EVALUATING THE EFFECT OF OVERNIGHT STAGNATION ON THE MICROBIAL QUALITY OF DRINKING WATER

By

Mehreen Khan

NUST201261038MSCEE65212F

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)
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It is certified that the contents and forms of the thesis entitled

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

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Table of Contents

List of Abbreviations

APHA	American Public Health Association
API	Analytical Profile Index
BGLB	Brilliant Green Bile Broth
CFU/Ml	Colony Forming Unit per milliliter
CICL	Cement Lined Cast Iron
Cl_2	Chlorine
CWS	Continuous Water Supply
DD C & M	Deputy Director Construction and Maintenance
DICL	Cement Lined Ductile Iron
DO	Dissolved Oxygen
DWDS	Drinking Water Distribution System
EC broth	*Escherichia coli* broth
Fe	Iron
Fe^+	Ferrous Ion
GI	Galvanized Iron
H^+	Hydrogen ion
HCl	Hydrochloric acid
HOCl	Hypochlorous acid
HPC	Heterotrophic Plate Count
IOB	Iron Oxidizing Bacteria
IRB	Iron Reducing Bacteria
IWS	Intermittent Water Supply
k_w	Wall decay constant
LTB	Lauryl Tryptose Broth
MAF	Million Acre Feet
MDPE	Medium Density Polyethylene

MF	Membrane Filtration
mg/L	Milligram per liter
MPN	Most Probable Number
NaOH	Sodium Hydroxide
NTM	Nontuberculosis Mycobacterium
NUST	National University of Sciences and Technology
OCl⁻	Hypochlorite ion
PCRWR	Pakistan Council for Research in Water Resources
PPR	Polypropylene
PVC	Polyvinyl Chloride
TA	Total alkalinity
TDS	Total Dissolved Solids
TH	Total Hardness
TLY	Tryptic Soy Broth
UV	Ultra violet
WHO	World Health Organization

List of Tables

List of Figures

ABSTRACT

Water may remain stagnant for extended periods in the premise plumbing during which, depending upon the pipe age, complex interactions occur between pipe material, disinfectant residual and supply patterns which deteriorate its quality. The study aimed to evaluate the effect of overnight stagnation on the quality of chlorinated and non-chlorinated drinking water in residential buildings in an educational institution. Galvanized iron and polypropylene pipes with service age ranging from one month to six years were taken into account. Free chlorine and dissolved oxygen were taken as indicators of stagnation. Pipes with service age more than 5 years exhibited high heterotrophic plate count (HPC) growth ($p<0.05$) as compared to those with service age less than 5 years. Both chlorinated and non-chlorinated water were found to show similar trend of bacterial regrowth. In pipes with service age equal to or more than 5 years, however, HPC reached their peak values at 18 hours stagnation in non-chlorinated water while in chlorinated water peak growth was observed at 24 hours. It was observed that GI pipes with service age equal to or more than 5 years of service age and receiving intermittent supply of water lead to highest chlorine decay, depletion of dissolved oxygen and HPC proliferation. Running taps for approximately 10 seconds resulted in 93-95% reduction in HPC in both chlorinated and non-chlorinated water which shows that bacterial growth was localized near taps in the small diameter pipes. In contrast to HPC, Total coliform and E. *coli* were suppressed as a result of stagnation in both chlorinated and non-chlorinated water. Pipe material, age, supply patterns and usage frequency are complex interrelated factors that affect water quality. Pipe material and age were found to have higher impact on bacterial regrowth during stagnation as compared to water supply patterns.

Chapter 1

INTRODUCTION

1.1. BACKGROUND

Access to safe and clean drinking water is a basic human right. A major portion of the world population suffers from health problems either due to lack of ample availability of drinking water or its microbial contamination. Contamination of water due to anthropogenic activities is a major issue which poses serious threat to human health and environment.

Approximately 70% of the population of Pakistan relies on ground water for their household water supply. The water bodies are contaminated due to the discharge of domestic and industrial wastewater (approx. 4 Million Acre Feet (MAF) per year. Faulty drainage system accompanied by poor supply lines result in the supply of unsafe drinking water to households (Mohsin *et al*., 2013).

Water supply through piped networks is an advancement in the drinking water distribution. As of 2012, water supply through piped networks in developing countries contributed to 73% urban and 24% rural water supply (Haydar *et al*., 2009). Change in the microbial processes within the distribution network can have significant impact on the water quality supplied at the households. Growth of microorganisms in the distribution system lead to corrosion and roughness of the pipes and impart bad taste and odor to the water. According to a research by Pakistan Council for Research on

Water Resources (PCRWR), water supplies in 21 cities of Pakistan are found bacteriological contaminated (Kalim *et al*., 2007).

Bio stability of water implies that concentration and composition of the microbial community in the distribution system should remain unchanged (Lautenschlager *et al*., 2013). Various factors in the distribution network limit the growth of microorganisms in the water such as low nutrient concentrations, adequate disinfectant residuals, short residence times and low temperatures. However, drinking water distribution systems have been reported to cause major changes in water quality during transportation which results in contamination at taps and subsequent outbreak of water borne illnesses.

In the premise plumbing, water is used or supplied at varying frequencies as a result of which water stagnates in pipes overnight or even for days (Haider *et al*., 2002). More than half of the water supply in Asia and approximately one third in Africa is supplied intermittently. When the supply is turned off, pressure is reduced in the pipes which results in the inflow of contaminants from the surrounding environment (Kumpel *et al.,* 2013).

Generally there are two approaches to minimize bacterial regrowth during distribution. First, maintain effective disinfectant residual. Second, limit growth supporting nutrients (Lu *et al*., 2014). Addition of disinfectants is the most widely used technique (Berry *at al*., 2010).

Chlorine is the most widely used disinfectant. However, the disinfectant residual reacts with the substances left in the water after treatment resulting in decay. Chlorine decay is dependent upon its residence time in the distribution system. Longer residence times, particularly in the extremities of the distribution system, result in higher chlorine decay (Blokker *et al*., 2014). Disinfectant residuals decay in the distribution system due to interaction with the pipe material, biofilm or the tubercles formed in the pipe walls, resulting in increased microbial concentration (Clark *et al.,* 1994; Al-Jasser, 2007).

Decline in disinfectant residual followed by increased microbial concentration promote the formation of biofilm which protect and nourish many microorganisms (van der Kooij, 2003; Parsek and Singh, 2003; Lethola *et al*., 2007). Most microbes that enter the water during stagnation in the distribution system come from the biofilms formed on the inner surface of the pipes. Thus, pipe material tends to play a key role in the extent of bacterial regrowth (Inkinen *et al*., 2014). The age and maturity of biofilms increase with the increase in service age of the pipes. (Martiny *et al.*, 2003; LeChevallier *et al.,* 1987; van der Wende *et al.,* 1989).

1.2. THE PRESENT STUDY

In the present study, water samples were collected from the drinking water distribution network of National University of Sciences and Technology (NUST) and analyzed for changes in the physicochemical and microbiological parameters as a result of overnight stagnation. Heterotrophic plate count (HPC) and most probable number (MPN) techniques were performed to evaluate bacterial growth in galvanized iron (GI) and polypropylene pipes (PPR) with service age ranging from one month to

six years as a result of overnight stagnation. Analytical Profile Index (API) was performed to identify predominant microorganisms in stagnant and flushed samples.

1.3. AIMS AND OBJECTIVES

The objectives of the study were to:

1. Evaluate the effect of overnight stagnation on the microbiological parameters of piped water.

2. Compare the extent of changes in chlorinated and non-chlorinated water.

3. Isolate and identify predominant bacterial species in stagnant and flushed samples.

LITERATURE REVIEW

2.1. DRINKING WATER DISTRIBUTION SYSTEM

Drinking water distribution system (DWDS) comprises of a complex network of pipelines, storage tanks and treatment plants that are used to carry potable water to consumers. The integrity of these systems is vital in supplying clean water to end users (Whittle *et al.,* 2013). In addition to leaks and bursts, bacterial regrowth in drinking water distribution systems is a problem that can effect large water supply utilities. Regrowth is said to occur when treated water that enters the distribution system with very few bacteria is found to have high amount of bacteria which makes water in a distribution system unstable. (Srinivasan and Harrington, 2007).

The potential for the water in the distribution system to transport microbial pathogens is found in different countries (Shakya *et al*., 2012). WHO requires that water that enters the distribution system should be microbiologically safe and biologically stable (WHO, 2006). For this purpose a disinfectant residual is usually maintained in the distribution system (Lautenshclager *et al.,* 2013). However, multiple factors during distribution such as temperature changes, pipe material, biofilms, intrusion of untreated water and stagnation zones can affect the quality of water (Laurent *et al.,* 2005a; 2005b; WHO, 2006).

2.2. INDICATORS OF WATER QUALITY

Many kinds of bacteria can grow in the drinking water distribution systems which include general heterotrophic plate count bacteria e.g. *Aeromonas* and *Pseudomonas* etc and indicator bacteria such as E. *coli* termed as coliforms.

2.2.1. Heterotrophic plate count bacteria

Heterotrophic plate count bacteria are general indicators of useful water quality that are different from bacteria having fecal origin and can undergo multiplication in drinking water when chlorine levels are dissipated. HPC bacteria can grow and produce visible colonies on media after incubation at 22 and 37°C (Sakyi and Asare, 2012). These bacteria may be present in distribution system even in the presence of a disinfectant residual and the water may still be free of any health risks. However, excessive activity may lead to deterioration of water quality such changes in color, taste and odor.

Opportunistic pathogens that can grow in water supplies include *Legionella* spp., non-tuberculosis *Mycobacterium* spp., *Mycobacterium avium* and *Helicobacter* (Pryor *et al.,* 2004; Thomas *et al.,* 2006). Studies report the association of HPC with gastrointestinal illnesses in humans and the role of some bacteria in causing cell cytotoxicity and invasiveness with particular threat to immunocompromised individuals. HPC bacteria may shelter opportunistic pathogens which are a threat to human health (Payment *et al*., 2003; Pavlov *et al*., 2004; Allen *et al*., 2004).

2.2.2. Coliform bacteria

Presence of various kinds of pathogens in drinking water require need of different detection methods. For simplicity, fecal coliforms are used as indicator of fecal contamination in drinking water (WHO, 2006). Coliform are gram negative, spore forming, rod shaped bacteria that ferment lactose with gas and acid formation at 35-37° within 24-48 hrs.

Fecal coliform bacteria are sub group of this family and include *Escherichia coli* which is the most common member of the group found in the fecal excrement of warm blooded animals. Other members of the group include species of *Klebsiella*, *Enterobacter* and *Citrobacter* which are responsible for causing a wide variety of diseases like cholera, typhoid, dysenteries and bacillary dysentery etc. These pathogens are highly resistant to disinfectant residuals and their presence in drinking water is an indication of fecal contamination (Anderson *et al.,* 2005; Ashbolt, 2004; Singh and McFeters, 1992).

2.3. CHLORINE DISINFECTION

Disinfectants are added in the distribution system to prevent water borne diseases. In addition to removing pathogens, disinfectants also serve to prevent bacterial regrowth. Chlorine is the most widely used disinfectant due to its low cost, stability and effectiveness against many pathogens.

Chlorine reacts with water to form hydrochloric acid (HCL) and hypochlorous acid (HOCL).

$$Cl_2 + H_2O \leftrightarrow HOCl + HCl \qquad \text{[Eq 2.1}$$

HOCL is a weak acid and further dissociates to H^+ and OCL^-.

$$HOCl \leftrightarrow H^+ + OCl^-$$ [Eq 2.2

HOCL and OCl⁻ species are commonly referred to as free chlorine and are highly reactive with numerous components of the bacterial cells. HOCL is much stronger than OCl⁻ and a much stronger disinfectant. It can result in oxidation, hydrolysis and deamination reactions with a variety of chemical substrates, and produces physiological lesions that may affect several cellular processes

High chlorine is added in the distribution system to maintain a detectable level at the end points. . Chlorine concentration at stand points and wells should be about 1 mg/L so that sufficient chlorine remains in distribution system pipes to minimize the effects of recontamination by killing or inactivating microbes. A dead end chlorine residual should be maintained at 0.2 to 0.5 mg/L (Blokker *et al*., 2014; Pickard, 2006).

However, high amount of chlorine may result in taste and odor problems (Ohar and Ostfeld, 2014; Song *et al.,* 2014). Disinfectants have been known to oxidize the natural organic matter present in water and as a result provide more substrate for bacterial regrowth to occur. Thus, a trade off exists between ensuring a high amount of disinfectant residual and a low substrate for bacterial regrowth (Liu *et al.,* 2002, Harrington *et al.,* 2003).

2.4. PREMISE PLUMBING

The portion of the drinking water distribution system between water main and the point of use in buildings is termed as ''premise plumbing'. Disease causing bacteria are often present in the distribution system water as well as the pipe walls where they

can reside in biofilms. Thus, premise plumbing serves as an ideal ecological niche for opportunistic pathogens and also as source from where various negative issues impacting human health arise (Wang *et al.,* 2013).

2.4.1. Stagnation of water

Due to varying frequencies with which water is used in different buildings, longer retention times are known to occur in premise plumbing (Haider *et al.,* 2002). Low velocity of water during higher retention times lead to biofilm detachment, negative pressures and subsequent microbial regrowth. In addition intermittent supply also lead to storage of water in tanks which also promotes bacterial regrowth (Ayoub and Malaeb, 2006).

Kumpel *et al.* (2013) compared the microbial water quality in intermittent and continuous water distribution networks. Higher concentration of indicator bacteria were observed in intermittent water supplies where 31.7% samples were found E.*coli* positive while 0.7% were found positive in continuous water supplies.

Andy and Kelkar (2007) evaluated the impact of intermittent water supply in four cities of India. Water samples collected from various locations receiving either continuous or intermittent supply were tested for total coliform. 90 to 100% of the samples were found coliform negative in case of CWS while for IWS the number of coliform negative samples varied from 24 to 73%.

