DETECTION OF MICROBIAL PATHOGENS IN DRINKING WATER BIOFILM USING PCR

By

Maryam Zafar

NUST201362291MSCEE65213F

A thesis submitted in partial fulfillment of requirements for the degree of

Master of Science

in

Environmental Science

Institute of Environmental Sciences and Engineering (IESE)
School of Civil and Environmental Engineering (SCEE)
National University of Sciences and Technology (NUST)
Islamabad, Pakistan
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It is certified that the contents and forms of the thesis entitled

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Has been found satisfactory for the requirements of the degree of Master of Science in Environmental Science

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I dedicate this thesis to my family & friends for their endless support and encouragement

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MARYAM ZAFAR

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List of Abbreviations

APHA	American Public Health Association
BGLB	Brilliant Green Bile Broth
BLAST	Basic Local Alignment Search Tool
BSE	Back-scattered electrons
CFU/Ml	Colony Forming Unit per milliliter
CICL	Cement Lined Cast Iron
DD C & M	Deputy Director Construction and Maintenance
DICL	Cement Lined Ductile Iron
DWDS	Drinking Water Distribution System
EC broth	*Escherichia coli* broth
EDTA	Ethylenediaminetetraacetic Acid
EDS	Energy Dispersive Spectroscopy
GI	Galvanized iron
IWS	Intermittent Water Supply
LTB	Lauryl Tryptose Broth
MAF	Million Acre Feet
MDPE	Medium Density Polyethylene
Mega4	Molecular Evolutionary Genetics Analysis.V4
NCBI	National Center for Biotechnology Information
PPR	Polypropylene
PVC	Polyvinyl Chloride

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ABSTRACT

Bacterial contamination in drinking water biofilm provides a potential source for bacteria to grow and increase rapidly. To understand the microbial density in drinking water distribution system (DWDS), a four month study was carried out. The aim of this study was to examine biofilm in three different pipe materials including Polyvinyl Chloride (PVC), Polypropylene (PPR) and Galvanized Iron (GI) in an educational institution. A set of all these pipe materials was installed in DWDS at eight different locations and analyzed after every fifteen days. Among different parameters measured, dissolved oxygen, organic carbon and residual chlorine were found to be the main indicators of microbial presence. Microbial identification made by molecular methods of pathogenic detection (PCR based gene sequencing) detected *Bacillus cereus* strain SH42, *Lactobacillus crispatus* ST1, *Lactobacillus jensenii* 269-3, *Staphylococcus aureus* A9719, *Paenibacillus sp.* P22, *Staphylococcus haemolyticus* strain KJ1-5-97, *Pseudomonas sp.* Lc31-4, *Bacillus sp.* BAB-3357, *Paenibacillus sp.* P22, *Achromobacter sp.* BQNT2, *Taylorella asinigenitalis* and *Tetrathiobacter kashmirensis* strain 3T5F as prominent species and their accession number were obtained from NCBI (National Center for Biotechnology Information). Significant increase in bacterial population was observed in GI pipes biofilm while least in PPR pipes. The quantity of DWDS bacteria was directly depended on biofilm growth and its increase was correlated with presence of organic matter on pipes. Pipe material also affected the biofilm thickness while similarity in bacterial species was observed between system

due to same disinfectant dose, time period and plumbing pipes.

Chapter 1

INTRODUCTION

1.1. BACKGROUND

Moving towards advancement water is supplied through water distribution system. In 2008, piped network contributed to 73% urban and 24% rural water supply (Haider *et al*., 2009). Microbial processes within water distribution system may have adverse effect on water quality both at pipelines and consumers end. Microbial growth in these systems may lead to corroded surfaces, pipe roughness and enhance problems related to taste and odor. According to Pakistan Council for Research on Water Resources (PCRWR), water supply of 21 cities of Pakistan is found to be bacteriological contaminated (Kalim *et al*., 2007).

Mostly contamination of drinking water is triggered by inefficient distribution system, by ineffective maintenance of the sewage system and by deficiencies in the disinfection practices and this results in water borne endemic outbursts. Therefore, it is necessary to perform routine monitoring of the drinking water distribution system (Hashmi *et al.,* 2009). Inner surfaces of the distribution network are inhabited by microorganisms either in the form of single cell colonies or densely layered microorganism biofilm. According to an estimate only 5% of the biomass resides in water phase and remaining 95% is attached on pipe surface in the form of biofilm. Thus biofilms in distribution network cause microbial contamination, ensuing deterioration of drinking water quality (Wingender & Flemming 2011).

It has become obvious that drinking water biofilms in drinking water distribution

networks can develop temporary or durable habitats for waterborne microorganisms. Important microorganisms include *Escherichia coli, Campylobacter spp.*, enteric viruses and opportunistic bacteria. Mostly they remain unobserved by the approaches selected to their detection. Thus, biofilms may serve as an environmental reservoir for disease causing microorganisms and signify a possible source of water deterioration, thus ensuing significant health risk for humans (Wingender & Flemming 2011).

Among the most challenging habitats for pathogenic microorganisms are distribution network pipes that transport water. Distribution networks are oligotrophic and hold disinfectants; so far microbes habitually form biofilms which get attached to inside of DWDS pipes. Many opportunistic bacteria can exist in drinking water biofilms despite of chlorination e.g. *H. pylori* can exist in drinking water which is exposed to low level of chlorine (0.2-1.2 mg/L). Its presence indicates the occurrence of many other opportunistic bacteria in biofilm which are mostly not detected by the culture methods. Often the most challenging part of the distribution system is the domestic plumbing having increased temperature and metal concentrations while holding decreased level of disinfectants (Giao *et al*., 2011).

Typically household pipes are made of plastic or copper, in some cases of stainless steel. Studies shows that pipe material greatly influence the formation of biofilm and pathogenic survival (Niquette *et al*., 2000). Disinfection and temperature may greatly influence the formation of biofilm. Various pipe materials develop various amount of biofilm with different level of chlorine residuals. At low level there was highly production of biofilm while the rate reduced significantly on rechlorination. Similarly low temperature (5°C) reduces the threat of biofilm formation thrice less than that of high temperature (15°C) (Hallam *et al.,* 2001). Cast iron pipes can promote biofilm growth because of corrosion which cause higher level of humic substances; a leading

cause to promote biofilm while, maintenance of residual chlorine may decrease the threat (Camper, 2000).

Disinfection residues play a very important role especially in the developing countries where water has high contaminants during the distribution of network and where there are poor sanitary conditions. This is important mainly where water has to travel to a long distant to fulfill the needs of consumers and the chlorine residue level reduce when water travels more distant from where it is stored (Egorov et al. 2003).

Increased microbial population due to declined disinfectant residual can promote the biofilm formation protecting and nourishing the microorganisms (van der Kooij *et al.*, 2005). They can enter in distribution system both in stagnant and flowing water from biofilm formed on inner and outer surfaces of distribution pipes. Thus, pipe materials can extend the microbial attack in water systems (Lethola *et al*., 2004).

1.2. THE PRESENT STUDY

Present study is aimed to determine the contamination level in drinking water biofilm formed on DWDS pipes in National University of Sciences & Technology (NUST). For this purpose three different pipe materials i.e. Polyvinyl Chloride (PVC), Polypropylene (PPR) & Galvanized iron (GI) were used in order to determine the effect of these materials on biofilm formation. A set of these pipe materials (each pipe having one inch cross section) was fitted in distribution system for four months at emergency water plugs (commonly known as fire hydrants; connected to the water distribution line). Pipes were fixed into pilot scale water distribution system rather than lab scale so as to provide natural conditions for biofilm to grow and get analyzed. Each set removed after every 15 days was examined for physicochemical and microbial parameters. Most Probable Number test was used to assess *E. coli* distribution in water samples while

further bacterial identification was made by (Polymerase Chain Reaction) PCR based sequencing. Each pipe material was examined for biofilm growth by different techniques. It was hoped that information collected from this study might help govt. institutions and water protection departments in establishing guidelines for improving water quality and monitoring and surveillance. This study will also provide guidelines for Environmental Protection Agency (EPA), Water and Sanitation Agency (WASA) and Capital Development Authority (CDA).

1.3. AIMS AND OBJECTIVES

Present research work is based on following objectives.

1. Detection of microbes in water distribution network
2. PCR identification of isolated species
3. Characterization of biofilm

Chapter 2

LITERATURE REVIEW

2.1. DRINKING WATER DISTRIBUTION SYSTEM

Drinking water distribution systems (DWDS) include a complex system of pipelines, storage tanks and treatment plants that are utilized to convey potable water to consumers. The uprightness of these systems is indispensable in supplying clean water to end users. Along with releases and blasts, bacterial regrowth in drinking water networks is an issue that can impact expansive water supply utilities. Regrowth is said to happen when treated water that enters the distribution network with a few microscopic organisms is found to have high measure of microorganisms which makes water flimsy in a distribution network (Whittle et al., 2013).

The potential for the water in the distribution network to transport microbial pathogens is found in different countries (Shakya *et al*., 2012). WHO requires that water that enters the distribution network ought to be microbiologically safe and organically stable (WHO, 2004).

For this purpose a specific dose of disinfectant residual is usually sustained in the distribution system (Lautenshclager *et al.,* 2013). However, different elements during distribution, for example, temperature changes, pipe material, biofilms, interruption of untreated water and stagnation zones can influence the nature of water (Laurent *et al.,* 2005a; 2005b).

2.2. DISTRIBUTION SYSTEM AS REACTOR

Basically distribution system acts as a reactor where biofilm grow and gets mature. Bacteria attach to surface due to changes in gene expression and external stresses. They are connected within a matrix of polysaccharide that binds the cells together. Production of biofilms requires sufficient numbers of bacteria (quorum). When the colony size is large enough (quorum sensing), biofilms are produced. This adaptation protects the bacteria from external stresses and antibiotics and they start growing. Nutrients diffuse into the matrix and other secondary bacterial species incorporate themselves within the biofilm and they released because of shear force applied by water flow (Van der Wende *et al.*, 1989).

Biofilms grow virtually everywhere including inside your pipes and microbes start attaching, growing and releasing on a man-made surface. Each year, biofilms cause billions of dollars in damage to filters, tanks and pipes by promoting microbial growth that corrodes pipes and promotes scaling (Martiny *et al.*, 2003).

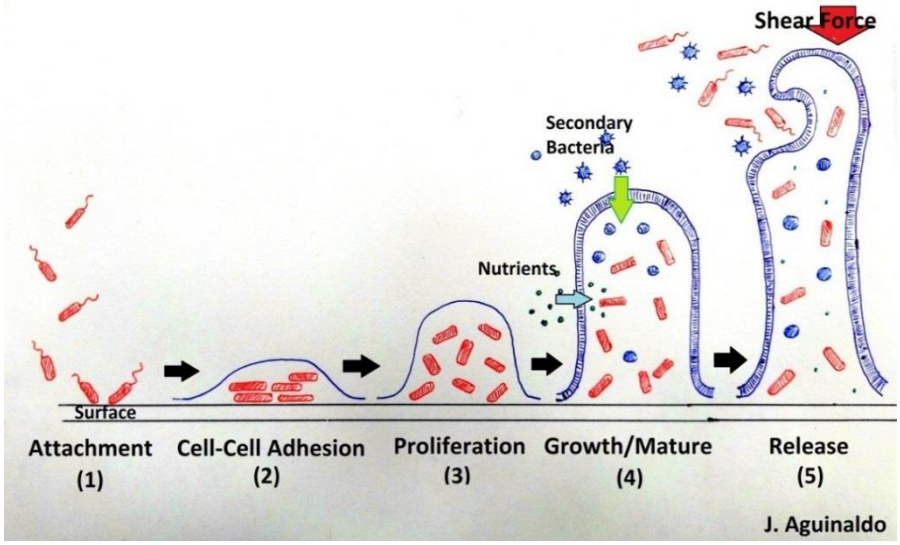


Figure 2.1. Biofilm formation in distribution network

2.3. GLOBAL DISEASE BURDEN

About twenty five percent of pathogenic infections are caused by water and amongst them drinking water contributes about sixty percent.

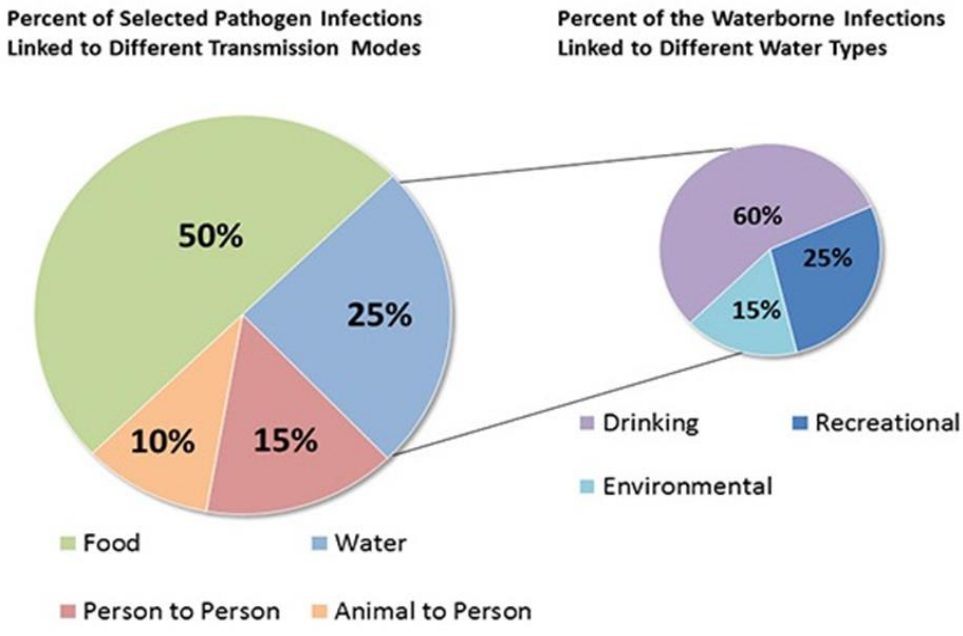


Figure 2.2. Pathogenic infections caused by drinking water

An estimate states that 1.1 billion people have lack of access to safe drinking water. Among them 1.7 million die every year mostly by the diarrheal diseases. Whereas, Pakistan has (84–89%) water below recommended standards due to DWDS problems which cause about than three million cases annually.

