INACTIVATION OF PATHOGENS BY CHLORINE IN A PROTOTYPE DISTRIBUTION NETWORK

By

Sidra-Tul-Muntaha

(2011-NUST-MSPhD-EnvS-22)

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

Master of Science

In

Environmental Sciences

Institute of Environmental Sciences and Engineering (IESE)

School of Civil and Environmental Engineering (SCEE)

National University of Sciences and Technology (NUST)

Islamabad, Pakistan

(2014)

INACTIVATION OF PATHOGENS BY CHLORINE IN A PROTOTYPE DISTRIBUTION NETWORK

By

Sidra-Tul-Muntaha

(2011-NUST-MSPhD-EnvS-22)

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

Master of Science

in

Environmental Sciences

Institute of Environmental Sciences and Engineering (IESE)

School of Civil and Environmental Engineering (SCEE)

National University of Sciences and Technology (NUST)

Islamabad, Pakistan

(2014)

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

INACTIVATION OF PATHOGENS BY CHLORINE IN A PROTOTYPE DISTRIBUTION NETWORK

Submitted by

Sidra-Tul-Muntaha

Has been found satisfactory for the requirements of the degree of

Master of Science in Environmental Sciences

Supervisor: _____
Dr. Imran Hashmi
Professor
IESE, SCEE, NUST

Member: _____
Dr. Ishtiaq A. Qazi
Professor & Associate Dean
IESE, SCEE, NUST

Member: _____
Dr. Zahiruddin Khan
Associate Professor & HoD-EE
IESE, SCEE, NUST

External Member: _____
Dr. Habib Nasir
Professor
SCME, NUST

I would like to dedicate my thesis to my beloved parents

For their love, support and prayers

ACKNOWLEDGEMENTS

In the Name of Allah, the Beneficent, the Merciful

First praise is to Allah, the Almighty, on whom ultimately we depend for sustenance and guidance. Second, my sincere appreciation goes to my honorable supervisor, Prof. Dr. Imran Hashmi, IESE, SCEE, NUST, Islamabad, for his dynamic supervision, able guidance and encouragement during my study and research work presented in this dissertation.

I am deeply indebted to Prof. Dr. Ishtiaq A. Qazi, Associate Dean, IESE, SCEE, NUST, Islamabad, for providing me valuable comments and suggestions. My special thanks are for Prof. Dr. Habib Nasir, SCME, NUST, Islamabad, without whose assistance and support it was not possible to accomplish the task. I am also grateful to the amicable and valuable suggestions by Dr. Zahiruddin Khan, HoD Environmental Engineering, IESE, SCEE, NUST, Islamabad.

I would also like to thank the Environmental Microbiology Research Group especially Ms. Sajida Rasheed for establishing lab scale distribution network at IESE, Microbiology Lab and Ms. Romana Khan for helping me out in gas chromatography analysis and its optimization. Sincere thanks to all my friends especially Sahaab Farooq, Sara Qaiser, Arish Naseem, Mehreen Khan, Khaula Balouch, and Amina Khalid for their kindness and moral support during my study.

I gratefully acknowledge here the funding received from NUST R &D project (2013-2014) entitled “Kinetics of drinking water disinfection byproducts (chlorine) at Lab Scale and within the distribution network” for necessary equipment.

My deepest gratitude goes to my beloved parents and family for their endless love, prayers and encouragement throughout my research work.

SIDRA-TUL-MUNTAHA

Table of Contents

LIST OF ABBREVIATIONS

µg/L	Microgram per Litre
mg/L	Milligram per Litre
CFU	Colony forming unit
CT	Chlorine dose x time
CSM	Charcoal Selective Medium
DBPs	Disinfection By-Products
EPA	Environmental Protection Agency
GC	Gas chromatography
HAAs	Haloacetic acids
HEC	Higher Education Commission
MCL	Maximum Contaminant Level
NOM	Natural Organic Matter
THMs	Trihalomethanes
TOC	Total Organic Carbon
UNDP	United Nations Development Programme
UNEP	United Nations Environment Programme
WHO	World Health Organization
WRI	World Resources Institute
WWF	World Wide Fund
XLD	Xylose lysine desoxycholate