Pepper *et al.* (2004) carried out a study to find the background HPC concentrations from source to tap. Samples were collected from kitchen and bathroom taps from the

first drawn water at 7 a.m in the morning. HPC in kitchen and bathroom taps were consistently above 500 CFU/mL in 68% of the samples. First drawn samples in house 1 had mean HPC 2.4×10^3 CFU/mL while after flushing for 30 seconds HPC reduced to 1.5×10^2 CFU/mL representing a reduction of one order of magnitude.

Siebel *et al.* (2008) carried out a study to determine correlations between total cell concentration, total adenosine tri-phosphate concentration and HPC during microbial monitoring of drinking water. Highest CFU/mL i.e. approx. 1.4×10^3 were found at 8 a.m in the morning indicating bacterial regrowth during night and it fell down to approximately 0.1×10^3 CFU/mL by 10 a.m after the tap used regularized.

Lautenschlager *et al.* (2010) while working on the effect of overnight stagnation on the microbial growth in drinking water quality reported upto 600 folds increase in HPC counts after overnight stagnation associated with significant changes in microbial community composition.

2.4.2. Factors affecting water quality in premise plumbing

A large number of factors in the premise plumbing are responsible for deteriorating the quality of water. A brief of all the factors is mentioned below.

2.4.2.1. <u>Disinfectant Residuals</u>

Addition of disinfectant eliminates the risk of water borne diseases resulting from the distribution system. However, during stagnation in the premise plumbing the disinfectant residual decays due to interaction with the pipe material, biofilm or the

tubercles formed on the pipe walls, resulting in increased microbial concentrations (Blokker *et al.,* 2014; Al-Jasser, 2007).

Chlorine is depleted at a faster rate by reaction with a corroded pipe wall. Bacterial regrowth is higher in such systems because the rust on pipe walls can alter the organic matter in water making it more available for bacterial growth and nourishment. The ferrous ion or hydrogen ion can also be utilized by bacteria for growth (Morton *et al.,* 2005; Zhang and Liu, 2014).

2.4.2.2. <u>Pipe Material</u>

Types of pipe material can play a key role in the bacterial regrowth by affecting the corrosion processes and biofilm formation.

Owing to the high porosity and the corrosion induced in iron pipes due to the reaction between pipe wall and disinfectant residual they are reported to support highest bacterial biomass as compared to PVC and are favorable for biofilm establishment because pipe sediments serve as a nutritional source for bacteria. . Biofilms serve as source of bacteria entering the distribution system (Inkinen *et al.,* 2014; Wang *et al.,* 2014). Norton *et al.* (2000) reported that bacterial densities in the biofilm formed in iron pipes is much higher (>100-fold) as compared to polyvinyl chloride pipes (PVC).

Niquette *et al.* (1999) carried out a study to examine the impacts of pipe materials on densities of fixed bacterial biomass in a drinking water distribution system. Densities of bacterial biomass in iron pipes were found 10 to 45 times higher than in plastic based pipes. The corrosion tubercles in the iron pipes provide increased surface area

and cracks and crevices to protect bacteria from disinfectant residual (LeChevallier *et al*., 1996).

Pederson (1990) reported that bacterial population on matt steel pipe surfaces were much higher i.e. 4.06×106 cells/cm^2 as compared to PVC pipe surfaces i.e. 2.8×106-3.3×106 cells/cm^2.

Lethola *et al.* (2007) observed that stagnation upto 16 hours resulted in 6 times increase bacterial cells in polyethylene pipes and 10 times increase in copper pipes.

2.4.2.3. Pipe Age

Pipe age has been experimentally shown to affect the decay of chlorine in a variety of ways (Al-Jasser, 2007; 2011). As the pipe service age increases, pipes experience wear, tear and corrosion due to water flow and environmental stress (Lin, 2001).

Hallam *et al.* (2002) carried out a study on the decay of chlorine associated with the pipe wall and found the average decay values as follows: 0.12 and 0.13 h^{-1} respectively for cement lined cast iron (CICL) and cement lined ductile iron (DICL), 0.09 and 0.05 h^{-1} for polyvinyl chloride and (PVC) and medium density polyethylene (MDPE).

Al-Jasser (2007) evaluated the effect of pipe age on chlorine decay in drinking water transmission and distribution. Pipe service age ranged from 0 to 55 years. It was observed that wall decay constant ranged from either an increase upto +431% or decrease upto -92%. For 25 mm cast iron (CI) pipes, wall decay constant (k_w) for

unused pipes was found to be 70 day^{-1} while for 40 years old pipe k_w was found to be 110 day^{-1}. Effect of service age on the wall decay constant was most evident in CI pipes while steel pipes were least affected.

2.4.2.4. Pipe Diameter

A very important characteristics of the premise plumbing which makes it susceptible to bacterial regrowth is its small pipe diameter (<0.5 in) compared to the distribution network (Edwards *et al*., 2003). As the pipe diameter decreases, surface-volume ratio increases and it provides larger surface area through which mass transfer can occur and thus greater the number of reactive site available for each unit volume of chlorinated water passing through with time in the pipe.

Ekeng and Agunwamba (2011) carried out a study to determine chlorine decay in aged and small diameter galvanized iron pipes. GI pipes with 10 to 40 years of age and following diameters were taken: 0.0217 (m), 0.01905 (m), 0.0254 (m), 0.0318 (m). Largest chlorine consumptions were observed in 40 years old pipes with smallest diameter. Wall decay constant (k_w) for 0.0127 m diameter pipes were found to be – 0.01 day^{-1} while for 0.0315 m pipes was 0.155day^{-1}. The rapid depletion of chlorine in ages and small diameter pipes represent that reaction occurs at the pipe walls.

2.4.2.5. Fluctuating Temperatures

Temperature is linked to bacterial regrowth and chlorine decay in a variety of ways (Hua *et al.,* 1999). Premise plumbing has the characteristic to experience temperature fluctuations different from those that naturally occur in the distribution system. These

fluctuating temperatures affect the rate of disinfectant decay during stagnation (Rushing and Edwards, 2004; Hua *et al.,* 1999).

LeChevallier *et al.* (1996) reported an 18-fold increase in coliform occurrences when water temperature changed from 0 to 5°C to >20°C. These fluctuating temperatures during stagnation affect the rate of disinfectant decay which may result in issues that may be attributed to public health risks.

2.4.2.6. Storage Patterns

Water is usually stored in the tanks that are made up of different materials and act as reserves during variable water supply periods. Overhead tanks are usually made up of steel and have inner lining of asbestos, coal tar, PVC, epoxy resin, acrylic or silicon while underground tanks are usually lined be concrete, asphalt, gunite or a plastic sheet. These coatings tend to cause bacterial growth problems during storage. Bituminous coatings cause the problem of organic polymer intrusion in water which serves as nutrient source for heterotrophic bacteria (Geldreich, 1996). Bacterial regrowth increases in slowly circulating and hot water tanks (Bagh *et al*., 2004)

2.4.2.7. Flow Rate

Pipe usage rates and flow rate of water can affect the biofilm formation in the pipes and thus the bacterial regrowth during stagnation. Cloete *et al*. (2003) carried out a study to evaluate the effect of fluid velocity on biofilm development. It was concluded that as the fluid velocity increased biofilm formation was limited. ±3 m/s and 4 m/s were observed as detaching velocities. Thus, velocities within this range would be

helpful in reducing the biofilm formation. As the flow rate increases, the rate of wall decay increases.

Shamsaei *et al*. (2013) observed that as the flow rate decreased in a distribution system, the retention time increased and so did the concentration of heterotrophic plate count (HPC) bacteria. At points along the distribution system, when the flow rate was found approx. 1.1 m^3/s, residual Cl_2 was found 1.2 mg/L and HPC were observed to be 50 CFU/mL. When the flow rate decreased to about 0.1 m^3/s, Cl_2 decreased to 0.6 mg/L and HPC were found to increase to 2900 CFU/mL.

2.5. CHLORINE DECAY

Chlorine may also react with natural organic matter and the pipe material resulting in formation of disinfection byproducts and corrosion of pipes. Iron pipes are subjected to corrosion in which iron ions are released into the water which can re-precipitate and form corrosion scales or tubercles. Thus, deteriorating the quality of water (Zhu *et al.,* 2014).

Iron corrosion corresponds to iron dissolution which is expressed as per below mentioned equation

$$Fe \leftrightarrow Fe^{2+} + 2e^{-} \qquad \text{[Eq 2.3}$$

When the medium is aerated or chlorinated either the reduction of dissolved oxygen as shown in equation 2

$$½ O_2 + 2H^+ + 2e^- \leftrightarrow H_2O \qquad \text{[Eq 2.4}$$

16
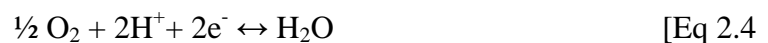
or that of free chlorine occurs as depicted in equation 3

$$HOCl + H^+ + 2e^- \leftrightarrow Cl^- + H_2O \qquad \text{[Eq 2.5}$$
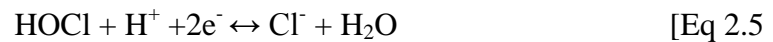
The Fe^{2+} produced in equation 1 can be oxidized by free chlorine into ferric ions as mentioned in equation 4

$$2Fe^{2+} + HOCl + H^+ \leftrightarrow 2Fe^{3+} + Cl^- + H_2O \qquad \text{[Eq 2.6}$$
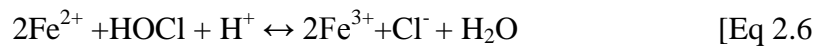
In this way corrosion can induce consumption of FCL at the interface of the metal or electrolyte as shown in equation 3 and also chemical consumption in the solution as shown in equation 4 (Frateur *et al.,* 1998).

Galvanized iron (GI) pipes with longer service age show larger chlorine consumption as compared to those with small age (Ekeng and Agunwamba, 2011).

2.6.   BACTERIAL SPECIES IN DIFFERENT PIPES

Stagnant waters provide nutrition deficient environment which cannot support growth of all the microorganisms. Oligotrophic bacteria are found to grow best in such conditions.

Jaeqqi and Schmidt-Lorenz (1990) analyzed two weeks stagnant water in six dead end water distribution pipes installed in Zurich. 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 were

found dominating in the water which proved to be oligotrophs in the nutrient tolerance test.

Different pipe materials favor growth of different bacteria in the drinking water. Stagnation of water in pipes can induce release of nutrients that can favor bacterial growth. Chen *et al.* (2013) 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.

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

Norton *et al.* (2000) observed the bacterial species accumulating biofilms 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%). *Nocardia* spp. has been known to degrade polyethylene terephthalate of plastic pipes by releasing enzyme esterase (Sharon and 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).

MATERIALS AND METHODS

3.1. STUDY SITE

New campus of National University of Sciences and Technology, Pakistan was taken as the study site. It is a residential public sector research university established in 1991 while its new campus was established in H-12 sector, Islamabad in 2008. It is spread over 707 acres, has more than 15 schools and institutes as well as hostels for both male and female students including faculty residence.

The distribution system of the university is fed with ground water sources. Water from catchment areas is pumped through 9 tube wells which is then transferred to 3 underground storage tanks or 2 overhead storage tanks where it is either supplied directly or stored in overhead storage reservoirs overnight, however, storage time sometimes extend up to 2-3 days. The tube wells have a pumping capacity of 0.2 million gallons per day (MGD) which serves a population of about 11400 people. Figure 3.1. shows the layout of university distribution system.

The distribution system pipes are primarily unplasticized Polyvinyl chloride (uPVC) mains and Polypropylene (PPR) and Galvanized iron (GI) service lines. As the campus is not too old, the pipe service ages in the area range from as one month to six years old. Prior to distribution water is treated with chlorine. University has a total of 6 water filtration plants which are located at various points throughout the campus. Details of buildings, pipe materials and storage tanks are shown in Table 3.1.

Figure 3.1: Layout of NUST

*Red circles represent sampling sites

Table 3.1: Characteristics of the components of university distribution system

Component	Number/ Pipe diameter	Material/ Well depth	Storage capacity/ Pumping capacity/Service age
Underground storage tanks	3	RCC(reinforced cement concrete)	1 lac gallon each
Overhead storage tanks	2	RCC	6 lac and 9 lac gallon
Overhead storage containers	62	PVC; RCC	2500-5000 gallons
Tube wells	6	300 ft	0.2 MGD
Main Supply lines	6 ″, 8 ″	uPVC, GI	One month to six years
Premise plumbing service lines	0.65 ″, 0.83 ″,1 ″,3 ″,4 ″ (in order from tap to tank for 4 story buildings)	PPR, GI	One month to six years

3.2. SITE SURVEY AND BACKGROUND DATA ANALYSIS

An extensive site survey was carried out to locate sampling sites. Background data was analyzed (reports of Environmental Microbiology Lab, IESE) to identify those locations where previous issues of repeated contamination have been reported. Sampling sites were identified in consultation with Deputy Director Construction and Maintenance (DD C & M NUST) Col. Sajjad Afzal and his team.