Drinking water is likely to be contaminated *by Escherichia coli, Salmonella enterica, Shigella, Pseudomonas, Anabaena sp., Rotifers, Giardia and Cryptosporidium* (Beikler *et al.,* 2011).

2.4. INADEQUACIES OF DISTRIBUTION SYSTEM

A number of failures in the distribution system, namely loss of adequate disinfectant residual, low water pressure, intermittent service and ageing of infrastructure can result

in the declining quality of the water supply (WHO, 2004). Pathogen interruption may occur in these conditions in case of poor sanitary conditions because of improper wastewater collection and leakages in the network. Consequently, these various deficiencies can result in the cross-contamination of a clean water supply which, in some cases, has led to outbreaks of waterborne and water related diseases.

2.4.1. Insufficent Distribution System

Main Problems faced by distribution system causing poor quality of water are irregular service, inadequate disinfection, less water pressure, and very old distribution system (WHO, 1997). Waste water leakage and inappropriate collection of waste in the system cause production of pathogen. Thus leakage of waste water and other discrepancies in drinking water supply led to the occurrence of many water linked diseases like diarrhea, gastroenteritis, and cholera.

2.4.2. Inadequate Disinfection Residual

Public health is one of the main problems that can be cured by appropriate disinfectants in the water treatment (Gadgil 1998; Ford 1999). There are many reasons because of which water supply is not treated with disinfections and some of them are bad taste of drinking water complains by consumers (Diergaardt & Lemmer 1995), and formation of many byproducts in drinking water system (van DijkLooijaard & van Genderen 2000). Chlorine is one of the effective and cheaper source of disinfection renowned globally (Baxter, 1995). But due to bad taste, corrupt odor and its reactivity with some other components may affect the choice to choose chlorine as a disinfection (Besner *et al.*, 2002).

Disinfection may also occur due to the carelessness, for example the supply of disinfectant chemicals in the DWDS may reduce and cannot be altered or even it can occur due to the human error like mechanical error or some other failure during the process of disinfection (Diergaardt & Lemmer 1995). This failure to treat water through disinfections can cause serious harm to public health so this disinfection has a significance role to stop waterborne disease and other outbreaks that has been occurring in both developed and less developed countries in the global water supply system. (Ca´ rdenas *et al*. 1993; Rab *et al*. 1997; Craun *et al*. 2002).

Disinfection residues play a very important role especially in the developing countries where water has high contaminants during water distribution and possess poor sanitary conditions. This is important mainly where water has to travel to a long distant to fulfill the needs of consumers and the residual chlorine level reduces when water travels more distant from where it is stored (Egorov *et al*. 2003).

If the pipelines are not well maintained it may have greater risk to be microbiologically contaminated as these contaminants require water disinfectant residue to be sustained in that pipeline in order to reduce these contaminants. The recommended value for disinfectants according to WHO is 0.2 to 0.5 mg that has to be maintained if the water distributing system has to operate normally (WHO, 1997).

2.4.3. Less Water Pressure

After sustaining the disinfection residue the next important part to protect the good quality of water is to maintain and normalize the pressure of water throughout the whole water supply network (Geldreich *et al*., 1996). The scientists say that the less flow of water due to less pressure and back washing or reverse flow cause the most

contamination in water supply system worldwide (Trussell *et al.,* 1998). Whenever water scarcity occurs, company employs use the approach in which they reduce the pressure of water throughout the pipelines so each household can use minimal water (delCarmen G. M. *et al.,* 1998). When the water level is decreased, it reflects to move the water towards the less pressure or result in reverse flow. This back flow of water causes unwanted water to flow back into the drinking water supply system (Herrick *et al.,* 1997). Two main kinds of back flow can occur. The leading one is back-siphonage, that happened when the pressure level reduced so much to create the vacuum influence in the pipes, which help to add contaminants from pipes from where the pipes are leaking (Geldreich 1996; Mermin *et al.* 1999; Kelkar *et al*., 2001).

2.4.4. Intermittent Water Supply

Due to less resources a limited number of treatment systems are present, according to the population (Ford, 1999), these water supply systems are not able to provide a continual supply of water. Thus, intermittent water service has become the norm, rather than the exception in many developing countries (Kumar, 1998). In Latin America and the Caribbean, it is estimated that 60% of the population are served by household connections having intermittent service (PAHO & WHO, 2001). In Africa and Asia, it is estimated that more than one-third and one-half of urban water supplies, respectively, operate intermittently (WHO, 2004). Water treatment plants are often encountered with water shortages, because consumers get services within a limited time period each day. Sporadic water supply meant that most of the water is not provided to families or household, and drastic drop in pressure in the system is dead time in around pipes to draw pollutants into drinking water supplies (Gadgil, 1998; delCarmen G. M, 1998; Ford 1999; Mermin *et al.*, 1999). Either the waste water drains or open drain is located

next to the pipeline, there is a high risk of contamination of drinking water and sewage (Moe *et al.,* 1991; Mermin *et al.,* 1999) Thereby increasing the risk to public health. In addition, the intermittent supply pressure surge leads to non-uniform strain on piping and connections, so that they are more likely to leak (Al-Ghamdi & Gutub 2002; Chowdhury *et al.,* 2002).

2.4.5. Leakages in Pipes

Unaccounted water, also known as the local water, is water in many developing countries. There is a lot of unaccounted water, which is defined as the amount of water delivered to the system and water sold to customer number differences. This loss of water consists of two parts: commercial losses and material damage (such as leaks) from the system, which accounted for the majority of non-revenue water (WHO, 2004; Chowdhury *et al.,* 2002).

There is evidence that the oil quantity level in water is extremely high in developing countries (Table 4). Line break and damages in Minsk, Belarus, and Bogota and Colombia, is expected to reach an annual break every 100 kilometers and 187 per 100 km, about 70 a year to rest, respectively, compared to the United States average per 100 km off every 17 years (WHO, 2004). The Water and Sewer Company in Sa˜ o Paulo, Brazil, has projected an astonishing 35,000 leaky situations per month (Massato & Thornton 1999). Furthermore, in Hyderabad city, India, approximately 181 leaks are reported each day, and these refer only to leaks that are visible to the consumer (Mohanty *et al.,* 2002).

2.4.6. Corrosion and Ageing Infrastructure

All distribution systems are deteriorating after some time; corrosion is a basic factor in the natural aging process. Corrosion is defined as a partial solubilization of distribution system materials (delCarmen G. M, 1998), and it means that adding of organic and inorganic substances into water supplies. No matter what their composition, all materials will be worse, with the passage of time corrosion (Agard *et al.*, 2002). There is evidence that distribution systems corrosion occur in many parts of the world due to the ageing process. Even in developed countries, it is not uncommon to use distribution pipes for at least a century old (Hass, 1998). In Hyderabad, India, one of the oldest parts of the urban power distribution system is about 100 years old; another 60-70 years old and they all are corroded (Mohanty, 2002).

Chowdhury and his colleagues (2002) found that a region in Bangladesh, about 20% from the beginning of the last century pipe corrosion and spills more than 50% for gate valves and fittings were badly rusted.

2.5. BIOFILM FORMATION AND DIFFERENT PIPE MATERIALS

In drinking water distribution systems, mostly biofilm grows on pipes as they contribute greatest surface area for biofilm attachment and growth. According to a study, no single material has been developed so far which can show resistivity to biofilm attachment (Rogers *et al.*, 1994; Camper, 2000), even high disinfectant doses haven't proved beneficial in retarding biofilm growth (Momba *et al*., 2004).

Variations in surface roughness, chemical activities of pipe materials play a major role in biofilm growth and detachment. Metallic pipes can form corrosion products on their

surfaces and release metals in water because of chemical and biological activities (Calle *et al*., 2007; Edwards *et al*., 2000).

Various studies have proved links in pipe materials and opportunistic pathogens in many field surveys and laboratory examinations. Some specified pipe materials can decay disinfectants, leading to increased microbial growth in distribution systems. Among them many have been found to release various organic compounds in water such as organic carbon (Hallam *et al.*, 2001; Lehtola *et al*., 2004).

It was studied that biofilm formation was slower in copper pipes than polyethylene pipes and copper can tend to lower microbial growth. It was found that plastic pipes being the replacement of metal pipes can release biodegradable organic compounds and phosphorous in water which further contribute to high microbial growth (Lehtola *et al.*, 2004).

Rough surface pipes can promote biofilm regrowth on their surfaces as compared to smooth surface pipes. Finally, structure of microbial community is also affected by pipe materials. It is revealed by a recent study of phospholipid analysis that more gram negative bacteria attract towards biofilm of galvanized iron pipes (Chang *et al.*, 2003).

2.6. BIOFILM FORMATION POTENTIAL OF DIFFERENT PIPE MATERIALS

According to one of the research of Knowledge Institute for Drinking Water (KIWA), it was concluded that chlorinated PVC outperforms than many other non-metallic pipe materials because it can resist biofilm formation. It was further stated that no statistical difference is found between chlorinated PVC and copper pipes in resisting biofilm (KIWA, 2007).

In plastic pipes it was documented that major portion of organic carbon is present already in water which is responsible for biofilm formation. In most cases organic carbon present in water is greater than what could percolate from plastic pipes interior.

Effect of different pipe materials was analyzed in biofilm formation and microbial communities. Pipe coupons made of six different pipe materials were inserted in drinking water distribution network. Highest biofilm formation potential was found in steel pipes while lowest in copper (Zacheus *et al.*, 2000).

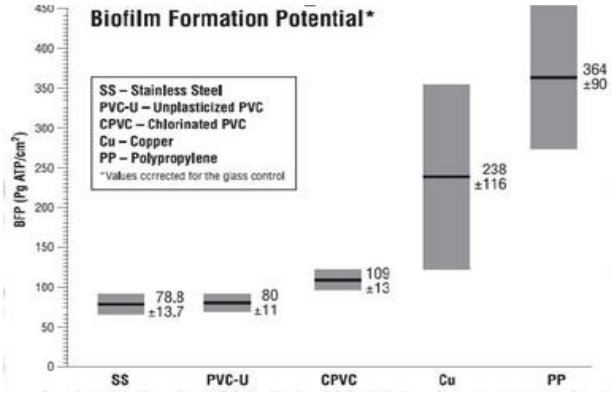


Figure 2.3. Biofilm formation potential by different pipe materials

ATP (Adenosine Triphosphate) values of pipes were in increasing order of copper, chlorinated PVC, polyethylene, stainless steel, polybutylene and coated steel.

Molecular analysis showed that pipe materials not only affect biofilm formation potential but also microbial densities.

2.7. BACTERIAL SPECIES IN DIFFERENT PIPES

Stagnant water provides nutrition deficient environment which cannot support growth of all the microorganisms. Oligotrophic bacteria were found to grow better in such conditions. Two weeks stagnant water in six dead end water distribution pipes was analyzed and fresh water samples were found to harbor species of the genera *Pseudomonas*, *Azotobacter* and *Actinobacteria* each contributing 30% to the total population. After two weeks stagnation in pipes, *Pseudomonads* we found dominating in the water which proved to be oligotrophs in the nutrient tolerance test (Jaeqqi & Schmidt-Lorenz, 1990).

Different pipe materials favor growth of different bacteria in the drinking water. Stagnation of water in pipes can induce release of nutrients that may favor bacterial growth. It was observed that bacterial community in stagnant water from iron pipes was found to be dominated *by Rhizobium, Pseudomonas, Lactococcus, Brevundimonas, Rheinheimera, Arthrobacter, Bacillus, and Herbaspirillum* (Chen *et al.*, 2013).

Steel pipes were found to support 100 times more HPC than PVC pipes when observed in an annual reactor. It was reported that dominance of *Sphingomonas, Acinetobacteria, Bacilli* and *Sphingomonas* were found to be the dominant species in all biofilms regardless of pipe materials (Jang *et al*. 2011).

Bacterial species accumulating biofilms were observed in PVC and iron pipes following conventional treatment. PVC pipes were reported to have highest percentage

of *Nocardia spp.* i.e. 38% followed by *Acidovorax spp.* (13%) and *Hydrogenophaga spp.* (11%) (Norton *et al*., 2000). *Nocardia spp.* has been known to degrade polyethylene terephthalate of plastic pipes by releasing enzyme esterase (Sharon & Sharon, 2012).

Iron pipes are reported to support growth of iron oxidizing bacteria (IOB) and iron reducing bacteria (IRB). *Acidovorax, Galionella, Leptothrix* and *Sphaerotilus* have been reported as IOB while *Bacillus, Clostridium, Escherichia coli* and *Pseudomonas spp.* have been reported in literature as IRB. *Bacillus infernus* has the ability to reduce ferric ion to ferrous ion (Sun *et al.*, 2014).

Chapter 3

MATERIALS AND METHODS

Methodology is the systematic, theoretical analysis of the methods applied to a field of study. It comprises the theoretical analysis of the body of methods and principles associated with a branch of knowledge. Methodology designed to achieve research objectives is based upon following phases.

