the normal body functions of an organism, in routine examination glucose and protein level are important parameters used to determine the normal bodily function in toxicology studies (Vutukuru, 2003). Fig 4.30 shows the effect of SMX on glucose levels of fish. When compared with control the values for exposed group shows a decline in trend for glucose levels. An overall decrease was observed in the values (49.7 - 29.55 mg/L) as compared to control (56.7 mg/L). A lowest value recorded on the 28th day of exposure (29.55 mg/L).

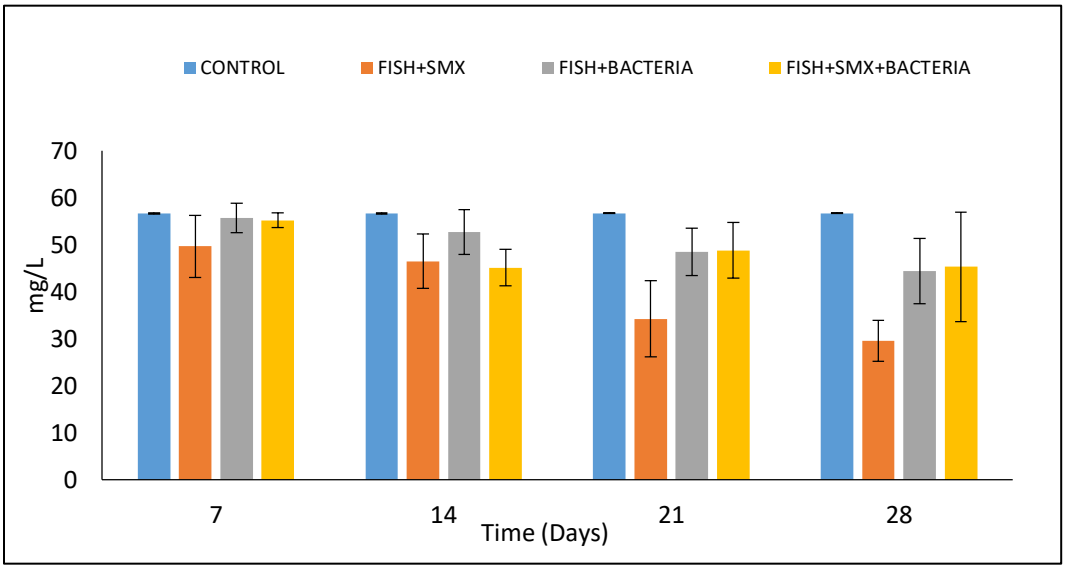


Fig 4.30: Effect of SMX on glucose level of fish

The breakdown of glucose in the body is a major process for energy production; glucose level in the body may vary if the fish is under stress and requires high energy needs. The conditions may

occur if the fish is under external or internal stress which may be caused by any xenobiotic which lead to a natural stress response in which the body secretes hormones such as corticosteroids, epinephrine and dopamine as a primary component to reactivate glycogenesis to overcome the high energy demand (Schwab *et al*., 2018). The increase and decrease in glucose levels due to the exposure of fish to xenobiotic may be due to the influence on carbohydrate metabolism which may cause an increase or decrease in the cortisol production which is a primary stress response. In the present study a decrease in glucose level maybe due to high metabolic demand caused by SMX. Similar trend was also observed by Ambili *et al*., 2013.

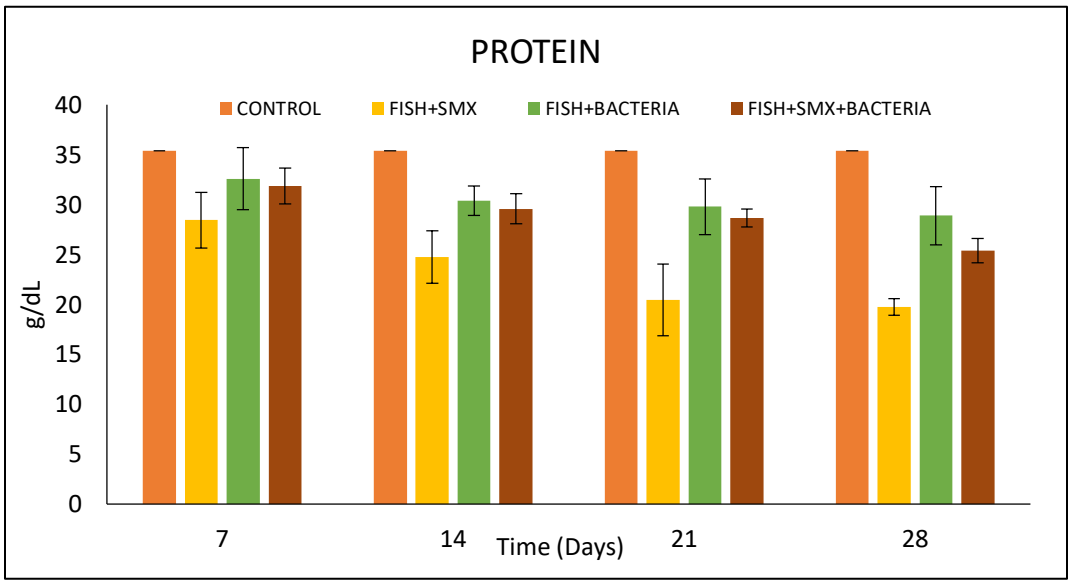


Fig 4.31: Effect of SMX on Protein level of fish

Lowered protein levels were also observed with time indicated in Fig 4.31. An overall decrease ranging from 28.45-19.75 g/dL was observed in the protein level compared with control values (35.4 g/dL). SMX significantly lowered glucose and protein level with time ($p<0.05$). Protein is an important component of every cell in the body. The body uses protein to build and repair tissues, to make enzymes, hormones, and other body chemicals. Protein is an important building

block of bones, muscles, cartilage, skin, and blood (Kristinsson *et al*., 2000). Fish when exposed to stress may undergo oxygen deficient conditions which may affect the protein content of body (Ramesh *et al*., 2018). The decrease in protein level may indicate the inhibition of protein synthesis and an increase in protein levels may be due to the adaptation of fish towards the contaminant. In this study the fish exposed to SMX shows a decrease in protein level as compared to the 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* (Saglam and Yonar, 2009) exposed to SMTZ showed a decreased in protein content which may be attributed to stress caused by the antibiotic or reduced protein synthesis.