3.2.1. Sampling site categories

In phase 1, sampling sites were broadly categorized on the basis of pipe age, pipe material and supply patterns (Figure 3.2.)
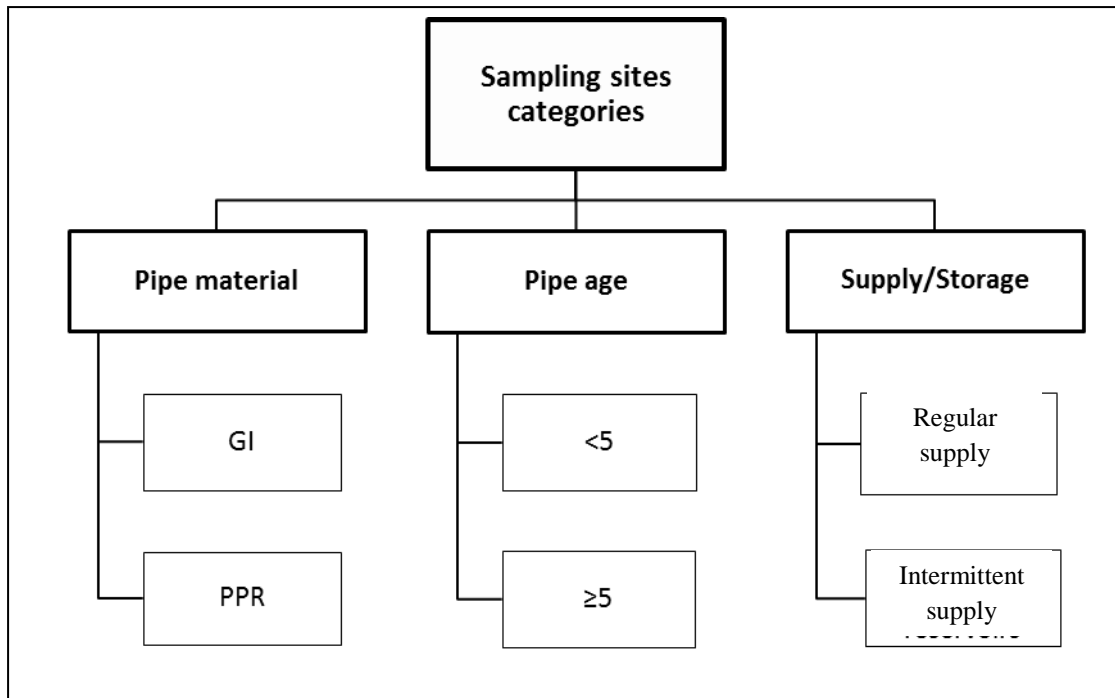
Figure 3.2: Categories of sampling sites

3.2.2. Volume Determination

Measurements at site were made with the help of C& M branch NUST to determine variation in diameters and pipe lengths from overhead storage tanks to taps in different buildings. Pipe diameter and lengths were used to determine the volume of water in different sections of the pipe. Mean flow rate was determined from different taps by opening the taps and letting the water flow gently in graduated tub while noting the time.

3.3. SAMPLING

3.3.1. Preparation of Glassware

Sterile leak proof 250 mL Schott (glass) bottles were used for sampling. All bottles were washed with detergent, rinsed with distilled water and autoclaved at 121°C, 15

psi for 15 minutes and then oven dried at 105°C for one hour. Following this treatment bottles were tightly capped and wrapped.

3.3.2. Sample Collection, Transportation and Storage

Tap were allowed to remain stagnant overnight (14-20 hours). Prior to sample collection, taps were cleaned with alcohol and flamed to avoid any chances of aerial or tap outlets contamination. Two samples were collected from each sampling station. (1)200 ml of first drawn tap water in the morning hours after overnight stagnation. (2)200 ml after flushing the tap for approximately 2.5 to 3 minutes. In case of chlorinated water 0.08% sodium thiosulfate was added in bottles to neutralize residual chlorine.

Samples were analyzed within one hour of their collection or stored in refrigerator at 4°C and analyses within 4 hours. All the collection, transportation and storage procedures were carried out as prescribed in the Standard Methods for the Examination of Water and Wastewater (APHA, 2012).

A total of 70 samples of chlorinated and 45 samples of non-chlorinated water were collected from November 2013 to May 2014. Triplicates were collected for each sample on different days to validate the results. Comparison of changes were made between chlorinated and non-chlorinated water.

3.4. WATER QUALITY ANALYSIS

3.4.1. Physicochemical Analysis

3.4.1.1. <u>On site Analysis</u>

pH, temperature, dissolved oxygen and free chlorine were measured on site using HACH 156 pH meter, Crison Oxi 45 DO meter and Spectroquant Colorimeter Picco

respectively. All the analysis were performed as per the Standard Methods for the Examination of Water and Wastewater (APHA, 2012).

3.4.1.2. Analysis in Laboratory

Conductivity, turbidity, total dissolved solids (TDS), hardness and alkalinity were measured in the laboratory within four hours of sample collection. WTW series pH/ Cond 720 meter and HACH 2100N turbidimeter were used for measuring conductivity and turbidimeter respectively. Total dissolved solids were measured using gravimetric analyses while hardness and alkalinity were determined through titrimetric analysis.

3.4.1.3. Free Chlorine, Dissolved Oxygen and Temperature Profiles

Taps that were left stagnant overnight were gently flushed and temperature, dissolved oxygen and free chlorine were determined incrementally. Temperature was measured by placing a digital thermometer under the tap when it was flowing. Water was allowed to flush until the chlorine and DO reached the network level. At this point water was considered fresh.

3.4.2. Microbiological Analysis

3.4.2.1. Spread Plate Count

3.4.2.1.1. *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.

3.4.2.1.2. *HPC Enumeration*

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 colony counter (560 Suntax Colony Counter).

3.4.2.2. Most Probable Number Technique

3.4.2.2.1. *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.

3.4.2.2.2 *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 vigorously shaking, the sample 10 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).

3.4.2.3. HPC and Coliform Interaction

Experiment was performed to identify interaction between E. *coli* and HPC bacteria following modified methodology of LeChevallier and McFeters (1985). 200 mL tap water was collected and split into two parts. 0.01 % sodium thiosulfate was added to one part to dechlorinate it immediately. While 2 mg/L of free chlorine was added to the other part and left for one hour at room temperature. After one hour this part was again dechlorinated. This resulted in compete removal of microorganisms from this part.

Side by side a culture of E. *coli* was grown in TLY broth overnight. TLY broth without dextrose was taken and supplemented with 1% lactose and 0.3% yeast extract. After 24 hours the culture of E. *coli* was washed and added to both samples. Sample that was treated with additional chlorine to remove all detectable microorganisms from it resulted in a pure E. *coli* culture after this step while the other sample comprised of a mixture of E. *coli* and HPC that were naturally present in tap water.

HPC and E. *coli* from sample containing both of them while E. *coli* from post chlorinated samples were enumerated through spread plate count technique using nutrient agar for HPC and eosin methylene blue (EMB) agar for E. *coli*. Samples were incubated at room temperature and readings were taken at 0,2,4,6,24,48 and 72 hours.

3.4.2.4. Impact of Stagnation Time on HPC and Coliforms

In order to determine the impact of stagnation time on HPC and coliform growth, samples from ten taps were taken after keeping them closed for varying intervals. Samples were collected at 0, 2, 4, 6, 18, 20, 24, 48 and 72 hours. Tap was flushed gently before keeping closed for each interval. Free chlorine, temperature and DO were measured for each sample.

3.4.2.5. Concentration of Bacteria in Small Diameter Pipes

Ten taps were used to collect samples to evaluate the localization of bacterial growth. Water samples were collected step wise from 0 to 5 L. Free chlorine, temperature and dissolved oxygen were measured for each sample.

3.4.3. Isolation of Bacteria

3.4.3.1. Streak Plate Technique

Pure cultures of bacteria were obt/ained from samples of GI and PPR pipes by repeatedly streaking them on nutrient agar plates. As major changes in bacterial counts were observed with increasing age, therefore, water samples taken from pipes with service age equal to or more than 5 years were only taken and analyzed to identify the presence of predominant bacterial communities.

3.4.4. Identification

For the identification of isolated bacterial strains following morphological, physiological and biochemical tests were performed (Table 3.2)

Table 3.2: Tests for identification of bacteria

Morphological	Physiological	Biochemical
Colony Morphology Cell Morphology Motility test	Optimum temperature Optimum pH	Oxidase Catalase Differential media Oxidation/Fermentation API test

3.4.4.1. <u>Morphological Identification</u>

Colony morphology of the isolated strains were observed to identify and characterize them. All physiological and morphological identification were performed as per Bergey's Manual of Determinative Bacteriology (Holt *et al*., 1994). Following morphological characteristics are usually observed (Pelczar, 1957).

Table 3.3: Morphological characteristics of bacteria

Morphological Characteristics	Description
Size	Small, large, punctiform
Margins	Entire, curled, lobate, undulate, filiform
Texture	Creamy, dry, mucoid
Color	Yellow, orange, off white, pale yellow
Elevation	Convex, umbonate, raised, flat
Form	Rhizoid, circular, filamentous, irregular

Grams staining was used to identify cell morphology. Prepared slides were observed under 100X oil immersion with a light microscope. Cells were identified as either gram positive or gram negative cocci, bacilli or cocco-bacilli.

Motility of the cells was determined using a hanging drop technique. In short, a drop of bacterial culture was placed under a cover slip in the concave depression of a slide. The slide was inverted so that the drop hangs in the concave well. A drop of oil immersion was added and slide was observed under 100X resolution using a light microscope.

3.4.4.2. Physiological Identification

3.4.4.2.1. *Optimum pH*

Nutrient broth was prepared as per manufacturer's instruction and adjusted to different pH ranging from 5 to 10 using 1N NaOH and 1N HCl. Broth was added in test tubes which were plugged and autoclaved at 121°C for 15 minutes.

Loop full of fresh bacterial culture was taken and inoculated in one mL distilled water to make bacterial suspension. Suspension was vortexed and 50µL was added in six test tubes containing broth of different pH for each strain. The test tubes were then incubated at 37°C for 24 hours. After 24 hours optical densities were measured at 600 nm using UV-visible spectrophotometer.

3.4.4.2.2. *Optimum Temperature*

Nutrient broth was prepared as per manufacturer's instruction and pH was adjusted to 7. 4 mL broth was poured in 3 test tubes for each strain. The tubes were properly plugged and autoclaved.

Bacterial suspension was prepared and after properly vortexing inoculated in broth. Tubes were then incubated at 25, 30, 37 and 45°C for 24 hours. Optical densities were measured after completion of incubation period at 600 nm using a UV-visible spectrophotometer.

3.4.4.3. Biochemical Characterization

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

3.4.4.3.1. *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.

3.4.4.3.2. *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.

3.4.4.3.3. *Growth on Differential Media*

Bacterial isolates were streaked on EMB and MacConkey agar. EMB is a selective and differential agar which inhibits the growth of gram positive bacteria and allows differentiation between organisms that do and do not ferment lactose. Lactose fermenting bacteria give colored colonies on EMB agar while non-fermenting bacteria give colorless colonies.

Isolated pure cultures were streaked on EMB agar and plates were placed in an incubator at 37°C and results were noted after 24 hours. Rapid fermenters appeared as dark colonies with metallic sheen which inicated presence of fecal coliform, while less fermenting showed brown-pink colonies and non-fermenters appeared as colorless colonies

MacConkey agar is used for the isolation and differentiation of gram negative rods from gram positive ones by selective growth of gram negative bacteria. Strong lactose fermenters result in the formation of pink halo around the colonies while weak fermenters appear pink without halo and non-fermenters appear colorless. Bacterial cultures were streaked on agar plates and results were noted after incubation at 37°C for 24 hrs.

3.4.4.3.4. *Oxidation/Fermentation test*

Following composition was used for this test (Table 3.4)

Table 3.4: Composition of medium for oxidation/fermentation test

S.No	Component	Quantity (g/L)
1.	Peptone water	2.0
2.	Sodium Chloride	5.0
3.	Dipotassium hydrogen phosphate(K_2HPO_4)	1.0
4.	Bromothymol blue	0.3
5.	Agar-Agar	10.0
6.	Glucose	10.0

All the ingredients were mixed well and pH was adjusted at 7.0±2.0. The mixture was then boiled and 5 mL was added to two test tubes designated for each strain. Agar was prepared for giving plug. Test tubes and agar were autoclaved at 121°C for 15 minutes. After this the medium was allowed to solidify in test tubes. Fresh bacterial culture was inoculated in the medium through stabbing using an inoculating loop. As mentioned above two test tubes were used for one strain. Cotton plug was added to one test tube to provide aerobic conditions to bacteria while 3mL agar was added to other tube to serve as plug which resulted in anaerobic conditions. All the test tubes incubated at 37°C for 24 to 48 hours.

Change in medium color from green to yellow in both plugged in non-plugged test tubes indicated acid production by fermenting bacteria while only slight change in medium color in non- plugged tube indicated oxidative bacteria. This lead to identification of bacteria as aerobic, anaerobic or facultative anaerobic.

In negative result, alkaline production resulted in change of medium color to blue which occurred due to break down of peptone. No color also indicated negative result. (Leboffe and Pierce, 2006; MacFaddin, 2000).

3.4.4.3.5. *API test*

API 20 E

API 20 E (Biomeriux, Canada) is used to identify gram negative rods i.e. members of the family *Enterobacteriaceae*. It allows 18-24 hours identification of bacteria. API 20 E kit consists of 20 cupules which contain dehydrated reagents that are rehydrated by adding distilled water.

0.85% saline solution was prepared and 7 mL of this solution was added in each test tubes (one tube corresponding to each strain). The tubes were autoclaved at 121°C and 15 psi. A loop full of 24 hour fresh bacterial culture was inoculated in each tube containing autoclaved saline solution and vortexed. The cupules in the kit were filled by this suspension. The strips were then incubated at 37°C for 24 hours. Reagents were added in some cupules after 24 hours and results were noted. Results were interpreted into numerical values and codes generated were interpreted through API web software.

API 20 Staph

API 20 Staph (Biomeriux, Canada) is used for the identification of Staphylococci and micrococci. The kit consists of 19 cupules that are filled with dehydrated medium. Bacterial suspension is prepared and inoculated in cupules as mentioned in API 20 E method and incubated at 37°C for 24 hours.

API 20 NE

API 20 NE is used for non-fastidious gram negative rods that do not belong to the family *enterobacteriaceae*. It consists of 20 cupules that were filled and the strip was incubated at 29±2 °C as per manufacturer's instructions. Results were noted after 24 hours.

3.4.5. Statistical Analysis

t-test assuming unequal variances was employed to determine statistical significance of the results. $P<0.05$ was taken to determine the significance.

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 are listed in Table 4.1. All the parameters were found within limits.