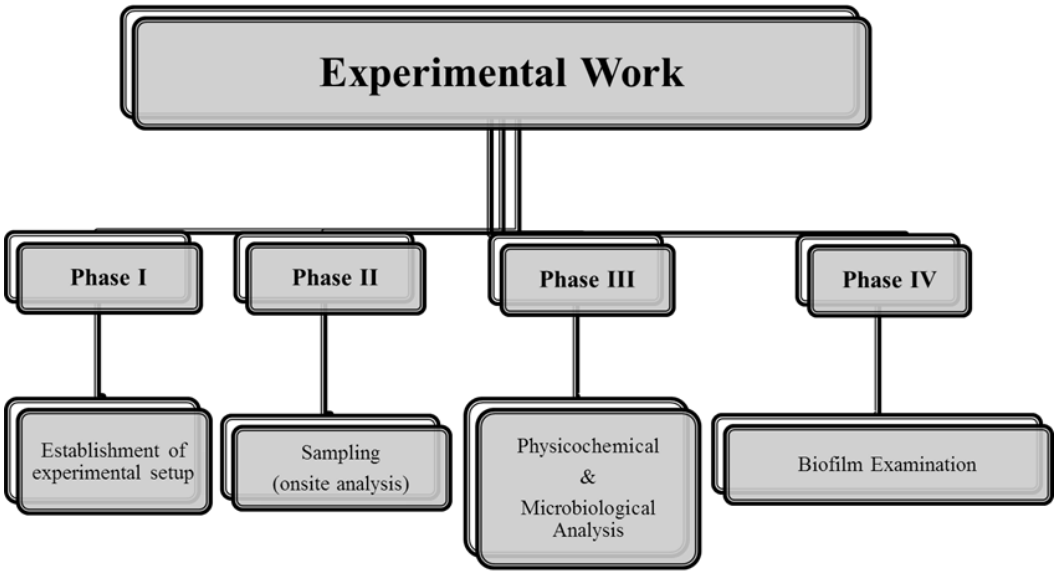


Figure 3.1: Methodology phases

3.1. ESTABLISHMENT OF EXPERIMENTAL SETUP

Experimental setup was made after selection of study area and sampling sites. It describes the study area along with its characteristics from which samples were taken

and get analyzed.

3.1.1. Preliminary Survey

Preliminary surveys were made to get handoff training and to assess sampling network. It was decided to insert pieces of pipe materials in water distribution system rather than cutting the actual distribution pipelines. Pipe pieces were inserted for biofilm to grow on them and get analyzed. Sampling points were finalized at distribution lines and fire hydrants (joining distribution lines) were selected as points to take sample. This was done in order to prevent leakage and making sure the feasible sampling.

3.1.2. Description of Study Area

New campus of National University of Sciences and Technology, Pakistan was taken as the study site. It was established in 1991 while its new campus was recognized in 2008 in H-12 sector, Islamabad. Covering an area of about 707 acres, it has more than 15 schools and institutes, faculty residence as well as male and female students' hostels.

Water distribution system of the university is served with ground water sources. Water is pumped through 9 tube wells from catchment areas and transferred to 3 underground storage tanks or 2 overhead reservoirs. These storage locations are either supplied directly or stored in overhead reservoirs overnight with storage time extending up to 2-3 days. Water is supplied to three locations (shown as blue box in map) which further serve to their designated areas. Tube wells having a pumping capacity of 0.2 million gallons per day (MGD) serve a population of around 11400 people. Figure 3.2. shows the layout of NUST water distribution system and location of sampling points.

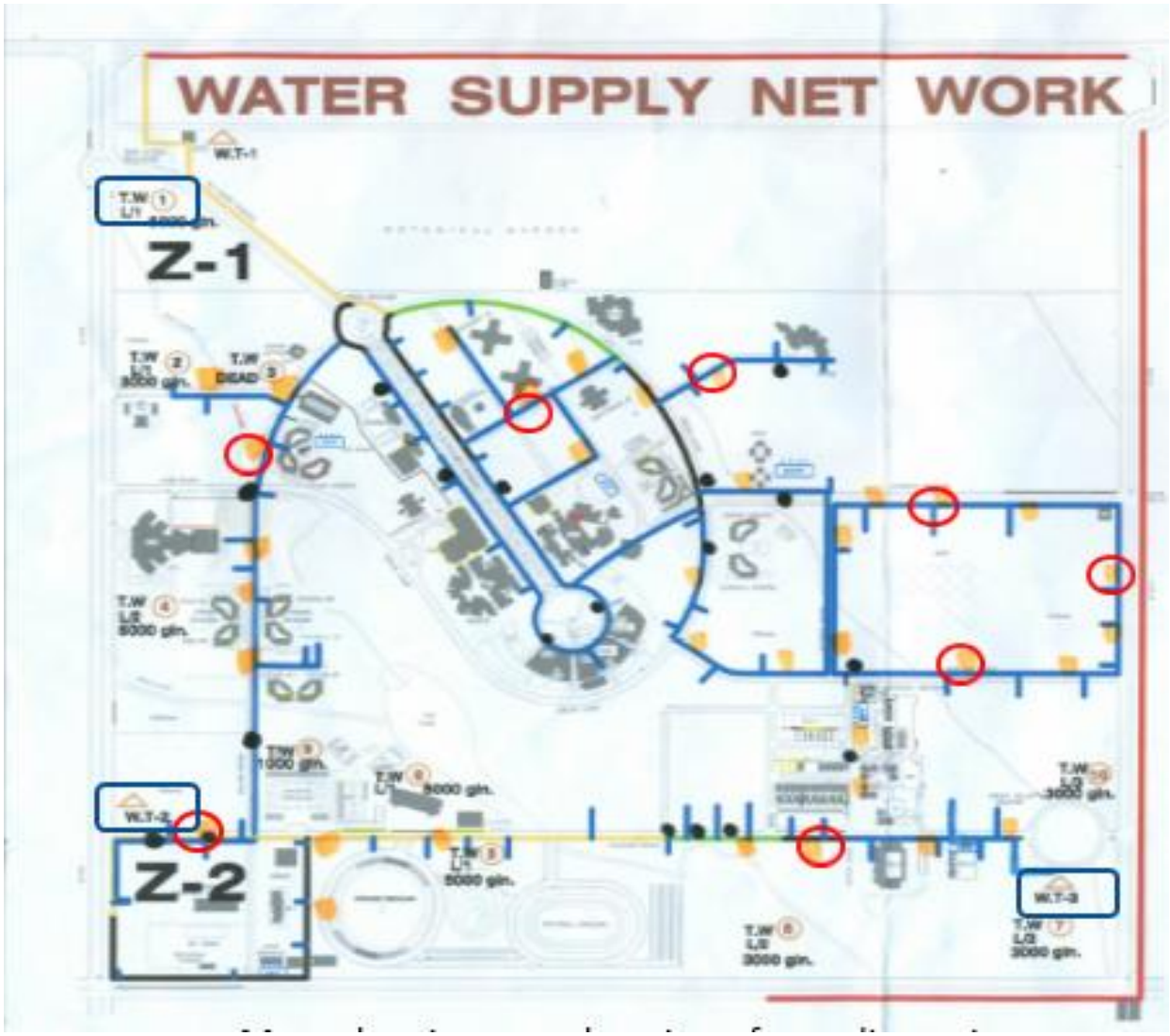


Figure 3.2: Layout of NUST water distribution system

****Red circles represent sampling sites and blue squares representing water locations***

Red lines along border show the CDA water lines while blue, green and yellow lines represent NUST water distribution lines with different diameters. Mostly these distribution lines are made of Galvanized iron (GI) which turn to unplasticized Polyvinyl chloride (uPVC), Polypropylene (PPR) and Galvanized iron (GI) service

pipelines (C&M NUST). University has a total of 6 water filtration plants which are located at various points throughout the campus.

3.1.3. Conditions and Processes

Some natural processes occurring in study area may affect the water quality. In NUST, important processes effecting water are shown in table 3.1.

Table 3.1: Major processes effecting water in distribution network

Type of process	Major process in water body	Water body
Hydrological	Dilution	All water bodies
	Percolation and leaching	Ground water
Physical	Adsorption/desorption	Ground water
	Diffusion	
Chemical	Acid base reaction	All water bodies
	Redox reaction	All water bodies
	Dissolution of particles	All water bodies
	Mineral precipitation	All water bodies
	Ion exchange	Ground water
Biological	Microbes die-off and regrow	All water bodies
	Decomposition of organic matter	All water bodies

These processes (occurring within underground storage tanks and distribution pipelines) led to high level of hardness (carbonates) and salinity in water. Further

biofilm formation occurred due to excessive organic matter and biological processes occurring within water bodies.

3.1.4. Meteorological Information

Since precipitation rates ensuring run-off are vital in determining water quality

because they erode land surfaces and increase concentration of suspended solids and phosphates in water. Sampling was made from April to July with a precipitation rate of 2.50 to 2.63 mm respectively. Increased precipitation in summer led to higher TSS (Total Suspended Solids) and nitrites concentration in water.

3.1.5. Frequency and Number of Samples

Eight sampling sites were selected on NUST main distribution lines receiving water from underground water resource and overhead reservoir. Reason behind selection of eight sampling sites lie in the WHO guidelines (table 3.2.) which state that one sample per thousand population should be monitored for monthly basis.

Table 3.2: Frequency of samples from distribution network

Population served	No. of monthly samples
<1000	1
1000-100000	1 per 1000
>100000	1 per 10000 population, plus 10 additional samples

***World Health Organization, 2011**

Each sample taken from these locations were representative of the water source, treatment plant, storage facilities, distribution network. At sampling points pieces of

different pipe materials were inserted for biofilm to grow and get analyzed. Fire hydrants were selected as pipe insertion points as it was convenient to remove them for laboratory analysis. Since the biofilm formation starts within two weeks so the samples were taken after every fifteen days. Sampling points along with their location and time are mentioned in table 3.2.

Table 3.3: Water distribution system sampling sites

Sample ID	Module Description	Location	Time	Data Points
S1	PVC, PPR & GI	L2 near SMME	April	8
S2	PVC, PPR & GI	L2 near Rumi hostel	15-April	8
S3	PVC, PPR & GI	L3 near residential area	May	8
S4	PVC, PPR & GI	L3 near medical center	15-May	8
S5	PVC, PPR & GI	L3 near gate-10	June	8
S6	PVC, PPR & GI	L1 near Iqra hostel	15-June	8
S7	PVC, PPR & GI	L1 near Ayesha hostel	July	8
S8	PVC, PPR & GI	L1 near IGIS	15-July	8

***L1, L2 & L3 representing three water locations of NUST**

3.1.6. Pipe Materials Selected for Study

At designated sampling sites three type of pipe materials namely Polypropylene (PPR), Polyvinyl Chloride (PVC) and Galvanized Iron (GI) were inserted in the distribution network. These pipes were selected as they were mainly used in DWDS. Table 3.4 focuses on the properties of these pipe materials especially those ones which contribute in biofilm attachment and growth.

In drinking water distribution systems, mostly biofilm grows on pipes as they contribute greatest surface area for biofilm attachment and growth. According to a study, no single material has been developed so far which can show resistivity to biofilm attachment (Rogers et al., 1994; Camper, 2000), even high disinfectant doses haven't proved beneficial in retarding biofilm growth (Momba et al., 2004).

Table 3.4: Pipe materials selected for this study along with their properties

Properties	Polyvinylchloride (PVC)	Polypropylene (PPR)	Galvanized Iron (GI)
Surface	Smooth	Smooth	Rough
Corrosive	Anti corrosive	Anti corrosive	Highly corrosive
Liberation	Assimilable organic carbon	--	--
Opposing nature	--	--	Anti bacterial property
Discharge	Biodegradable organic compounds	Biodegradable organic compounds	--
Bacterial attachment	--	--	Gram negative
Strength	Brittle	Brittle	Robust

3.2. Experimental Setup

These pipe materials were cut into one inch cross section with the help of SMME workshops. Curved shape of pipe (same as in distribution system pipelines) was maintained in order to provide the real conditions for biofilm growth. They were hanged through a string and inserted in distribution pipeline via fire hydrants points (joining distribution line). Eight sets of all these pipe materials were installed at every sampling site. After every fifteen days one set of each pipe material was removed and analyzed for various physiochemical and microbial analysis. Following the same steps were repeated for every sampling site.

3.3. SAMPLING

3.3.1. Preparation

Sterile leak proof glassware e.g. 250 mL Schott bottles, 100 mL beakers were used for sampling. All glassware was washed, rinsed with distilled water and autoclaved at 121°C, 15psi for 15 minutes and oven dried at 105°C for two hours. Bottles were tightly capped and beakers were covered. Prior the visit of sampling site, DO meter, temperature & pH meter and chlorimeter were kept along.

3.3.2. Sample Collection, Transportation and Storage

Prior to sample collection, fire hydrants were cleaned with alcohol and flamed in order to avoid contamination from air. Two samples were taken into account; one in 250 mL bottles for laboratory analysis and other one in beaker for onsite analysis. One set of each pipe material (carrying biofilm) was cut from string and placed in glass bottle. 0.08% sodium thiosulfate was added in bottles in case of chlorinated water so as to neutralize residual chlorine.

On site analysis was made and samples were transferred to laboratory for analysis within 4 hours. Standards methods were used for collection, transportation as prescribed in the *Standard Methods for the Examination of Water and Wastewater* (APHA, 2012).

After every fifteen days sample were collected from each sampling site and results were observed. Triplicate water samples were taken and monitored for result validation.

3.4. WATER QUALITY ANALYSIS

3.4.1. Physicochemical Analysis

3.4.1.1. On site Analysis

On site analysis were made for some parameters because they may change with time.

Temperature and pH were measured with potable HACH 156 pH meter. Dissolved Oxygen (DO) and disinfectant residuals were monitored with Crison Oxi 45 DO meter and Hanna HI 96734 chlorimeter respectively (APHA, 2012).