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Chlorine may kill microbes in contaminated water by disrupting their metabolism and protein synthesis processes but it does not kill all pathogenic bacteria, cysts, viruses and protozoa at normal dosages. The present study was designed to evaluate the inactivation efficiency of chlorine doses against selective pathogenic bacteria in a prototype distribution network. *Shigella flexneri* causative agent of *Shigellosis* and *Campylobacter jejuni* causative agent of *Campylobacteriosis* were selected as model organisms. Isolation and purification of *S. flexneri* and *C. jejuni* was carried out from Ratta Amral, a site associated with serious water quality issues according to an earlier study conducted for HEC project no. 20-874/HEC/R&D/07/379 entitled as "Monitoring of drinking water within the distribution network of Rawalpindi and Islambad". Chlorine disinfection studies were conducted in two phases i.e. Bench Scale and Lab Scale. For Bench scale studies, selected chlorine dosages against *S. flexneri* (10^7 CFU/mL) were 0.5, 1, and 1.25 mg/L whereas for *Campylobacter jejuni* (10^7 CFU/mL) selected chlorine dosages were 0.5 and 1 mg/L. Samples were taken at different time intervals of 0, 10, 15, 30, and 45 minutes. For lab scale studies survival of *S. flexneri* and *C. jejuni* was investigated at 1.25, 1.5 and 1 mg/L, respectively. Samples were taken at time intervals of 0, 30, 45, 60, 75 and 90 minutes. Bench scale results indicated that *S. flexneri* and *C. jejuni* showed 7-log reduction at CT values of 56.25 and 15 mg-min/L. Lab scale studies revealed that addition of humic matter increased the chlorine demand of water and both bacteria showed complete inactivation at CT value of 90 and 45 mg-min/L. TOC determination of organic matter at different time intervals revealed that bacterial load decreases with decrease in organic load. Detection of trihalomethanes including chloroform, bromodichloromethane and dibromochloromethane was also determined using gas chromatography. Chloroform concentration was highest among total trihalomethanes formed at different time intervals and their formation increases with increase in contact time and dissolved organic carbon as determined by absorbance of chlorinated water samples at UV_{254} nm using UV-Vis spectrophotometer.

Chapter 1

INTRODUCTION

Access to safe drinking water is the basic human right but water and sanitation are the most overlooked sectors in Pakistan thus making it stand at 80th among the states as regards the availability of safe and clean drinking water to its people (Azizullah *et al.*, 2011). As of 2005, about 38.5 million people lack access to clean drinking water causing many households to suffer from water borne illnesses. If this trend continues, further 52.8 million people will be deprived of safe drinking water by year 2015 (Sadia *et al.*, 2011). Inadequate treatment of surface and ground water, flawed sewage collection systems, and malfunctioned water distribution pipelines have led to contamination of potable water by fecal *coliform* and other pathogens (Wullings *et al.*, 2011). Contaminated water results in gastroenteritis and diarrhoea that are specified as the leading cause of mortality among infants and children in the developing world (Haydar *et al.*, 2009).

According to World Health Organization (WHO) Drinking Water Guidelines, *coliform* and *fecal coliforms* should be 0 CFU per 100 ml of water sample (WHO, 1993). Worldwide drinking water is routinely monitored for fecal contamination by testing for fecal-indicator organisms including *E. coli* and thermotolerant *coliforms* to ensure microbial safety (Van der Wielen and Medema, 2010). Though these indicator organisms based microbial safety evaluation measures have been used for many decades, yet there are other emerging water borne pathogens of concern including

Shigella, *Campylobacter*, *Legionella* and *Helicobacter* that are known to cause adverse health effects (Taylor *et al.*, 1991).

Among all commonly used disinfection practices, chlorine is so far the most cost-effective disinfectant. Its ability to destroy the outer surfaces of bacteria and viruses helped to eradicate water borne diseases such as cholera, typhoid and dysentery (ADS, 2005). Only chlorine-based disinfectants leave a favorable residual level within the water systems thus protecting it during distribution and storage. It has, therefore, been considered as a cost-effective disinfection approach especially for developing countries like Pakistan.

Water supplies containing natural organic matter (NOM), when disinfected with chlorine, result in the formation of chlorinated, brominated and, in much lesser levels, iodinated by-products known as disinfection by-products (DBPs). Two major classes of chlorinated disinfection by-products (DBPs) include trihalomethanes (THMs) and haloacetic acids (HAAs). These compounds have been reported to cause cancer, retard growth, trigger abortion, and promote congenital cardiac effects among consumers (Cedergren *et al*., 2002).

In Pakistan, only little work has been reported regarding the bacteriological quality of water. In addition, no proper conditions have ever been testified at any treatment plant for effective chlorination system to meet the drinking water standards.

The present study aimed at observing the effect of chlorine and contact time against survival of pathogenic *microorganisms* and formation of trihalomethanes at lab scale distribution network. To observe the effect of drinking water variables on chlorine

decay and bacterial regrowth, selected indigenous *microorganisms* like *Shigella flexneri* and *Campylobacter jejuni* will be isolated from the distribution network of Rawalpindi. After isolation and purification, these cultures will be used as pure culture for the chlorine disinfection studies with various variables like pH, turbidity, electrical conductivity, etc.

1.1. OBJECTIVES OF THE STUDY

Deterioration of water quality, within water distribution networks, has a great impact on human health and public acceptance of tap water reaching the consumers. Residual chlorine should be maintained through network pipes to prevent contamination and microbial regrowth to levels designated harmless by public health standards. Chlorine being a strong oxidant, however, combines with inorganic and organic material in water, thus increasing the chlorine demand of water. Hence, the chlorine dosage should be sufficient enough to fulfill the chlorine demand of source water. Also natural organic matter compounds are undesired in drinking water because they have a tendency to react with chlorine. Process of chlorine inactivation of *microorganisms* is affected by various factors within water distribution network like chlorine dose, temperature, residence time, organic matter concentration and pH.