Table 4.1: Physicochemical parameters compared with WHO and PSDWQ

| Parameter	Mean Value (Min-Max)	WHO guidelines	PSDWQ limits
pH	7.4 (7.2-7.7)	6.5-8.5	6.5-8.5
Temperature (°C)	17.63 (13.9-26.3)	-	-
Conductivity (μS/cm)	822 (777-880)	2500	-
Turbidity (NTU)	0.38 (0.28-0.54)	<5	<5
TDS (mg/L)	542.5 (512.8-580.8)	<1000	<1000
Alkalinity (mg/L)	317.66 (300-340)		
Hardness as $CaCO_3$(mg/L)	349 (328-376)	-	<500
Residual Chlorine (mg/L)	0.26 (0.05-0.45)	0.2-0.5	0.2-0.5 * 0.5-1.5 **
Dissolved Oxygen (mg/L)	8.06 (7.1-9.1)	-	-

*at consumer end   **at source

4.1.1. Effect of stagnation on pH and temperature

pH and temperature profiles were developed by flushing taps that were allowed to remain stagnant overnight. No significant variation in pH was observed between stagnant and flushed samples (Figure 4.1)
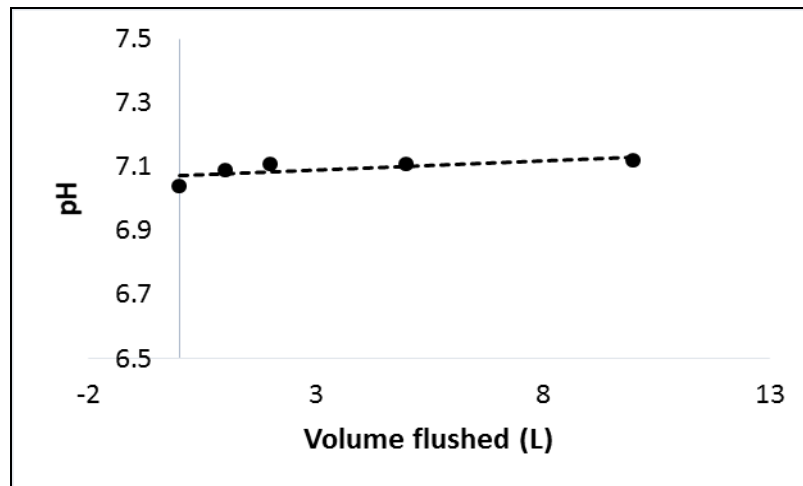
Figure 4.1: Effect of stagnation on pH

Depending upon ambient temperature and supply patterns, water temperature either increased, decreased or remained the same on flushing of taps. therefore, for further analysis these two parameters were not used as indicators of bacterial regrowth during stagnation (Figure 4.2)
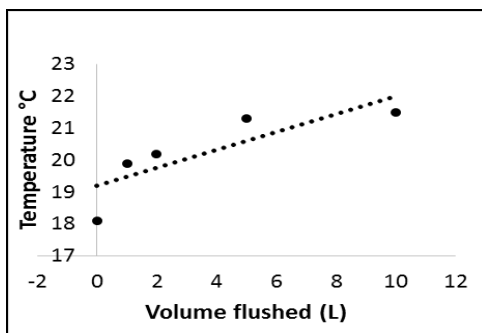
(a)
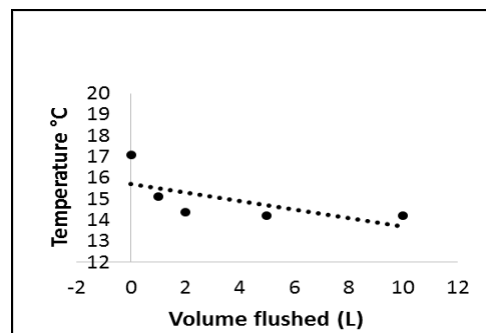
(b)
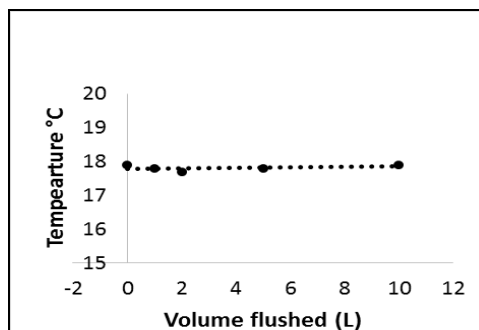
(c)

Figure 4.2: Variation in water temperature on flushing of taps. Temperature either (a) increases; (b) decreased or (c) remained the same

4.2. EFFECT OF STAGNATION ON HPC REGROWTH
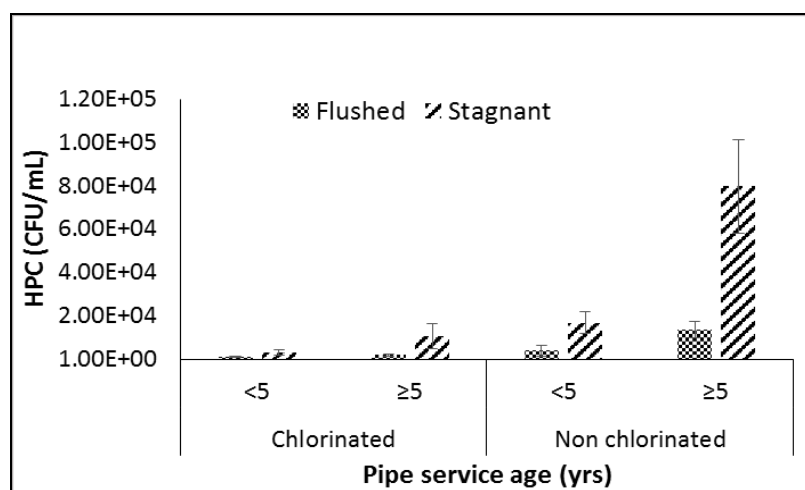
4.2.1. Effect of Pipe Age

Effect of pipe age on microbial regrowth was determined by dividing pipe service age in two groups i.e. less than five and equal to or more than five years. In case of chlorinated water, when pipe age was less than 5 years stagnant samples had 3.1 times (p=7.43e-07) more HPC growth as compared to flushed samples. However, when the service age was equal to or more than 5 years stagnant samples showed 5.7 times (p=1.15e-11) more HPC than flushed samples.

Similar trend was observed in case of non-chlorinated water. Pipes with less than 5 years of age had 4.4 folds (6.73e-18) more HPC as compared to flushed water. Highest HPC growth i.e. 5.82 folds (4.83e-22) in stagnant samples was observed in non-chlorinated water when the pipe age was equal to or more than 5 years (Figure 4.3)
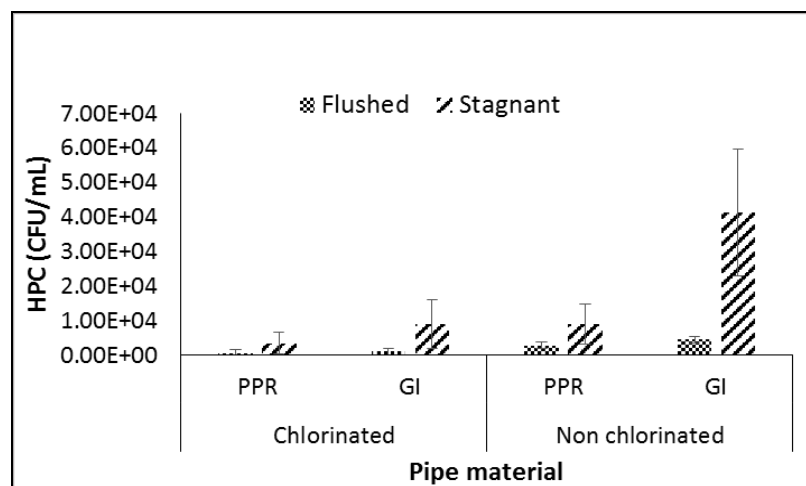

Figure 4.3: Effect of pipe age on HPC

Decline in HPC upon flushing may be attributed to the fact that as the water is moving, relatively small amount of bacteria enter the water then when it is stagnant.

Moving water receives a smaller amount of bacteria from biofilm then when the water is stagnant.

4.2.2. Effect of Pipe Material

In case of both chlorinated and non-chlorinated water GI pipes induced higher HPC growth in stagnant water as compared to PPR. In chlorinated water, stagnant water from GI pipes had 8.2 folds more HPC than in flushed samples. While PPR pipes had 5.1 folds more counts in stagnant samples. Highest HPC were observed in stagnant GI pipes of non-chlorinated samples which induced 9 folds more counts as compared to flushed samples (Figure 4.4)

Figure 4.4: Effect of pipe material on HPC

4.2.3. Effect of Supply Patterns

Chlorinated samples taken from buildings that receive regular daily had 8 folds more HPC growth in stagnant samples while those that receive intermittent supply had 8.55 folds more growth in stagnant samples. Non-chlorinated samples were also found to show similar. Stagnant samples taken from buildings that receive intermittent supply had 8.9 folds more growth in stagnant water (Figure 4.5).

39
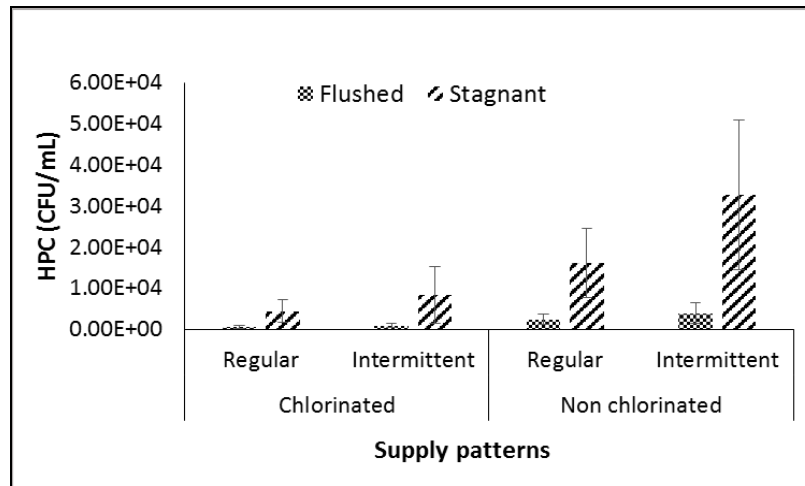
Figure 4.5: Effect of supply patterns on HPC

The increased rate of microbial growth in stagnant water in samples taken from sites where water is stored in tanks may be due to the fact that water may remain stagnant in tanks allowing time for disinfectant residual to decay and microbial water quality to deteriorate (LeChevallier *et al.*, 1996).

4.2.4. Effect of Pipe Usage Frequency

In some cases, water samples collected from same buildings, receiving water from same source and similar pipe materials showed different patterns of HPC growth. Upon further investigation, it was found that pipe usage plays a significant role in microbial regrowth. GI pipes with less than 5 years of age that are used less frequently showed more growth in stagnant samples as compared to those with higher service age. While pipes that are more flown showed more growth in when service age was equal to or more than 5 years (Figure 4.6)
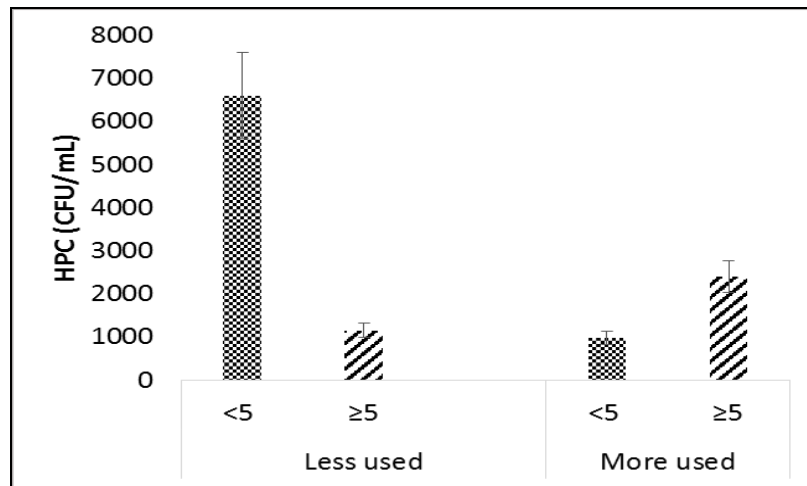
Figure 4.6: HPC regrowth in GI pipes

PPR pipes also showed similar trend (Figure 4.7). Biofilm is less developed in less flown pipes and more bacteria enter the water from the biofilm during stagnation (Rubulis *et al*., 2007)
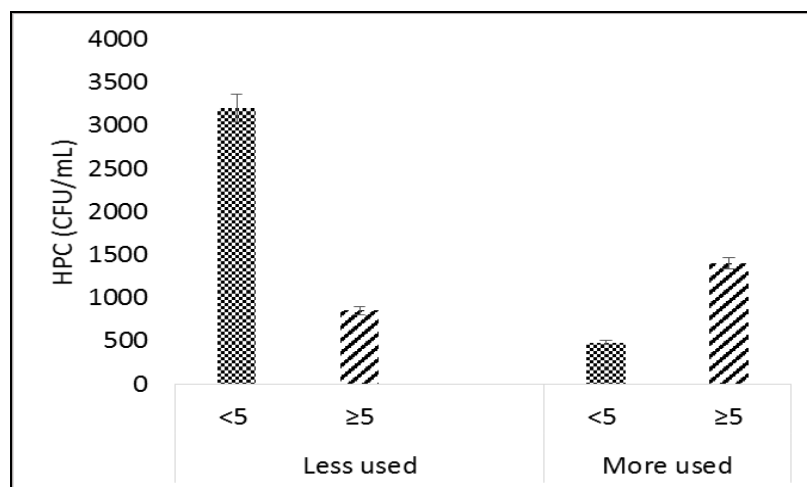
Figure 4.7: HPC regrowth in PPR pipes

