POTENTIAL CO-RESISTANCE OF MICROBIAL ISOLATES TO HEAVY METALS AND ANTIBIOTICS IN WASTEWATER SAMPLES

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CERTIFICATE

It is certified that the contents and form of the thesis entitled "Potential Co-resistance of Microbial Isolates to Heavy Metals and Antibiotics in Wastewater Samples", submitted by Ms. Shanza Bashir, has been found satisfactory for patrial fulfillment of requirements for the degree of Master of Science in Environmental Science.

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DEDICATION

This thesis is dedicated to my dear and beloved parents, for their unconditional and endless love, care, and support, since forever.

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Shanza Bashir

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LIST OF ABBREVIATIONS

AGRs	Antibiotic Resistance Genes
BOD	Biological Oxygen Demand
COD	Chemical Oxygen Demand
CLSI	Clinical and Laboratory Standards Institute
CFU	Colony Forming Unit
DO	Dissolved Oxygen
EC	Electrical Conductivity
FAO	Food and Agriculture Organization of the United Nations
GDP	Gross Domestic Product
m^3/day	Meter cube per day
µg/L	Micrograms per Liter
µS/cm	Micro Siemens per Centimeter
mg/L	Milligrams per Liter
mm	Millimeter
NEQS	National Environmental Quality Standards
NTU	Nephelometric Turbidity unit
Pak-EPA	Pakistan Environmental Protection Agency
TDS	Total Dissolved Solids
TKN	Total Kjeldahl Nitrogen
US EPA	United States Environmental Protection Agency
WHO	World Health Organization

ABSTRACT

Existence of correlation between heavy metals and antibiotic resistance in bacteria is well-known and is being studied for a while now, but due to complex mechanisms occurring at molecular and genetic level, well established understanding of the topic is yet to be found. Heavy metals are known to act as a co-selecting agent for antibiotic resistance, and co-resistance mechanisms also exist between the two at microbial level, bringing about multidrug resistance in bacteria. The study aimed at assessing the wastewater characteristics within the channel running through the twin cities of Islamabad and Rawalpindi in terms of physicochemical parameters, heavy metal contamination, and co-resistance of heavy metals and antibiotics. The study area was comprised of water tributaries preceding Nullah Lai, Nullah Lai itself, followed by Soan River. The physicochemical analysis provided varying trends for all the parameters, with electrical conductivity, total dissolved solids, nitrate-nitrogen, and sulphates being within the NEQS, FAO and US EPA guidelines, whereas the rest violating the standards and guidelines. Selected heavy metals, cadmium (Cd), copper (Cu), lead (Pb) and zinc (Zn) were quantified in the collected wastewater samples. Zn concentration of all the samples was found to be highest, whereas that of Cu to be the lowest. Chaklala Cantt site reported the highest contamination of the selected heavy metals, immediately followed by the adjacent site of Saddar, whereas the last sampling location near Jamia Masjid recorded the lowest heavy metal pollution. Bacterial isolation from the samples provided majority of gram-negative strains. Heavy metal resistance test of the isolated bacterial strains proved 59.2 percent to be resistant against all the heavy metals tested, i.e., Cd, Cu, Pb and Zn. The antibiotics namely amoxicillin, ciprofloxacin, levofloxacin, and ofloxacin, resistance testing concluded 22.2 percent strains to have resistance against these selected antibiotics. Investigation on co-resistance of heavy metals and antibiotics in the isolated bacteria suggested the correlation of co-resistance existing between the two. To understand the interactions fully, detailed mechanistic studies are required in the future.

Chapter 1

INTRODUCTION

1.1 Background

The necessity of water for survival of any living being cannot be overlooked in any given scenario. Life on earth is dependent upon a continuous water supply, and humans, being the most significant species of the planet, are the ones consuming the most water, and at the same time are also the ones disturbing the natural waters most due to their unchecked activities. Humans and all other beings require a certain level quality of water for proper functioning of their systems, presence of any unwanted material above a certain level becomes a cause of hazard and toxicity to living beings.

As with the ever-increasing development in all fields of life, intensive urbanization, industrialization, and agriculture for the sake of economic growth have become mandatory for any nation's development, which are all water intensive activities, and thus are putting great pressures on the natural water resources, altering its natural makeup, and contaminating it to dangerous levels.

Contamination of water with high concentrations of heavy metals is among the major issues that the increasing development has brought with itself. Although, the natural waters contain some amounts of heavy metal in them, which are not harmful, from some natural sources such as weathering of rocks and soils, volcanic eruptions, etc.; but the main source of heavy metals in water is the intensive anthropogenic activities in the form of urbanization, industrialization, and other such developmental activities. Such excessive use of heavy metals allowed for evolution of the microorganisms to develop resistance against them, which further adds to the hazards and toxicity (De Wet et al., 2020; Tchounwou et al., 2012).

Another such contaminant that has become a major highlight in recent decades especially, is the antibiotics. Since their discovery, their use has altered the pattern of life for humans, and has revolutionized pharmaceutics, animal husbandry, food production, and agriculture for the better, but their over and misuse has brought about contamination of the environment and has led to rapid emergence of resistance in bacteria, opening a whole new era of crisis affecting the world. Records have shown there to be a significant relationship between heavy metals and antibiotics' microbial resistance, which is not yet fully understood, but is of utmost importance in the way for dealing with worldwide crisis of antimicrobial resistance (Gothwal & Shashidhar, 2015; Ventola, 2015).

Pakistan, for the most part, depends heavily upon agriculture for its economy, which means a continuous supply of high amounts of water is essential to meet the irrigation demands of the country. But as environmental problems like pollution, climate change, etc. are aggravating, water sources are also being depleted, and less and less freshwater is available for meeting the demands of agriculture, domestic and industrial needs. Thus, for meeting such demands, the concept of wastewater reuse is becoming more of a common practice. The wastewater is sometimes treated, but usually is used untreated, thus becomes a source of contamination for the various environmental mediums, e.g., untreated wastewater having high concentrations of contaminants when used for agriculture deposits heavy metals and antibiotics to soil and plants, contaminating the soil and affecting the health of the plant negatively as well due to presence of unnaturally high concentrations. From here, heavy metals tend to enter the food chain as well, where they cause harm to organisms, and can lead to human exposure, risking human health (Ensink et al., 2004; van der Hoek et al., 2002; Waseem et al., 2014).

Islamabad and Rawalpindi, together known as the twin cities, one being the federal capital, and latter being one of the most significant metropolitan cities, make up for great importance to the country. As of the latest census, Islamabad has a population of 2 million, whereas Rawalpindi has 2.09 million (Pakistan Bureau of Statistics, 2018). Such huge number of people do require continuous water supply for continuity of life, but with growing water shortage throughout the country, twin cities face the challenge equally, requiring for water reuse practices to be implemented. Therefore, monitoring of water quality is necessary to check for maintenance of basic parameters, as well as contaminants of significance, such as heavy metals, antibiotics, and their microbial resistances.

1.2 Problem statement

The ever-increasing population, industrialization, urbanization, and development lead to an inevitable release of pollutants like heavy metals and antibiotics into environmental components including water bodies. Increased heavy metal pollution has been reported to have an association of co-resistance with antibiotic resistance in bacteria, leading to aggravation of the multi-drug resistance in microorganisms. So, there is need to explore potential co-resistance to heavy metals and antibiotics in bacteria.

1.3 Significance of the Study

Data on relation of co-resistance between heavy metals and antibiotics has been observed to be comparatively scarce in Pakistan. Furthermore, Pakistan, for the most part is an agricultural country, and requires huge water resources for this reason, but depletion of freshwater resources presents a need of reusing wastewater for irrigation purposes. Therefore, monitoring of such wastewater for its quality parameters, including heavy metal and antibiotic resistance is necessary to prevent such contamination from further spreading into the environmental components, as well as the food chain, and ultimately leading to human exposure. Thus, the study is aimed at assessing physicochemical parameters and heavy metal contamination of such a wastewater body and determining the relationship between heavy metal and antibiotic resistance in bacterial strains obtained.

1.4 Objectives

Based on the surveyed literature and information obtained from it, the objectives of the study were designed as follows:

- Assessment of physicochemical parameters of the wastewater samples.
- Evaluation of heavy metal concentration in the samples.
- Establishment of relationship between heavy metal and antibiotic resistance.

Chapter 2

LITERATURE REVIEW

2.1 Water Pollution

Water is abundant and the most important commodity, which covers two-third of the surface of the earth (Wang et al., 2011). Water is imperative for the sustenance of life and also equally important when it comes to development of society and economy (Daud et al., 2017). Owing to the high indulgence and exploitation of natural resources by humans, the global environment is changing every day (Hussain & Khoso, 2014). Anthropogenic activities like intensive livestock farming, application of fertilizers and pesticides, deforestation, industrial effluent discharge, domestic sewage, inefficient irrigation practices, etc., have greatly influenced the quality of water (Khatri & Tyagi, 2015). Therefore, the gradual depletion of resources of water is happening.

The increasing scarcity of freshwater all around the world and the relative food security owing to the growing populations led to making fresh water a priority for usages other than agriculture. In developing regions, the issues related to water are highly escalating owing to the deficiency of resources, poor management systems for the resources available, and the limitations in financial resourcefulness (Raza et al., 2017). As indicated by the United Nations, about 780 million people lack access to drinkable water, comparative to the very lower number of 6.6 billion individuals in 2000 (Connor, 2015). Estimates have predicted more than half of the world’s population to suffer from moderate or severe water shortage by 2025 (Raza et al., 2017). In the meanwhile, a developing country like Pakistan suffers from extreme water shortage crisis for the last decade, as the availability of water for particular uses is becoming increasingly inadequate. Rapid population growth, expanding irrigation facilities, and elevated urbanization and industrialization are further stressing out aquatic reserves (Khoso et al., 2015).

It is estimated that 60 percent of Pakistan’s freshwater is wasted as a result of inadequate and irresponsible management, while the rest is only implied for the water needs (Raza et al., 2017). According to the Pakistan Bureau of Statistics, only about half of the country's population, i.e., 56 percent have availability of clean potable water (Farooq et al., 2008). Hence, the use of water with deteriorated quality causes the spread of various diseases. Daud and fellows (2017) reported that approximately 50 percent of ailments, and 40 percent of demises in Pakistan occur from consumption of low-quality water.

Agriculture sector consumes the most water worldwide, and withdraws nearly 70 percent of the freshwater (Winpenny et al., 2010). This provides for a competition of water resources between agricultural and industrial sector, further enhancing the water demand. In this scenario, wastewater has progressively become the cheap and more readily available alternative for irrigation needs throughout the world, particularly in the semi-arid and arid regions. It is assessed that about 10 percent of the population of the world consumes plant based food that is irrigated with wastewater, and more than 20 million hectares agriculture is irrigated with wastewater, either treated or untreated (Jiménez et al., 2009). Literature suggests that nearly 44 countries are relying on treated wastewater (reusing over 15 million m^3/day of reclaimed water) for irrigation purposes (Winpenny et al., 2010). Considerably, wastewater could be considered as a reliable water source that is available throughout the year and its accessibility and nutrient contents are important factors that make it a precious resource predominantly in water deprived regions of the world (Elgallal et al., 2016). But, such wastewater reusage brings with itself a whole new set of issues and complexities that are yet to be fully overcome, or even completely understood to be dealt with, and are threatening the well-being of humans, as well as other living beings.

2.2 Heavy Metals

Heavy metals are metallic elements of higher atomic mass, having a density of up to five times more than that of water. They are ubiquitous to the earth's environment and occur naturally but are categorized as contamination when their concentrations reach to unnatural amounts, leading to issues to harmful exposure to living beings. Therefore, heavy metal contamination origins from natural as well as anthropogenic sources (Dickinson et al., 2019; Masindi & Muedi, 2018).

Although, normal conditions do not cause significant environmental contamination of heavy metals, but certain processes and conditions have reported to be a reason for release of unwanted amounts of heavy metals into environment, e.g. weathering of rocks and sediments, volcanic eruptions, forest fires, sea salt sprays, and other such biogenic sources. These sources have reportedly led to arsenic (As), copper (Cu), chromium (Cr), cadmium (Cd), lead (Pb), mercury (Hg), nickel (Ni), and zinc (Zn) contamination among others, which even at low concentrations can cause toxicity (Masindi & Muedi, 2018).

2.2.1 Heavy Metal Pollution

Over the years, heavy metals have found an extremely wide array of applications throughout human life activities, including industrial (including mining, smelting activities), technological, domestic, health and pharmaceutical, agriculture, fossil fuel burning, and more. Although, all these utilities and applications of heavy metals have brought advancements, conveniences, and improved quality of life, but a major downside it brought with itself is an unwanted amount of heavy metals into environmental components, leading to contamination referred to as anthropogenic (meaning human generated) (Masindi & Muedi, 2018; Tchounwou et al., 2012).

Such heavy metal pollution, as a result of anthropogenic activities, leads to their bioaccumulation in ecosystems of the earth, causing affects like reduced biomass, harmful impacts on microbial communities, negatively altered plant growth, diseases and abnormalities in animals, and at times leading to biodiversity reduction. Thereof, providing ways for heavy metal contamination to enter the food chains, further providing exposure pathways to humans (Dickinson et al., 2019).

Some heavy metals are required for normal functioning of living beings, including humans, as they regulate a number of metabolic, biochemical, and physiological bodily functions, and are considered as essential nutrients, e.g., cobalt (Co), Cu, Cr, iron (Fe), magnesium (Mg), manganese (Mn), molybdenum (Mo), Ni, selenium (Se) and Zn, etc. Lack of these nutrients can even lead to distortion of body functions, diseases, and abnormalities. But owing to their persistence and toxic nature even at low concentrations, exposure to living being in unwanted amounts can lead to major hazards and toxicities, even at very low concentrations for some heavy metals. Therefore, risen unwanted amounts of heavy metals in the environmental components is a problem of major concern and implications throughout the world (Arshad et al., 2020).

Among major sources of heavy metal contamination's exposure to human are the wastewater sources, including municipal as well as industrial, and agricultural. As has been discussed earlier, water scarcity issues have led to prevalence of practices of wastewater reuse. Such heavy metal contaminated wastewater when reused for various purposes, especially agriculture, contaminate the plant bodies; animals when grazed on such land and plants, are also exposed to heavy metal contamination, which further provides more avenues for human exposure to such unwanted concentrations and types of heavy metals.

Numerous studies have provided evidences for the alarming amounts of heavy metals in water bodies throughout the globe. Zhou and collegues, (2020) reported concentrations of heavy metal in lakes and water bodies throughout the globe (Africa, Asia, Europe, North America, and South America) to have significantly increased, especially leading up after the 2000s. Comparing to World Health Organization (WHO) and United States Environmental Protection Agency (US EPA) standard threshold limits of heavy metals in water, developing regions appeared to exceed both number of heavy metals and concentrations in comparison to the developed countries. Studies have shown South Asian region's waters to be under stress of heavy metal pollution due to discharges of urban wastes into freshwater bodies (Subramanian, 2004).

Over the years, water bodies all over Pakistan have been found to have enhanced in their heavy metal concentrations. High levels of As have been found to be specifically prevalent in surface and ground waters throughout the country (Waseem et al., 2014). Imran alongwith his fellows (2019) studied levels of Cd, Cu, Fe, Pb, Mn, and Zn, in surface water from regions of Sindh. They found most of the heavy metals to be in concentrations higher than WHO's guidelines for drinking water quality, especially during the pre-monsoon season. Water from tube wells in Punjab has been found to exceed in WHO limits for permissible concentrations of As, Cd, Fe, and Pb (Rasool et al., 2016). River Kabul, Khyber Pakhtunkhwa has been observed to be high in levels of Cd, Pb, and Ni concentrations

(Khan et al., 2011). Furthermore, drinking water samples from the province, Nowshera District were revealed to be high in As, Cd, Cr, Pb, and Ni, concentrations (Khan et al., 2015). Cd and Pb amounts in drinking water of District Jhal Magsi of Baluchistan were reported to be higher than the thresholds given by WHO and Pakistan Environmental Protection Agency (Pak-EPA) (Mustafa, 2017).

2.2.2 Heavy Metal Resistance

Although, bacteria are generally regarded as very sensitive to heavy metal exposure, but, over time, as heavy metal contamination increased, bacteria, as a self-protection mechanism, evolved and adapted to have resistance mechanisms against heavy metal exposure toxicity, by means of mutations, or by uptake mobile genetic elements containing genes encoding for resistance (Dickinson et al., 2019). Cai alongwith his fellows (2019) were able to isolate bacterial strains from *Acinetobacter*, *Bacillus*, *Lysinibacillus* and *Shewanella* genera, from electroplating wastewater treatment plant, that showed resistance to high concentrations of Cr, Co, Cu, Ni, and Mn. *B. cereus* BDBC01, *B. cereus* AVP12 and *B. cereus* NC7401 resistant against concentrations of Cd, Cr, and Ni were obtained from rhizosphere of Southern Cone Marigold plant (*Tagetes minuta*) growing near automobile workshops (Akhter et al., 2017). Such heavy metal resistant microbial species are widely employed for various helpful purposes, especially phytoremediation, but if not taken care of, the issue can aggravate, and can be a serious threat to the future of implying heavy metals' antimicrobial properties to use.