3.4.1.2. Laboratory Analysis

Conductivity, total dissolved solids (TDS), oxidation reduction potential (ORP) and salinity were measured in the laboratory with Consort 1020 multi parameter analyzer within four hours of sample collection. While turbidity was monitored with HACH 2100N turbidimeter. Total phosphates (TP) and total nitrates (TN) were measured with Hach DR/2400 spectrophotometer at 480 P and at 351 N wavelength respectively.

3.5. MICROBIOLOGICAL ANALYSIS

3.5.1. Spread Plate Count

3.5.1.1. Preparation of Agar Plates

For the enumeration of heterotrophic plate counts (HPC), 20 g nutrient agar was mixed in 1 L distilled water and autoclaved at 121°C and 15 psi for 15 minutes. Molten agar was then poured in autoclaved petri plates and incubated at 37°C for 48 hours to check sterility.

3.5.1.2. HPC Count

Heterotrophic plate counts from stagnant and flushed water samples were analyzed using spread plate count technique as per standard procedures (APHA, 2012). 0.5 mL of the samples was spread plated onto sterile nutrient agar plates. The plates were incubated at 37°C for 24 hours and counted with 560 Suntax Colony Counter.

3.5.2. Most Probable Number Technique

3.5.2.1. Preparation of Media

For each sample, 10 tubes of Laural Tryptose Broth (LTB), Brilliant Green Bile Broth (BGLB) and Escherichia coli (EC) broth each were prepared. For preparation of LTB tubes, 36.5 g of media was mixed in 1L distilled water. 10 mL of the mixture was added in 10 tubes each containing an inverted durham tube. The tubes were then autoclaved at 121°C and 15 psi for 15 minutes and placed in incubator at 37°C for 24 hours to check sterility. BGLB and EC broth were prepared following similar procedure by dissolving 40 g BGLB in 1L and 37 g EC broth in 1L.

3.5.2.2. Enumeration of Total Coliform and E. coli

Total *coliforms* and *E.coli* were enumerated using Most Probable Number (MPN) or Multiple Tube Fermentation Technique. In the presumptive phase, 10 fermentation test tubes each containing 10 mL LTB and an inverted durham tube were used. After vigorous shaking, the sample 1 mL was added to each tube and the tubes were kept at 37°C for 24 hours. Production of gas in the tubes showed a positive presumptive reaction and gave an indication of presence of total *coliforms.*

Positive tubes were further subjected to confirmation phase. Positive LTB tubes were shaken slightly and a small inoculum using wire loop was transferred to BGLB tubes. BGLB tubes were then placed in an incubator at 37°C for 24 hours. Production of gas after 24 hours in BGLB tubes confirmed presence of total *coliforms.*

Positive tubes from previous phase were taken and after gently shaking a small amount using wire loop was added to EC broth tubes and incubated at 37°C for 24 hours. Production of gas confirmed the occurrence of fecal *coliforms* (*E. coli*) (APHA, 2012).

3.5.3. Bacterial Isolation

3.5.3.1. Sonication and Dilution

Each cross section of all pipe materials (supporting biofilm) was put into 100 mL autoclaved glass beaker containing 50 mL distilled water and sonicated at 36°C for 15 min. Separate beaker was used for each pipe material. This allowed the biofilm dissolution in distilled water which was further used to make serial dilutions.

3.5.3.2. Streak Plate Technique

Bacterial pure cultures obtained from serial dilutions of all pipe materials was streaked on nutrient agar plates. Process was continued for weeks until pure colonies (isolated ones) were appeared on nutrient agar plates.

3.5.4. Identification of Bacteria

3.5.4.1. Morphological Identification

Morphological identification of isolated strains was made on the basis of their surface, margins, elevations, size, form and texture. All tests for morphological identification were executed as per Bergey's Manual of Determinative Bacteriology. Cell morphology was identified by gram staining. Prepared slides observed under 100X resolution identified the morphology of bacterial cells, while motility was observed by a hanging drop technique. A drop of bacterial culture was positioned under a cover slip in the concave depression of slide which was inverted so that the drop hangs in the concave well. After adding a drop of oil immersion slide was observed under 100X resolution using a light microscope.

3.5.4.2. Biochemical Characterization

Different biochemical tests following standard procedures were carried out to identify bacterial strains. These are mentioned below

3.5.4.2.1. Oxidase Test

Strips of filter paper were taken and loop full of inoculum of a 24 hour fresh culture was placed on one paper. On the inoculum one drop of 1% N, N-dimethyl-p-phenylenediamine dihydrochloride solution was added. Appearance of blue or purple

color within seconds indicated the presence of enzyme cytochrome oxidase and hence oxidase positive test.

3.5.4.3.2. Catalase Test

Inoculum from a 24 hour fresh culture was placed on a clean slide using a sterilized wire loop. A drop of 3% hydrogen peroxide was then added to it. Bubble formation confirmed catalase positive test and thus presence of enzyme catalase which breaks hydrogen peroxide into molecular oxygen and water. This enzyme is produced by bacteria to neutralize toxic forms of oxygen.

3.5.5. PCR Based Gene Sequencing

Bacterial identification was made by Sanger's method of reading linear DNA (Deoxyribonucletide acid) sequences using special nucleotide bases. PCR was based on 16S rRNA using 27F and 1492R primers; and yielding of 1,300bp or more sequencing data. Basic processing involved using two internal primers 785F and 907R.

3.5.5.1. Sample Preparation

2 µl of deionized water (DI) was poured on 24 hours fresh culture and allowed to stay for 30-40 sec until all bacterial colonies were dissolved in DI water. Further it was transferred in 3 mL autoclaved eppendorf tubes and centrifuged at room temperature for about 5 min. Supernatant thus formed was discarded and loopful of nutrient agar and glycerol was added to remaining solution making the volume up to 20ul. Prepared samples should exhibit the following characteristics:

- High purity

- Suitable concentration
- No secondary priming sites
- No mismatches
- A length of 18-25 bases.
- GC% content between 40% and 60%.
- A Tm (melting temperature) between 55°C and 60°C
- No significant hairpins (>3bp)
- Free of salts, EDTA, or other contaminants

3.5.5.2. Sample Submission

19 samples were submitted to Macrogen clinical lab (South Korea) for nucleotide sequence of bacterial DNA vie Fedex (located in G-9 Islamabad).

3.5.6. Sequence Analysis

Nucleotide sequence sent by Macrogen were run on BLAST software in order to confirm the similarity with other bacterial species. In case of 99% similarity nucleotide sequences were sent to NCBI by creating account on NCBI Banklt and submitting nucleotide sequences. Sequences showing least similarities were trimmed manually by removing repeated bases as they represent noise. Accession number were received which were run on Mega4 software for generating phylogenetic tree.

3.5.7. Phylogenetic Tree

A phylogenetic tree or evolutionary tree is a branching diagram or "tree" showing the inferred evolutionary relationships among various biological species or other entities-their phylogeny-based upon similarities and differences in their physical or genetic characteristics. The taxa joined together in the tree are implied to have descended from a common ancestor. Phylogenetic trees are central to the field of phylogenetics.

3.6. BIOFILM CHARACTERIZATION

Biofilm was characterized by using these three main procedures.

Table 3.5: Techniques involving Biofilm depiction

Biofilm characterization		
Surface Topography	**Elemental Composition**	**Biofilm Depth**
Scanning Electron Microscopy (SEM)	Energy Dispersive Spectroscopy (EDS)	Optical profilometer

3.6.1. Scanning Electron Microscopy (SEM)

Scanning electron microscopy is focused on producing images of a sample by scanning it with a focused beam of electrons. The electrons interact with atoms in the sample, producing various signals that may be detected and that contain information about the sample's surface topography and composition.

3.6.1.1. Working Principle

Back-scattered electrons (BSE) are beam electrons that are reflected from the sample by elastic scattering. BSE are often used in analytical SEM along with the spectra made from the characteristic X-rays, because the intensity of the BSE signal is strongly related to the atomic number (Z) of the specimen. BSE images can provide information about the distribution of different elements in the sample. For the same reason, BSE imaging can image colloidal gold immuno-labels of 5 or 10 nm diameter, which would

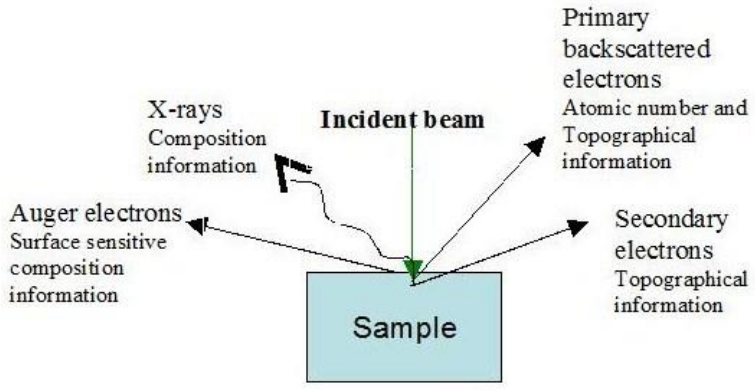
otherwise be difficult or impossible to detect in secondary electron images in biological specimens. Characteristic X-rays are emitted when the electron beam removes an inner shell electron from the sample, causing a higher-energy electron to fill the shell and release energy. These characteristic X-rays are used to identify the composition and measure the abundance of elements in the sample.

Figure 3.3: Working principle of Scanning Electron Microscopy

3.6.1.2.

Microanalysis of Pipe Materials by SEM

Slices of all pipe materials removed from water were air dried and dried specimen were them mounted on a stub using an adhesive (such as epoxy resins) or sometimes by electrical double sided adhesive tape. Surface of attached specimen is coated with gold (sometimes gold/platinum alloy) and then examined with microscope. Biofilm images were obtained at different resolutions (from 50-500) and at power ranging from 5-50kV.



3.6.2. Energy Dispersive Spectroscopy (EDS)

Energy dispersive X-ray spectroscopy (EDS, EDX, or XEDS), sometimes called energy dispersive X-ray analysis (EDXA) or energy dispersive X-ray microanalysis (EDXMA), is an analytical technique used for the elemental analysis or chemical characterization of a sample. It relies on an interaction of some source of X-ray excitation and a sample.

3.6.2.1. Working Principle

To stimulate the emission of characteristic X-rays from a specimen, a high-energy beam of charged particles such as electrons or protons or a beam of X-rays, is focused into the sample being studied. At rest, an atom within the sample contains ground state (or unexcited) electrons in discrete energy levels or electron shells bound to the nucleus. The incident beam may excite an electron in an inner shell, ejecting it from the shell while creating an electron hole where the electron was. An electron from an outer, higher-energy shell then fills the hole, and the difference in energy between the higher-energy shell and the lower energy shell may be released in the form of an X-ray. The number and energy of the X-rays emitted from a specimen can be measured by an energy-dispersive spectrometer. As the energies of the X-rays are characteristic of the difference in energy between the two shells and of the atomic structure of the emitting element, EDS allows the elemental composition of the specimen to be measured.

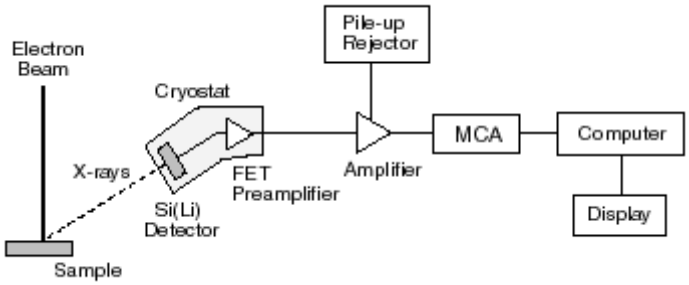


Figure 3.4: Working principle of Energy Dispersive Spectroscopy

3.6.2.2. Elemental Composition of Pipe Materials by EDS

Dried samples coated with gold were placed in sample chamber of instrument which is cooled with liquid Nitrogen. Detector is placed at the end of a long arm in sample chamber which at the end of a long arm, which is itself cooled by liquid Nitrogen. The most common detectors are made of Si(Li) crystals that operate at low voltages to improve sensitivity, but recent advances in detector technology make available so-called "silicon drift detectors" that operate at higher count rates without liquid nitrogen cooling.

A typical EDS spectrum is portrayed as a plot of x-ray counts vs. energy (in keV). Energy peaks correspond to the various elements in the sample. Generally they are narrow and readily resolved, but many elements yield multiple peaks.

3.7. Optical Profilometer

Biofilm thickness was measured by optical profilometer which is used to analyze biofilm surface profiles and roughness.

3.7.1. Working Principle

A vertically moving stylus remains in contact with sample and gradually moves it for specified contact force and distance. Small surface variations are measured as a function of position. Highest position of stylus generating an analog signal starts converting it into digital signals which are displayed further. Horizontal resolution is controlled by data signal sampling rate and scanning speed. Stylus tracking force ranges from less than 1 to 50 mg.

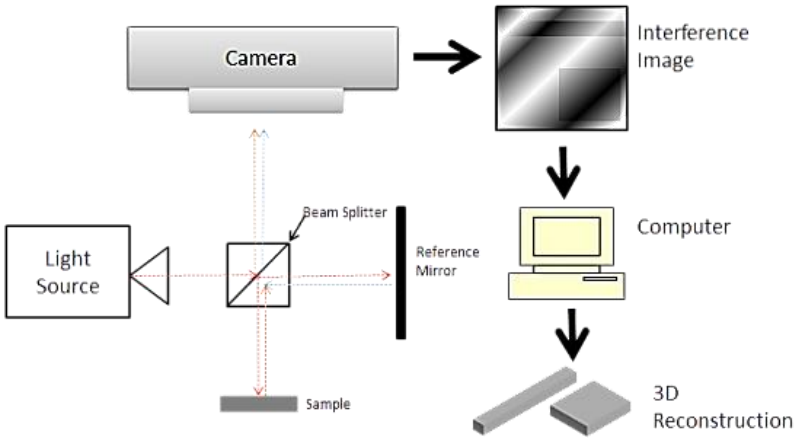


Figure 3.5: Working principle of optical profilometer

3.7.2. Thickness & Roughness Profiles of Pipe Materials

Dried pieces of pipe materials were swabbed from an edge in order to provide reference surface from which thickness of entire biofilm was measured. Prepared pieces were placed on movable slide; which gave peaks showing maximum and minimum heights of biofilm. Difference between average values of maximum and minimum heights gave the depth of biofilm. Same process was repeated for each pipe material slice.