4.3. EFFECT OF STAGNATION ON COLIFORM

4.3.1. Effect of Pipe Age

The results of total coliform were surprising and in total contrast to HPC. Total coliform in stagnant samples with pipe service age equal to or more than 5 years was

found 2.2 as compared to flushed samples where MPN index was found 5.1. As opposed to HPC, lowest total coliform i.e. 1.1 MPN index/100 mL were observed in stagnant non-chlorinated samples with service age equal to more than five years while in flushed samples MPN index was found to be 12 (Figure 4.8)
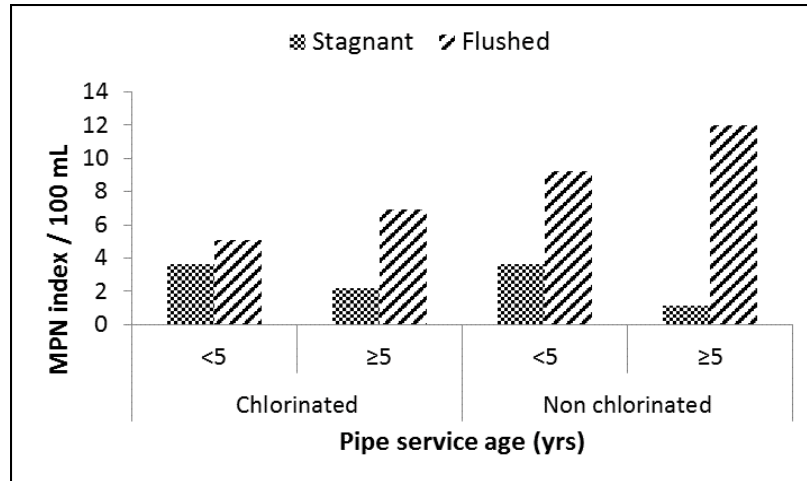
Figure 4.8: Effect of pipe age on total coliform

Similar trend was observed for E. *coli*. MPN index was found 5.1 in flushed sample, while in stagnant it was found to be 2.2 in non-chlorinated samples taken from pipes with higher service ages (Figure 4.9)
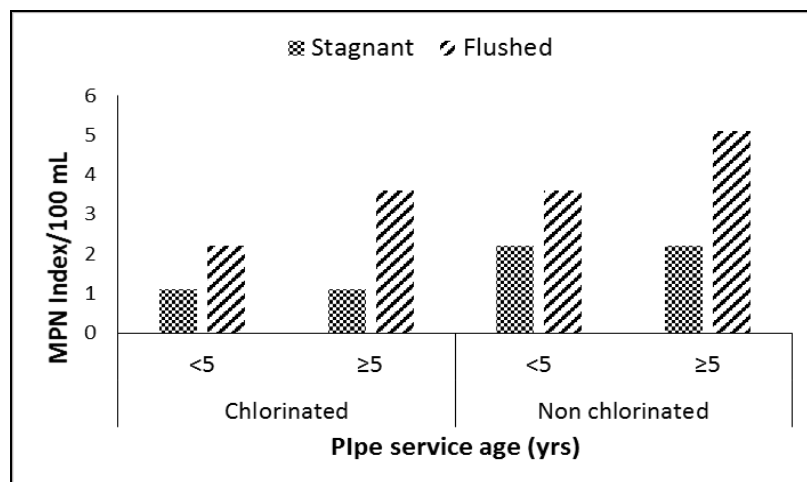
Figure 4.9: Effect of pipe age on E. *coli*

4.3.2. Effect of Pipe Material

Similar trend of reduction of total coliform and E. *coli* like pipe age was observed. GI pipes had more reduction in MPN Index of stagnant samples as compared to flushed for both total coliform and E. *coli*.

Highest reduction was observed in non-chlorinated GI samples. Total coliform in non-chlorinated GI samples was found 2.2 as compared to 12 in stagnant while E. *coli* was found 2.2 as compared to 6.9 in flushed (Figure 4.10 and 4.11)
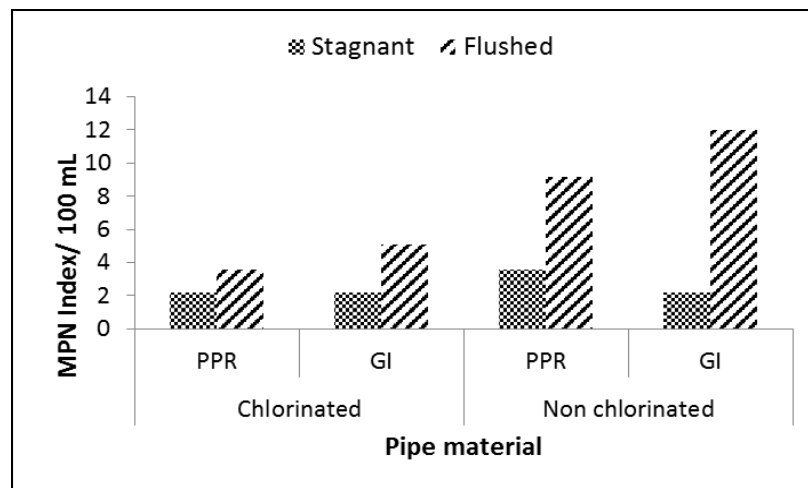
Figure 4.10: Effect of pipe material on total coliform
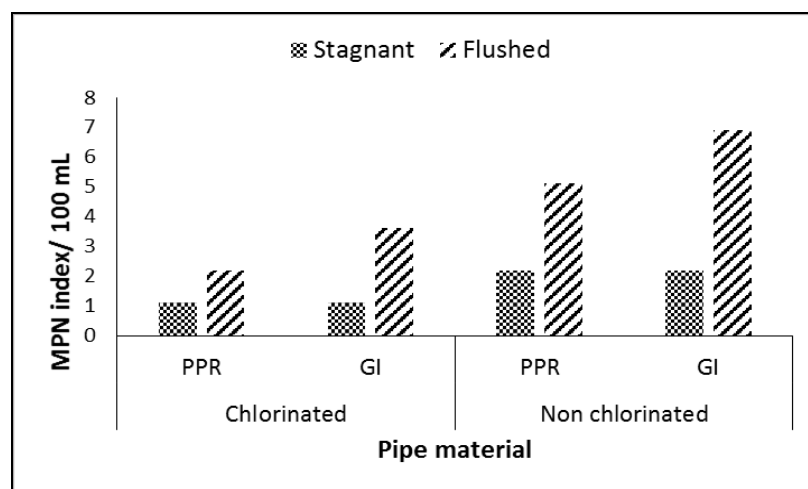
Figure 4.11: Effect of pipe material on E. *coli*

43

4.3.3. Effect of Water Supply Patterns

Stagnant samples collected from buildings that receive intermittent supply lead to higher suppression of total coliform than those which receive regular supply. MPN index/100 ml was found 1.1 in stagnant samples of intermittent supply as compared to 3.6 MPN index/100 ml in flushed. Stagnation samples from non-chlorinated intermittent supply had highest reduction of total coliform (Figure 4.12)
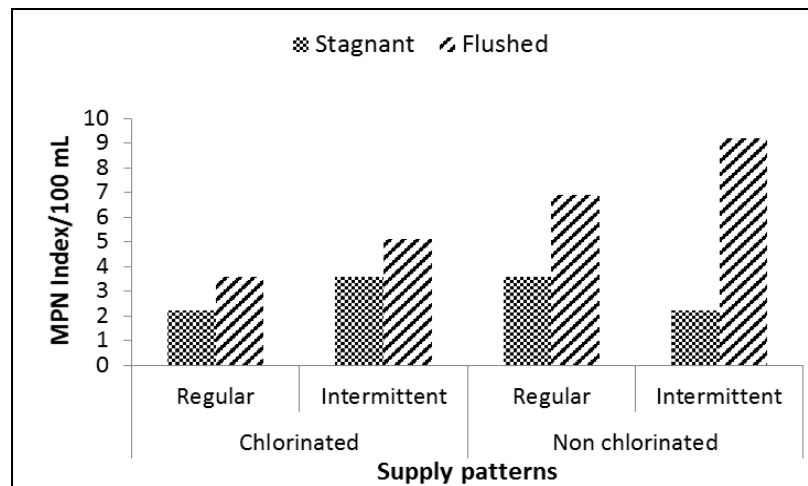
Figure 4.12: Effect of supply patterns on Total coliform

Stagnant water samples collected from buildings that receive intermittent supply lead to higher suppression of E. *coli* than those which receive regular supply (Figure 4.13)
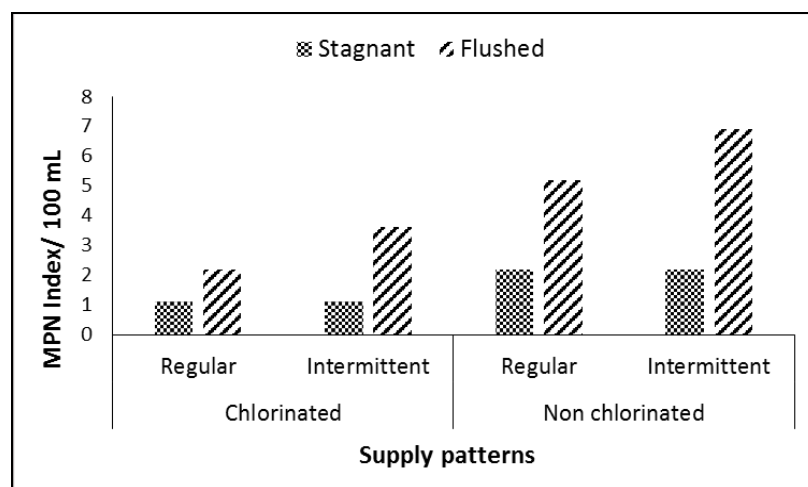
Figure 4.13: Effect of supply patterns on E. *coli*

4.4. CUMULATIVE EFFECT OF FACTORS

4.4.1. Effect on HPC

In order to determine which factor contributed most to microbial regrowth in stagnant water, samples were collected from different combinations of pipe material, age and supply patterns. Results of only stagnant samples were compared to identify the combination that resulted in highest HPC. In case of chlorinated water, highest growth (7.83×10^3 CFU/mL) was observed in GI pipes with service age equal to or more than five years and that receive intermittent supply (Figure 4.14)
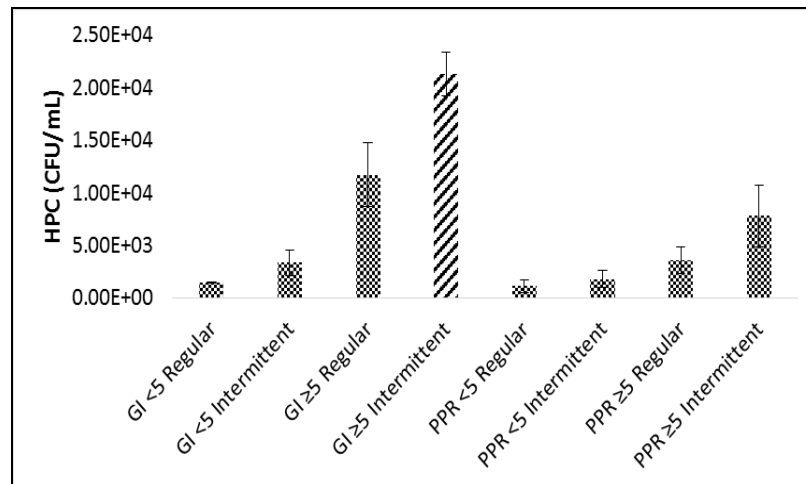
Figure 4.14: Cumulative effect on HPC growth in chlorinated water

Similar trend was observed for non-chlorinated water (Figure 4.15). Highest HPC i.e. 2.41×10^5 CFU/mL were observed in GI pipes with service age equal to or more than 5 years and that receive intermittent supply.
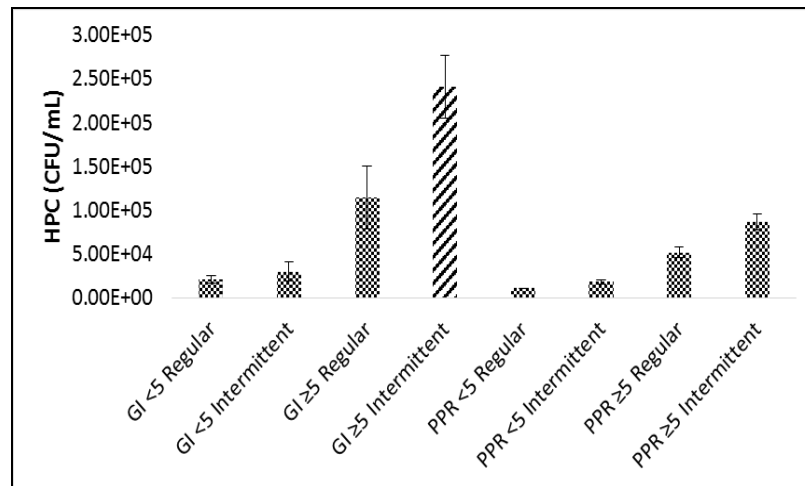
Figure 4.15: Cumulative effect on HPC growth in non-chlorinated water

4.4.2. Effect on Total Coliform

Figure 4.16 and 4.17 represent variation in MPN index/100 mL in stagnant samples for varying pipe material, age and supply patterns for total coliform in chlorinated and non-chlorinated water respectively. Highest reduction was observed in samples collected from GI pipes with service age equal to or more than 5 years and that receive intermittent supply.