2.3 Antibiotics

Antibiotics are antimicrobials that work by killing the bacteria or inhibiting bacterial growth, as is reflected is the name. Antibiotics are one of the most revolutionary discovery in the history of mankind. Since their discovery in 1929 by Alexander Fleming, antibiotics took over the modern pharmaceutical industry, and a huge number of new antibiotics kept adding to the list soon after, improving health care and reducing morbidity and fatality rates all around the world. Antibiotics are widely implied in pharmaceuticals. (Al-Riyami et al., 2018; Kraemer et al., 2019)

2.3.1 Antibiotics' Misuse

Initially they were utilized for disease treatment and prevention, but as more and more depth and knowledge into this revolutionary drug enhanced, their application spectrum widened for various purposes, including growth promotion and enhancement, and various other commercial and consumerism related purposes. Reports have shown a high percentage of antibiotics, i.e., more than 50 percent about are utilized in veterinary purposes (Zhou et al., 2016). WHO (2017) claimed that as much as 80 percent of the antibiotics sold in United States of America (USA) are used for food- producing animal industry.

This extremely wide application spectrum led mankind to have a great confidence in antibiotics' beneficial abilities, bringing about what can be called as an overuse of the products. People have found a reliance in antibiotics ability to cure them, even for the slightest of an issue that might not necessarily require antibiotic. It has inculcated so much so that people often ask of healthcare practitioners/doctors to prescribe them some antibiotic and tend to be upset when it isn't done so. Furthermore, practice of self-medication has also become common. Antibiotics' inappropriate and irresponsible use has led to an aggravation in the issue of environmental contamination. It has been estimated that in USA, about 30 percent of antibiotic prescriptions to outpatients are without a necessary need for antibiotic (Fleming-Dutra et al., 2016). Such anthropogenic action for the sake of various healthcare, pharmaceutical, commercial, and food security purposes has been a cause of significant release of excess antibiotics into environmental components, specially, water and soil, thus contaminating them.

2.3.2 Antibiotic Pollution

Concentrations of antibiotics in water bodies have been reported from all over the world. Analysis has provided that enhanced levels of erythromycin, roxithromycin, sulfameter, sulfamerazine, sulfamethoxazole, , tetracycline, and oxytetracycline are found in lake waters all around the globe (Yang et al., 2018). Danner et al., (2019) reported mean antibiotic concentrations for Asia to be 17.7 µg/L, Africa to be 11.3 µg/L, Europe to be 0.4 µg/L, whereas, USA was reported with 0.9 µg/L of mean antibiotic concentration. They further revealed Spain and China to top the list of countries with highest occurrences of antibiotics in rivers and streams. Studies from the African continent present sulfamethoxazole concentration of as high as 53.8 μg/L in Mozambique (Segura et al., 2015), followed by 38.9 μg/L concentration in Kenya (Madikizela et al., 2017). Additionally, analysis of data of wastewater treatment plants from around the world (Africa, Asia, Europe, North America) has revealed macrolides, quinolones, sulfonamides, and trimethoprim to be the highest prevalent classes of antibiotics detected in influent, as well as the effluents (Wang et al., 2020).

Just as the world is faced with alarming amounts of antibiotics in their waters, Pakistan does not fall behind. Numerous investigations have shown varying concentrations of variety of antibiotics to be present in water bodies. While studying the incidence of personal care products and pharmaceuticals in canal surface water and wastewater in Lahore, Ashfaq along with his fellows (2019) found the samples to be predominantly contaminated with antibiotics, including oxytetracycline being the most abundant with average amount of 486 ng/L, followed by ofloxacin, ciprofloxacin, sulfamethoxazole, and norfloxacin. High levels of antibiotic contamination were found in downstream of River Ravi (Khan et al., 2013). Riaz and his fellows (2017) have recorded high levels of ciprofloxacin, enrofloxacin and levofloxacin in wastewater samples of the Kahuta industrial zone in Islamabad, as well as Hattar industrial zone in Haripur, Khyber Pakhtunkhwa.

2.3.3 Antibiotic Resistance

Along with contamination of the environmental components, another catastrophic effect, the inappropriate and irresponsible use of antibiotics brought with itself, is the emergence of antibiotic resistance in bacteria, which comes as a significant threat to humans in terms of health, food security and development (Zhao et al., 2019). The problem has turned to severity, as even the newly developed generations of antibiotics are losing their efficacy, and there remains a need for new and more effective drugs to tackle the ever-increasing storm of bacteria related infections and diseases.

Antibiotic resistance develops owing to the intrinsic and ubiquitous resistance found in bacteria in terms of gene encoded resistance, and mobile genetic elements such as plasmids, integrons, or transposons, and can be because of natural mutations (Nguyen et al., 2019; Zhao et al., 2019). Additionally, the extraordinarily huge number of bacterial cells being present on earth provide massive opportunities for genetic variability, mutations, rearrangements, and horizontal gene transfer (Bengtsson-Palme et al., 2018).

Since its emergence, it has been a reason for acute concern worldwide due to the devastating affects it has had and is estimated to be even more harm causing. WHO (2015) has recognized antibiotic resistant bacteria (ARB) as major danger to modern medicine and healthcare, and is affecting the prevention and treatment from the ever increasing number of bacteria caused infections and diseases, and has been estimated to become the lead cause of death by 2050 (Zhao et al., 2019). In addition to being a hazard to human health, it has produced massive economic losses throughout the world as well. Centers for Disease Control and Prevention (CDC) (2013) has reported that antibiotic resistance (ABR) has costed US alone more than $20 billion in healthcare. Immediate and rapid actions are required, and efforts are being put towards combating this global catastrophe, which otherwise can lead to even more havoc, such as effects for humankind as well other living beings on the plants, including terrestrial as well as marine animals. World Bank in 2014, estimated global Gross Domestic Product (GDP) to fall by 2050, by 1.1 percent in case of low impact antimicrobial resistance (AMR), whereby, 3.8 percent fall would be experienced if AMR remains high impact, which would cause low income countries to reduce more in GDP every year (Ahmad & Khan, 2019). Pakistan already suffering from harms of ABR, further, being ranked 6th amongst 22 of the high disease burden countries in the world (Khan, 2018), does not look to be in a good position to handle the effects in case of high impact AMR.

Although, misuse of antibiotics in pharmaceuticals and healthcare, agriculture, animal husbandry, food security, and other commercial setups has been under view and attention as the leading reason for rapid development and spread of antibiotic resistance, the environment's role in development and dissemination has come to light and is being studied upon more than ever now (Ashbolt et al., 2013). Various environmental pathways including food water sources, animal waste, food materials etc., are found to be sources of dissemination of resistant microbes from humans to animals, and vice versa (Finley et al., 2013). Physical environmental forces, e.g. wind and water movements can move bacteria over large distances (Allen et al., 2010). Also, sewage, wastewater treatment plants, water bodies, air-borne aerosols, dust, and food colonized by bacteria, have been proven to be vital vectors allowing for bacterial transmission between hosts through the environment (Bengtsson-Palme et al., 2018).

2.4 Association between Heavy Metal and Antibiotic Resistance

There being a relation and/or association between heavy metal resistance and antibiotic resistance in bacteria have been a focus of research for a while now yet remains unclear and there still remains a whole lot of understanding and mechanisms to unfold. Thus, issue at hand is being investigated in all parts of the world.

It has been found that antibiotics are not the only influence pressuring bacteria to develop resistance, but other environmental pollutants, e.g. heavy metals can play a role in adding to the bacterial stress of self-defense/self-protection, and further development and dissemination of resistance through co-selection mechanisms of cross-resistance and co-resistance, etc., as heavy metal are also well known for their antimicrobial properties (Grass et al., 2011). Furthermore, human pathogens are found as resistant to several antimicrobials, including heavy metals, suggesting there to be a relation between microbial resistance to heavy metals and antibiotics. Involvement of heavy metal in antibiotic resistance spread first came to light in areas of high pollution that have reportedly been treated with wastewater, as well as biosolids, and provided for an indication of antibiotics being impacted by heavy metal concentrations (Knapp et al., 2017). Bacterial resistance to As, Cd, Co, Cr, Cu, Hg, Ni, Pb and Zn has been found to co-select for antibiotic resistance (Zhao et al., 2019). Speculations of genes for heavy metal and antibiotic resistance to be present on same mobile genetic elements provides reasoning for there to be a physical relation between the two, that can provide for co-resistance mechanisms to be carried out.

Various approaches being applied for investigating the problem at hand include (Dickinson et al., 2019):

- microorganism growth in presence of varying or defined heavy metal concentrations (Stepanauskas et al., 2006).
- establishment of correlation between heavy metal concentrations and environmental samples containing antibiotic resistance genes (ARGs) (Zhao et al., 2019).
- quantification of microbes with resistance to both heavy metals and antibiotics, obtained from samples of lands treated with heavy metals, or from samples of livestock that has been fed with heavy metal contaminated feed (Bednorz et al., 2013).
- analysis of genetic linkage between heavy metal resistance and antibiotic resistance genes by means of techniques from bioinformatics (Pal et al., 2015).

The approach that has received quite a lot of attention is the quantification of heavy metal resistance and antibiotic resistance within the environmental samples under consideration (Dickinson et al., 2019), and is the one implied in the current research.

2.3.1 Mechanisms of Association

Various complex mechanisms undergo in bacterial cells for the purpose of development of resistance to heavy metals, similar to the mechanisms undergone for development of resistance against antibiotics. It has been established that heavy metals can act as co-selecting agent for antibiotic resistance by means of co-resistance, whereby genes for heavy metal resistance and antibiotic resistance are linked on mobile genetic elements, and they serve to transfer these gene between microorganism during conjugation; or cross-resistance, which entails changes at genetic levels in response to same bacteria being exposed to heavy metals as well as antibiotics, initiating common pathway response (Baker-Austin et al., 2006; Dickinson et al., 2019).

2.3.1.1 Co-resistance

Co-resistance at bacterial level occurs when gene encoding for heavy metal resistance and those encoding for resistance against antibiotics exist on single genetic elements, which are usually the mobile genetic elements, including plasmids, transposons, and integrons (Figure 2.1). Plasmids and transposons are very transferable, providing for easy transfer of these resistance genes to other bacterial cells, proving that horizontal gene transfer plays a highly significant part in mechanisms of co-selection between heavy metal and antibiotic resistance. Plasmid pU302L from *Salmonella*, when studies, revealed to contain genes that encode for resistance to many antibiotics, and also for resistance to Hg. Furthermore, *Salmonella Typhimurium* also revealed to have resistance genes sul1, qacED1, aadA1, blaOXA-1 for resistance to antibiotics sulfonamide, quaternary ammonium compounds, streptomycin, spectinomycin and penicillin respectively, on the plasmid pUO-StVR2 that also encoded gene merRTPCADE to Hg resistance (Yu et al., 2017). Collembolan's gut microbiota was found to have abundance of blaCTX-M and mphA genes in presence of increased Cu and Zn concentrations. The respective genes have a strong relationship to responsible for resistance to heavy metals, suggesting for co-resistance mechanisms to be involved in gut-associated antibiotic resistance genes, under stress of heavy metals. Thus, Ding and fellows (2019) concluded plasmid mediated co-resistance of heavy metals and antibiotics under heavy metal stress.

2.3.1.2 Cross-resistance

Bacteria tends to develop resistance against heavy metals, which can be in terms of membrane transport systems, but fails to specify the substrate, therefore, providing for resistance to antibiotics as well, or the other way around, which is referred to as cross-resistance (Figure 2.1). It can potentially occur as a result of enhanced efflux systems (Pal et al., 2017; Yu et al., 2017). Hayashi et al. (2000) recorded *Burkholderia cepacia* to experience cross resistance to Cd, Zn and beta-lactams, ofloxacin, erythromycin, novobiocin, and kanamycin, as a result of formation of the DsBA– DsbB disulphide bond formation system. Flach et al., (2017) also found cross-resistance mechanisms between Cu and Zn, and tetracycline to be present in biofilms.

2.3.1.3 Co-regulation

Co-regulation is secondary mechanism of resistance. Bacteria when exposed to different concentrations of heavy metals, can undergo alterations in the expression of genes encoding for resistance to antibiotics. It initiates as a transcriptional or translational response during replication of deoxyribose nucleic acid (DNA) (Nguyen et al., 2019; Yu et al., 2017). An example of such co-regulation mechanism has been found between Cd, Co and Zn, and imipenem, which belongs to carbapenem class of antibiotic. Transcription of the czcCBA operon (gene encoding for an efflux pump- RND-type) in *Pseudomonas aeruginosa* has been found to be influenced by cscRS gene that encodes for a dogmatic system consisting of two components, encoded by, which provides resistance to Cd, Co and Zn. But, CscRS in this situation, also reduces entry of imipenem into bacterial body by reducing the expression of a porin- OprD (Caille et al., 2007).

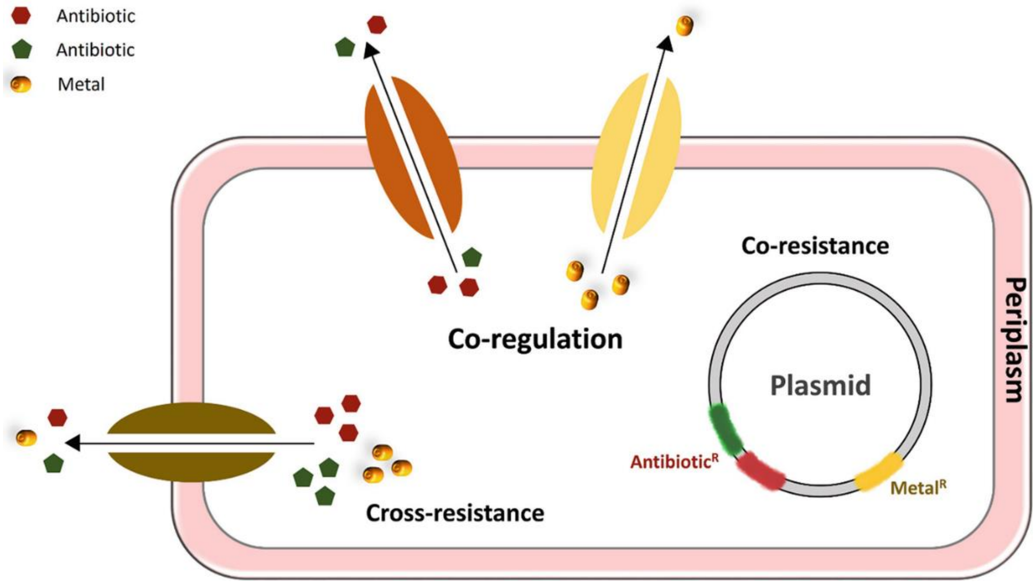

Figure 2.1: Mechanisms of co-selection between heavy metals and antibiotics; co-resistance, cross-resistance, and co-regulation (Figure adopted from Baindara, 2019).

Chapter 3

METHODOLOGY

For carrying out the research in order to achieve the objectives, various experimental sequences, procedures, protocols, and methods were adopted and implied. The details of which are explained in this chapter. The quality control and quality assurance of the sampling and analysis to obtain reliable results were maintained by adopting standard operating procedures.

3.1 Study Area

The methodology took a spatial approach, aiming at analyzing the quality of the main water channel passing through the cities of Islamabad and Rawalpindi. The cities, due to rapid and extreme development and urbanization have been highly connected and sort of merged into being one, thus called as the twin cities. They lay at 72°45′ and 73°30′ E longitude and 33°30′ and 33°50′ N longitude. Of the two, Rawalpindi, sitting at an area of 5258 km^2 is the older and the larger city, known for its high industrial and commercial value, and has special significance for being the core for military activities of the country. On the other hand, Islamabad, located to the north of Rawalpindi, at an area of 906 km^2, is more modern, newly planned and built mainly in and after the 1960s. Being the capital, a huge number of prominent federal and government office reside in the city. (Fareed et al., 2016; Sajjad et al., 2020)

The twin cities sit on the Potowar plateau, with an elevation of 545 m for Islamabad and 497 m for Rawalpindi. Northern part of the twin cities, specifically Islamabad, resides the luscious Margalla Hills at 1600 m altitude. Climate of the metropolitan area has a mean annual average temperature of 21 °C, and can be categorized as monsoonal, where the summers are more rainy than winters, with monsoon starting from June and ending in September with its peak being in the month of August (ICTA; Sheikh et al., 2007).

There are two main water drains running through area of the twin cities, namely, Soan River and Kurang River. The focus of the current study is the Soan River, whose major tributary is the Nullah Lai that enters southward into the River. Nullah Lai is further backed by the streams coming from the mountain front in Islamabad, running through urban and industrial areas, and entering into Nullah Lai in Rawalpindi at Katarian. Soan River furthers adds into the Simli lake, that is used as domestic and drinking water supply source for the urban population. Owing to the combined population of more than 3 million of the twin cities, and the rapid and huge urbanization, development, and industrialization, there is bound to be a lot of waste generation. Following from streams in Islamabad, this water channel receives the most municipal as well as industrial waste, at most times untreated, from both the cities. Carrying forward into the Nullah Lai, most of the liquid as well as the solid waste of Rawalpindi is put into Nullah Lai, which carries it with itself into the Soan River, polluting it to unhealthy and unacceptable levels. Thus, the Soan River, which at some point used to be categorized into freshwater, now, can fall into the category of wastewater (Sheikh et al., 2007).