Therefore, the present study focused on the following objectives;

- Isolation and Identification of *Shigella flexneri* and *Campylobacter jejuni*.
- Optimization of chlorine dosages for inactivation of *microorganisms*.
- Detection of trihalomethanes formation.

Chapter 2

LITERATURE REVIEW

Inactivation of bacteria and viruses is accomplished through a variety of chemical disinfection methods like chlorine, ozone and UV radiation. Among all these, chlorine has been recognized as an efficient and effective disinfectant. At the turn of 20^{th} century, water supplies were first chlorinated, and over the following two decades chlorination was introduced to disinfect water supplies in most industrialized countries (IARC, 1991). As a result of increased chlorination practices around the world, the outbreaks of cholera, typhoid and dysentery decreased in the earlier part of the twentieth century (Calomiris and Christman, 1998).

Chlorine and chlorine-based compounds are the only disinfectants that can kill micro-organisms in water and maintain its quality from the treatment plant to the consumer's tap. For effective chlorine disinfection, there must be a residual concentration of 0.5 ppm after at least 30 minutes contact time at pH<8.0 (UNICEF, 2005).

2.1. CHLORINE BACTERICIDAL ACTION

Leading advantage of chlorine usage is its broad-range germicidal potency and persistence in water supply systems. However, mechanism of this activity has not been fully determined despite much research.

When chlorine is added to water, hypochlorous acid is formed:

$$Cl_2 + H_2O \rightarrow HOCl + H^+ + Cl^-$$

Hypochlorous acid partly dissociates into hypochlorite ions depending on water pH.

$$Cl_2 + 2H_2O \rightarrow HOCl + H_3O^+ + Cl^-$$

$$HOCl + H_2O \rightarrow H_3O^+ + OCl^-$$

Hypochlorous acid molecules penetrate the cell membrane of bacteria; inhibit metabolic reactions and enzymes within cell results in bacterial death. Whereas hypochlorite ions cannot penetrate the cell membrane and acts only from outside causing damage to bacteria. Therefore, hypochlorous acid is 100 times more effective antimicrobial agent as compared to hypochlorite ions. It also indicates that microbial cell death results from chlorine attacking variety of bacterial molecules including lipids, enzymes and nucleic acids (Calomiris and Christman, 1998).

2.2. BENEFITS OF CHLORINATION

Current research studies show that the benefits of chlorinating drinking water are much greater than any health risks from trihalomethanes (THMs) and other by-products. Although various alternative disinfectants are available, chlorine will remain the choice of water treatment experts. When combined with modern water filtration methods, chlorine is effective against almost all microorganisms. Chlorine is easy to apply and small amounts remain present in the water supplies as chloramines thus

ensuring that microbes cannot re-contaminate the water after it leaves the treatment plant (Health Canada, 2009; Hafeez, 2008) Chlorine is also helpful to control taste and odor problems by oxidizing foul-smelling algae, decaying vegetation, hydrogen sulfide and ammonia (Calomiris and Christman, 1998).

2.3. SELECTED ENTERIC PATHOGENS

Shigella flexneri, a facultative anaerobe belonging to the family *Enterobacteriaceae*, is the causative agent of diarrhea and dysentery in humans. Potentially life-threatening, *S. flexneri's* effects include bacteremia, hemolytic uremic syndrome (HUS) and toxic megacolon (Lew *et al.,* 1991). The principle disease of diarrhea and dysentery caused by this pathogen is known as *Shigellosis*. 10-100 organisms are sufficient to cause disease, and transmission is generally from person-to-person by way of fecal-oral route (Beletshachew *et al.,* 2004). Typically, *Shigellosis* caused by *S. flexneri* is considered as a third world disease. Approximately 150 million cases per year of *Shigellosis* are reported in third world countries as compared to about 1.5 million cases per year in developed countries (Schaechter *et al.,* 1999). It accounts for approximately 1 million deaths per year worldwide. *Shigellosis* can be characterized as a disease with over 60% incidences in children age 1-5 (Schaechter *et al.,* 1999).

Campylobacter jejuni is a microaerophilic organism, which means it has a requirement for reduced levels of oxygen. It is relatively fragile, and sensitive to environmental stresses. Because of its microaerophilic characteristics the organism requires 3 to 5 % oxygen and 2 to 10 % carbon dioxide for optimal growth conditions (Parkhill *et al.,* 2000). This bacterium is now recognized as an important enteric pathogen. Surveys

have shown that *C. jejuni* is the leading cause of bacterial diarrheal illness worldwide. It causes more diseases than *Shigella spp.* and *Salmonella spp.* combined.