Thus, it was observed that as the service age of the premise plumbing increased, effect of pipe material and supply patterns on microbial growth became more pronounced. Pipe age was therefore found to be the most critical factor affecting water quality. It was concluded that no factor acts independently. Rather a complex interaction exists between factors that promote bacterial regrowth in buildings
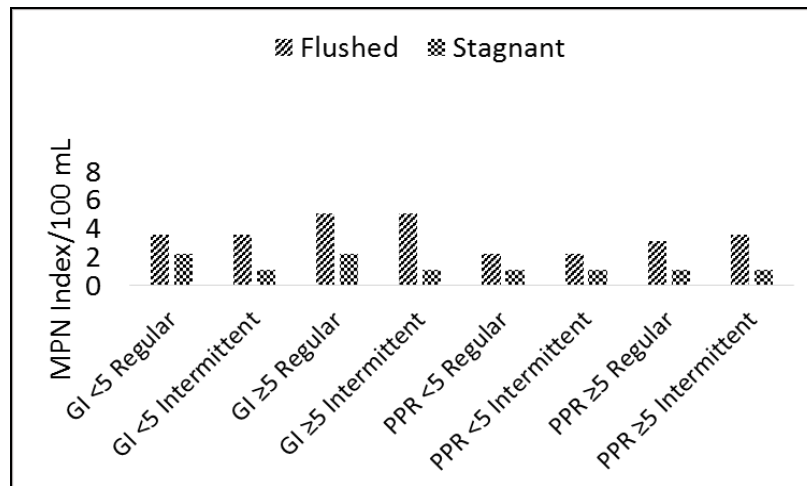
Figure 4.16: Cumulative effect on total coliform in chlorinated water
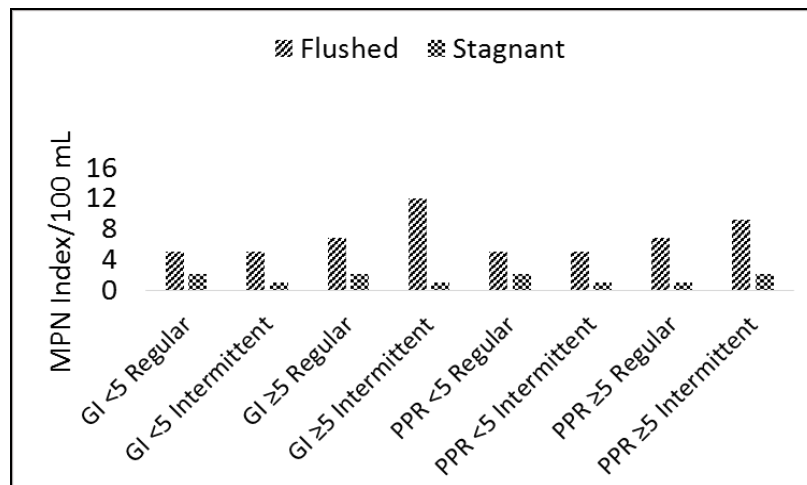
Figure 4.17: Cumulative effect on total coliform in non-chlorinated water

4.5. HPC AND COLIFORM INTERACTION

Antagonistic effect of HPC on E. *coli* was determined which showed that in sample that contained pure culture of E. *coli*, counts kept on increasing uptil 72 hours while in sample that contained a mixture of HPC and E. *coli*, HPC counts were observed to increase uptil 72 hours. However, E. *coli* counts were observed to increase upto 6 hours where they reached their maximum growth and declined afterwards. Decline of E. *coli* in mixed cultures represent suppression of coliforms by HPC (Figure 4.18)
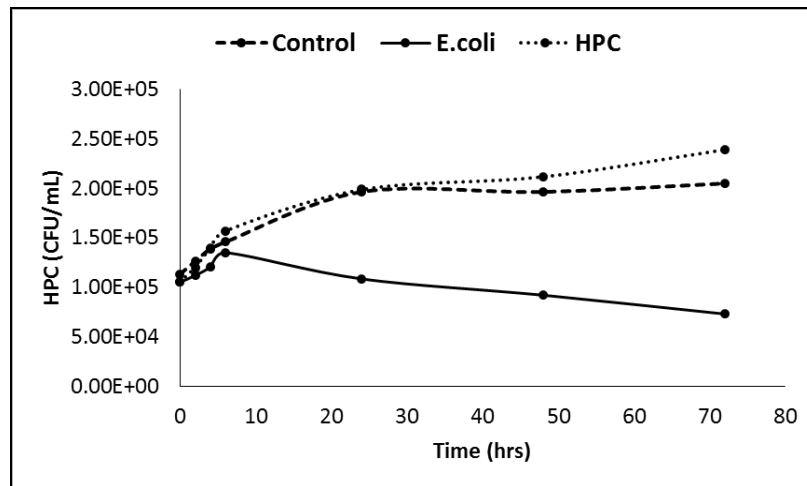
Figure 4.18: HPC and coliform interaction

These contrasting results for HPC may be due in part to the fact that HPC tend to inhibit and suppress the growth of coliform. Coliform detection decreases when the HPC levels exceed 500 or 1,000 CFU/mL due to competition of nutrients between the two groups (LeChevallier and McFeter, 1985). Secondly, HPC bacteria grow in the distribution system while coliform do not grow while travelling in the distribution system. In contrast, higher HPC values might increase the risk of pathogen contamination as they make the detection of coliform difficult and hence, leading to lesser attention and treatment that they actually deserve (van der Kooij, 2003).

4.6. EFFECT OF STAGNATION TIME

4.6.1. Effect of Stagnation Time on HPC

Effect of stagnation time was determined by keeping taps closed for varying intervals. For chlorinated water, HPC in GI and PPR pipes of age group less than 5 years were observed to increase gradually from 0 to 72 hours at a rate of 21.12 and 19.65 CFU/mL per hour respectively while for GI and PPR pipes with longer service age the HPC were observed to increase sharply uptil 24 hours at a rate of 38.85 and 34.53 CFU/mL per hour respectively after which the increase became more gradual (Figure

4.19) this may be due to the fact that hig her substrate in pipes of higher age provide more nutrients for growth of bacteria and HPC reach their maximum concentration at a faster rate.
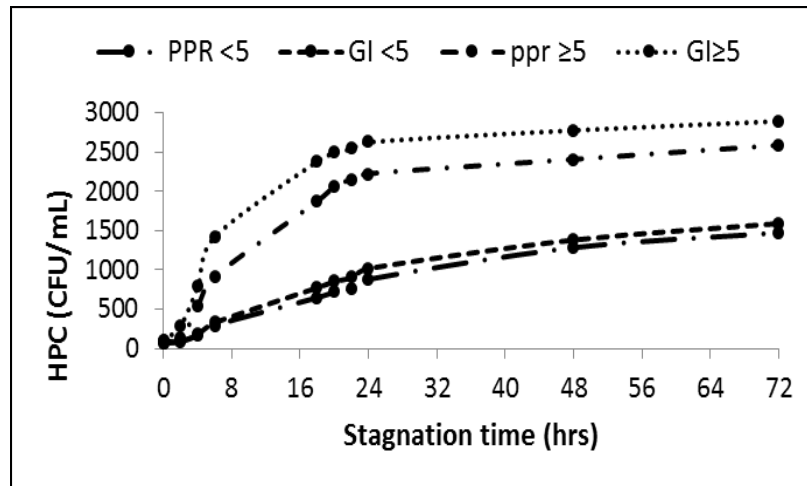
Figure 4.19: Effect of stagnation time on HPC growth in chlorinated water

However, in case of non-chlorinated water highest increase in HPC uptil 24 hours of stagnation was observed in GI pipes (17.95 folds) followed by PPR pipes (15 folds) with service age equal to or more than 5 years. Least growth (7.38 folds) uptil 18 hours was observed in PPR pipes with less than 5 years of service age (Figure 4.20).
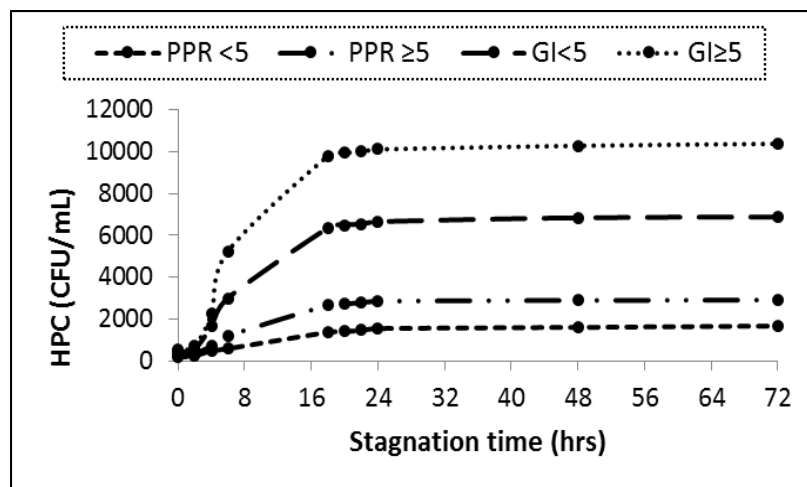
Figure 4.20: Effect of stagnation on HPC growth in non-chlorinated water

Negligible increase in HPC after 18 hours of stagnation for both GI and PPR pipes of different age groups shows that the HPC in non-chlorinated tap water are limited due to substrate and assimilative organic carbon (AOC) (Lautenschlager *et al.,* 2010). HPC reach their maximum growth faster in non-chlorinated water due to absence of disinfectant residual.

4.6.2. Effect of Stagnation Time on Cl_2 and DO

This pattern of HPC increase with pipe age and material corresponds to the chlorine decay rate in the pipes. Highest decay (72%) uptil 18 hours of stagnation was observed in GI pipes with service age equal to or more the 5 years while lowest (23 %) was observed for PPR pipes with less than 5 years of service (Figure 4.21).
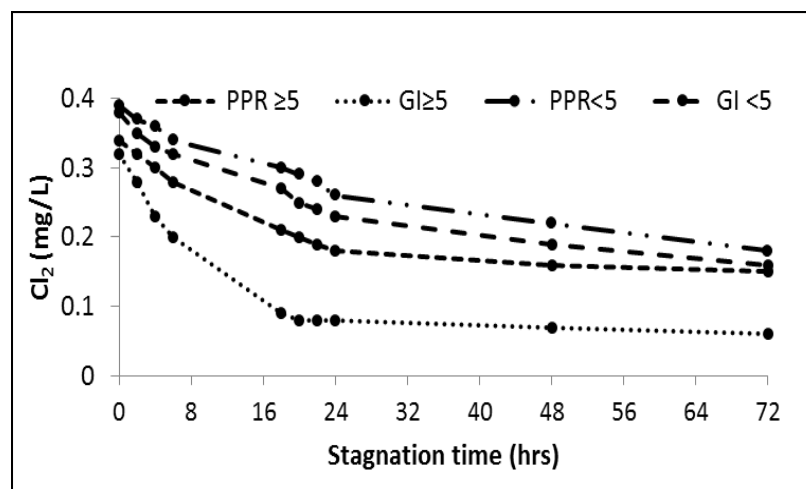
Figure 4.21: Effect of stagnation time on chlorine decay

As the disinfectant travels through the pipes, it results in oxidation of materials present in the water and the pipe wall (Vikesland *et al.,* 2001). However, most of the losses of disinfectant occur due to reaction with pipe walls (Hallam *et al.,* 2002).

Dissolved oxygen also showed a pattern similar to chlorine decay (Fig. 4.22). Highest DO dissipation (22%) uptil 20 hours was observed in older GI pipes while lowest (10%) in young PPR pipes for chlorinated water (Figure 4.20). DO was found higher in taps with zero hour stagnation, however, it started decaying when the taps were allowed to remain stagnant. Decay was found higher uptil 24 hours stagnation because bacterial growth was high in this time period. Gradual decay of DO on stagnation beyond 24 hours corresponded to HPC reaching their maximum growth at 24 hours.
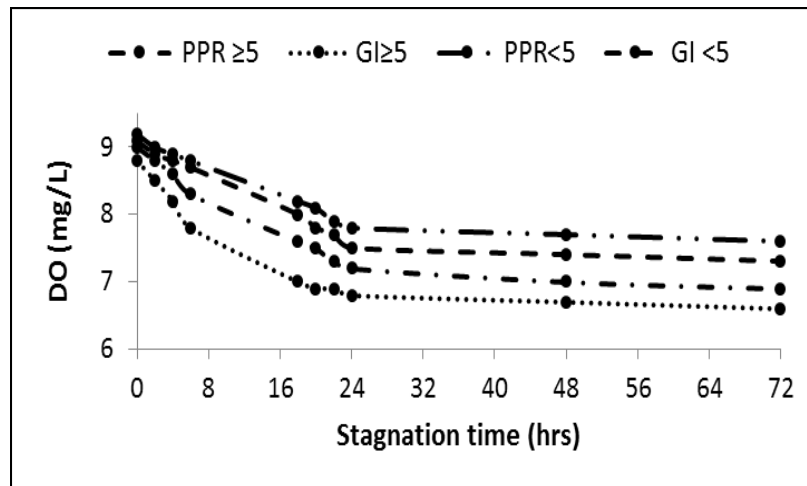
Figure 4.22: DO decay in chlorinated water

While for non-chlorinated water, 18 hours stagnation in oldest GI pipes resulted in 19.5% dissipation of chlorine and youngest PPR resulted in 11% dissipation (Figure 4.23)

51
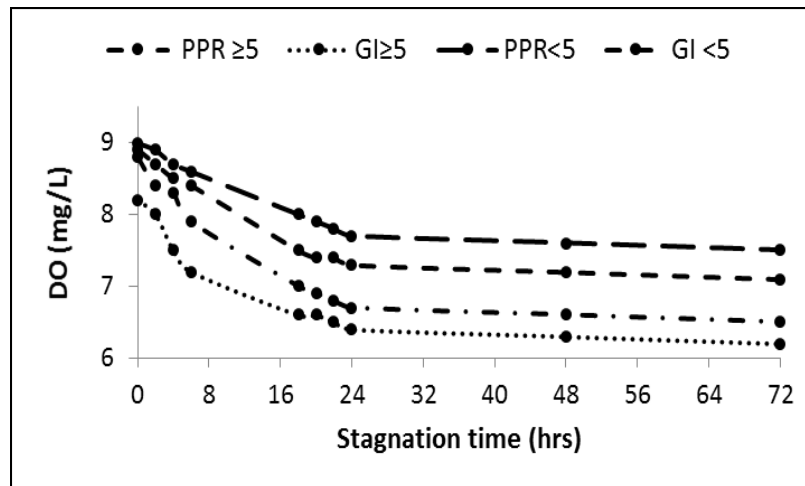
Figure 4.23: DO decay in non-chlorinated water