For sampling point selection, the site was divided into three main regions; water channel of Islamabad, followed by Nullah Lai in Rawalpindi, that further falls into the Soan River, also in Rawalpindi. These three regions were each categorized for their up-stream, mid-stream, and down-stream. Therefore, for the sake of ease, the upstream, mid-stream and down-stream of the Riverbed in Islamabad are labeled as I-1, I-2, and I-3 respectively, N-1, N-2, and N-3 for Nullah Lai; and S-1, S-2, and S-3 for Soan River. The sampling points along with their respective longitudes and latitudes have been mentioned in Table 3.1 and are displayed in Figure 3.1.

Table 3.1: Sampling locations with their longitudes and latitudes

Points	Location	Latitude	Longitude
I-1	11 Street 3D, F-10, Islamabad, Islamabad Capital Territory, Pakistan	33.6932	73.0057
I-2	626 Soan Rd, G-10, Islamabad, Islamabad Capital Territory, Pakistan	33.6791	73.0169
I-3	58 Service Rd North, I-10, Islamabad, Islamabad Capital Territory, Pakistan	33.6573	73.0370

N-1	Katarian Pull, IJP Road, Islamabad	33.6467	73.0537
N-2	Nullah Lai Bridge, City-Saddar Road, Rawalpindi	33.6046	73.0544
N-3	Bridge Nallah Lai, Chaklala Cantt., Rawalpindi, Punjab, Pakistan	33.5798	73.0871
S-1	Soan Adha Road, Rawalpindi, Punjab, Pakistan	33.5550	73.0967
S-2	Kahuta Road, Miana Thub, Islamabad, Islamabad Capital Territory, Pakistan	33.56758	73.2572
S-3	Bridge near Jamia Masjid Bait-ul-Mukaram, Islamabad Capital Territory, Pakistan	33.5923	73.2988

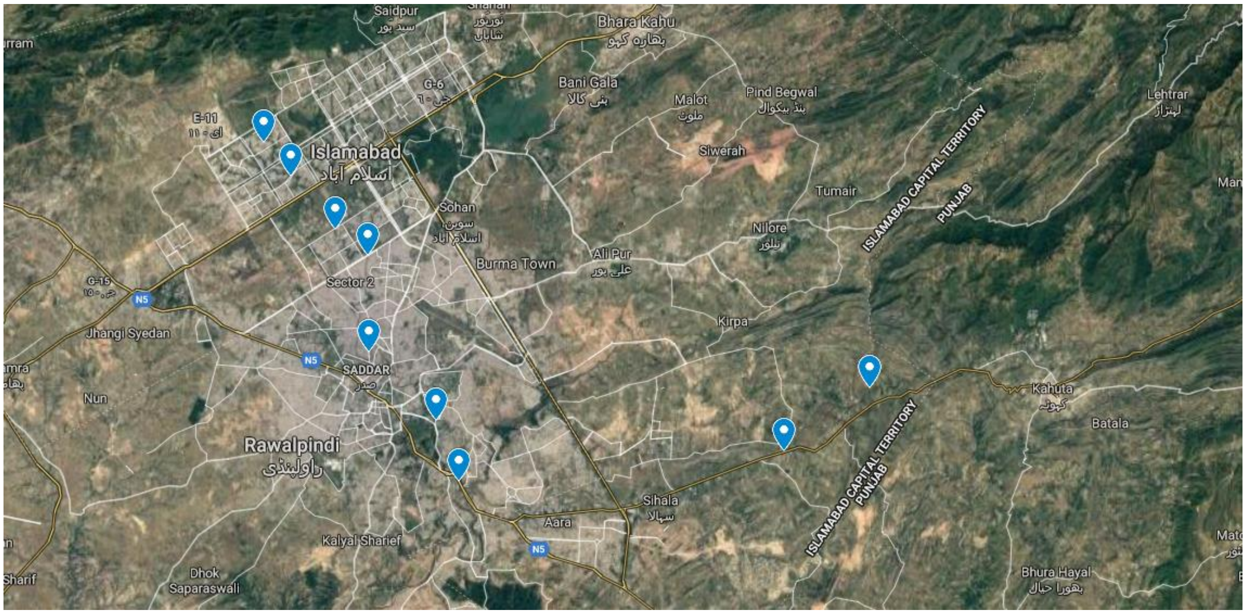

Figure 3.1: Map showing the sampling locations

3.2 Sampling

For sample collection, grab sampling technique was implied. Two types of samples were collected from each site, one for analysis of physicochemical parameters, and the other for heavy metal quantification. For physicochemical parameters' analysis, the samples were collected in triplicates for each sampling point. Samples were collected each in a 1000 mL glass sampling bottle, that were cleaned thoroughly and sterilized before heading out for sampling. For the sake of heavy metal analysis, water samples were collected in 500 mL clean, acid washed plastic bottles, and the samples were preserved with addition of 0.75 mL Nitric Acid immediately after collection, to maintain the pH of the sample below 2. The samples were collected carefully in order to avoid as much of the debris. After collection, all the samples were immediately put into an icebox in order to maintain standard preservation conditions of 4 °C for transporting them back the laboratory for further assessment. The samples were stored in refrigerator also at 4 °C, until further analysis.

3.3 Physicochemical Parameters' Analysis

A number of physicochemical parameters were analyzed, for assessing the quality of the samples collected form the water bodies, the list along-with the methods and protocols implied, are provided in the Table 3.2.

Among physicochemical parameters, temperature, pH, and EC of the samples were recorded on-site using portable meters, while the rest parameters were analyzed using standard examination protocols after bringing the samples into the laboratory.

Table 3.2: Physicochemical parameters and their analysis techniques

Parameter	Units	Analysis Technique / Protocol
Temperature	°C	Portable meter
pH	-	Portable meter
EC	µS/cm	Portable meter
Turbidity	NTU	Turbidity meter
TDS	mg/L	Gravimetric method
DO	mg/L	Wrinkler method
COD	mg/L	Titration
BOD	mg/L	Titration
TKN	mg/L	Kjeldahl apparatus

Nitrate-Nitrogen	mg/L	Colorimetric method / Spectrophotometer
Nitrite-Nitrogen	mg/L	Colorimetric method / Spectrophotometer
Phosphate-Phosphorus	mg/L	Colorimetric method/ Spectrophotometer
Sulphates	mg/L	Colorimetric method/ Spectrophotometer
Hardness	mg/L as $CaCO_3$	Titration
Alkalinity	mg/L as $CaCO_3$	Titration
Chlorides	mg Cl^-/L	Titration

Source of Protocols: Baird et al., 2017.

3.4 Heavy Metal Analysis

The preserved samples were analyzed for quantification of selected heavy metals through Atomic Absorption Spectrophotometer (Model SHIMADZU, AA-7000). It is based on the principle of absorption of electromagnetic radiations by free atoms in the gas phase, by atomizing the liquid phase of the sample, using thermal energy. Hallow cathode monochromatic lamps bombard the atoms with specific radiations, causing excitation by absorption of radiations. The absorption is proportional to the concentration of free atoms available. Rest of the radiations are detected, and to provide the exact concentration of the detected element (Ivanova, 2004).

3.5 Microbiological Analysis

The microbiological analysis was carried out for the purpose of isolating bacterial isolates from the collected samples and to test their resistance against selected heavy metals and antibiotics. Standard microbiological protocols and methods were implemented to ensure quality and reliability of the results obtained.

3.5.1 Isolation of Bacterial Strains

Prior to carrying out all microbiological process, all the required glassware was autoclaved at 121 °C temperature and 15 psi pressure, for 15 minutes, for achieving sterilization. For isolation, Nutrient agar was used as the growth medium. For preparing the petri plates, agar was prepared and sterilized, after which the agar was poured into sterile glass petri plates in a sterile environment in a laminar flow hood to avoid any chances contamination. After pouring, the plates were set aside for the agar to cool down and solidify, after which they were inverted to avoid condensation on to the surface of the agar to prevent contamination, and were incubated at 37 °C for 24 hours to check the sterility of the plates.

For isolation of bacterial strains, the collected samples were processed within 24 hours of collection. Serial dilution method was adopted, whereby, each collected wastewater sample was diluted 10 times, up to 10 folds each time. 0.2 mL of each dilution was poured onto prepared Nutrient agar plate each and was spread uniformly on the agar using a glass spreader. The plates were incubated at optimum conditions of 37 °C for 24 hours. Of all the plates, the ones with countable colonies (1- 300) were selected, and the colonies were counted using colony counter. The process for repeated for each collected sample.

Representative colonies were selected from petri plate of each dilution of each sample, based on the colonies' varying morphological traits, and were picked on a sterile wire loop, and were sub-cultured on a fresh Nutrient agar petri plate. A total of 27 isolates were obtained. The isolates were subject streak plate method for 6-7 repeated cycles, until pure colonies were obtained, and were then stored in refrigerator at 4 °C until further processing.

3.5.2 Characterization of the Isolated Bacterial Strains

For characterizing the isolated bacterial strains, they were examined morphologically and biochemically. Morphological parameters of the bacterial colonies were studied with naked eye, comprising of shape/form, color, elevation, margin, texture, opacity, and pigmentation. For studying the cell morphology of the bacterial isolates, gram staining using standard protocol (American Public Health Association et al., 2005) was performed to identify bacteria as gram positive or negative. Biochemical parameters of the isolated bacterial strains were studied for, including catalase, oxidase, Simmons citrate agar and Mannitol salt agar tests.

3.5.3 Identification of Heavy Metal Resistant Bacteria

To screen the bacterial isolates for their heavy metal resistance, four heavy metals Cd, Cu, Pb, and Zn, and their concentration/dosage (1000 mg/L) to be applied were selected based of the information obtained by studying and investigating the available literature on the bacterial resistance against heavy metals (Abd Elhady et al., 2020; Gupta et al., 2014).

Ability of the bacterial isolates to resist heavy metal stress was investigated using the well diffusion technique. Nutrient agar petri plates were prepared, and fresh (overnight) bacterial culture of each isolate was inoculated on to the plate to create a lawn. The salts of the selected metals available (cadmium sulphate, copper sulpahte, lead nitrate, zinc sulphate) were utilized to prepare the solutions for each heavy metal. They were already prepared and sterilized by autoclaving. 7 mm wide wells were dug into the agar using sterile cork borer, and 100 µL of the respective heavy metals' solutions were poured into well using micropipette for each of the isolate. Incubation of plates was carried out at 37 °C for 24-48 hours, after which the results were recorded by measuring the inhibition zone in mm, using a scale. Inhibition zone of less than 1 mm was recorded to be resistant (Gupta et al., 2014; Rani & Moreira, 2010).

3.5.4 Identification of Antibiotic Resistant Bacteria

The literature was surveyed thoroughly to identify the most commonly used antibiotics, in order to select the antibiotics and their respective concentrations to be applied for the study. The selected antibiotics include amoxicillin (10 µg), ciprofloxacin (5 µg), levofloxacin (5 µg), and ofloxacin (5 µg) (Humphries et al., 2019; Khademi et al., 2013; Sjölund-Karlsson et al., 2014; Tang et al., 2020).

To identify antibiotic resistance capability of the bacterial isolates obtained from the collected wastewater sample, Kirby Bauer disk diffusion technique was applied. Readily available antibiotic disks of the required concentrations supplied by oxoid were used. Mueller Hinton agar is the standard agar for this method, as it is a non-selective agar, providing that all organisms can grow on it, and it also has constituents that provide for optimum conditions and do not interfere with antibiotic's activity (Wayne, 2013). Petri plates were prepared with Mueller Hinton agar using the standard procedure.

Bacterial inoculums were prepared in 10 mL sterile Nutrient broth in culture tubes with 24 hours of incubation to allow for the bacterial growth to reach the log phase. Then, turbidity of the bacterial inoculum was altered to reach that of 0.5 McFarland standard, i.e., 1.5 x

10^8 colony forming units per mL (CFU/mL). The broth was then used to culture the bacteria in lawn technique, on the previously prepared Mueller Hinton agar plates. Each antibiotics' disks were positioned onto the surface of the agar with help of sterile forceps, and the plates were incubated at 37°C for 24 hours. Results were recorded by measuring the inhibition zone in mm, using a scale and the results were interpreted using Clinical and Laboratory Standards Institute (CLSI) guidelines (Cockerill, 2011).

3.5.5 Co-resistance between Heavy Metal Resistance and Antibiotic Resistance

For finding out the potential association between heavy metal resistance and antibiotic resistance, present in the bacterial isolates obtained from the collected wastewater samples, statistical correlation between the two was studied. XLStat 2020 and Microsoft Excel Office-365 were used to calculate the various parameters.

Chapter 4

RESULTS AND DISCUSSION

This section presents all the results obtained through the experimental work, and their interpretations and implications onto the problem stated earlier.

4.1 Physicochemical Parameters of the Wastewater Samples

As has been mentioned earlier, the target water body was divided into three territories, and each was sampled from its initial, middle, and final point. Each sample was tested for a set of physicochemical parameters. These physicochemical parameters were studied in light of water quality standards and guidelines provided internationally and nationally in order to determine their relative quality for various intents and purposes. Considered water quality standards are mentioned in Table 4.1.

Table 4.1: Wastewater quality standards and guidelines

Parameter	Units	NEQS (municipal)	FAO (irrigation water)	USE EPA (wastewater reuse in agriculture)
Temperature	°C	43	-	-
pH	-	6-9	6-8.5	6-9
EC	µS/cm	-	3000	-
Turbidity	NTU	-	-	2
TDS	mg/L	3500	2000	-
DO	mg/L	-	-	-
COD	mg/L	150	-	-
BOD	mg/L	80	-	10-30
TKN	mg/L	-	-	-
Ammonia-Nitrogen	mg/L	40	5	-
Nitrate-Nitrogen	mg/L	-	10	-
Nitrites-Nitrogen	mg/L	-	-	-
Phosphate-Phosphorus	mg/L	-	2	-

Sulphates	mg/L	600	-	-
Hardness	mg/L as $CaCO_3$	-	-	-
Alkalinity	mg/L as $CaCO_3$	-	-	-
Chlorides	mg Cl^-/L	1000	-	-

Sources: Pakistan Gazette (2000); Ayers & Westcot (1985); Bastian & Murray (2012).

4.1.1 Temperature

Temperature of the collected samples was mainly a function of the season the samples were collected in. Sampling was carried out through the months of October, November, and December 2019, which are the months of winter season in Pakistan and the reason, starting from October, the cold gradually strengthens until February. This explain the temperature drop, as the sampling for the Nullah Lai was done in October 2019, followed by the Islamabad region sewage nullahs in November 2019, and lastly, the Soan River in December 2019 (Figure 4.1). The ranges in water samples' temperatures can somewhat potentially also be attributed to the chemical and biological interactions and reactions taking place.

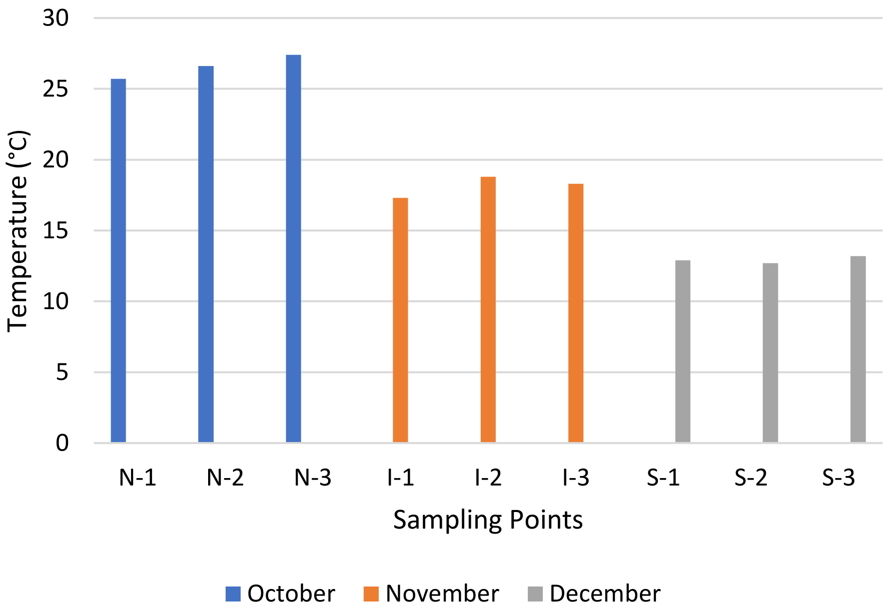

Figure 4.1: Temperature of the Samples

4.1.2 pH

pH is a vital parameter when it comes to water quality assessment and provides a quick view into the pollution level. pH of the samples varied to both spectrums of the pH scale, ranging from 5-8. F-10, G-10, and Katarian pull sites violate the NEQS, FAO and US EPA guidelines by falling below the range, whereas the rest are within the provided standards and guidelines. Figure 4.2 shows that the initial sampling points had the lowest pH and were only ones falling below the standards under consideration, followed by Nullah Lai sampling points, whereas the Soan River had the highest pH. pH variation can tend to impact the biological and chemical process occurring in the water body.