Campylobacteriosis is the name of the illness caused by *C. jejuni*. It is also often known as *campylobacter* enteritis or gastroenteritis. Infection causes diarrhea, fever, abdominal pain, nausea, and headache and muscle pain. The infective dose of *C. jejuni* is considered to be small; about 400-500 bacteria may cause illness in some individuals (Friedman *et al.,* 2000).

2.4. DRINKING WATER CHLORINATION IN PAKISTAN

Pakistan is facing critical water scarcity and deteriorating water quality issues. In year 2007, it was reported by World Wild Fund (WWF) that 70 percent of the drinking water supplied to public by water supply authorities was contaminated with pathogenic *microorganisms* (WWF, 2007). Also UNICEF in 2002 reported that only 54% of Pakistan's population had access to appropriate sanitation facilities with more percentage of urban residents (92%). Mortality among children under five years was 101 per 1000 live births in the year 2004 (Shehzad, 2006). High rate of water borne illnesses is mostly associated with shiga toxin (STEC) and enterotoxin produced by *Escherichia coli* (ETEC) (Ram *et al.,* 2009).

Chlorination of drinking water is the most common method applied in Pakistan at treatment plants. Most water supply systems in Pakistan are not working exactly the way they are meant to perform disinfection and many are completely dysfunctional.

2.5. CHLORINE DISINFECTION OF MICROORGANISMS IN DRINKING WATER

Campylobacter jejuni was identified as a significant enteric pathogen of humans. Wang *et al.,* (1983) investigated the effect of hypochlorite ion on the survival of *Campylobacter jejuni* in drinking water using 1.25 and 5 mg/L of hypochlorite ion. 1.25 mg/L of dose destroyed three strains of inoculum size ranging from 10^3 to 10^4 CFU/mL in 1 min of contact time. 5 mg/L of hypochlorite killed three strains with an inoculum size of 10^6 to 10^7 in 15 minutes. Results demonstrate that, under the conditions tested (pH 7.0; 24 to 26^{o}C), the recommended standard concentration of disinfecting agent was adequate to destroy *C. jejuni*.

In another study by Blaser *et al.,* (1986), vulnerability of three strains of *C. jejuni* and *E. coli* were compared against chlorine and chloramine disinfectants. Selected bacterial strains were inactivated using chlorine and monochloramine doses of 0.1 and 1.0 mg/L, respectively. Inactivation studies were done at pH 6 and 8 and at temperatures 4 and 25^{o}C. All the three *C. jejuni* strains were more susceptible than the *E. coli* strain with more than 99% of inactivation for every condition being tested. Optimized contact time for 1 mg/L of monochloramine and 0.1 mg/L of chlorine was 15 and 5 mins, respectively. The study concluded that normal disinfection procedures carried out for drinking water treatment are adequate to eliminate *Campylobacter jejuni*.

Vulnerability to chlorine disinfectant for two enteric pathogens, *Campylobacter jejuni* and *Yersinia enterocolitica,* and one indicator bacterium *Eschericiha coli*, was

investigated by Lund (1996) using autoclaved lake water at two temperatures 4 and 10°C. Exposure to 0.2 mg/L of chlorine resulted in 3-log reduction of *C. jejuni* in 10–15 seconds, *Y. enterocolitica* in 20–180 seconds, and *E. coli* in 20–25 seconds. *C. jejuni* being more sensitive to chlorine showed same log reduction at 0.02-0.04 mg/L of free chlorine in 12 min at 4°C and 2 min at 10°C. *Y. enterocolitica* and *E. coli* showed same sensitivity to chlorine. It was also revealed that *E. coli* is not a safe indicator for the occurrence of *Y. enterocolitica* under all studied conditions.

A study was conducted by Rice *et al.,* (1999) to determine the resistance of extremely pathogenic avian influenza (H5N1) virus to chlorination. On exposure of two virus strains to chlorinated buffer solution at pH 7 or 8 at 5°C it was found out that free chlorine concentrations normally used in drinking water treatment was adequate to inactivate the respective virus by greater than 3 orders of magnitude.

Park *et al.,* (2002) studied effectiveness of electrolyzed (EO) and chlorinated water for killing *Campylobacter jejuni*. It was revealed that *C. jejuni* inactivation occurred within 10 seconds of exposure to EO or chlorinated water with 50 mg/L of residual chlorine. Diluted EO water containing 25 mg/L of residual chlorine also showed strong bactericidal activity and mean population of *C. jejuni* reduced to 10 CFU/mL after 10 seconds of exposure. Diluted chlorine water was less effective than the diluted EO water for inactivation of *C. jejuni*.

Gagnon *et al.,* (2004) evaluated disinfection potential of chlorine dioxide, free chlorine and chloramines on bacterial water quality within model distribution system. Two disinfectant doses were evaluated for the mentioned disinfectants. Bacterial

inactivation varied as a function of disinfectant type, dose, sample type (bulk water or biofilm bacteria) and pipe material under specific environmental circumstances. Disinfectants were ranked according to their efficiency in the following order: chlorine dioxide > chlorine > chloramines. Continuous disinfectant application on young biofilms of 10^6–10^7 CFU/cm^2 resulted in log removal of heterotrophic bacteria concentrations from 1.1 to 4.0 and from 0.2 to 2.5, respectively. An additional log inactivation of 1–2.5 of heterotrophic bacteria counts occurred on doubling the amount of disinfectant dose. Outcomes of the study demonstrated that bacterial inactivation in distribution systems was merely governed by numerous inter-related parameters.