4.6.3. Effect of Stagnation Time on Total Coliform and E. *coli*

In contrast to HPC, total coliform and E. *coli* showed a different behavior. For both chlorinated and non-chlorinated water, MPN index/ 100 mL increased uptil 6 hours after which it started to decline and reached minimum level uptil 24 hours when the HPC attained the maximum count (Figure 4.24a and 4.24b)
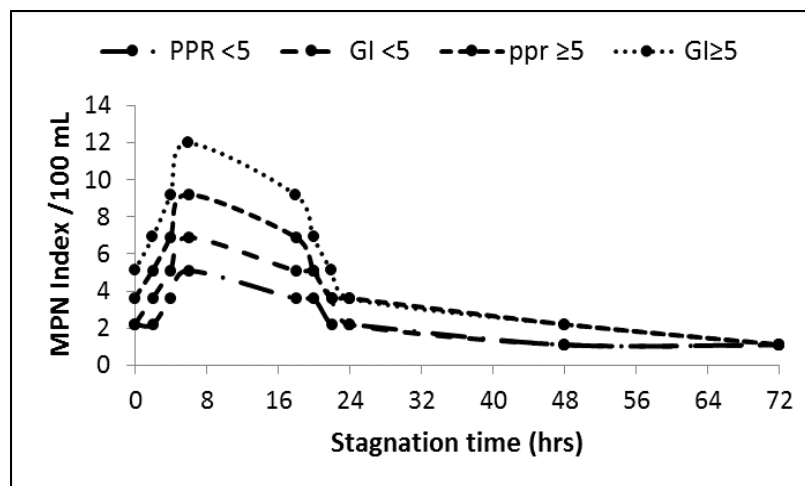
Figure 4.24a: Effect of stagnation time on total coliform in chlorinated water
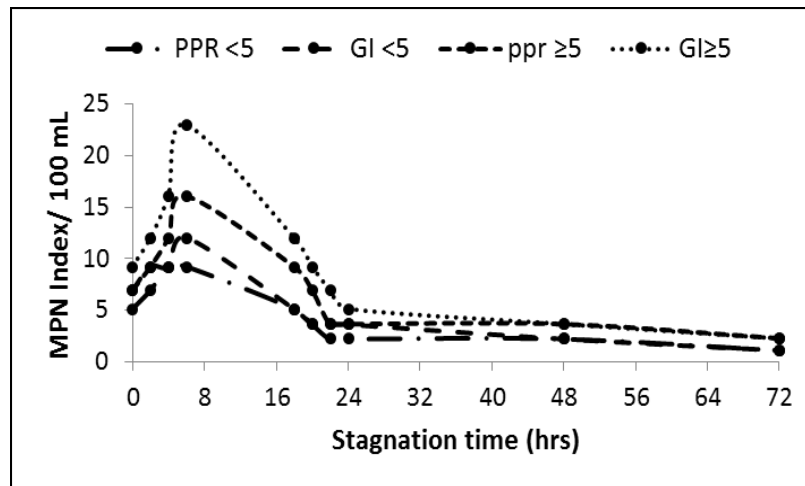
Figure 4.24b: Effect of stagnation on total coliform in non-chlorinated water

When HPC reached their peaks they may actually interfere in the detection of coliform which may be the decline of coliform after 6 hours of stagnation. Coliforms continue to grow until they consume all the nutrients and begin to starve and die off. These dead bacteria are then used by HPC as nutrient source which explains why HPC continue to grow after coliforms have declined (Sakyi *et al*., 2012).

4.7. BACTERIAL GROWTH IN SMALL DIAMETER PIPES

4.7.1. Concentration of HPC

To evaluate flushing of taps as a mitigation strategy mean flow rate was determined and found to be 0.21L/s. Water samples from GI and PPR pipes of varying ages were collected step wise to identify localization of bacterial growth. For chlorinated water, it was observed that a sharp drop in HPC from 4.62×10^3 to 2.12×10^3 CFU/mL occurred during the first 20 ml followed by further decline to 9.60×10^2 CFU/mL in the subsequent 200 ml. Finally after 2 L flushing the HPC were found to drop down to 3.20×10^2 CFU/mL representing a 93% decrease on 2L flushing. Further flushing of

tap to 5 L resulted in change of only 20 CFU/mL. This was found comparable to Cl_2 and DO concentrations. Cl_2 concentrations increased from 0.05 to 0.15 mg/L in the first 200 mL and 0.15 to 0.33 from 200mL to 2 L. Flushing 5 L resulted in increase to only 0.34 mg/L (Figure 4.25)
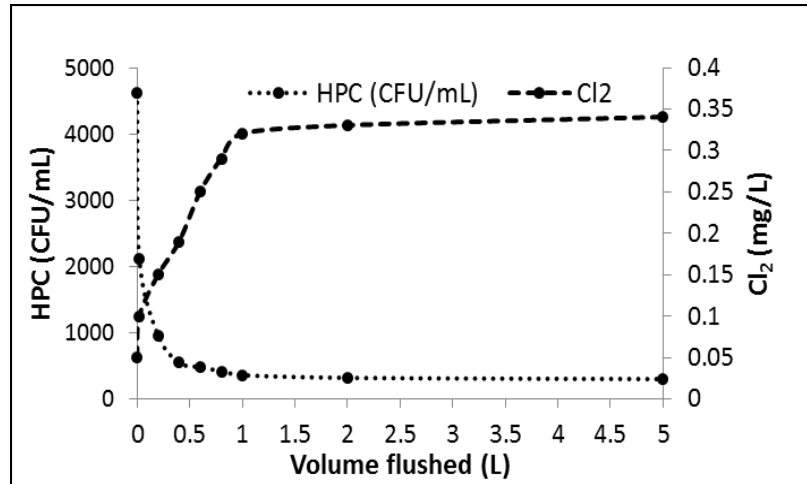
Figure 4.25: HPC and Cl$_2$ profiles

DO increased from 7.4 to 7.9 in the first 200 mL and reached 8.67 in the first 2 L. Finally 8.69 mg/L was detected on flushing 5 L (Figure 4.26)
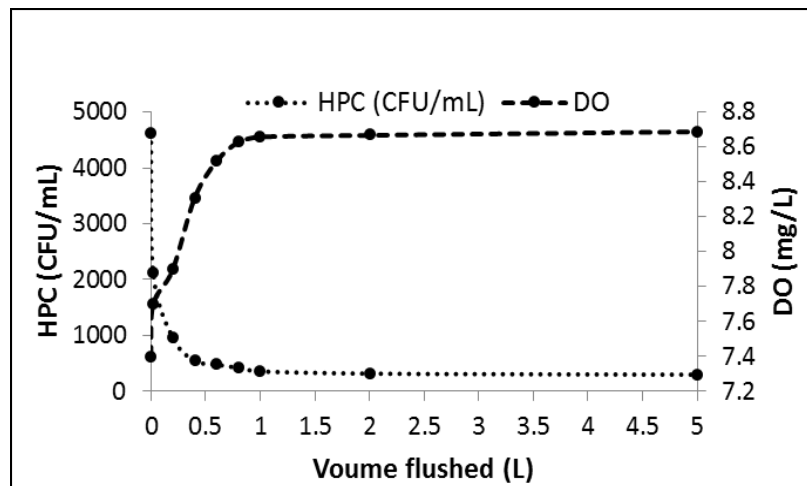
Figure 4.26: HPC and DO profiles for chlorinated water

Similarly for non-chlorinated water HPC dropped from 1.27×10^4 to 8.64×10^3 CFU/mL in initial 20 mL which dropped to 4.64×10^3 on flushing 200 mL and 8.56×10^2 on flushing 2L. Thus, representing a 95% decrease in HPC on 2L flushing. Further flushing to 5 L resulted in decrease of only 1.96×10^2 CFU/mL. No relation between HPC and temperature could be determined. Similar results have been reported in a study carried out by Lautenschlager (2010). DO was also observed to increase from 7.3 to 8.63 in the first 2 L and reached 8.64 on flushing 5 L. (Figure 4.27). Flushing only 2L of water was thus found to significantly lower HPC levels and at a rate of 0.21L/s it took only 9.52 seconds. Rapid decline in HPC on flushing only 2 L of water indicates that bacterial growth mainly occurs on the inner surface of small diameter pipes which may be due to sharp decline of chlorine with decreasing pipe diameters (Ekeng and Agunwamba, 2011).
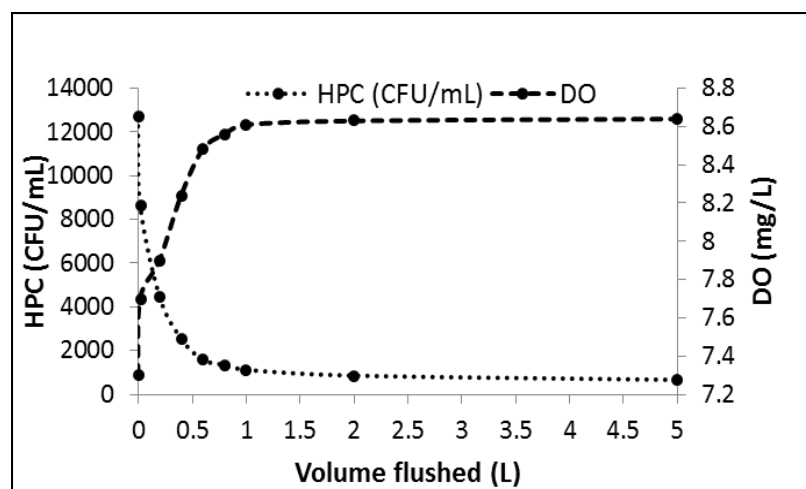
Figure 4.27: HPC and DO profiles for non-chlorinated water

4.7.2. Concentration of Total coliform and E. *coli*

As HPC continued declining, Total Coliform and E. *coli* were observed to increase following the similar pattern. Similar trend was observed for chlorinated and non-chlorinated water. Pipe material and age did not seem to effect the localization of

bacterial growth. Therefore, a general graph depicting change in Total Coliform and E. *coli* index is given below (Figure 4.28). As HPC declined upon flushing 2 L, Total Coliform increased from 1.1 in stagnant sample to 9.2 MPN Index/100 mL. Index remained 9.2 on flushing 5 L.
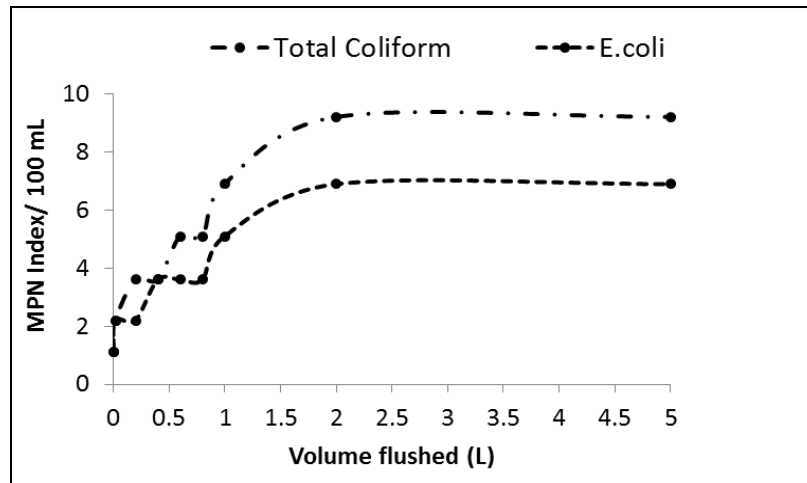
Figure 4.28: Total coliform and E.*coli* profiles

4.8.   EFFECT OF PIPE DIAMETER ON CHLORINE

While the chlorine profiles were being determined it was observed that changes in chlorine concentrations occurred at points where the pipe diameter changes (Figure 4.29)
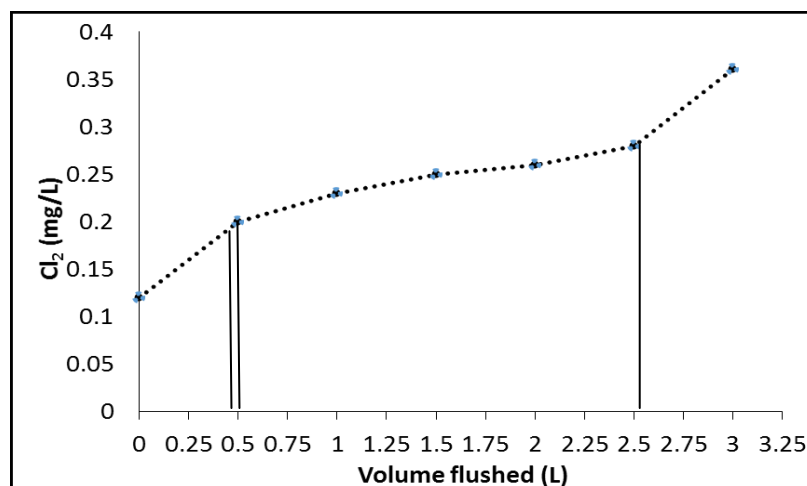
Figure 4.29: Changes in chlorine concentrations with pipe diameter

Starting from tap it was observed that when 0.4 to 0.5 L of water were flushed, major changes in chlorine concentrations were observed. Chlorine concentration again changed majorly when 2.5 to 3 L of water was flushed. Considering the pipe diameters, lengths and flow rate 0.21 L/s, it was concluded that pipe diameter changes at these points which accounts for changes in chlorine concentration. As the diameter decreases in the pipes to about 0.5 inches, the chlorine consumption increases. This is because higher S/V ratio provides higher surface area of pipe that is in contact with chlorine.