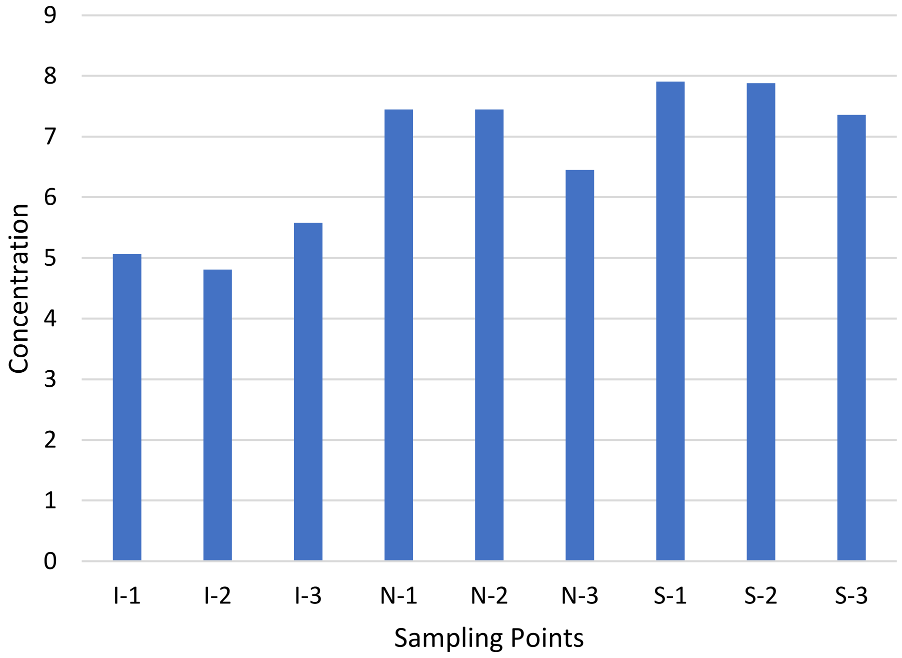

Figure 4.2: pH of the Samples

4.1.3 Electrical conductivity

Electrical conductivity (EC) provides for an estimate of the concentration of dissolved mineral salts in the water, in form of electrolytes, in any given sample. EC of the samples under study ranged from as low as 551.6 µS/cm to as high as 1446 µS/cm, starting from a relatively low EC on the initial most sampling point and increasing and fluctuating, but finally decreasing at the last two sampling points, i.e., Kahuta Road and near Jamia Masjid. All the EC values were within the limits that standards provided (Figure 4.3).

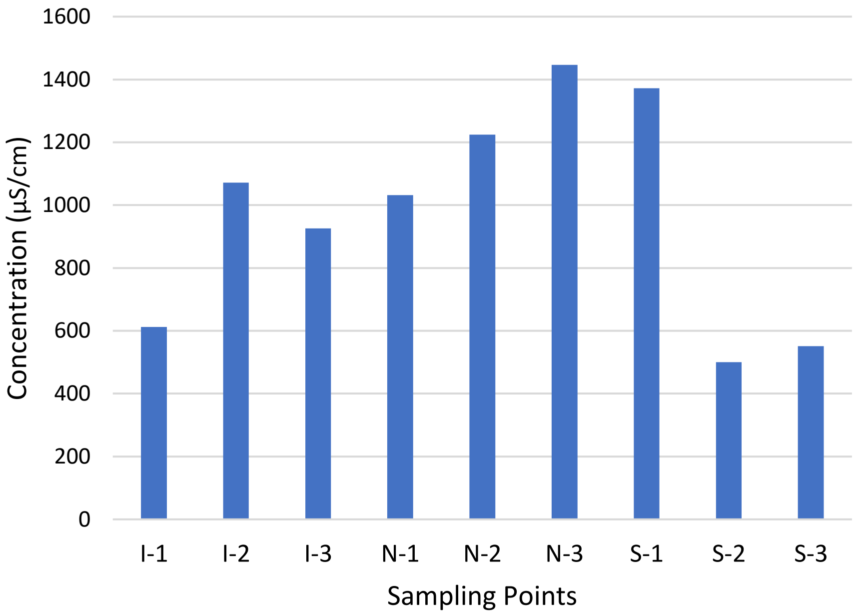

Figure 4.3: Electrical Conductivity of the Samples

4.1.4 Turbidity

Turbidity provides a quick assessment of water quality because of it being visible to the naked eye, and human can assess how dirty the water is for their daily activities. All samples were observed to be relatively high in turbidity, with only the sample from the Jamia Masjid point to have lowest turbidity of 3.6 NTU, but still higher than the US EPA guidelines for water reuse for irrigation. Figure 4.4 shows an increasing trend from the initial point of F-10 till Chaklala Cantt and a decrease from there on. Sources of turbidity are industrial as well as domestic effluent containing dissolved, as well as insoluble particulate matter into the water streams.

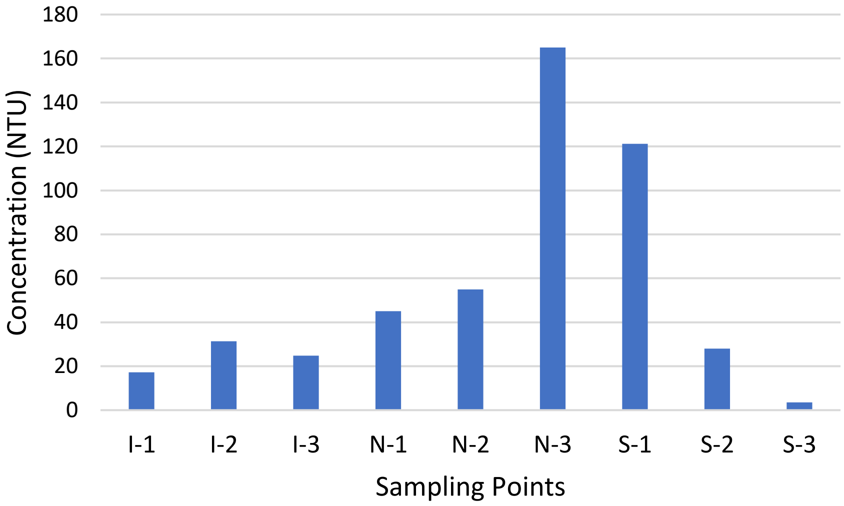

Figure 4.4: Turbidity of the Samples

4.1.5 Total Dissolved Solids

Total dissolved solids, abbreviated as TDS, refer to the total amount of inorganic and organic matter dissolved in water. TDS has a linear and direct relation with electrical conductivity. It is also visible in the similar trend followed in Figure 4.3 and Figure 4.5. TDS values (Figure 4.5) for all the samples were within the standards and guidelines of NEQS, FAO, as well as US EPA.

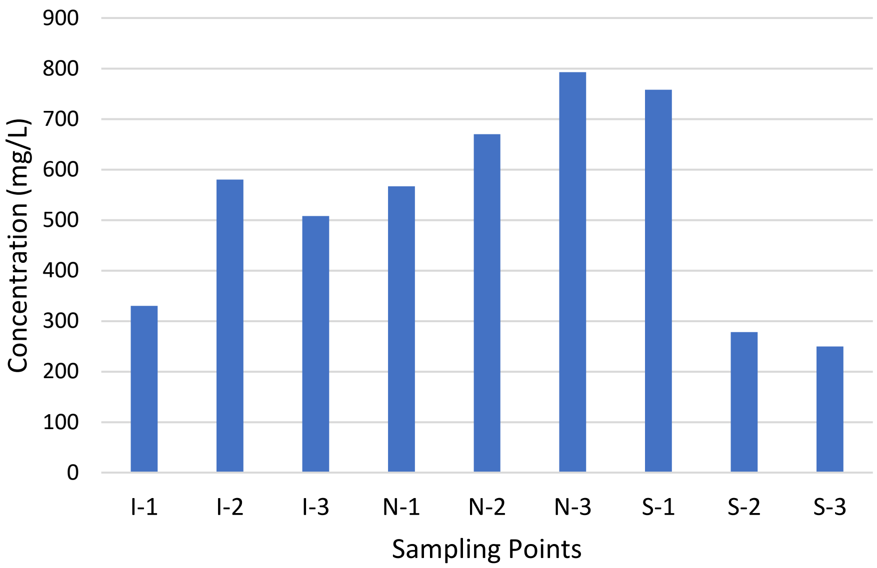

Figure 4.5: Total Dissolved Solids of the Samples

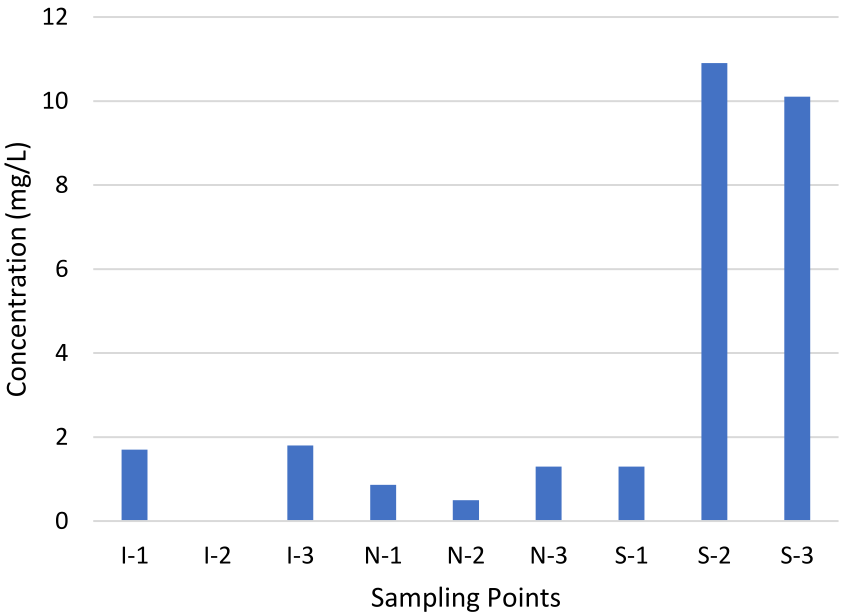

Figure 4.6: Dissolved Oxygen of the Samples

4.1.6 Dissolved Oxygen

Dissolved oxygen (DO) is the total oxygen present in water in dissolved form and indicates the oxygen quantity available to the living organisms in water, therefore, more polluted the water, less is the amount of DO available. Range for DO of the samples was found to be between 0 and 10.9, with almost all of the samples falling in lower spectrum of the range, but only the last low sampling sites, i.e., the Kahuta Road and the Jamia Masjid sites showing a relatively high DO of 10.9 and 10.1 mg/L, respectively (Figure 4.6).

4.1.7 Chemical Oxygen Demand

Chemical oxygen demand (COD) is a measure of the amount of oxygen required to chemically oxidize the organic as well as inorganic matter present in water. In the collected samples, COD was found to vary, following no specific trend, specifically for the Nullah Lai region of the water channel. COD for all the samples exceeded the NEQS standard of 150 mg/L, and ranged from 174.08 to 460.08 mg/L (Figure 4.7).

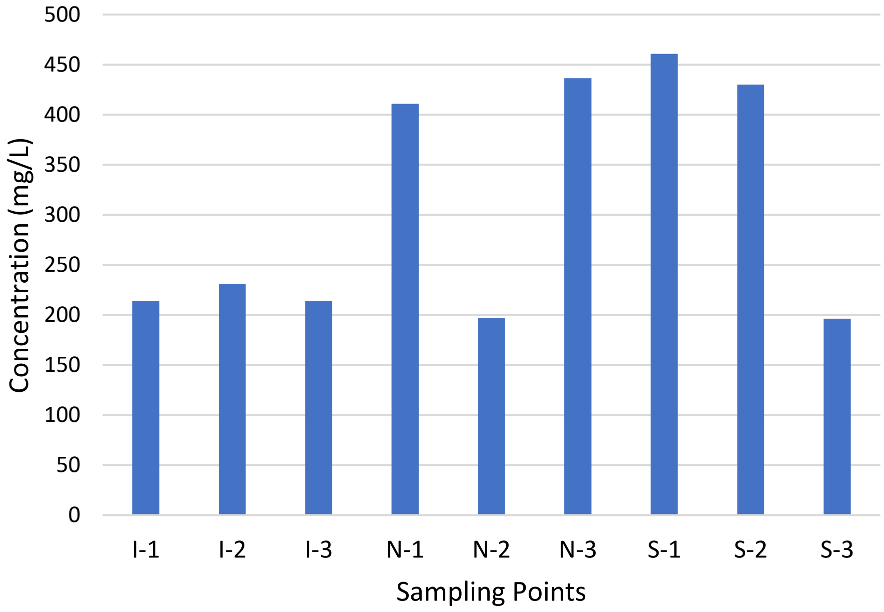

Figure 4.7: Chemical Oxygen Demand of the Samples

4.1.8 Biological Oxygen Demand

Biological oxygen demand (BOD), another important and common quantitative parameter for water quality estimation, is the total amount of oxygen consumed by bacteria and other microorganisms to oxidize the organic matter available. Of all the sampling points, four points i.e., Katarian pull, Chaklala Cantt, Soan Adha, and Kahuta Road samples had higher BOD compared to the rest, and the total range was observed to be 100.4 to 287.6 mg/L, whereas Jamia Masjid site had a comparatively low BOD value (Figure 4.8). BOD for all samples exceeded the NEQS.

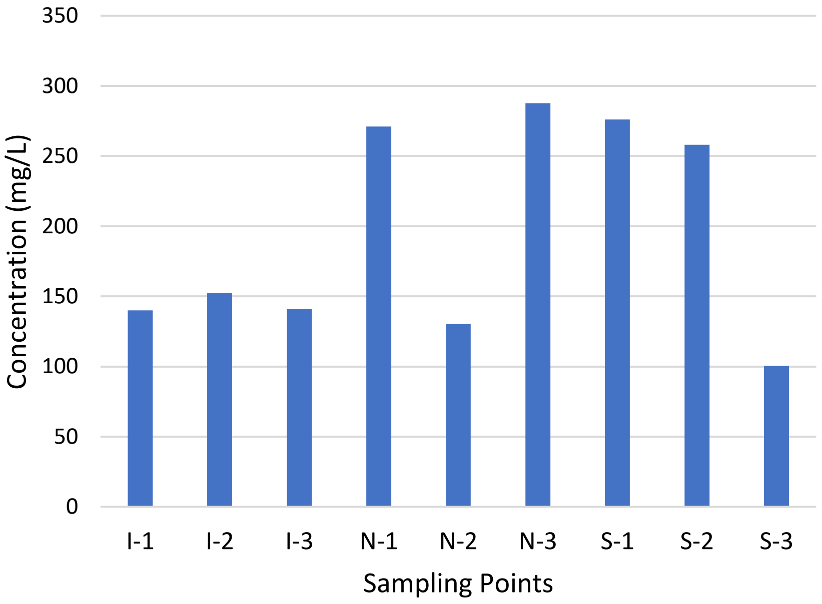

Figure 4.8: Biological Oxygen Demand of the Samples

4.1.9 Nitrate-Nitrogen

Nitrate content was recorded to be quite less with no amounts found in four of the samples including G-10, Katarian pull, Chaklala Cantt and Soan Adha, and that observed in the rest of the samples ranged from 0.269-2.33 mg/L (Figure 4.9), all within the FAO guidelines. Probable sources of such nitrates can be fertilizers, drainage from livestock areas, waste from some industries, as well as sewage effluent, and provide for nutrients for microbial growth and spread.