Salmonella typhi and *Shigella flexneri* due to their significant public health impacts were investigated for their survival in a number of aqueous media including distilled water, 0.9 % NaCl solution, and chlorinated water and at different temperatures by Uyanik *et al.,* (2008). It was observed that *S. flexneri* survived the longest in both 0.9 percent NaCl solution and distilled water (87 and 83 days, respectively) when kept in the refrigerator. In the same way, *S. typhi* was found to be viable for about 65 days in both media (0.9 % NaCl solution and distilled water), when stored in the refrigerator. However, both bacteria showed no growth at all in chlorinated water samples kept at all four temperatures in the first 24 hours. *S. flexneri* generally persisted longer than *S. typhi* in the different water media being tested. Thus, it was concluded that *S. flexneri* being more persistent would present more risk in the spread of waterborne infections than *S. typhi*.

Inactivation of infectious viruses against free chlorine and monochloramine was evaluated by Theresa *et al.,* (2010). Selected viruses were human adenoviruses 2, 40,

and 41 (HAdV2, HAdV40, and HAdV41), coxsackieviruses B3 and B5 (CVB3 and CVB5), echoviruses 1 and 11 (E1 and E11), and murine norovirus (MNV). Applied chlorine and monochloramine doses were 0.2 and 1 mg/L, respectively at pH 7 and 8 in buffered solution at 5°C. Enteroviruses took the longest time for chlorine inactivation followed by MNV. For 4 log removal, CVB5 took the longest contact time with CT (Concentration x Time) values of 7.4 and 10 mg-min/liter at pH 7 and 8 respectively. Monochloramine disinfection was found to be more effective for E1 as CT values ranged from 8 to 18 mg-min/liter for 2- and 3-log reductions, respectively. In case of E11 and HAdV2, 3-log reductions occurred at CT values of 1,300 and 1,600 mg-min/liter. At pH 7, monochloramine inactivation was most successful for the adenoviruses, CVB5, and E1. Viruses were least susceptible to monochloramine disinfection than chlorine disinfection.

Dupuy *et al.,* (2011) conducted study using three oxidizing disinfectants i.e. chlorine, monochloramine and chlorine dioxide against free-living amoeba as a reservoir for pathogenic *Legionella pneumophila*. For study purpose, three *Acanthamoeba* strains and *L. pneumophila* alone or in co-culture was used. Samples were treated with disinfectants for 1 hour and the concentration of disinfectant was followed to calculate disinfectant exposure (Ct). *Acanthamoeba* strains showed significant differences of susceptibility to the three disinfectants they were exposed to. However no profound difference was observed between infected and non-infected *Acanthamoeba*. Comparison between the three disinfectants showed that monochloramine was efficient at the same level towards free or co-cultured *L. pneumophila* while chlorine and chlorine dioxide were least effective towards co-cultured *L. pneumophila*.

Ten different genera of gram negative bacteria including *E. coli, Citrobacter spp., Shigella spp., Enterobacter spp., Providencia spp., Klebsiella spp., Salmonella spp., Pseudomonas spp., Proteus spp. and Edwardsiella spp.,* were investigated by Bishankha *et al.,* (2012) against the effect of chlorination. Higher bacterial growth was observed at higher temperature (p= 0.002), and in the same way greater level of residual chlorine reduced the bacterial growth (p= 0.037). Increase or decrease of pH (p= 0.454), turbidity (p= 0.164) and conductivity (p= 0.969) did not affect the microbial growth. Between heterotrophic plate count and residual chlorine a negative correlation (r= -0.162) was observed, with no statistical significance (p= 0.096). Correspondingly, negative correlation (r= -0.383) among total *coliform* count and residual chlorine was observed with statistical significance (p= 0.001). All the tested eight genera of gram negative bacteria were found to be chlorine resistant at 0.2 mg/l for a contact time of 30 minutes. Average time required for maximum log reduction of viable isolates was found to be less than 60 minutes and greater than 30 minutes respectively.

Inactivation experiments were conducted using seven strains of chlorine-resistant bacteria, isolated from a drinking water distribution system, with four different kinds of disinfectants by Chen *et al.,* (2012). All the tested seven bacterial strains exhibited high resistance to chlorine, especially *Mycobacterium mucogenicum*. 4-log reduction of *M. mucogenicum, Sphingomonas sanguinis* and *Methylobacterium* occurred at CT value of 120, 7 and 4 mg-min/L, respectively. Chlorine dioxide and potassium monopersulfate resulted in 5-log reduction of *M. mucogenicum* within 30 minutes. Chloramination was only effective as a disinfectant at higher concentration. Free chlorine was less effective because it decayed very rapidly. 99.9% inactivation of

highly chlorine-resistant *M. mucogenicum* occurred at 3.0 mg/L of monochloramine, 1.0 mg/L of chlorine dioxide, and 1.0 mg/L of potassium monopersulfate for exposure time of 60 minutes.