4.9. IDENTIFICATION OF BACTERIAL SPECIES

4.9.1. Bacterial species isolated form GI and PPR pipes

Bacterial strains were isolated and biochemical tests were conducted to characterize the microbial populations predominant in stagnant and flushed Galvanized Iron (GI) and Polypropylene (PPR) pipes. Figure 4.30 shows the presence of bacterial communities in stagnant and flushed GI pipes
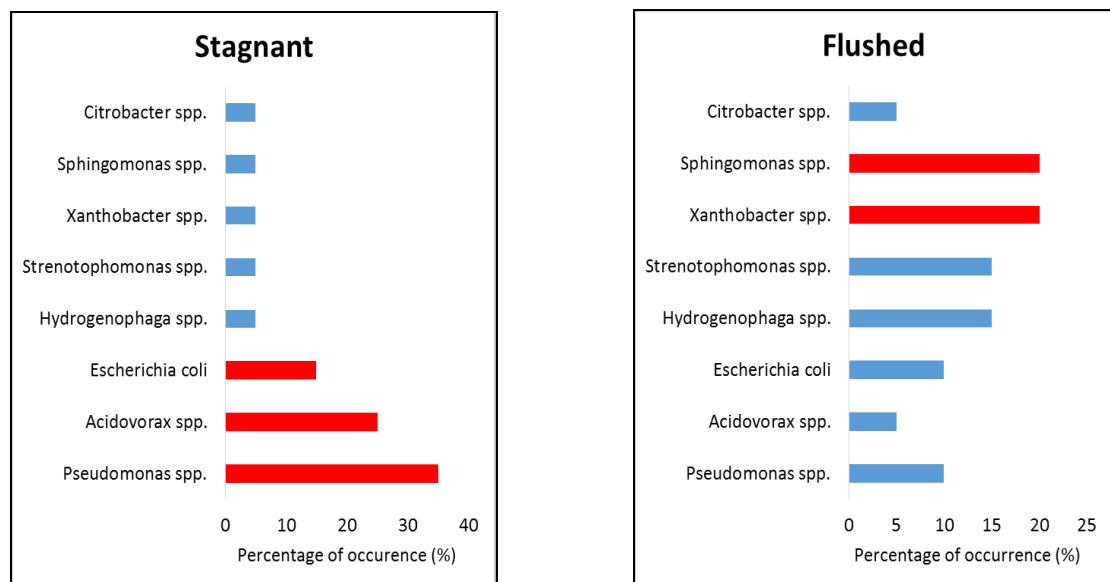
Figure 4.30: Bacterial species isolated from GI pipes

Pseudomonas spp., *Escherichia coli, and Acidovorax* spp were the predominant genera of bacteria that colonized stagnant Galvanized iron pipes while *Sphingomonas* and *Xanthobacter* spp. were found predominant in flushed samples. Presence of these species may be the reason of higher bacterial counts obtained in stagnant samples of GI pipes with longer service age as literature reports the role of some of these bacteria in corrosion of iron pipes as iron oxidizing bacteria (IOB) and iron reducing bacteria (IRB). *Acidovorax, Gallionella, Leptothrix, Sphaeotilus* are reported as IOB and *Bacillus, Clostridium, Pseudomonas* and *Escherichia coli* are reported as IRB (Sun *et al*., 2014). Biofilms formed on the inner surface of the pipes may shelter many opportunistic pathogens (Berry *et al*., 2010).

Principal species identified in stagnant polypropylene pipes were *Strenotophomonas* spp., *Acinetobacter* spp. and *Enterobacter* spp. while *flavobacterium* spp., *Methylobacterium* spp. and *Sphingomonas* spp. were isolated from flushed PPR pipes (Fig. 4.31*)*.
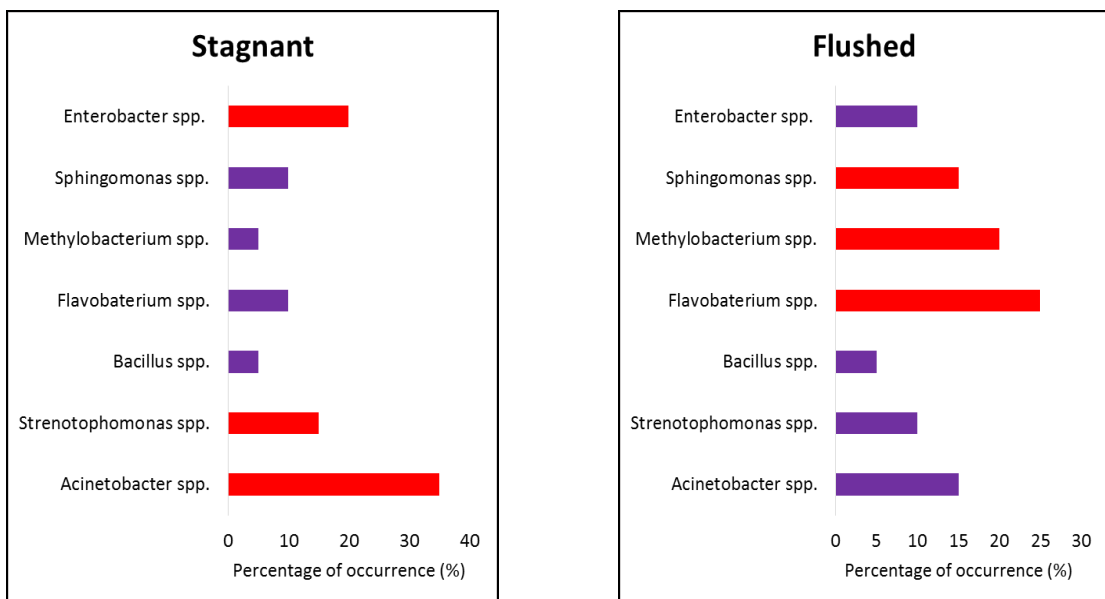
Figure 4.31: Bacterial species isolated from PPR pipes

Table 4.3: Characterization of isolated specie

Characteristics	Observations								
	MSI	**MS2**	**MS3**	**MS4**	**MS5**	**MS6**	**MS7**	**MS8**	**MS9**
Colony Morphology	Shiny, slight raised, entire margins, mucoid, creamy and dark center	Translucent, smooth convex, entire margins, pale yellow or reddish yellow	Oval, rough, convex, wavy margins, mucoid, pigmented green	Glistening, circular, pinpoint, entire, yellow to translucent	Translucent, Smooth, creamy white	Shiny, round, flat, undulate, umbonate, mucoid, white translucent	Circular, convex, smooth or rough, moist, gray-white	Circular, convex, irregular, outgrowths from margins, Gray-white	Opaque, Smooth, mucoid, spreading margins, pale colonies
Cell Morphology	Gram negative (rods)	Gram negative (rods)	Gram negative (rods)	Gram negative (rods)	Gram negative (rods)	Gram negative (rods)	Gram positive (rods)	Gram negative	Gram negative (rods)
Motility test	+	-	+	+	+	-	-	+	-
Optimum temperature	37°C	30-37°C	37°C	30-37°C	37°C	37°C	30°C	37°C	45 °C
Optimum pH	7	7	7	6	7	7	8	7	6
Oxidase	-	-	+	+	+	-	+	+	-
Catalase	+	+	+	+	+	+	+	-	+
Growth on EMB	+ (Green sheen)	+ (pink-purple)	+ (colorless)	-	-	+ (brown, dark centered)	-	-	+ (blue-blue gray)
Growth on MacConkey	+ (pink)	+ (red)	- (pale yellow colonies)	-	- (colorless-light pink)	+ (pink)	-	-	-
Oxidation /Fermentation	Facultative anaerobic	Aerobic	Aerobic	Aerobic	Aerobic	Facultative anaerobic	Aerobic	Aerobic	Aerobic

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

- Complex interaction exists among factors that degrade bacterial quality of water during stagnation.

- Stagnation of water leads to an increase in HPC and decrease in total and fecal coliform.

- HPC growth in non-chlorinated water reached its peak at 18 hours as compared to chlorinated water where bacterial growth continues uptil 72 hours.

- Flushing of taps resulted in 93-95% reduction in HPC upon 2L flushing. At a flow rate of 0.21 L/s it took approximately 10 seconds.

5.2 Recommendations

Following research studies may be carried out

1. Formation and composition of biofilm in other pipe materials like polyvinyl chloride, cast iron or copper pipes.

2. Advanced techniques such as Denaturing Gradient Gel Electrophoresis (DGGE) and Polymerase Chain Reaction (PCR) to identify changes in community composition during stagnation.

3. Other disinfectants like ozone and ultraviolet may be studied.

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

API 20 E results interpretation

Test	Substrate	Reaction tested	+ Results	-Results
ONPG	ONPG	Beta-galactosidase	Colorless	Yellow
ADH	Arginine	Arginine dihydrolase	Yellow	Red/Orange
LDC	Lysine	Lysine decarboxylase	Yellow	Red/Orange
ODC	Ornithine	Ornithine decarboxylase	Yellow	Red/Orange
CIT	Citrate	Citrate utilization	Pale green/yellow	Blue-green/Blue
H_2S	Na Thiosulfate	H_2S production	Colorless/gray	Black deposit
URE	Urea	Urea hydrolysis	Yellow	Red/orange
TDA	Tryptophan	Deaminase	Yellow	Brown-red
IND	Indole	Indole production	Yellow	Red (2 min)
VP	Na pyruvate	Acetoin production	Colorless	Pink/red (10 min)
GEL	Charcoal gelatin	Gelatinase	No black diffusion	Black diffuse
GLU	Glucose	Fermentation/Oxidation	Blue/blue-green	Yellow
MAN	Mannitol	Fermentation/Oxidation	Blue/blue-green	Yellow
INO	Inositol	Fermentation/Oxidation	Blue/blue-green	Yellow
SOR	Sorbitol	Fermentation/Oxidation	Blue/blue-green	Yellow
RHA	Rhamnose	Fermentation/Oxidation	Blue/blue-green	Yellow
SAC	Sucrose	Fermentation/Oxidation	Blue/blue-green	Yellow
MEL	Melibiose	Fermentation/Oxidation	Blue/blue-green	Yellow
AMY	Amygdalin	Fermentation/Oxidation	Blue/blue-green	Yellow
ARA	Arabinose	Fermentation/Oxidation	Blue/blue-green	Yellow
OX	Oxidase	Oxidase	Colorless/Yellow	Violet

API 20 Staph results interpretation

Tests	Active Ingredients	Reactions / Enzymes	-Results	+Results
0	No substrate	Negative control	Red	-
GLU	D-glucose	(Positive control) (D-Glucose)	Red	Yellow
FRU	D-fructose	Acidification (D-Fructose)		
MNE	D-mannose	Acidification (D-Mannose)		
MAL	D-maltose	Acidification (Maltose)		
LAC	D-lactose (bovine origin)	Acidification (Lactose)		
TRE	D-trehalose	Acidification (D-Trehalose)		
MAN	D-mannitol	Acidification (D-Mannitol)		
XLT	Xylitol	Acidification (Xylitol)		
MEL	D-melibiose	Acidification (D-Melibiose)		
NIT	Potassium nitrate	Reduction of Nitrates to nitrites	NIT 1+ NIT 2/10 mins	
			colorless-light pink	Red
PAL	ß-naphthyl phosphate	Alkaline Phosphatase	ZYM A + ZYM B / 10 min	
			Yellow	
VP	Sodium pyruvate	Acetyl-methyl-carbinol production	VP1 +VP2/ 10 mins	
			colorless-light pink	Violet pink
RAF	D-raffinose	Acidification (Raffinose)	Red	Yellow
XYL	D-xylose	Acidification (Xylose)		
SAC	D-saccharose (sucrose)	Acidification (Saccharose)		
MDG	Methyl-αD-Glucopyranoside	Acidification (Methyl-αD-Glucopyranoside)		
NAG	N-acetyl-glucosamine	(N-Acetyl-Glucosamine)		
ADH	L-arginine	Arginine Dihydrolase	Yellow	Orange-red
URE	Urea	Urease	Yellow	Red-violet

API 20 NE results interpretation

Tests	Active Ingredients	Reactions/Enzymes	- Results	+ Results
NO₃	Potassium nitrate	Reduction of nitrates to nitrites	NIT 1+ NIT 2 (5 min)	
			Colorless	Pink red
			Zn (5 min)	
			Pink	Colorless
TRP	L-trytophane	Reduction of nitrates to nitrogen	JAMES(immediate)	
			Colorless Pale green/yellow	Pink
GLU	D-glucose	Indole production (tryptophane)	Blue to green	Yellow
ADH	L-arginine	Fermentation (Glucose)	Yellow	Orange/pink/red
URE	Urea	Arginine dihydrolase	Yellow	Orange/pink/red
ESC	Esculin ferric citrate	Urease	Yellow	Gray/brown /black
GEL	Gelatin(bovine origin)	Hydrolysis (b-glucosidase)	No pigment diffusion	Diffusion of black pigment
PNGP	4-nitrophenyl-Bd-galactopyranoside	b-galactosidase(para-nitrophenyl-Bd-galactopyranosidase)	Colorless	Yellow
GLU	D-glucose	Assimilation (Glucose)	Transparent	Opaque
ARA	L-arabinose	Assimilation (arabinose)	Transparent	Opaque
MNE	D-mannose	Assimilation (mannose)	Transparent	Opaque
MAN	D-mannitol	Assimilation (mannitol)	Transparent	Opaque
NAG	N-acetyl-glucosamine	Assimilation (N-acetyl-glucosamine)	Transparent	Opaque
MAL	D-maltose	Assimilation (Maltose)	Transparent	Opaque
GNT	Potassium gluconate	Assimilation (Potassium gluconate)	Transparent	Opaque
CAP	Capric acid	Assimilation (capric acid)	Transparent	Opaque
ADI	Adipic acid	Assimilation (adipic acid)	Transparent	Opaque
MLT	Malic acid	Assimilation (malate)	Transparent	Opaque
CID	Trisodium citrate	Assimilation (trisodium citrate)	Transparent	Opaque
PAC	Phenylacetic acid	Assimilation (phenylacetic acid)	Transparent	Opaque
OX	Oxidase	Cytochrome oxidase	Colorless/light purple	Pink

ANNEXURE-II

Pipe diameter and length determination

Flushing of taps

Oxidase test

Catalase test