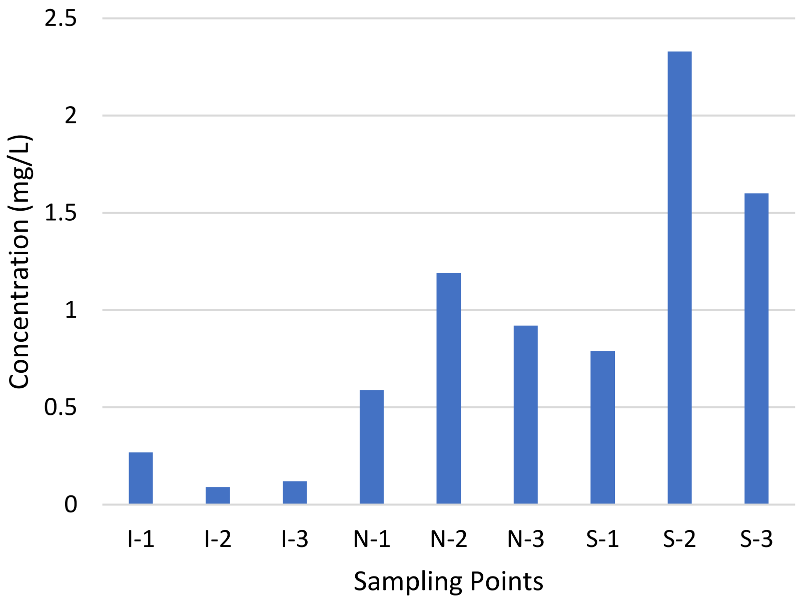

Figure 4.9: Nitrate-nitrogen concentration in the Samples

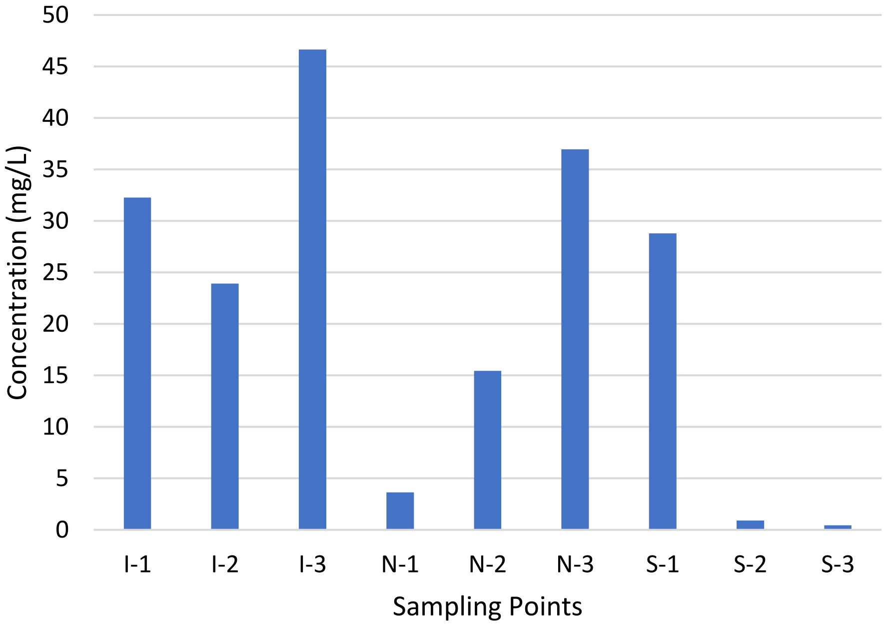

Figure 4.10: Nitrite-nitrogen concentration in the Samples

4.1.10 Nitrite-Nitrogen

Nitrites serve to be an intermediate between ammonia and nitrates. Concentration of nitrite ions showed a wide range of 0.45-46.66 mg/L in the samples, with Katarian pull, Kahuta Road, and Jamia Masjid location falling on the lower end (Figure 4.10). Sources of nitrites can be fertilizers, animal waste, septic tanks and sewage, and other mineral deposits.

4.1.11 Total Kjeldahl Nitrogen

Total kjeldahl nitrogen is a measure of ammonia and other organic nitrogen present. TKN ranged from 5.6-75.6 mg/L for the collected samples from all the sampling points. TKN values for all the samples exceed FAO guideline for ammonia nitrogen of 5 mg/L, while more than half of the sample are within the ammonia standard limit of NEQS of 40 mg/L, except for the consecutive sites of Katarian pull, City-Saddar Road, Chaklala Cantt, and Soan Adha (Figure 4.11). Fertilizers, animal waste, sceptic tanks and sewage, and industrial sources can be responsible for such ammonia and organic nitrogen contamination.

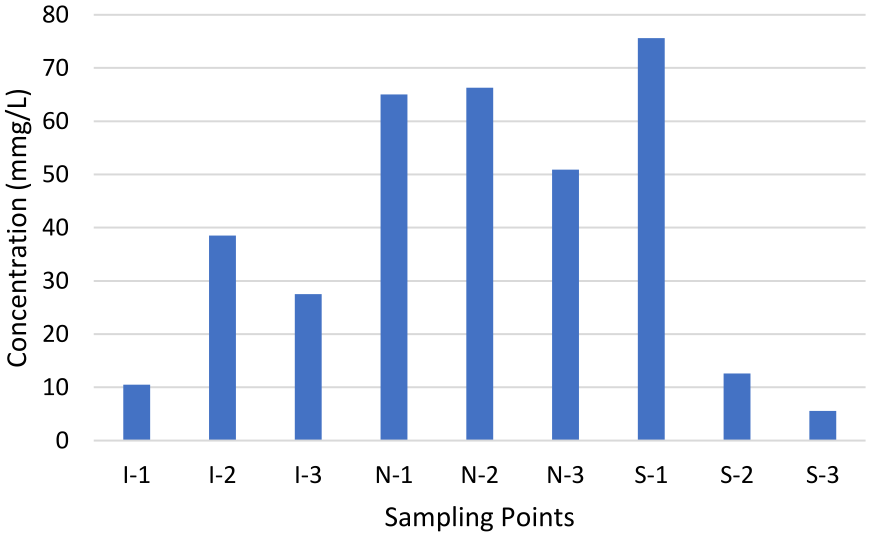

Figure 4.11: Total Kjeldahl Nitrogen concentration in the Samples

4.1.12 Phosphate-Phosphorus

Phosphorus in terms of phosphates was found to be present in all the samples, few being under the guideline of FAO, while rest exceeding the guidelines (Figure 4.12). The initial most sampling point of F-10, and the last two points of Kahuta Road and Jamia Masjid were the ones under 2 mg/L limit of FAO, whereas the rest being higher than it, with Chaklala Cantt sample topping among all. Phosphates are considered to be of benefit to microorganisms for their growth, thus indicating there to be high microbial and/or algal bloom/growth in the waters sampled. Human and animal waste, domestic effluent containing detergents etc., and industrial wastes can be the sources of phosphates.

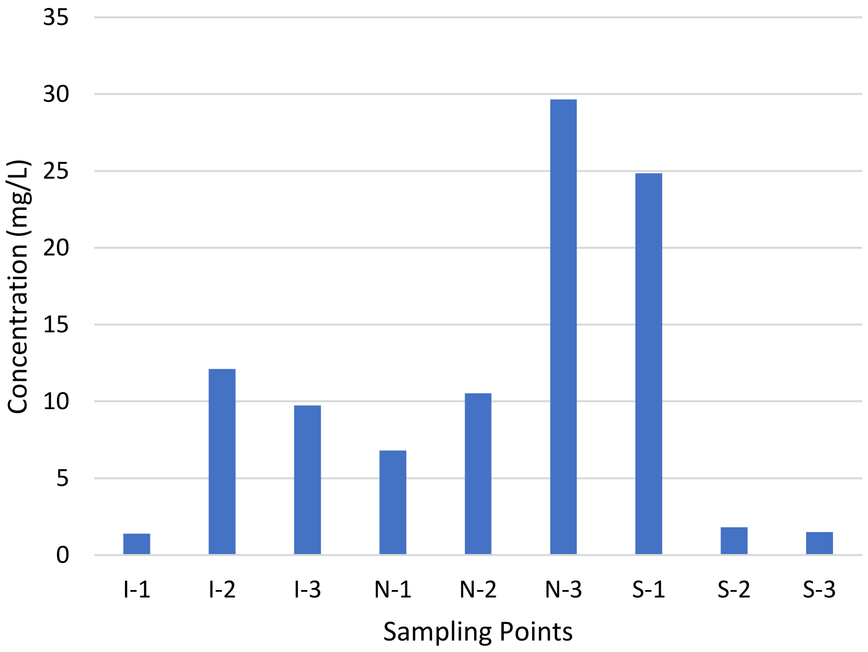

Figure 4.12: Phosphate-phosphorus concentration in the Samples

4.1.13 Sulphates

Amount of sulphates detected in the wastewater samples varied from 30-240 mg/L, with the lowest for F-10, and highest for Soan Adha location (Figure 4.13). All samples were within the NEQS. Detergents from domestic and/or industrial sources, industries using gypsum, irrigation runoff, and sewage water can be the sources for sulpahte contamination.

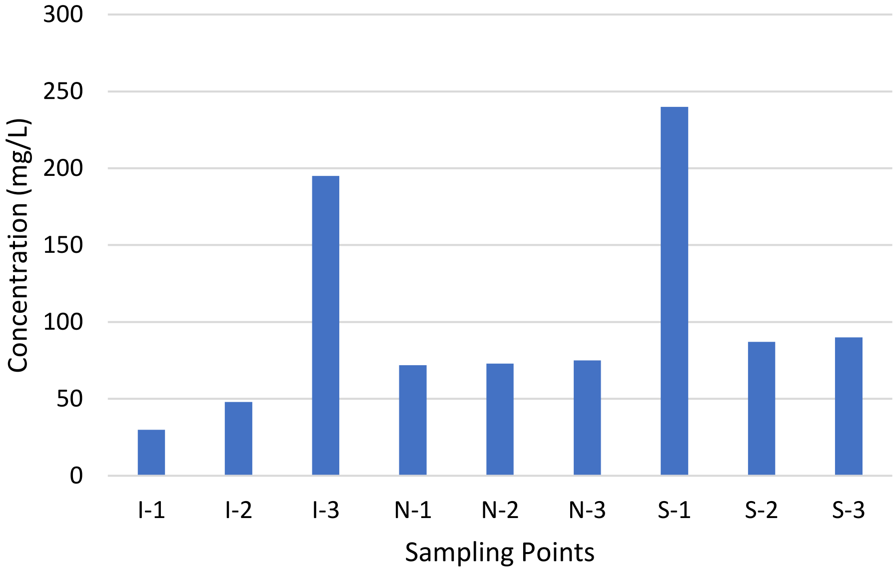

Figure 4.13: Sulphates concentration in the Samples

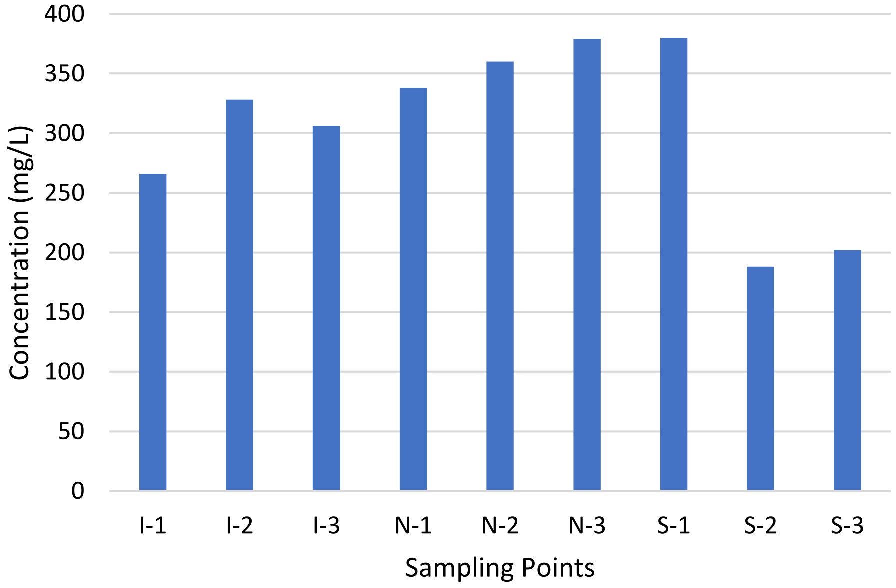

Figure 4.14: Hardness in the Samples

4.1.14 Hardness

Hardness provides the multivalent cation concentration in water, specially cations of calcium (Ca), Mg, among others including aluminum (Al), Fe, Mn, strontium (Sr), etc. It is measured in terms of mg of calcium carbonate ($CaCO_3$) in per liter of the sample. Hardness ranged from 180-360 mg/L (Figure 4.14) in the collected samples, indicating high content of carbonates, bicarbonates and other such multivalent cations of Ca, Mg, etc. Kahuta Road sample and Jamia masjid point sample were observed to have the lowest hardness among all. Weathering of sedimentary rocks, soil runoff, food waste and residue, human waste, etc. are the sources for hardness in wastewater.

4.1.15 Alkalinity

Alkalinity, also measured as mg of $CaCO_3$ per liter of the sample, indicates the ability of water to neutralize the acidity from various pollution factors, i.e., its ability to absorb H^+ ions; in other words, it can be referred to as water's acid buffering capability. It ranged from 200 mg$CaCO_3$/L being the lowest for F-10 sample, whereas 790 mg$CaCO_3$/L being the highest from two adjacent sites of Chaklala Cantt and Soan Adha (Figure 4.15). Carbonate mineral's weathering, domestic or industrial effluent containing limestone, can be the sources of alkalinity.

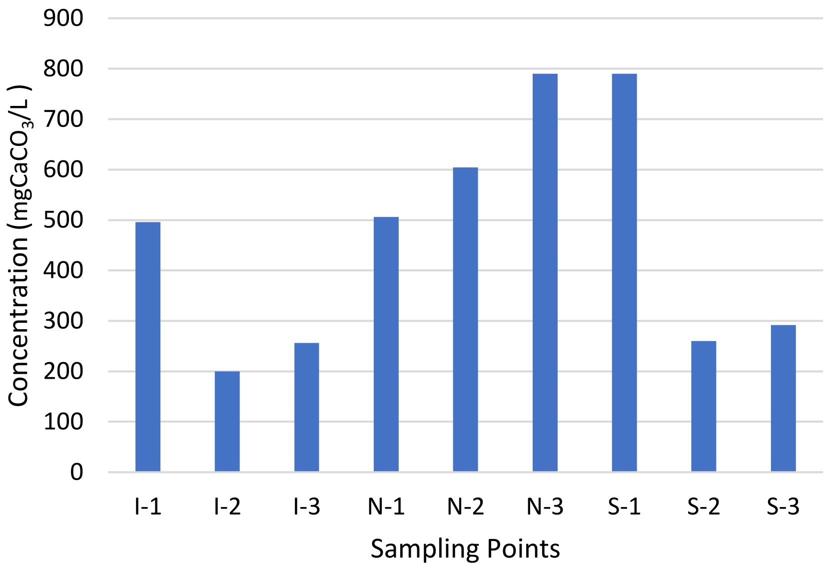

Figure 4.15: Alkalinity in the Samples

4.1.16 Chlorides

Concentrations of chlorides were found to be present in the samples. Chlorides ranged from 18-96.6 mg/L (Figure 4.16), all well below the NEQS standard. Natural mineral deposits, agricultural runoff, sewage, and industrial waste can be the sources of chlorides.

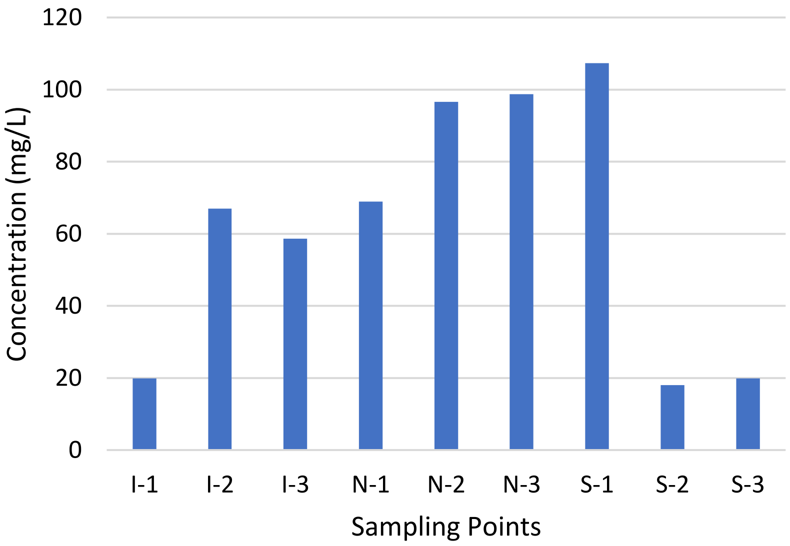

Figure 4.16: Chloride concentration in the Samples

4.2 Heavy Metal Contamination in the Wastewater Samples

It is established that the water streams under study, regularly receive industrial as well as municipal waste, making it prone heavy metal contamination. The heavy metal selected for investigation for the sake of this study are Cu, Cd, Pb and Zn, and the assessment was carried out using atomic absorption spectrophotometer (AAS). Detected concentrations of heavy metals were studied in comparison to the standards and guidelines available internationally and nationally (Table 4.2), for the purpose of assessing the quality of the sampled water, and its applicability.

Table 4.2: Heavy metal threshold limits

Heavy metals (mg/L)	**NEQS (municipal)**	**FAO (irrigation water)**	**US EPA (wastewater reuse in agriculture)**
Cadmium	0.1	0.01	0.01
Copper	1.0	0.20	0.20
Lead	0.5	5.0	5.0
Zinc	5.0	2.0	2.0

Sources: Pakistan Gazette (2000); Ayers & Westcot (1985); Bastian & Murray (2012).

Cadmium

Quantity of Cd was observed to be between 0.0362-0.0755 mg/L (Figure 4.17), with mean value being 0.0546 mg/L. All values exceeded the FAO and US EPA guidelines for wastewater reuse in irrigation, but are within the NEQS standard of municipal wastewater. Various industrial waste can be a contributor of Cd in wastewater. Presence of Cd, even at concentrations as low as 0.1 mg/L, can be toxic to various plants and crops, such as beets, turnips, etc. and many beans also, and has been found to have a high tendency of accumulation in plants as well as soil, and can further be of harm to humans through food chain exposure.