Chlorine inactivation of highly pathogenic free-living protozoa *Naegleria fowleri*, responsible agent of meningoencephalitis was assessed by Payal and Charles, (2012). CT factor for inactivation of *N. fowleri* at 1 mg/L was determined using the efficiency factor Hom model. EHM was applied to observe bench scale values to generate C × T values for 2, 3, and 4-log reductions of *Naegleria fowleri*.

Liu *et al.*, (2013) studied *Bacillus subtilis (*ATCC6633) as potential model for resistant microorganism against the effect of chlorine in drinking water and also factors that influence chlorine disinfection including chlorine concentration, contact time, pH, temperature, and initial density of spores. Selected chlorine concentration, reaction time, pH, temperature, and the initial density of spores for the study ranged from 2.06 to 10.3 mg/L, 0 to 166 min, 6 to 9, 1 to 30℃ and 102 to 1012 cfu/ml, respectively. Results indicated that the inactivation process of *Bacillus* spores with chlorine was characterized by a lag and a log phase of inactivation. Inactivation rate was higher at high concentration of disinfectant and longer reaction time. Under acidic conditions chlorine inactivating ability was stronger as compared to alkaline conditions. Increase in temperature also enhanced the inactivation capacity of chlorine but no profound effect was observed with increase in density of spores. This study also concluded that *Bacillus subtilis* spores were more resistant to chlorine than *Bacillus anthracis* spores.

2.6. CHLORINATION AND DISINFECTION BY-PRODUCTS FORMATION

Disinfection by-products are formed as a result of chlorine disinfectant reaction with an organic precursor, represented by various components of natural organic matter (NOM), and an inorganic precursor, most often certain halide ions. Chlorine oxidizes complex NOM molecules into simpler moieties, which are then reactive with additional chlorine acting as a halogen substitution agent and also oxidizes bromide into bromine which are less effective oxidants but more effective substitution agents than chlorine (Krasner, 2009). Other influential factors affecting the formation of halogenated DBPs include NOM concentration as dissolved organic carbon (DOC), pH, temperature, disinfectant concentration and reaction time.

Trihalomethanes are considered as carcinogens (National Cancer Institute, 1976; National Toxicology Program, 1986; National Toxicology Program, 1989) and among them chloroform also known as trichloromethane ($CHCl_3$) is of prime importance because of its severe impacts on the nervous system, kidney, liver and the reproductive system. Other trihalomethanes (THMs) include bromoform ($CHBr_3$), bromodichloromethane ($CHCl_2Br$), and dibromochloromethane ($CHClBr_2$). Maximum contaminant level (MCL) for total trihalomethanes (TTHMs) in drinking water is 0.08 mg/L (USEPA, 2002). WHO guideline values for $CHCl_3$, $CHCl_2Br$, $CHClBr_2$ and $CHBr_3$ are 200, 60, 100 and 100 ppb respectively.

Haloacetic Acids (HAAs) include monochloroacetic acid, dichloroacetic acid, trichloroacetic acid, and monobromoacetic acid. Environmental Protection Agency (EPA) reported that long term consumption of water with levels of HAA's above

maximum contaminant level (MCL) that is 0.06 mg/L may cause an increased risk of cancer. Haloacetic Acids are classified by the EPA as a Group 2B cancer classification (possibly carcinogenic to humans) because there is evidence of carcinogenicity in experimental animals, but there is either no evidence or not sufficient evidence of carcinogenicity in humans (USEPA, 2002; Health Canada, 1994).

Table 2.1 Maximum Contaminant Level Goals in Drinking water (USEPA, 1998)

Contaminant	Maximum Contaminant Level Goals (µg/l)
Chloroform	70
BDCM	0
DBCM	60
Bromoform	0

Seven different natural waters were collected and treated with five types of disinfectants including chlorine, chloramine, both with and without pre-ozonation, as well as chlorine dioxide. Disinfection by-product formation potential of all five disinfectants was investigated by Guanghui and David, (2007). Outcomes of the study showed that pre-ozonation diminish the formation of disinfection by-products for majority of waters. Water with low humic content showed increase THMs, HAAs and TOX concentrations. Pre-ozonation when done with chloramination showed increase or decrease formation of dihaloacetic acids and unidentified TOX (UTOX). Chloramines plus chlorine dioxide created a higher percentage of UTOX than free chlorine. They also produce more iodoform in addition to total organic iodine (TOI) than free chlorine in the existence of iodide. Free chlorine formed much advance level

of overall organic chlorine (TOCl) plus bromine (TOBr) than chloramines as well as chlorine dioxide in the existence of bromide.