Chapter 4

RESULTS AND DISCUSSION

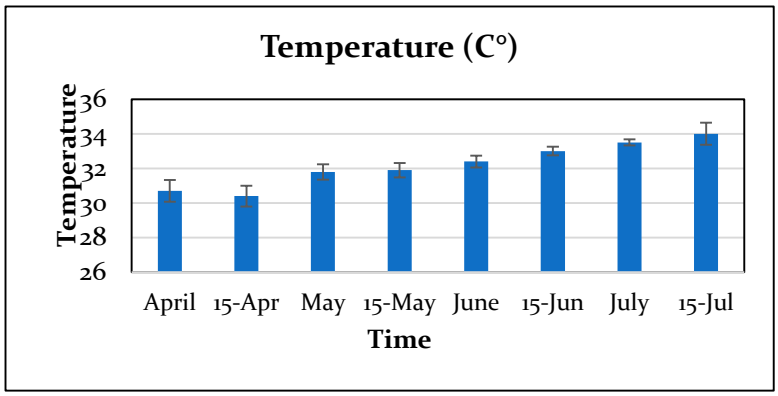
4.1. PHYSICOCHEMICAL QUALITY

Physicochemical parameters of water samples collected from the university distribution network compared with World Health Organization and Pakistan Standards for Drinking Water Quality.

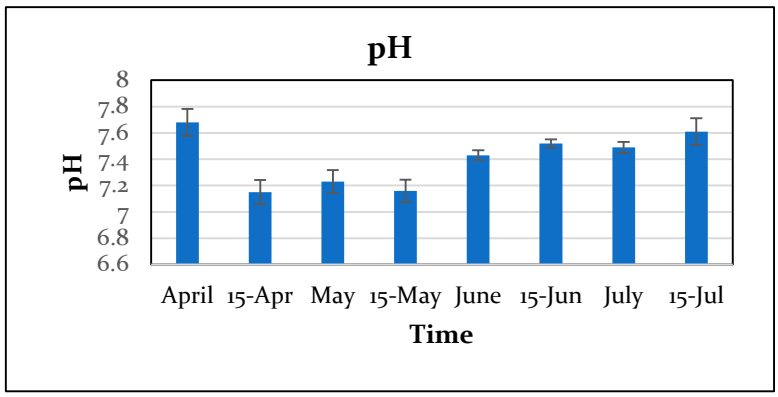
4.1.1 On site Analysis

Temperature, pH, dissolved oxygen (DO) and residual chlorine were measured onsite. Temperature profile shows that temperature increased from April to July as a consequence of seasonal change. Temperature has no direct effect on water quality, however according to WHO guidelines temperature above 15°C may enhances the growth of microorganisms and may increase problems related to taste, odor, color and corrosion. Fig. 4.1 (a) shows average temperature values obtained at different sampling points throughout the sampling duration.

Depending on a number of characteristics of the distribution system, pH may be a strong determining factor in the bacterial and chemical quality of water. Although pH usually has no direct impact on water consumers, it is one of the most important operational water quality parameters. pH measured was ranged from 7.1 to 7.7 (as shown in Fig. 4.1 (b)) which was found within desirable range set by for drinking water quality (NSDWQ, 2008).



(a)

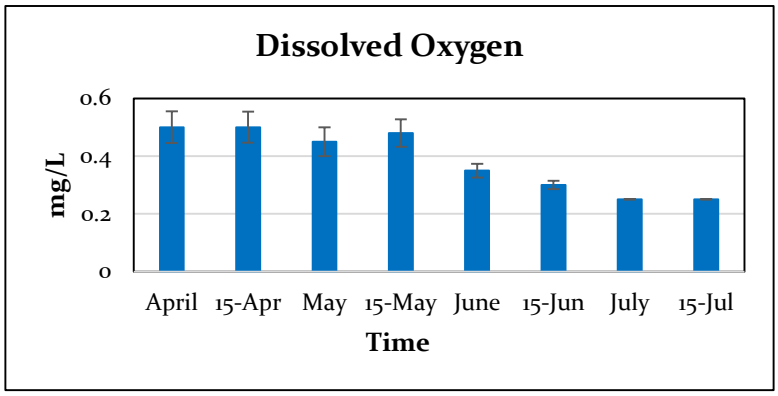


(b)

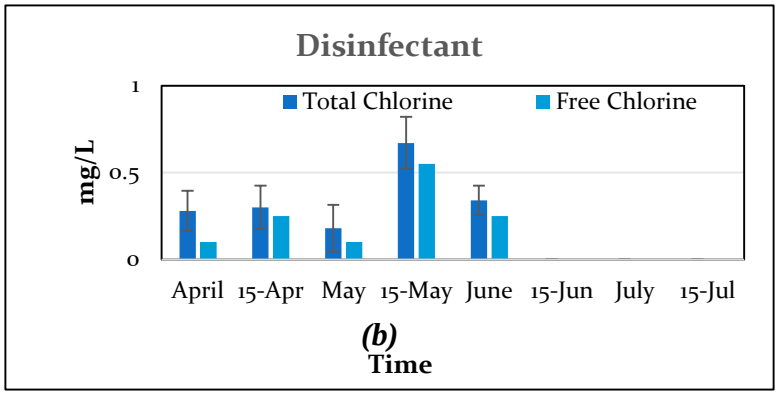
Figure 4.1: On site analysis performed (a) variations in temperature (b) pH variations

No health-based guideline value is proposed for pH, however it is said that it should range from 6.5 to 8.5. Dissolved oxygen decreased in July (Fig. 4.2 (a)) as a result of all oxygen being consumed by microbial activities occurring in water. DO is effected by temperature level in a water body and found to be critical for the survival of aquatic organisms for aerobic respiration.

For the estimation of total chlorine water samples were collected and observed only at distribution pipelines and the max value among all sampling points was 0.6 mg/L. All samples showed the residual chlorine below WHO limit of 0.2-0.5 mg/L.



(a)



(b)

Figure 4.2: On site analysis performed (a) variations in DO level (b) residual chlorine

Water temperature is an important parameter because it is a critical factor in determining the growth of the microorganisms (Ramteke *et al*., 1992). Bacterial growth rates, decay of disinfection residual, corrosion rates and even distribution hydraulics are all affected by water temperature (Kelin *et al*., 2005).

Careful attention to pH control is necessary at all stages of water treatment to ensure satisfactory water clarification and disinfection. For effective disinfection with chlorine, the pH should preferably be less than 8.0.

According to Ramteke *et al*., (1992), water supplies are normally well aerated, which reduces the risk of microbial-induced corrosion, denitrification, sulfide production and other consequences of anaerobic stagnation. However, oxygen may not penetrate to the

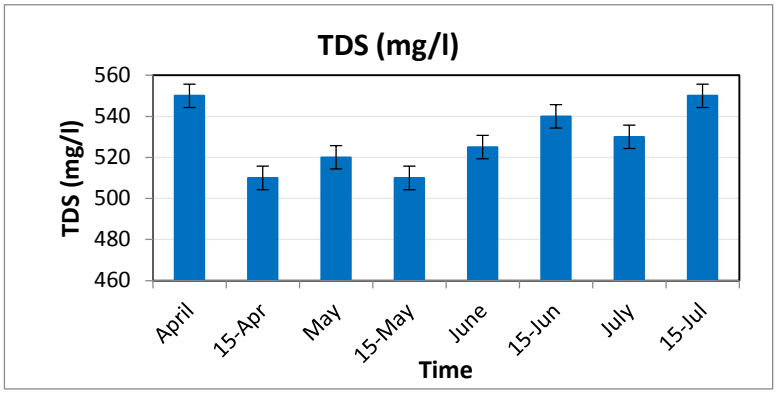
bottom layers of biofilms, corrosion tubercles, and other pipe deposits and reservoir sediments where anaerobes such as sulfate-reducing bacteria may proliferate.

Developing countries, like Pakistan, usually have an inadequate water distribution system; due to which the probability of recontamination is very high in both urban and rural areas. The existence of a disinfectant residual is especially important in such regions (Aziz, 2005). Greater concentration of residual chlorine means there is lesser microbial contamination. Hence, chlorine residuals of drinking water are incredible indicators of the microbial quality of water in distribution networks. The presence of any disinfectant residual reduces the microorganism level and frequency of occurrence at the consumer's tap (Olivieri *et al*., 1986).

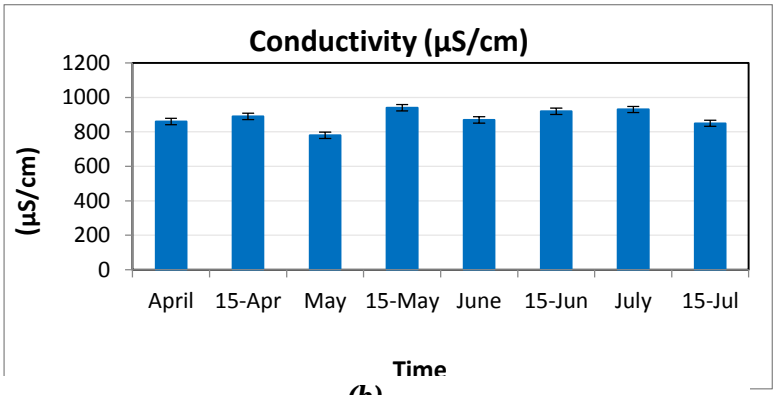
4.1.2. Analysis Performed in Laboratory

After transporting samples into laboratory various physicochemical parameters were performed which include conductivity, salinity, total dissolved solids (TDS), turbidity and oxidation reduction potential (ORP). Conductivity varies from 780 to 950 µS/cm at sampling duration from April to July as shown in Fig. 4.3 (b). There is a relationship between TDS and conductivity. As the concentration of dissolved salts (usually salts of sodium, calcium and magnesium, bicarbonate, chloride, and sulphate) increases in water, electrical conductivity increases (Kelin *et al*., 2005). The electrical conductivity is higher for water that has more dissolved ionic species. TDS vales ranged from 510 to 550 mg/L as shown in Fig 4.3 (a).

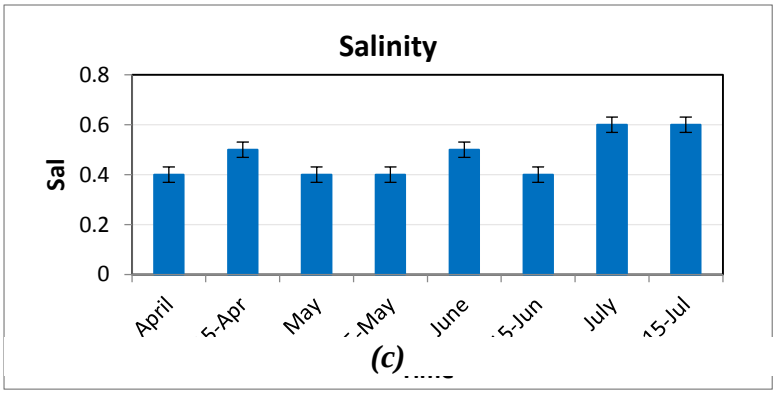
Salinity is the measurement of electrical conductivity found in water. Fig. 4.3 (c) shows the salinity level found within permissible limits set by WHO for drinking water quality.



(a)



(b)



(c)

Figure 4.3:

Variations in (a) TDS level, (b) conductivity & (c) salinity level

Water containing TDS concentrations below 1000 mg/L is usually acceptable to consumers, although acceptability may vary according to circumstances (WHO, 2004). The United States Environmental Protection Agency recommends treatment when TDS concentrations exceed 500 mg/L or 500 parts per million (ppm). Deterioration in

drinking water quality in distribution networks is probably due to an increase in microbial numbers, an elevated concentration of iron or increased turbidity, all of which affect taste, odor and colour in the drinking water. It may provide shelter for opportunistic microorganisms and pathogens. Hence, water with high turbidity, from organic sources, also gives rise to a substantial chlorine demand for disinfection purposes.