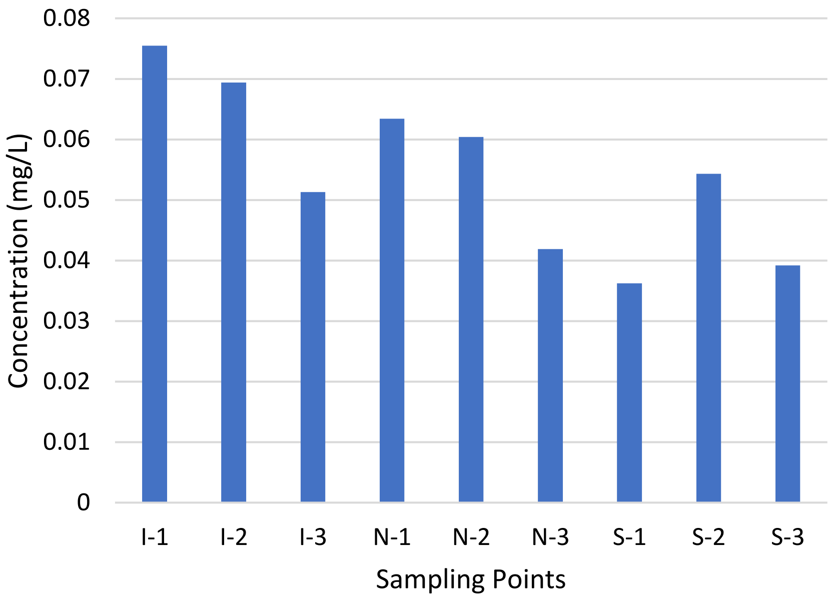

Figure 4.17: Cadmium concentration in the Samples

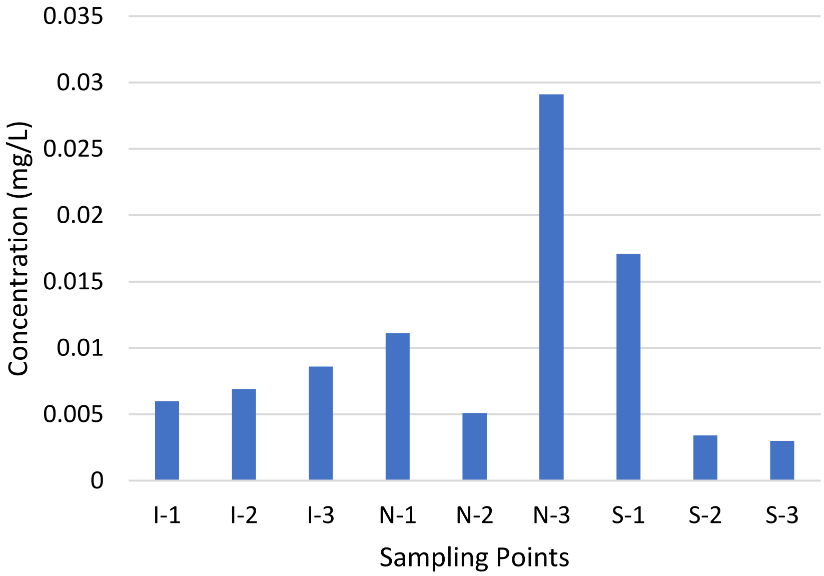

Figure 4.18: Copper concentration in the Samples

Copper

Average concentration of Cu in the samples came out to be 0.0100 mg/L, with highest concentration being 0.0291 mg/L for Chaklala Cantt site, while the lowest for Jamia Masjid site being 0.003 mg/L (Figure 4.18). All the values fall within the NEQS, FAO and US EPA guidelines also. Main sources of copper can be household as well as industrial or commercial plumbing and water fixtures, and other Cu containing materials' corrosion, as well as some industrial waste. Concentrations of Cu between 0.1 and 1.0 mg/L can cause toxicity in plants.

Lead

Pb is another heavy metal of high toxicity and can harm plant health and inhibit its growth. Pb contamination in the wastewater sites under study ranged between 0.0064 mg/L and 0.059 mg/L (Figure 4.19), with the average concentration being 0.0288 mg/L. Concentrations of all the sites fall within the NEQS, FAO and US EPA guidelines. Pipes, plumbing fixtures and other materials made of lead, metallurgy and mining and industrial wastes, as well as can be sources of this lead pollution.

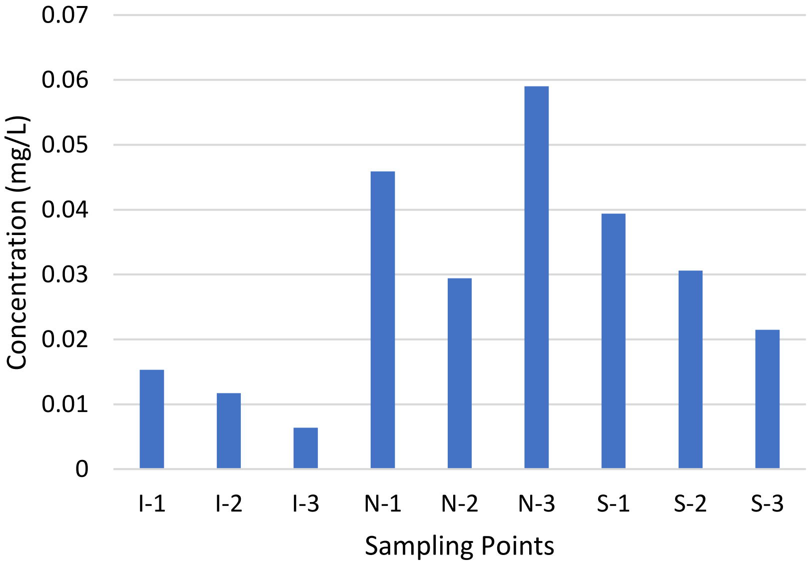

Figure 4.19: Lead concentration in the Samples

Zinc

The range for Zn contamination was recorded to be 0.0664-0.3297 mg/L, with the highest being for Saddar sample and lowest for Jamia Masjid site (Figure 4.20). The mean Zn concentration turned out to be 0.1808 mg/L. Zn contamination for all the sites fall within the NEQS, FAO and US EPA guidelines. Municipal waste, batteries, and various industrial activities including tanneries, mining, smelting, electroplating, etc. can be the potential sources of Zn. Varying concentrations of zinc can cause toxicity to several plants.

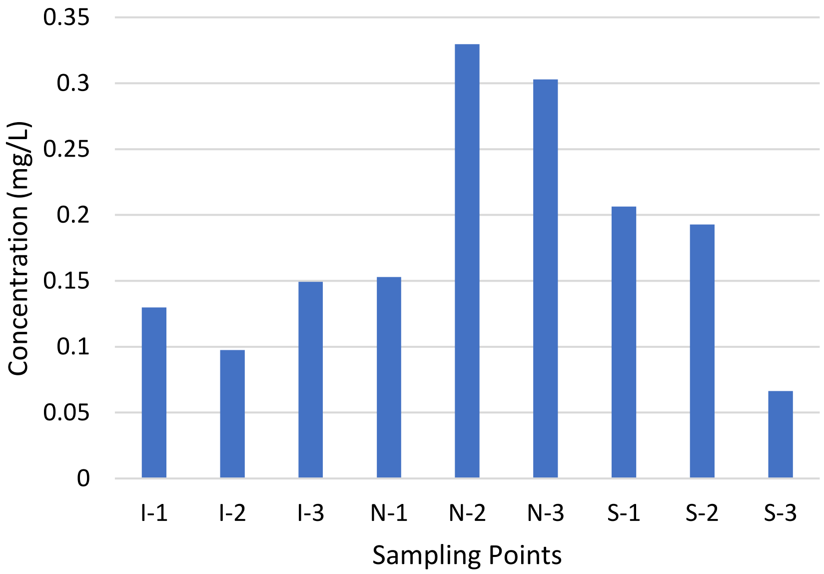

Figure 4.20: Zinc concentration in the Samples

Analysis of the physicochemical characteristics and heavy metal concentration of the collected wastewater samples as a representative of the individual water bodies, and their evaluation within the light of standards and guidelines available, provide an overall picture of the water quality of these water bodies. For physicochemical analysis, all the sampled sites vary in their concentration and constituent pollutants and indicators and follow no specific trend.

In terms of heavy metal pollution, Chaklala Cantt site has the highest concentration, it can be because of a brewing factor located at the site, that release its effluent into this water stream; second to it is the adjacent site of Saddar, both having highest Zn in comparison to Cd, Cu, and Pb. Jamia Masjid site had the least heavy metal pollution among all sites. Taking all sites under consideration, the overall order of heavy metal pollution follows Zn>Cd>Pb>Cu.

Overall analysis suggests there to be an inflow of domestic, municipal, industrial waste and effluent into these natural water streams, leading to them turning into wastewater, deteriorating their quality to the least. Furthermore, sewage, agricultural runoff specifically containing fertilizers, livestock and poultry waste from the twin cities dumped into the water channel provides for its high nutrient load, leading to high microbial pollution, algal blooms, and eutrophication. All these factors also contribute to high concentrations of various heavy metals, making it of toxic nature to living being including plants, animals, and especially human beings (Nazeer et al., 2014). The results are in consistence with previously reported investigations on the study area (Kanwal et al., 2012; Kalim et al., 2012; Perveen et al., 2017), and proposes that treatment of all the waste and effluents, industrial, domestic, sewage be carried out before their inflow into the natural water streams to maintain their quality, and to protect the environment from anthropogenic affect, in-turn protecting humans from its harmful impacts.

4.3 Microbiological Analysis

4.3.1 Isolation of Bacterial Strains

A total of twenty-seven bacterial strains were isolated from the collected wastewater samples on the basis of their varying morphologies, by culturing pure colonies obtained from the prepared dilution plates of the water samples. The strains were picked on random basis.

4.3.2 Characterization of the Isolated Bacterial Strains

The bacterial strains isolated from the collected wastewater samples varied in their morphological parameters of shape/form, elevation, margin, texture, opacity, and pigmentation. It was observed that most of the bacteria occurred in punctiform and the rest as circular. Furthermore, raised elevation, entire margin, smoothness in texture, and non-pigmentation appeared to be the most frequently seen traits in the bacterial strains.

Following morphological characterization, various biochemical tests were performed to obtain more knowledge of the isolated strains, which included gram staining, catalase test, oxidase test, Simmons citrate agar test, and mannitol salt agar test. The results reveal 81 percent of bacteria to be gram negative, while the rest being gram positive (Figure 4.21). Most of the bacteria proved to be cocci under the microscope, and the others were rod shaped.

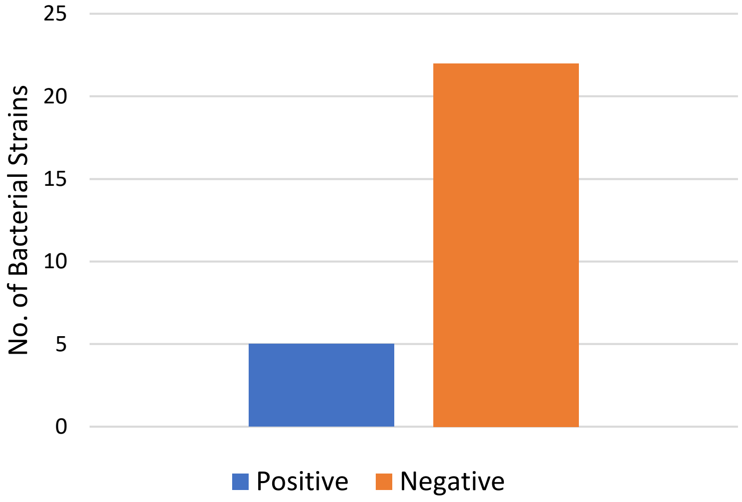

Figure 4.21: Gram stain results for the bacterial strains

Rest of the biochemical characterization revealed 85 percent and 66.6 percent of the bacterial isolates to experience catalase and oxidase positive behaviors, respectively. In addition, while examining the growth of isolated bacterial strains on different media, 92.5 percent were able to grow on Simmons citrate agar, however, only 66.6 percent could cultivate on mannitol salt agar, while the rest showed no growth on either. The results are presented in 4.22.

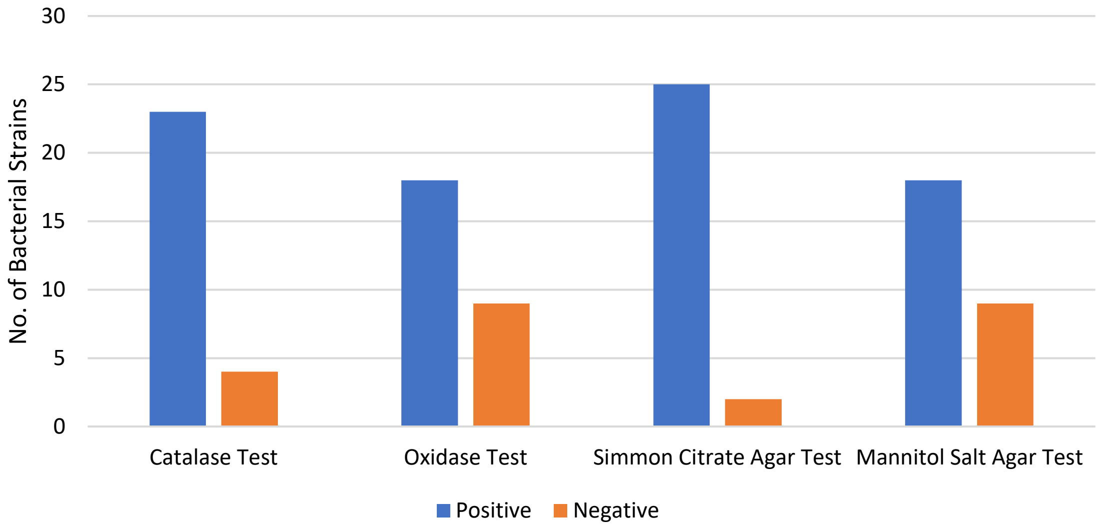

Figure 4.22: Results of biochemical tests for the bacterial strains

The tests were helpful in providing an insight into the bacterial characteristics and traits, hinting a faint idea to their identities. Catalase test proves the presence of the enzyme catalase in bacteria, that breaks down hydrogen peroxide, obtaining oxygen which bacteria uses for respiration, releasing water as a byproduct. This provides the idea of catalase positive bacteria to belong to aerobic and facultative type of microorganisms, while catalase negative bacteria to be from the anaerobic type. Streptococci bacteria are known to contain catalase enzyme, thus being catalase positive, however, streptococci have been shown to be catalase negative (Sizar & Unakal, 2020). Enterobacteriaceae species have also been able to be distinguished based on the catalase test (Taylor & Achanzar, 1972).

Oxidase test identifies bacteria that have the enzyme cytochrome oxidase, which catalyzes the electron transport chain in bacteria. The catalysis helps the transport of electrons from electron donating compounds in bacteria, e.g. NADH to electron acceptors present in the environment, usually oxygen, producing either water or hydrogen peroxide. In oxidase test, the reagent TMPD acts as the electron acceptor, producing a blue colored compound called indophenol, confirming the result to be positive. Usually aerobic bacteria are known to have cytochrome oxidase mechanism (Jurtshuk & McQuitty, 1976).

Simmons citrate agar supports the growth of microorganisms that can utilize citrate present in the media as a carbon source for their survival. This test can help differentiate specifically *Enterobacteriaceae*, but also C*itrobacter, Klebsiella, Proteus* and *Providencia* are been found to provide positive results on the test, whereas *Escherichia coli, Edwardsiella, Shigella,* and *Yersinia,* species have produced negative results.

Mannitol salt agar is a selective medium that contains high concentration of salt (sodium chloride) providing saline environment, thus testing microbes' ability to tolerate saline environments. It also contains mannitol, which is a carbohydrate, which if fermented by some bacteria, produce lactic acid, turning the media yellow. Mannitol salt agar supports the growth of various staphylococcus species, while suppressing that of others (Kateete et al., 2010).

4.3.3 Identification of Heavy Metal Resistant Bacteria

All obtained bacterial isolates were examined for their resistance against the four selected heavy metals, including Cd, Cu, Pb and Zn. Each isolate showed varying trends for resistance towards these heavy metals.

4.3.3.1 Cadmium

The isolated bacterial strains when tested for resistance against Cd, showed a higher percentage, i.e., 88.8 percent to be resistant to Cd, while the rest 11.1 percent experienced susceptibility to the heavy metal (Figure 4.23). Khan et al. (2015) isolated *Klebsiella pneumoniae* from industrial wastewater samples from some districts of Lahore, and identified it to be resistant against Cd, therefore, they further utilized it for environmental bioremediation. Various Cd resistance mechanisms adopted by bacteria include passive mechanisms like extracellular complexation through polymeric substances like polysaccharides, proteins, nucleic acids and/or siderophores, and cell wall binding by means of ion-exchange reactions, complexation with oxygen and nitrogen ligands, and destruction of complexating ligands. Whereas, active mechanisms may constitute metallothioneins, redox mechanisms, efflux pumps, precipitation, volatilization, intracellular sequestration and rhamnolipids (Abbas et al., 2018).