Extensive research was carried out by Hong *et al.,* (2007) to examine as well as model THM formation in Dongjiang river water samples after chlorination. Results showed that overall THMs formation varies between 11.7-91.8 mg/L due to controlled reaction time and bromine levels. Bromide concentration was a key factor in measuring bromine-containing THMs. Greater concentration of bromine results in more bromine-containing THMs.

Brominated DBPs are more carcinogenic than their chlorinated analogs. Role of bromide ion in the formation and speciation of disinfection by-products (DBPs) during chlorination of Buyukcekmece Lake Water (BLW) in Istanbul was evaluated by Uyak and Toroz, (2007). Chlorination of BLW samples was carried out at pH 7.0 with 5 and 12 mg/l of chlorine dosages. For each chlorine dosage, six bromide concentrations ranging from 0.05 to 4.0 mg/L were used. Increasing bromide concentration gradually shifted trihalomethanes (THMs) and haloacetic acids (HAAs) speciation from chlorinated species to the mixed bromochloro species during chlorination. The halogen substitution ability of HOBr and HOCl during the formation of THMs and HAAs was estimated through the use of probability theory. It was concluded that, in both halogen substitution for THM and dihalogenated HAA formation, HOBr was found to be 20 times more reactive than HOCl.

Abdel-Wahab *et al.,* (2010) investigated formation of halogenated organic compounds as a result of chlorination and found out that concentrations of THMs increased rapidly

with time during the first half hour. Chlorination of seawater has shown significant increase in total THMs (TTHMs) and in bromoform concentrations. Rapid decrease of UV absorbance at 254 nm was also observed during seawater chlorination which is indicative of natural organic matter degradation into small organic molecules including THMs and other by-products. The increase in chlorine dose was accompanied with an increase in TTHMs and bromoform concentrations. Linear relationships between total chlorine concentration and both TTHMs and bromoform concentrations were established.

Presence of THM's in tap water samples collected from different localities within Karachi city was assessed by Karim *et al.,* (2011). For the determination of THM's, liquid-liquid extraction and gas chromatography coupled with electron capture detector was used. Concentrations of investigated THMs were found to be within WHO limits i.e. 80 ppb. Mean concentration of chloroform, bromodichloromethane and dibromochloromethane were found as 30.40, 1.04 and 0.09 µg/L respectively. Bromoform ($CHBr_3$) was not detected.

Concentrations of trihalomethanes (THMs) and haloacetic acids (HAAs) were measured in tap water passing through water distribution systems of six water treatment plants in Seoul, Korea and estimated using a probabilistic approach by Lee *et al.,* (2013). The concentration ranges for total THMs and HAAs were 3.9–53.5 and <LOD–49.5 µg/L, respectively. Among DBPs, chloroform, bromodichloromethane, dichloroacetic acid, and trichloroacetic acid were the most frequently detected. Spatial and seasonal variations in concentrations of THMs and HAAs in the six water distribution systems were significant ($P < 0.001$).

Ozdemir *et al.,* (2013) conducted chlorination experiments using water samples from Terkos and Buyukcekmece Lakes, Istanbul, Turkey. Changes in UV absorbance of water samples were characterized using defined differential UV spectroscopy (DUV), a novel spectroscopic technique. The maximum loss of UV absorbance for chlorinated Terkos Lake (TL) and Buyukcekmece Lake (BL) raw water samples were observed at a wavelength of 272 nm. Interestingly, differential absorbance at 272 nm (ΔUV_{272}) was shown to be a good indicator of UV absorbing chromophores and the formation of trihalomethanes (THMs) resulting from chlorination. The concentration of THMs formed when natural organic matter is chlorinated increases with increasing time and pH levels. Among all THMs, $CHCl_3$ was the dominant species formed as a result of the chlorination of TL and BL raw water samples.

Chapter 3

MATERIALS AND METHODS

For disinfection studies, *Shigella flexneri* and *Campylobacter jejuni* were isolated, purified and characterized according to the method by Cappuccino and Sherman, (1996). Chlorine disinfection studies against isolated strains were conducted in two phases; (1) Bench scale (2) Lab scale.

3.1. ISOLATION

3.1.1. Sampling

Water samples were collected from Ratta Amral, Rawalpindi, which has been identified as a site subjected to severe microbial contamination of drinking water in a study conducted for the HEC project entitled as "Monitoring of drinking water with in the distribution network of Rawalpindi and Islamabad" (Farooq *et al.*, 2008). Samples were collected in clean and sterilized glass sample bottles and stored at 4^{o}C in refrigerator before bacterial isolation.

3.1.2. Isolation procedure

Approximately 100 ml of water sample was passed through 0.45 µm filter paper. After that the filter paper was placed in peptone water for *S. flexneri* and preston broth for *C. jejuni* for 3-4 days at 37^{o}C and 42^{o}C in an incubator shaker for enrichment. Enriched sample was then streaked on selective mediums including MLA, DCA, XLD, BA and

CSM agar plates for overnight at 37^{o}C. Suspected *Shigella* and *Campylobacter* colonies were selected and subjected to biochemical tests for confirmation (Khanzadi *et al.,* 2010).