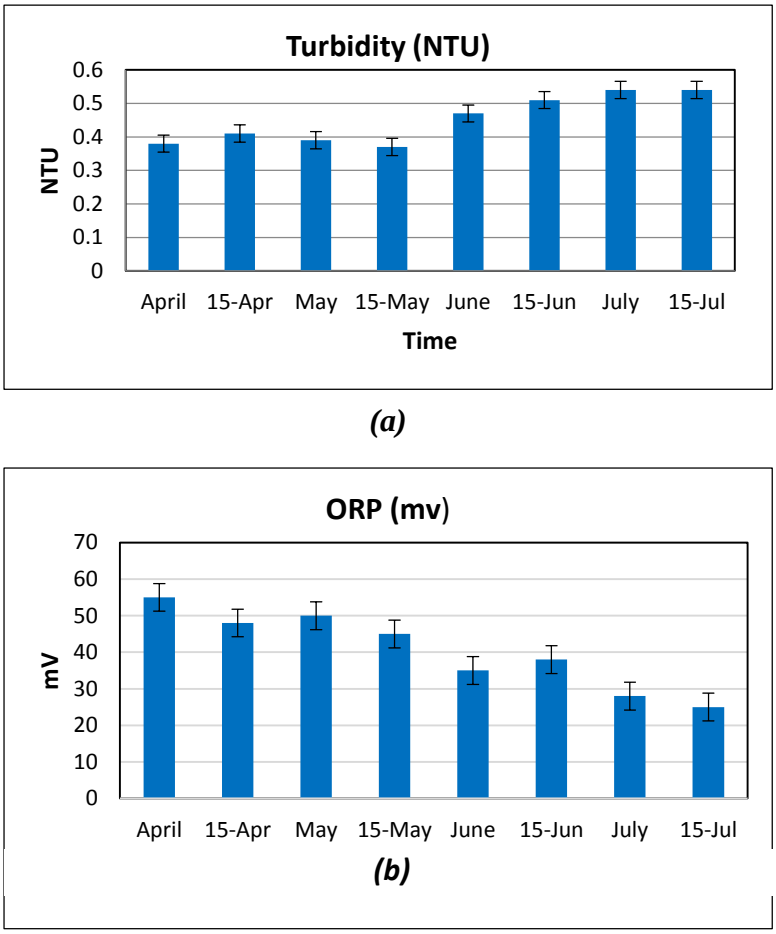


Figure 4.4. Variations in (a) turbidity and (b) Oxidation Reduction Potential

This could result in reductions in the free chlorine residual in distribution systems as protection against possible recontamination. All values were found within permissible range of WHO guidelines as shown in Fig. 4.4 (a). Continuous exchange of electrons that takes place between water bodies is known as ion exchange. A positive ORP

reading indicates that a substance is an oxidizing agent. The higher the reading, the more oxidizing it is and more DO. Fig. 4.4 (b) shows that all samples showed a positive ORP value, hence proving themselves as oxidizing agents. Haas *et al.*, (1983) noted that increased values of pH, temperature, and turbidity were associated with increased concentrations of microorganisms

4.1.3. Nutrients Analysis

Although some microorganisms may survive on mineral elements, they are of little significance in distribution networks. However, many microorganisms may proliferate if there is sufficient dissolved or particulate organic matter containing carbon, nitrogen or phosphorus.

4.1.3.1. Total Phosphates

Total phosphates have no direct effect on health, however higher level of phosphates are attributed to high rain fall leading to increased runoff in water bodies.

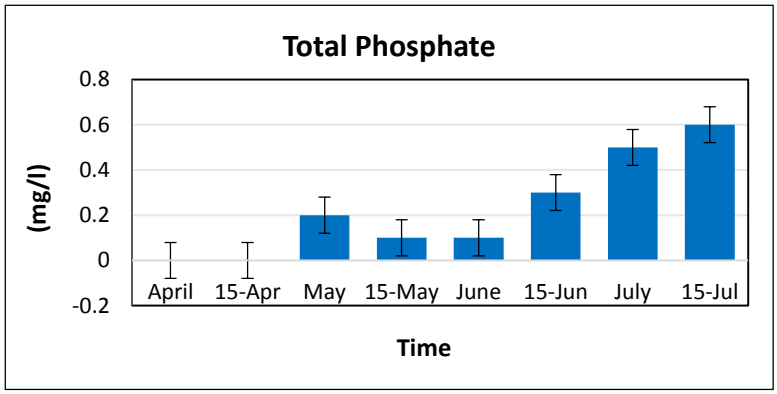


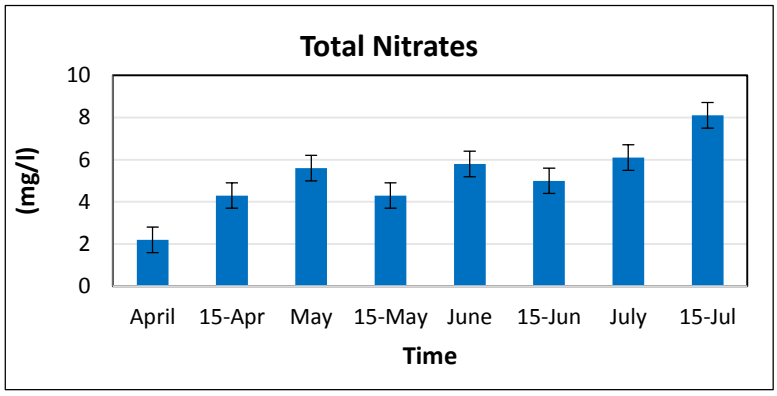
Figure 4.5. Average values of total phosphates obtained throughout the study period

In April, phosphate detected was below detectable limits; however it increased in summer due to excessive runoff. Fig. 4.5 shows the variations in phosphates level monitored throughout the sampling period. Same results were reported by M. Batte´*et*

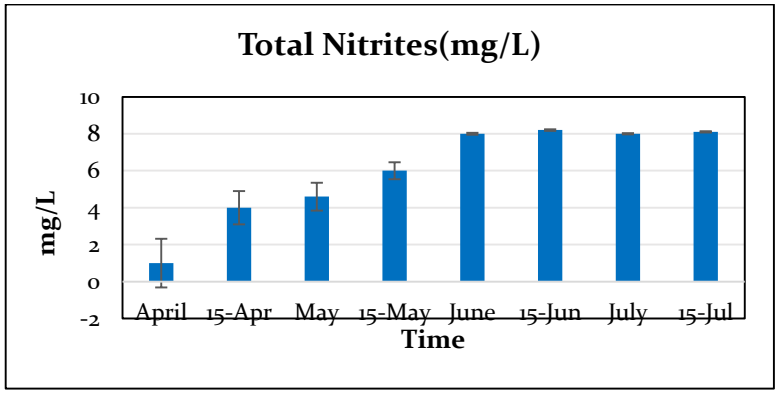
al., (2003) when bacterial density of phosphate treated (15 days) distribution network biofilm was compared with control with an increase in bacterial density from 2.84 $\times 10^7$ to 3.21$\times 10^7$ cells cm^{-2}.

4.1.3.2. Total Nitrates and Nitrites

Nitrates are naturally occurring ions that are part of nitrogen cycle. In general, vegetables are the main source of nitrate intake when level in drinking water is below 10 mg/L.



(a)



(b)

Figure 4.6. Average values of (a) total nitrates & (b) total nitrites

The presence of nitrate indicates an old contamination provided nitrites are absent. Total nitrates were found within WHO limit of 50 mg/L while nitrites were above WHO limit of 3 mg/L. Presence of high level of nitrites is attributed to excessive run off and presence of more detergents in domestic effluents. High level of nitrite in drinking water is due to excessive use of agriculture fertilizers, decayed vegetable matter, domestic effluent, sewage disposal industrial discharges, leachable from refuse dumps, atmospheric and atmospheric precipitation has become a serious problem. When nitrates level in drinking water exceeds 50 mg/L, drinking water becomes the main source of total nitrate intake. The presence of nitrate indicates a previous contamination provided nitrites are absent. Total nitrate were found within the WHO limit of 50 mg/L while nitrites were above the WHO limit of 3 mg/L.

Total nitrite measured were beyond WHO limits of 3 mg/L. Nitrites may reach both surface water and groundwater as a consequence of agricultural activity and from wastewater treatment. Further, it is described in the literature that nitrites may also be formed by chemical reactions in galvanized iron distribution pipes by *Nitrosomonas* bacteria during stagnation (Lee *et al.* 2004).

4.2. Microbial Analysis

4.2.1. Most Probable Number

The presence of *coliform* organisms indicates the biological contamination of drinking water (Le Chevallier *et al.*, 1991). It is not necessary to analyze drinking water for all pathogens. Escherichia coli are found in all mammal faeces but it does not multiply significantly in the environment. Hence, it is the most important biological drinking water indictor for public health importance (Edberg *et al*., 2000). To determine the

presence of total *coliform* and faecal *coliform* in drinking water samples, MPN test was conducted.

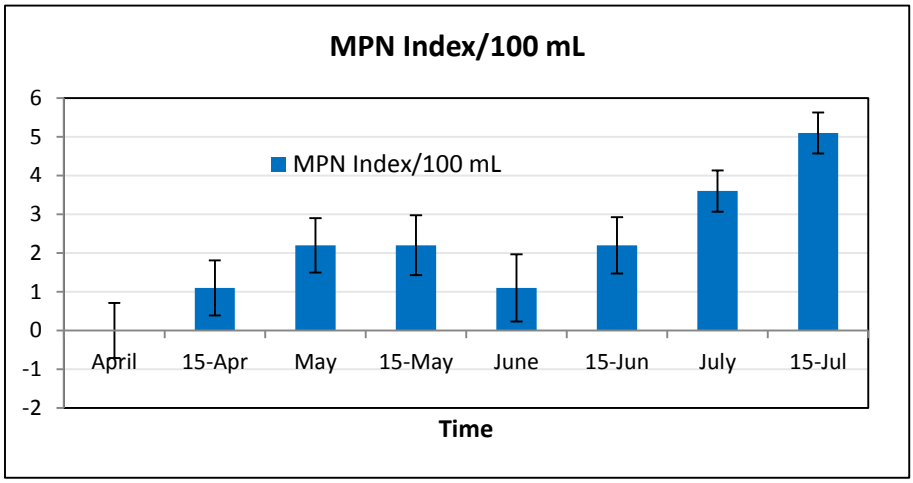


Figure 4.7. Average values of Coliform counts obtained throughout the study period

Assessment of MPN data attained revealed that the samples collected in April were less contaminated with fecal *coliforms* than those obtained in sampling period from May to July. As evident from Table 1, in April, the value of MPN index/ 100 mL for total *coliform* was <1.1 and probability ranged from 0 to 3.4 while in May sampling periods showed MPN index of 2.2 and probability ranged from 0.37 to 8.2. The highest MPN index was found in July depicting a value of 3.6 and 5.1 with a probability ranging from 0.91 to 9.70 and 1.6 to 13 respectively. Samples collected in April were found suitable because of low nutrient level and appropriate disinfection while all others were found above WHO guidelines.

4.2.2. Bacterial Identification

Samples sent to Macrogen clinical lab resulted into FASTA nucleotide sequence which gave PCR peaks.

First and last peak of samples were primer peaks while other one showed PCR products (Fig. 4.8)

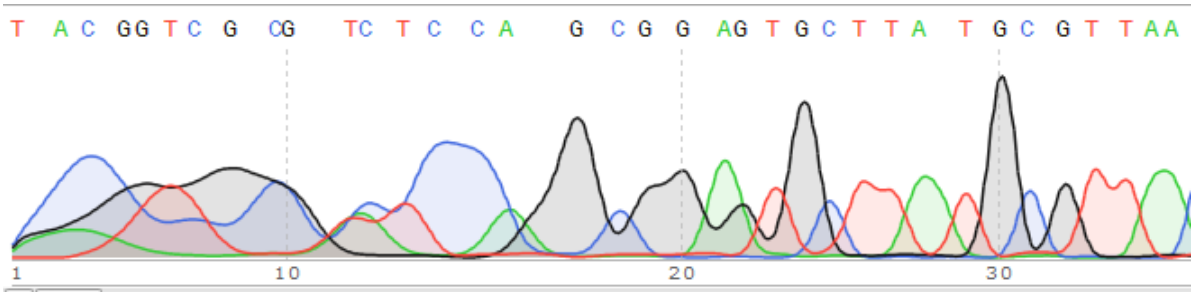


Figure 4.8. PCR peaks showing nucleotide bases

All samples showed their peaks at different angles along with their sequence of interest of nucleotide bases of Adeninie, Guanine, Cytocine and Thymine as shown in Fig. 4.9.

ACAGATGTCTTGTAATCCGGCCGTTGGTGGCATAGGGAAAGACA
TTTAGTGAAAGAAATTGATGCGATGGGTGGATCGATGGCTTATG
CTATCGATCAATCAGGAATTCAATTTAGAGTACTTAATAGTAGCA
AAGGAGCTGCTGTTAGAGCAACACGTGCTCAGGCAGATAAAATA
TTATATCGTCAAGCAATACGTAGTATTCTTGAATATCAAAAATTTT
TGTTGGTTATTCA

Figure 4.9. Alignment of nucleotide bases of bacterial isolates

Nucleotide sequence was run on software BLAST which showed similarity with all other bacterial species. BLAST sequence was sent to NCBI genbank which gave the accession number of isolated strains. Table 4.1 shows the isolated strains alongwith their accession number which were obtained first time from drinking water biofilm in Pakistan with accession number attained from NCBI (National Center for Biotechnology Information, USA) genbak.

Table 4.1. Bacterial isolates from biofilm alongwith their accession number

Seq ID	Accession Number	Bacterial Isolates	Length(bp)	Source
MZ1	KU180424	*Bacillus cereus* strain SH42	989	NCBI
MZ2	KU180425	*Lactobacillus crispatus* ST1	708	NCBI
MZ3	KU180426	*Lactobacillus jensenii* 269-3	1576	NCBI
MZ5	KU180427	*Staphylococcus aureus*	1468	NCBI
MZ6	KU180428	*Staphylococcus aureus* A9719	1495	NCBI
MZ7	KU180429	*Paenibacillus sp*. P22	1438	NCBI
MZ8	KU180430	*Achromobacter sp.* clone Cl-38	1495	NCBI
MZ9	KU180431	*Achromobacter insolitus* strain PBR-1	1487	NCBI
MZ10	KU180432	*Staphylococcus haemolyticus* strain KJ1599	1487	NCBI
MZ11	KU180433	*Staphylococcus haemolyticus* strain KJ1-5-97	1487	NCBI
MZ12	KU180434	*Staphylococcus epidermidis*	1480	NCBI
MZ13	KU180435	*Staphylococcus sp*. BAB-4187	1487	NCBI
MZ14	KU180436	Bacterium clone BN11	1480	NCBI
MZ15	KU180437	*Pseudomonas sp*. Lc31-4	1534	NCBI
MZ16	KU180438	*Bacillus sp.* BAB-3357	1480	NCBI
MZ17	KU180439	*Paenibacillus sp.* P22	1507	NCBI
MZ18	KU180440	*Achromobacter sp.* BQNT2	1499	NCBI
MZ19	KU180441	*Taylorella asinigenitalis*	1501	NCBI
MZ20	KU180442	*Tetrathiobacter kashmirensis* strain 3T5F	1333	NCBI

4.2.3. Phylogenetic Tree

A phylogenetic tree was constructed which showed the evolutionary relationship among different bacterial species. Decoding that information is not always straightforward and requires some understanding of the elements of a phylogeny and what they represent.