4.3.3.2 Copper

All of the isolated bacterial strains were tested against Cu for their resistance. The results revealed 77.7 percent strains to be resistant to the applied concentration of Cu as presented in Figure 4.23. However, 22.2 percent of the bacterial strains showed susceptibility. *Escherichia coli*, *Salmonella*, *Streptococcus* and *Vagococcus* species isolated from tanneries' wastewater in Kasur and Rohi Nala, were found be resistant to high concentrations of Cu, and had efficient Cu accumulation capabilities (Shakoori & Muneer, 2002). Cu resistance mechanisms in *Ochrobactrum* MT180101 were concluded to be cell wall binding of metal cations, chelation and stabilization of cations with proteins, bio-transportation from intramembrane to the outer membrane through various intracellular transport mechanisms, reduction of heavy metals through enzyme-mediated biotransformation (Peng et al., 2019).

4.3.3.3 Lead

While studying the resistance of all the isolated bacterial strains towards Pb, it was observed that 81.5 percent of the strains revealed to be resistant to Pb (Figure 4.23), however, the rest of 18.5 percent bacterial isolates showed susceptibility. Bacterial strains isolated from industrial wastewater samples were found to tolerate toxic concentrations of Pb (Chatterjee et al., 2012). Many Pb resistant bacterial strains were isolated from wastewater samples obtained from oil field, among which, *Bacillus cereus* could resist Pb concentration as high as 10 mM and showed high Pb accumulation as well (Utami et al., 2020). Bacterial species resistant to Pb have been observed to imply extracellular and intercellular accumulation, precipitation, biosorption, alteration in morphology of the cell, high production of siderophores, and different efflux mechanisms to confer resistance against high Pb concentrations (Naik & Dubey, 2013).

4.3.3.4 Zinc

When exposed to concentrations of Zn, all 100 percent of the bacterial strains experienced complete resistance to the applied concentration of Zn as is shown in Figure 4.23. Resistance to Zn can be due to extracellular accumulation of Zn, sequestration by metallothioneins, storage within the cell, or efflux systems. A gram-negative bacterium which is ubiquitous to soil environments, *Ralstonia*, is found to have Czc system to provide for mechanism for Zn resistance. Czc systems provides for resistance against Zn, Cd and Co, by working as a cation antiporter that effluxes cations from the cells (Choudhury & Srivastava, 2001). Various bacterial species including *Bacillus*, *Pseudomonas*, and *Staphylococcus* isolated from soil irrigated with metal contaminated wastewater, were found be to Zn resistant (Ahemad & Malik, 2011).

It has been determined that all the bacteria isolated from the collected wastewater samples show some extent of resistance to the applied concentration of selected heavy metals, i.e., Cd, Cu, Pb and Zn. The results also reveal that the resistance percentage displayed by the isolates towards each heavy metal is in accordance with the concentration of those respective heavy metals detected in the wastewater, i.e., Zn was found to be of highest (65.9 percent) in total aggregate heavy metal concentration of all the collected samples, and the resistance test also revealed highest percentage (100 percent) of the isolates to be resistant to Zn; followed by Cd having 19.9 percent contribution in the heavy metal concentration and falling second in the trend of bacterial resistance with 88.8 percent; Pb being 10.4 percent in heavy metal concentration with 81.5 bacteria being resistant to it; and lastly Cu concentration being 3.65 percent with 77.7 percent of the bacterial strains being resistant to it. Similar reports of concentration of heavy metal being in correspondence with the resistance experienced by the isolated bacteria was expressed by Nasrazadani et al., (2011).

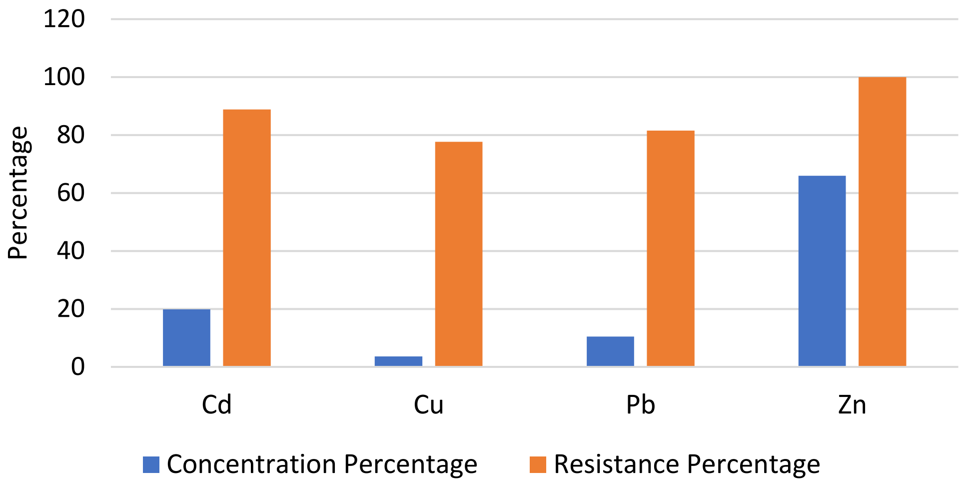

Figure 4.23: Heavy metals and their corresponding resistance

4.3.4 Identification of Antibiotic Resistant Bacteria

The isolated bacterial strains were also screened for their resistance towards selected class fluoroquinolones' antibiotics. The antibiotics selected for this study are amoxicillin, ciprofloxacin, levofloxacin, and ofloxacin.

4.3.4.1 Amoxicillin

All the isolated bacterial strains were tested for resistance against amoxicillin, and the results revealed 70.4 percent of strains to be completely resistant to the applied amoxicillin (Figure 4.24), 7.4 percent showed intermediate resistance, while 22.2 percent were susceptible. An increase in the β lactam resistance genes was recorded in presence of increasing concentration of amoxicillin, indicating the antibiotic to have a significant role in resistance genes' functionality and dissemination. Vertical horizontal gene transfer was found to be more active in this case in comparison to horizontal gene transfer (Meng et al., 2017). Higher resistance towards amoxicillin among other antibiotics was observed in bacterial isolates obtained from wastewater systems, especially among *Escherichia coli* species (Teshome et al., 2020). In *Staphylococcus aureus*, mecA and femX genes have been found to have a major role in conferring resistance against amoxicillin (Yao et al., 2019).

4.3.4.2 Ciprofloxacin

When tested for resistance against ciprofloxacin, 59.3 percent of the isolated bacteria were resistant to the applied concentration of ciprofloxacin, 33.3 percent were intermediately resistant, while the rest 7.4 percent showed susceptibility towards the antibiotic (Figure 4.24). A high percentage of *Gammaproteobacteria* resistant to ciprofloxacin were isolated from wastewater treatment plant, and its receiving river body (Marti et al., 2014). While studying ciprofloxacin resistant *Escherichia coli* obtained from hospital wastewater, most of the isolates were observed to contain large plasmids, while all the resistant isolates had gyrA double mutations (Akter et al., 2012). *Peudomonas aeruginosa* has been reported to use various mechanisms for resistance against ciprofloxacin including target site modification by mutating the gyrAB and parCE genes, thereby decreasing the ability of the enzymes gyrase and topoisomerase to attract ciprofloxacin. In addition to this, efflux mechanism is also adopted, whereby an overexpression of the efflux pumps causes ciprofloxacin to expel from the cell (Rehman et al., 2019).

4.3.4.3 Levofloxacin

Test for bacterial resistance against the antibiotic levofloxacin discovered 55.6 percentage of bacteria to be resistant to it, 29.6 percent to have intermediate resistance, and the remaining 14.8 percent of the isolated bacterial strains to be susceptible to the antibiotic. Figure 4.24 presents the resistance percentages. It has been found that bacterial resistance to antibiotics was observed to emerge the same year levofloxacin was introduced into medical and clinical prescription and practice (Sengupta et al., 2013). A high percentage of *Helicobacter pylori* strain isolated clinically were found to be resistant to levofloxacin, and most of those strains were observed to have mutation in their gyrA gene at Asn-87 and Asp-91 (Miyachi et al., 2006). Levofloxacin resistance in *Vibrio alginolyticus* have been recorded to also be a function of proteomics, whereby a reduction in central carbon and energy metabolism happens to provide resistance (Cheng et al., 2018).

4.3.4.4 Ofloxacin

Figure 4.24 shows that 51.9 percent of the bacterial strains displayed complete resistance to the applied concentration of ofloxacin, while 25.9 percent had intermediate resistance, however the rest portion of the bacterial isolates, i.e., 22.2 percent were susceptible to ofloxacin. Ofloxacin resistant *Escherichia coli* species were obtained from stream receiving high loads of wastewater effluent (Akiyama & Savin, 2010). *Salmonella typhimurium* strains resistant to ofloxacin were also isolated from River Ravi, owing to the release of effluents from various hospitals into the river without any treatment, and further investigation revealed the isolated *Salmonella typhimurium* to disseminate the resistance to other species cultured with it (Ahmad et al., 2014). OtrC protein has been reported to confer resistance to ofloxacin and other antibiotics (Peterson & Kaur, 2018).

Thus, all the bacterial isolates obtained from the collected wastewater samples were screened for their resistance abilities towards selected antibiotics, i.e., amoxicillin, ciprofloxacin, levofloxacin, and ofloxacin. The bacterial isolates expressed varying responses in terms of resistance against all four antibiotics (Figure 4.24). Saima et al., (2020) have reported presence of multidrug resistant bacteria in wastewater in Pakistan.

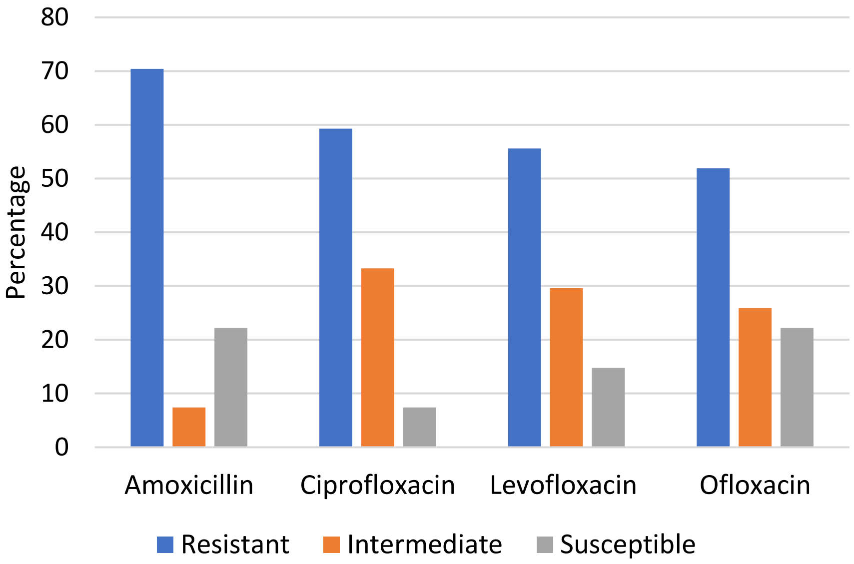

Figure 4.24: Varying bacterial resistance towards selected antibiotics

4.3.5 Co-resistance between Heavy Metals and Antibiotics

It has been observed that the isolated bacteria have high resistance towards heavy metals and antibiotics. Bacterial strains showed co-resistance within the metals as well, as 92.5

percent of the bacteria were resistant to 3 or 4 of the tested metals, i.e., Cd, Cu, Pb and Zn. Whereas, 51.8 percent of the isolates were resistant to 3 or 4 of the applied antibiotics, including, amoxicillin, ciprofloxacin, levofloxacin and ofloxacin. Table 4.3 shows each bacterial strains' resistance to respective heavy metals and antibiotics.

The results have revealed that the heavy metal resistant strains experienced resistance to multiple antibiotics they were tested against as well, proving them to possess multi-drug resistance, and suggesting towards the potential of there being an association between intrinsic resistance towards both types of antimicrobials. Isolates N3-S3, N3-S6, N3-S7, S2-S1, and S3-S8 were resistant to all eight antimicrobials, i.e., the four heavy metals and four antibiotics their resistance was tested for, while in total, as much as 92.5 percent of the strains were resistant to at least half or more of the antimicrobial tested, providing evidence of bacteria being strong in defending themselves, posing threats to other living beings, specifically to human well-being, especially when it comes to pathogenic microbes. Similar results of heavy metal resistance and antibiotic resistance have also been recorded by Yamina et al. (2012).

Table 4.3: Co-resistance of bacterial isolates to multiple antimicrobials

Sr. No.	**Strain**	**Resistance to metals**	**Resistance to Antibiotics**
1	I1-S9	Cd, Pb, Zn,	Amoxicillin, Levofloxacin, Ofloxacin
2	I2-S4	Cd, Cu, Zn	Ciprofloxacin, Levofloxacin, Ofloxacin
3	I2-S10	Cd, Cu, Zn	Amoxicillin, Ciprofloxacin, Levofloxacin
4	I2-S15	Cd, Cu, Zn	Amoxicillin, Ciprofloxacin, Levofloxacin, Ofloxacin
5	I3-S1	Zn	Levofloxacin, Ofloxacin
6	I3-S2	Cd, Cu, Pb, Zn	Amoxicillin, Ciprofloxacin, Ofloxacin
7	I3-S3	Cd, Cu, Pb, Zn	-
8	I3-S4	Cd, Cu, Zn	-
9	I3-S5	Cd, Cu, Pb, Zn	Amoxicillin, Ciprofloxacin, Levofloxacin
10	I3-S15	Cu, Pb, Zn	Ciprofloxacin
11	I3-S16	Cd, Pb, Zn	Amoxicillin
12	N1-S1	Cd, Pb, Zn	Amoxicillin, Ciprofloxacin

13	N1-S4	Cd, Pb, Zn	Amoxicillin, Ofloxacin
14	N2-S5	Cd, Cu, Pb, Zn	Amoxicillin
15	N2-S6	Cd, Cu, Pb, Zn	Amoxicillin, Ciprofloxacin
16	N3-S4	Cd, Cu, Pb, Zn	Amoxicillin
17	N3-S5	Cd, Cu, Pb, Zn	Amoxicillin, Ciprofloxacin, Levofloxacin, Ofloxacin
18	N3-S6	Cd, Cu, Pb, Zn	Amoxicillin, Ciprofloxacin, Levofloxacin, Ofloxacin
19	N3-S7	Cd, Cu, Pb, Zn	Amoxicillin, Ciprofloxacin, Levofloxacin, Ofloxacin
20	N3-S8	Cd, Cu, Pb, Zn	Amoxicillin
21	N3-S9	Cd, Cu, Pb, Zn	Amoxicillin, Levofloxacin, Ofloxacin
22	S1-S2	Pb, Zn	Ciprofloxacin, Levofloxacin
23	S1-S4	Cd, Cu, Pb, Zn	Ciprofloxacin, Levofloxacin, Ofloxacin
24	S1-S12	Cd, Cu, Pb, Zn	Ciprofloxacin, Levofloxacin, Ofloxacin
25	S2-S1	Cd, Cu, Pb, Zn	Amoxicillin, Ciprofloxacin, Levofloxacin, Ofloxacin
26	S2-S3	Cd, Cu, Pb, Zn	Amoxicillin
27	S2-S8	Cd, Cu, Pb, Zn	Amoxicillin, Ciprofloxacin, Levofloxacin, Ofloxacin

Statistical correlation (Pearson) obtained between the various heavy metals and antibiotics' resistance, under study is represented in Table 4.4. Zn is omitted from the statistical correlation because of there being no variation present in its results, with all the bacterial strains being completely resistant to it. Among all the antibiotics, amoxicillin has been observed to have positive correlation with all the heavy metals, as well as the other antibiotics, immediately followed by ciprofloxacin to have positive with all except Cd. Strong correlation of amoxicillin to the heavy metals is also reiterated in the biplot obtained from Principal Component Analysis (PCA) (Figure 4.25). A good percentage of positive correlation is also seen among the heavy metals, as well as among antibiotics. Sinegani and Younessi (2017) informed Zn, Cd, Pb and Cu resistance to have a positive to relation with resistance to several antibiotics in soil isolated bacteria.

Table 4.4: Statistical correlation (Pearson) between heavy metal and antibiotic resistance

Variables	Cd	Cu	Pb	Amoxicillin	Ciprofloxacin	Levofloxacin	Ofloxacin
Cd	1	0.378	0.135	0.487	-0.083	-0.125	0.130
Cu	0.378	1	-0.025	0.095	0.157	-0.067	-0.024
Pb	0.135	-0.025	1	0.276	0.090	-0.125	-0.061
Amoxicillin	0.487	0.095	0.276	1	0.230	0.043	0.008
Ciprofloxacin	-0.083	0.157	0.090	0.230	1	0.664	0.425
Levofloxacin	-0.125	-0.067	-0.125	0.043	0.664	1	0.734
Ofloxacin	0.130	-0.024	-0.061	0.008	0.425	0.734	1

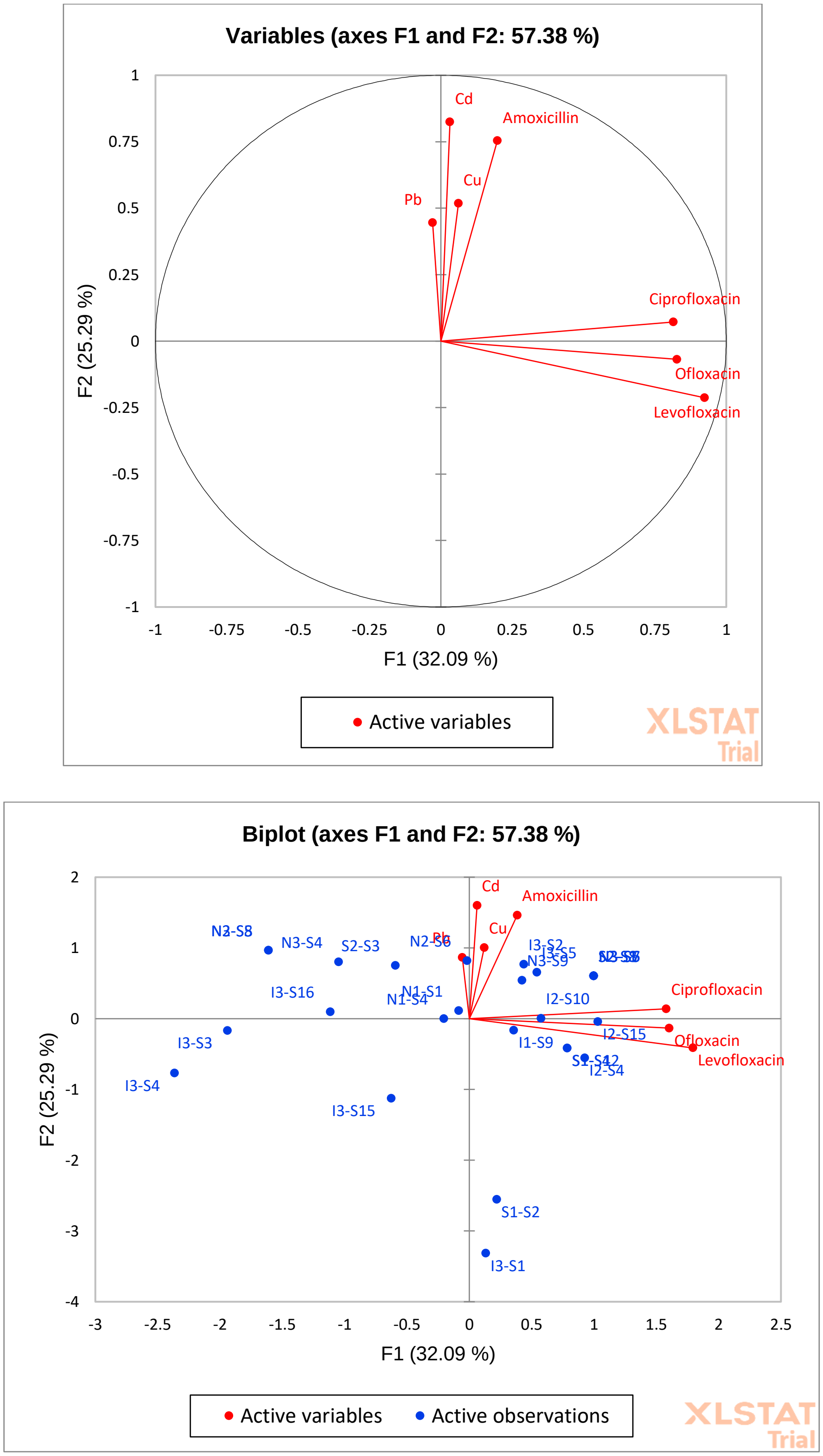

Figure 4.25: Biplots presenting variable projection of F1 (heavy metals) and F2 (antibiotics)

Various bacterial studies have provided evidence that bacterial exposure to one kind of antimicrobials can tend to affect is ability to respond to, and its susceptibility towards other types of antimicrobials (Wales & Davies, 2015). Correlation between heavy metal and antibiotic resistance in terms of co-selection is a topic of interest all over the world, and is well known, but still not fully understood. However, heavy metal pollution’s role in it is has been reported (Knapp et al., 2017). Ji and fellows (2012) reported ARGs in animal manure and agricultural land to be more significantly correlated to heavy metal content of the soil in comparison to the respective antibiotics. Several investigations have reported environmental bacterial isolates to confer resistance to both heavy metals and antibiotics. Simultaneous existence of heavy metal and antibiotic resistance within microbial organisms has been reported in *Bacillus cereus* and *Pseudomonas aeruginosa*, and researchers have established that it is not a by chance occurrence, rather suggests towards existence of co-selection mechanisms between the two resistances, and can be attributed to various environmental pollutions as a consequence of anthropogenic activities (Nath et al., 2019). Dweba and his collegues (2019) presented an account isolation of multi drug resistant; heavy metal and antibiotic resistant and pathogenic strains of *Stephalococcus aureaus* from livestock production systems and proposed co-selection mechanisms between heavy metal and antibiotic resistance. Bacterial isolates resistant to Zn, obtained from environmental samples have been found to correlate through mechanism of co-selection to antibiotic resistance against oxacillin, cefotaxime and trimethoprim (Dickinson et al., 2019).

The results obtained from the current research by assessing the resistance of isolated environmental bacterial strains present co-occurrence of heavy metal and antibiotic resistance in a very high percentage, and suggests that there can be a genetic association existing between the two resistances, i.e., co-resistance. Sair and Khan (2018) also recorded evidences of co-resistance mechanisms in bacterial isolates obtained from Soan River and Ravi River. As the water channels under study are of high significance, are used highly for irrigation and other purposes, and even lead into the drinking water supply, the high pollution in terms of heavy metal pollution and multi drug resistant bacteria, and the rapid and high dissemination of such resistance can lead to uncontrollable havocs, if timely measures are not put in place.

Chapter 5

CONCLUSIONS AND RECCOMENDATIONS

5.1 Conclusions

The comprehensive study on a major metropolitan area of the country, i.e., Islamabad and Rawalpindi, also known as the twin cities, provides with an overall assessment of the quality of water running through the streams of the area, and into the Soan River. Physicochemical analysis of the wastewater the parameters of quality revealed a mixed trend when compared with NEQS, FAO and US EPA guidelines.

Quantification of the selected heavy metals, Cd, Cu, Pb and Zn revealed that concentrations of Cu and Pb at some sites exceeded the thresholds provided by NEQS for wastewater quality. However, except Cd, concentrations of the other three heavy metals are within the FAO and US EPA guidelines provided to regulate wastewater reuse for agriculture. Thus, it can be concluded that heavy metals are within the guidelines/ regulatory limits, and the water quality is suitable for reuse in agriculture.

The results obtained propose there to be an inflow of untreated municipal, as well as industrial waste into these water bodies, degrading the quality. Such water when adds into the water sources supplying for drinking water can be a threat for human health, and cause issues of severity.

Bacterial isolation and studying their resistance towards antimicrobial agents discovered majority of the isolates to be resistant against the applied heavy metal and antibiotic concentrations. The investigation also supported the concept of correlation between heavy metal and antibiotic resistance, and heavy metal contamination to further function as a selective agent when it comes to co-resistance of heavy metals and antibiotics.

5.2 Recommendations

Based upon the knowledge obtained by carrying out the current research, there appears to be a desperate need to take care of the natural environmental sources and to protect them from the irresponsible and inappropriate anthropogenic activities, in order to sustain them and maintain their quality. It has been seen that the harms of such anthropogenic activities tend to impact the human race in unprecedented ways, along with being threatening and harmful to other living species in animals as well as plants.

The water bodies of the twin cities, that once used to be freshwaters, are now categorized to be wastewater due to unregulated liquids and solid waste dumping. Proper and regulated treatment and screening of industrial as well as municipal waste before being released into the natural waters is an urgent need. Responsible authorities should take the responsibility for maintaining the water quality by regular monitoring and quality assurance. Pak-EPA should make sure that the policies made are being implemented and followed.

As wastewater reuse practice became a need due to water shortage and increasing scarcity, treatment of wastewater before being used for various purposes, specially irrigation should be made sure in order to prevent harmful contaminants including heavy metals from harming the life and health of the crops and soil, and to inhibit them from entering into the food chain, which otherwise has been seen to be a great threat to living beings.

The ever-increasing multidrug resistance of the microbiological organisms, especially the pathogenic species, is emerging as an issue of concern throughout the world. The unregulated misuse of antibiotics is a major factor to blame for development of such resistances in bacterial species. This requires for a more vigilant and regulated use of such antibiotics in pharmaceuticals as well as in livestock industry, as a high percentage of antibiotics is consumed by livestock systems. Heavy metal and heavy metal resistance acting as a co-selecting agent in antibiotic resistance is further aggravating the problem, where it can reach to the point of being uncontrollable. Therefore, it is the need of the hour to put effective measures in place, and to regulate the use and disposal of such antimicrobial agents into the environmental components. Effective and efficient policies should be made and implemented by the regulatory authorities for this purpose.

Co-resistance between heavy metals and antibiotics is known for a quite some time now, and is being studied upon throughout the world, but the complex mechanisms underlying are still not fully recognized. More research on the topic is therefore recommended, focusing on more extensive spectrum of heavy metals and antibiotics. Mechanisms taking place at the molecular, biochemical, and genetic levels need further insight for understanding the working of co-selection and co-resistance in order to be fully able to come up with more effective strategies to combat the issue.

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APPENDICES

Table A.1: Quantitative analysis of the physicochemical parameters of the collected samples

Parameter	Units	I-1	I-2	I-3	N-1	N-2	N-3	S-1	S-2	S-3
Temperature	°C	17.3	18.8	18.3	25.7	26.6	27.4	12.9	12.7	13.2
pH	-	5.06	4.81	5.57	7.45	7.47	6.45	7.91	7.88	7.36
EC	µS/cm	612	1072	926	1032	1224	1446	1372.6	500.3	551.6
Turbidity	NTU	17.26	31.3	24.86	45	55	167	121.2	28	3.6
TDS	mg/L	330	580	508	567	670.3	793.2	753	278	300
DO	mg/L	1.7	0	1.8	0.86	0.50	1.3	1.3	10.9	10.1
COD	mg/L	214	231.12	214	410.88	196.88	436.56	460.8	430.08	174.08
BOD	mg/L	140	152.3	141.2	271.1	130.2	287.6	276	258	100.4
TKN	mg/L	10.5	38.5	25.7	63.05	66.3	50.9	75.6	12.6	5.6
Nitrate-Nitrogen	mg/L	0.269	0.09	0.12	0.59	1.19	0.92	0.79	2.33	1.60
Nitrite-Nitrogen	mg/L	32.26	23.93	46.66	3.631	15.45	36.96	28.78	0.9	0.45
Phosphate-Phosphorus	mg/L	1.4	12.11	9.75	6.81	10.54	29.67	24.84	1.801	1.49
Sulphates	mg/L	30	48	195	72	73	75	240	87	90
Hardness	mg/L as $CaCO_3$	266	328	306	338	360	379	380	188	202
Alkalinity	mg/L as $CaCO_3$	496	200	256	506	604	790	790	260	292
Chlorides	mg Cl^- /L	19.9	66.97	58.9	68.9	96.9	98.7	107.3	18	19.9

Table A.2: Heavy metal concentration in the collected samples

Sample	Cd (mg/L, ppm)	Cu (mg/L, ppm)	Pb (mg/L, ppm)	Zn (mg/L, ppm)
I-1	0.0755	0.0060	0.0153	0.1298
I-2	0.0694	0.0069	0.0117	0.0974
I-3	0.0513	0.0086	0.0064	0.1493
N-1	0.0634	0.0111	0.0459	0.1529
N-2	0.0604	0.0051	0.0294	0.3297
N-3	0.0419	0.0291	0.059	0.3029
S-1	0.0362	0.0171	0.0394	0.2064
S-2	0.0543	0.0034	0.0306	0.1927
S-3	0.0392	0.003	0.0215	0.0664

Table A.4: Morphological Parameter of Bacterial Strains

Strain	Shape/Form	Color	Elevation	Margin	Texture	Opacity	Pigment
I1-S9	Punctiform	Creamy white	Raised	Entire	Smooth	Opaque	Non-pigmented
I2-S4	Punctiform	Creamy white	Flat	Entire	Smooth	Opaque	Non-pigmented
I2-S10	Punctiform	White	Flat	Entire	Smooth	Opaque	Non-pigmented
I2-S15	Punctiform	Yellow	Flat	Entire	Smooth	Translucent	Yellow
I3-S1	Punctiform	Creamy white	Flat	Entire	Smooth	Opaque	Non-pigmented
I3-S2	Punctiform	Cream	Raised	Entire	Smooth	Opaque	Non-pigmented
I3-S3	Punctiform	Cream	Raised	Entire	Smooth	Opaque	Non-pigmented
I3-S4	Punctiform	Creamy white	Raised	Entire	Smooth	Opaque	Non-pigmented
I3-S5	Punctiform	Cream	Flat	Entire	Smooth	Translucent	Non-pigmented
I3-S15	Punctiform	Yellow	Flat	Entire	Smooth	Translucent	Yellow
I3-S16	Punctiform	Creamy white	Raised	Entire	Smooth	Opaque	Non-pigmented
N1-S1	Punctiform	Cream	Flat	Undulate	Rough	Slightly opaque	Non-pigmented
N1-S4	Circular	Creamy white	Convex	Undulate	Smooth	Opaque	Non-pigmented
N2-S5	Circular	Creamy white	Raised	Entire	Smooth	Slightly opaque	Non-pigmented
N2-S6	Circular	Creamy white	Raised	Entire	Smooth	Opaque	Non-pigmented
N3-S4	Circular	Creamy white	Raised	Entire	Smooth	Opaque	Non-pigmented
N3-S5	Circular	Creamy white	Raised	Entire	Smooth	Opaque	Non-pigmented
N3-S6	Punctiform	Creamy white	Raised	Entire	Smooth	Translucent	Non-pigmented
N3-S7	Punctiform	Creamy white	Raised	Entire	Smooth	Opaque	Non-pigmented
N3-S8	Punctiform	Cream	Raised	Entire	Smooth	Translucent	Non-pigmented
N3-S9	Punctiform	Creamy white	Flat	Entire	Smooth	Translucent	Non-pigmented
S1-S2	Circular	Creamy blackish	Raised	Entire	Smooth	Opaque	Non-pigmented

S1-S4	Circular	White	Flat	Entire	Rough	Opaque	Non-pigmented
S1-S12	Punctiform	Creamy white	Flat	Entire	Smooth	Opaque	Non-pigmented
S2-S1	Punctiform	Creamy white	Raised	Entire	Smooth	Opaque	Non-pigmented
S2-S3	Circular	Cream	Flat	Entire	Smooth	Opaque	Non-pigmented
S2-S8	Circular	Creamy white	Flat	Entire	Smooth	Slightly opaque	Non-pigmented

Table A.3: Biochemical Characteristics of Bacterial Strains

Strain	Gram Staining Test	Catalase Test	Oxidase Test	Simmon Citrate Agar Test	Mannitol Salt Agar Test
I1-S9	Negative/ Rods	+	+	+	-
I2-S4	Positive/ Coccobacilli	+	+	+	-
I2-S10	Positive/ Coccobacilli	+	+	+	+
I2-S15	Negative/ Coccobacilli	+	-	+	+
I3-S1	Negative/ Coccobacilli	+	+	+	+
I3-S2	Negative/ Coccobacilli	-	+	+	-
I3-S3	Negative/ Rods	-	+	+	-
I3-S4	Negative/ Rods	-	+	+	-
I3-S5	Negative/ Coccobacilli	+	-	+	-
I3-S15	Negative/ Coccobacilli	+	-	+	+
I3-S16	Negative/ Coccobacilli	+	+	+	-
N1-S1	Negative/ Rods	+	-	+	+
N1-S4	Negative/ Coccobacilli	+	-	+	+
N2-S5	Negative/ Rods	+	-	+	+
N2-S6	Negative/ Rods	+	+	-	+
N3-S4	Positive/ Rods	+	+	+	+
N3-S5	Negative/ Rods	+	+	+	+
N3-S6	Negative/ Rods	+	+	+	+
N3-S7	Negative/ Rods	+	+	+	+
N3-S8	Negative/ Coccobacilli	+	+	+	+
N3-S9	Negative/ Rods	+	-	+	+
S1-S2	Positive/ Rods	-	+	+	-
S1-S4	Negative/ Coccobacilli	+	+	+	-
S1-S12	Negative/ Coccobacilli	+	+	+	+
S2-S1	Negative/ Rods	+	+	+	+
S2-S3	Positive/ Coccobacilli	+	-	-	+
S2-S8	Negative/ Coccobacilli	+	+	+	+