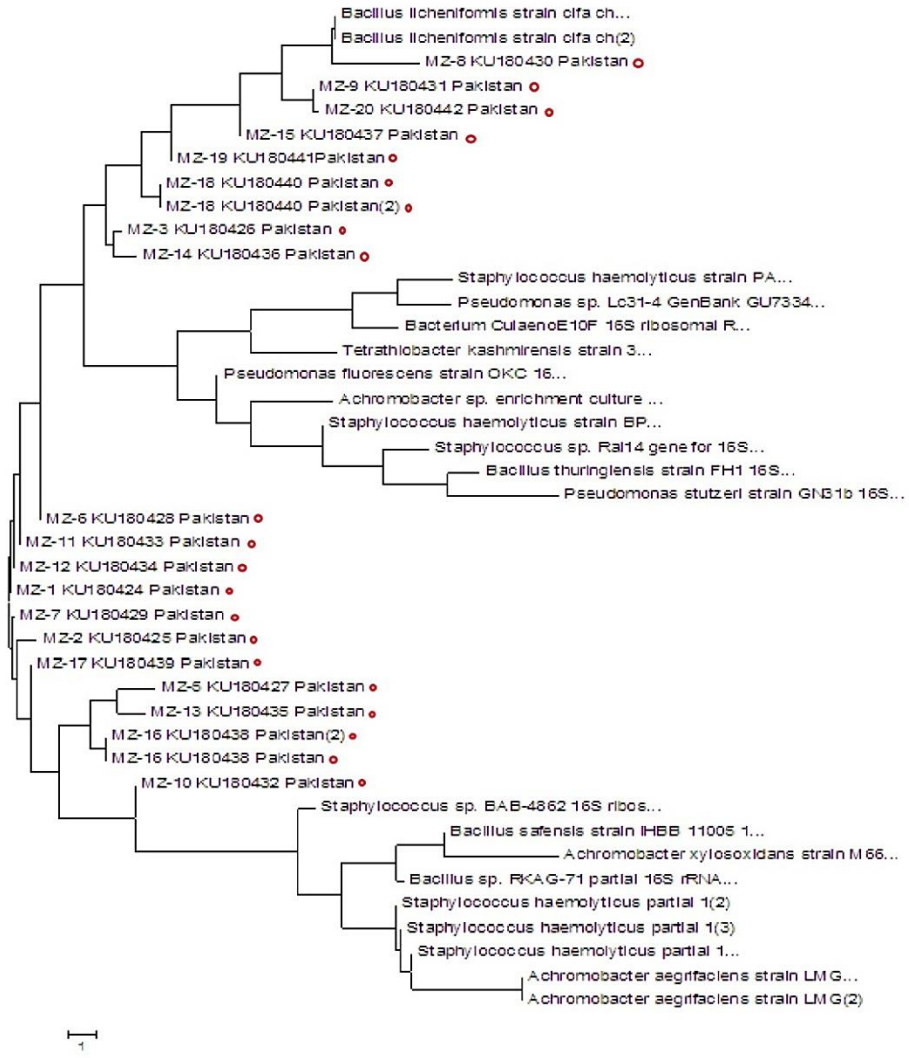


Figure 4.10: Evolutionary relationship among bacterial isolates shown by phylogenetic tree

*Highlighted species isolated from microbiology lab

A neighbour joining tree was constructed which showed the evolutionary relationship among different bacterial species. Phylogenetic analysis of 16S rRNA gene sequence

of isolated species was compared with closely related species and at least one top BLAST hit was included for each specie.

In Figure 4.10. the horizontal dimensions give the amount of genetic change. The horizontal lines are branches and represent evolutionary lineages changing over time. The longer the branch in the horizontal dimension, the larger the amount of change. The vertical dimension in this figure has no meaning and is used simply to lay out the tree. The bar at the bottom of the figure provides a scale for this. The highlighted sp. isolated in this study has been added for the first time in Genbank from Pakistan.

It reveals that bacteria MZ1, MZ3, MZ7, MZ17 and MZ18 belong to the phylum Firmicutes. MZ1 is closely related to the MZ17 with a bootstrap confidence of 30%. The RDP Naive Bayesian rRNA Classifier Version 2.10 designates MZ2 and MZ16 as unclassified and MZ2 is closely related to the phylum Firmicutes and class Staphylococcaceae with 3% bootstrap value. MZ1 and MZ7 are also included in this group. MZ8, MZ9, MZ19 and MZ20 are classified as Betaproteobacteria while, MZ14 and MZ15 as Gammaproteobacteria. Top BLAST hits of 16S rRNA gene sequence belong to phylum Firmicutes and Proteobacteria and they are most closely neighbour in phylogenetic relations. Substitution per nucleotide position is represented as bar scale on corner of tree (representing a value of 1). It shows the length of a branch that represents genetic change of one.

4.3. Biofilm Characterization

The biofilm structure was investigated using scanning electron microscopy (SEM). The SEM revealed the existence of bacterial cells within the biofilm matrix as shown in Fig. 4.11.

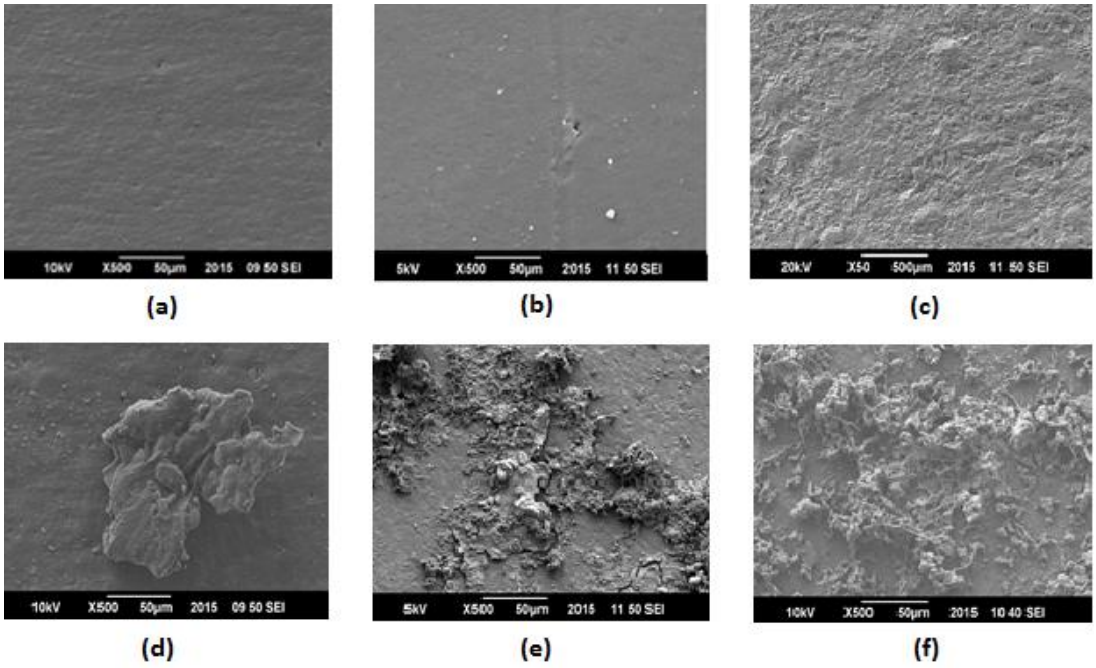


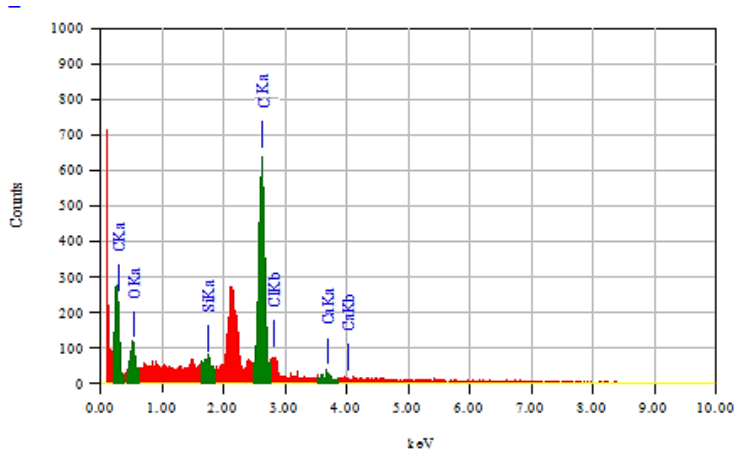
Fig. 4.11:. Resolution images of pipe materials, (a) Reference PVC (b) reference PPR, (c) reference GI (d) contaminated PVC, (e) contaminated PPR & (f) contaminated GI

Greater amount of bacterial cells were found on the galvanized iron pipe than on the PPR and PVC coupons. If biofilms contain any pathogenic bacteria, the detachment of biofilms could release these bacteria into drinking water and affect risk levels of consumers. Taughels *et al.*, (2006) found that biofilm regrowth on pipes made of rough surface materials such as cast iron, concrete-lined cast iron, and galvanized steel was greater than that on smooth-surface polyvinyl chloride (PVC) pipe.

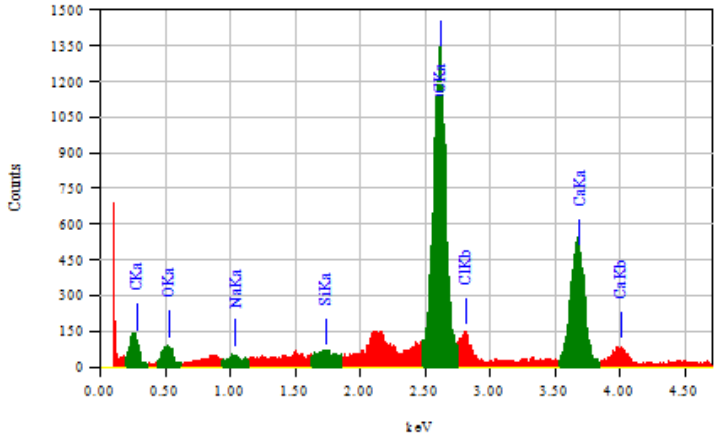
4.4. Elemental Composition of Different Pipe Materials

4.4.1. Elemental Composition of PVC Pipe

Elemental composition of PVC pipe was evaluated by measured by SEM-EDS. Green peaks showed the elemental compositions while red ones were representative of gold coating.



(a)



(b)

Figure 4.12. Elemental composition of PVC (a) reference, (b) contaminated

There was a marked difference in carbon and calcium composition when compared with reference. Results were found in correlation with Chowdhary (2012) studies where plastic pipes such as PVC, which have recently been used as cost-effective replacements of traditional metal plumbing, may release biodegradable organic compounds and phosphorus, which may arouse microbial regrowth and biofilm formation.

Table 4.2: Percentage difference in mineral composition of PVC pipe

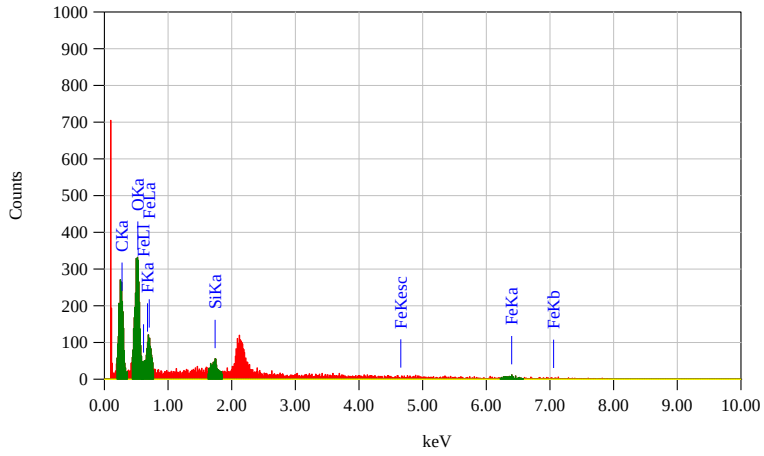
	C%	Si%	Ca%
Reference	33.72	0.23	0
Contaminated	45.33	0.82	2.27
Percentage Difference	11.61	0.59	2.27

EDS data demonstrate that biodegradable organic carbon present on pipe cross sections were found to be the essential nutrient in promoting biofilm growth in drinking water regardless of chlorine presence. Thus, it was concluded that removal of organic carbon may reduce the biofilm growth by retarding the availability of nutrients to microbes for their growth and maintenance. It's reported earlier that high level of phosphorous such as detected in the summer (0.60 mg/L in July) may lead to build an interaction with biodegradable organic carbon and thus effecting biofilm formation (Park & Hu 2010).

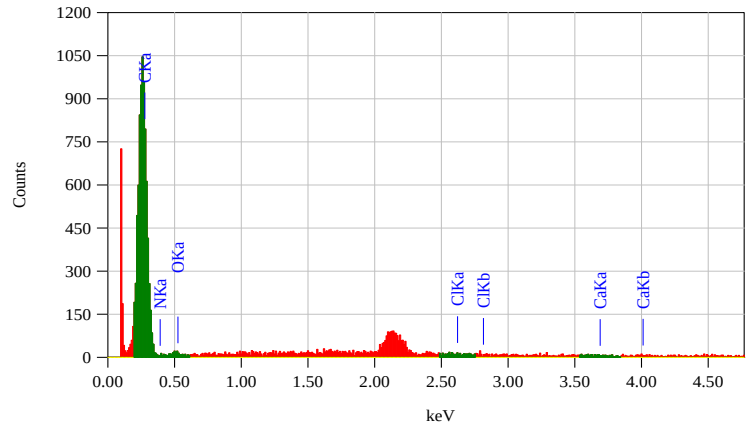
Presence of high silicon level was supposed to nourish more silicon utilizing bacteria such as *Bacillus* and *Coccus.* Silicon utilizing organisms are defined as organisms with utilizing high silicon level (≥ 1% dry weight) and metabolizing silicon with silicon transporter genes (SIT) found in them (Das & Das 2010). So, increased level of about 0.59% silicon in PVC pipe supported more growth of *Bacillus spp.*

4.4.2. Elemental Composition of PPR Pipe

Elemental composition showed a marked difference in carbon and calcium level.



(a)



(b)

Fig. 4.13: Elemental composition of PPR (a) reference, (b) contaminated

Davey (2000) has documented that a majority of the organic carbon responsible (such as shown in Table 4.3) for biofilm formation already exists in the water being tested before it flows through any piping material. In most cases, the amount of organic carbon in the water is significantly greater than what could percolate from the interior of the pipe.

Table 4.3: Percentage difference in mineral composition of PPR pipe

	C%	Ca%
Reference	32.79	0
Contaminated	39.32	0.11
Percentage Difference	4.53	0.11

4.4.3. Elemental Composition of GI Pipe

Elemental composition of GI pipe showed a marked difference in carbon, calcium and zinc level.

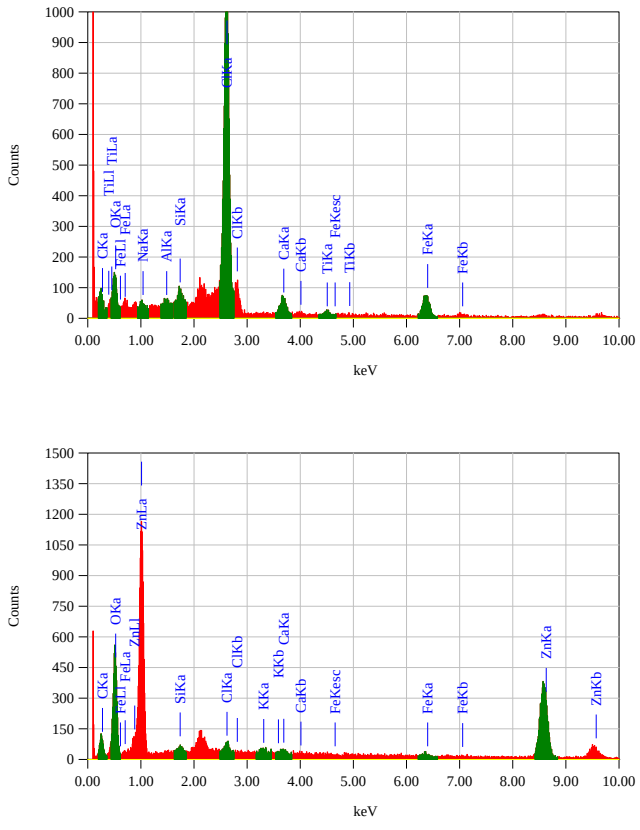


Fig. 4.14: Elemental composition of GI (a) reference, (b) contaminated

Table 4.4: Percentage difference in mineral composition of GI pipe

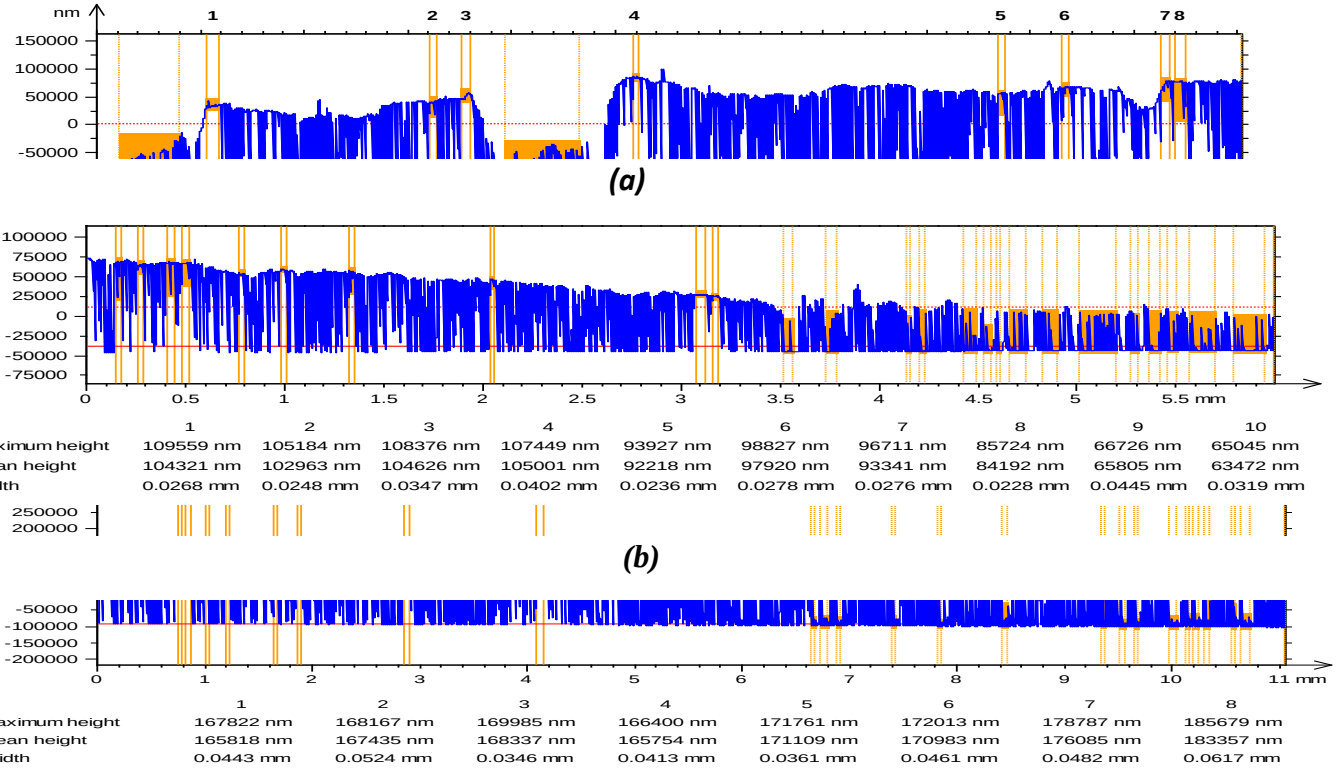
	C%	Zn%	Ca%
Reference	12.38	0	0.56
Contaminated	15.53	50.80	2.66
Percentage Difference	3.15	50.80	2.1

Chang *et al.,* (2003) found that biofilm regrowth on pipes made of rough surface materials such as cast iron, concrete-lined cast iron, and galvanized steel was greater than that on smooth-surface polyvinyl chloride (PVC) pipe. Increased zinc concentration (50.80%) on GI pipes is attributed to microbiologically influenced corrosion (MIC) and it is described in the literature that pure cultures with high zinc content tend to accelerate the corrosion of GI pipes which further promotes the biofilm growth by making rough surface availability for biofilm attachment (Bolton *et al*., 2010).

4.5. Biofilm Thickness of Pipe Materials

Biofilm thickness was measured by optical profilometer which showed the maximum and minimum peaks in terms of thickness.

Average difference of all maximum and minimum heights gave the value of biofilm thickness which came out to be 0.0938 nm in GI pipe (Fig. 4.15 (a). Similarly in PVC pipe average width was found to be 0.0405 nm while in PPR pipe it came out to be 0.0617 nm (Fig.4.15 (b) & (c)).


nm
150000
100000
50000
0
-50000
1 2 3 4 5 6 7 8
(a)
100000
75000
50000
25000
0
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-50000
-75000
0 0.5 1 1.5 2 2.5 3 3.5 4 4.5 5 5.5 mm
1 2 3 4 5 6 7 8 9 10
Maximum height 109559 nm 105184 nm 108376 nm 107449 nm 93927 nm 98827 nm 96711 nm 85724 nm 66726 nm 65045 nm
Mean height 104321 nm 102963 nm 104626 nm 105001 nm 92218 nm 97920 nm 93341 nm 84192 nm 65805 nm 63472 nm
Width 0.0268 mm 0.0248 mm 0.0347 mm 0.0402 mm 0.0236 mm 0.0278 mm 0.0276 mm 0.0228 mm 0.0445 mm 0.0319 mm
250000
200000
(b)
-50000
-100000
-150000
-200000
0 1 2 3 4 5 6 7 8 9 10 11 mm
1 2 3 4 5 6 7 8
Maximum height 167822 nm 168167 nm 169985 nm 166400 nm 171761 nm 172013 nm 178787 nm 185679 nm
Mean height 165818 nm 167435 nm 168337 nm 165754 nm 171109 nm 170983 nm 176085 nm 183357 nm
Width 0.0443 mm 0.0524 mm 0.0346 mm 0.0413 mm 0.0361 mm 0.0461 mm 0.0482 mm 0.0617 mm

(c)

Figure 4.15. Biofilm thickness measured by optical profilometer

(a) GI (b) PVC and (c) PPR

The results were found in correlation with studies conducted by Niquette *et al.*, (2000) who found that biofilm thickness depends on pipe materials used for that specific study and plastic based materials (PVC & Polyethylene) support less attach biomass as compared to steel and iron (showing high susceptibility to biofilm attachment).

4.6. Roughness Profile of Biofilm

Roughness of biofilm was measured by optical profilometer and gave the average and root mean square value (table 4.2)

Table 4.5: Average & root mean square roughness values of biofilm

Roughness (average & Root mean value)		
Galvanized Iron	Ra	32432 nm
	Rq	37226 nm
Polyvinyl Chloride	Ra	24616 nm
	Rq	28237 nm
Polypropylene	Ra	63703 nm
	Rq	73386 nm

Analysis of pipe material showed that PPR supported most rough biofilm while PVC having the least.

Van der Wende *et al*., (1989) stated that ratio between biofilm surface loading and shear rate determines the biofilm structure and roughness. When shear forces are relatively high only a patchy biofilm will develop, whereas at low shear rates the biofilm becomes highly heterogeneous with many pores and protuberances. In case of a right balance smooth and stable biofilms may be obtained.

Chapter 5

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

The following conclusions were drawn from the present study:

1. Most of parameters, i.e. disinfectant concentration, total phosphates and total nitrites were found unsatisfactory when compared with WHO guidelines.
2. *Coliform* count of all water samples (except in April) were above the permissible limit of WHO and USEPA.
3. 19 bacterial isolates were isolated from drinking water distribution network biofilms. On the basis of 16S rRNA customized sequencing, isolated bacterial species were identified as *Bacillus cereus* strain SH42, *Lactobacillus crispatus* ST1, *Lactobacillus jensenii* 269-3, *Staphylococcus aureus, Staphylococcus aureus* A9719, *Paenibacillus sp.* P22, *Achromobacter sp.* clone Cl-38, *Achromobacter insolitus* strain PBR-1, *Staphylococcus haemolyticus* strain KJ1599, *Staphylococcus haemolyticus* strain KJ1-5-97, *Staphylococcus epidermidis, Staphylococcus sp.* BAB-4187, *Bacterium clone* BN11, *Pseudomonas sp.* Lc31-4, *Bacillus sp.* BAB-3357, *Paenibacillus sp.* P22, *Achromobacter sp.* BQNT2, *Taylorella asinigenitalis* and *Tetrathiobacter kashmirensis* strain 3T5F.
4. Accession number of isolated strains was obtained from NCBI and phylogenetic tree was prepared and most of isolated species showed resemblances with Firmicutes and Proteobacteria.
5. Of selected pipe materials GI supported more HPC and hence, more biofilm growth due to more mineral deposition on pipe surface (Carbon: 3.15%; Calcium: 2.1%; Zinc: 50.80%). Surface mineralogy evaluated increased carbon concentration (11.61% in PVC; 4.53% in PPR & 3.15% in GI) which is reported

to stimulate biofilm formation.

5.2. Recommendations

Following research studies are suggested:

1. Biofilm effect on corrosion and scale formation in pipelines should be monitored.
2. Biofilm formation in lab scale set up and pilot scale should be compared.
3. Effect of other disinfectants like ozone and ultraviolet toward biofilm monitoring should be carried out.
4. Regular monitoring of chlorine along the distribution network should be ensured to supply water that meets the WHO drinking water quality criteria.

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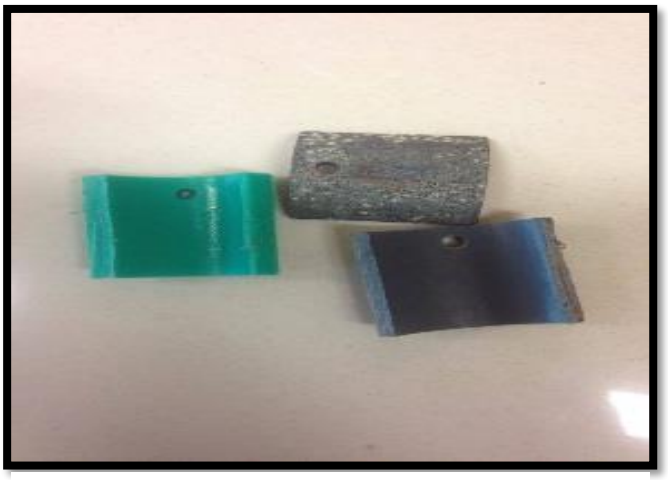
ANNEXURE-I



Curved shaped pipe materials

Fire hydrants as pipe insertion point

Pipes hanged through a string

Pipe material holding biofilms: Removed for analysis