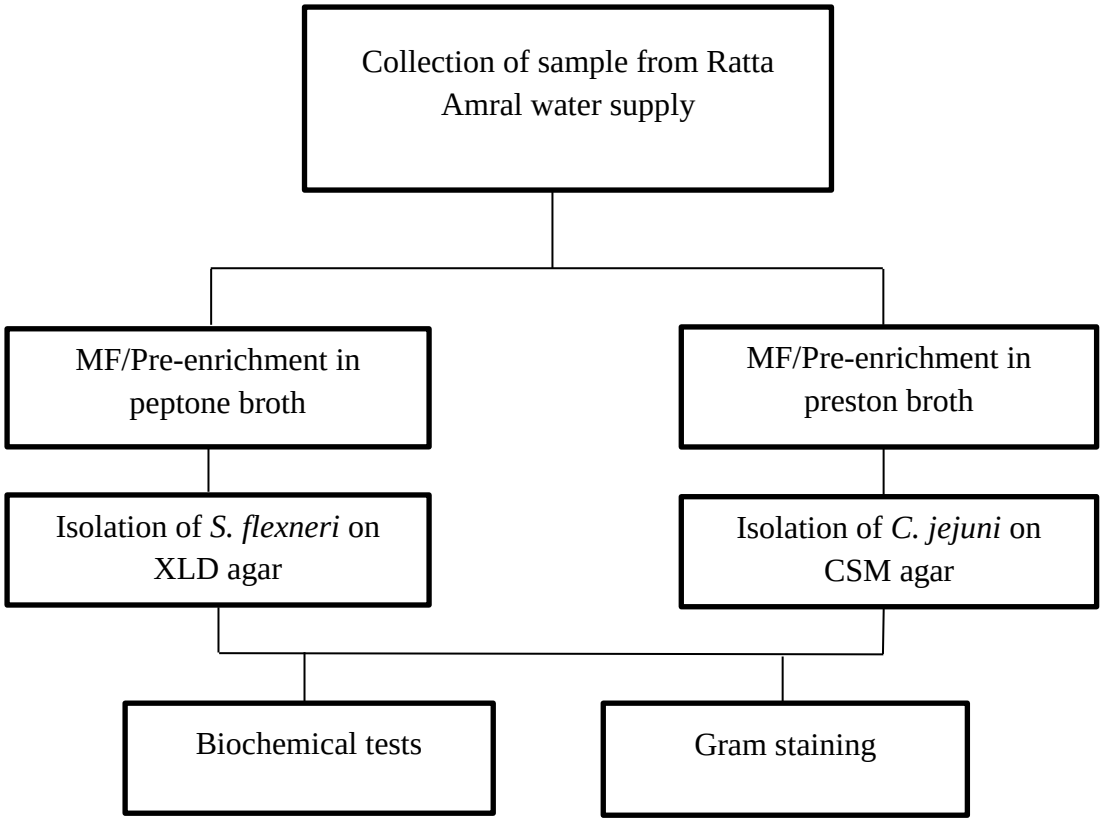


Figure 3.1 Schematic diagram of isolation of *Shigella* and *Campylobacter*

3.2. IDENTIFICATION

Identification of isolated strains was performed with the help of colony morphology, gram-staining, biochemical tests and API20E as per standard methods outlined in Bergey's Manual of Determinative Bacteriology (Holt *et al.,* 1994).

3.2.1. Morphological characteristics

Colony morphological characteristics of isolated strains grown on selective agar mediums were observed to identify and classify the strains. Cell morphology was observed through gram staining method (Beveridge, 1990) under oil immersion at 100X resolution. Observed morphological characteristics are stated in Table 3.1.

3.2.2. Biochemical tests for confirmation of *S. flexneri* and *C. jejuni*

Biochemical tests were performed to confirm *Shigella flexneri* and *Campylobacter jejuni* suspected colonies using different standard biochemical tests and API20E identification kit as given below;

3.2.2.1. Catalase production

Using sterile inoculating loop, suspected colony was picked and transferred on a clean slide and few drops of 3 percent hydrogen peroxide was then added on the slide. Bubbling showed positive result for catalase test.

Table 3.1: Common Description of Colony Morphology

Morphological characteristics	Description
Colony size	Punctiform, small, large
Margins	Entire, Undulate, filiform, curled, lobate
Forms	Circular, irregular, filamentous, rhizoid
Texture	Creamy, mucoid, dry
Elevation	Raised, convex, flat, umbonate
Colour	Yellow, orange, pale yellow, off-white

(CDC and WHO, 2003)

3.2.2.2. Simmon citrate test

The citrate test identifies the use of citrate as a sole carbon source by the bacteria. The basic end products will cause the bromo thymol blue indicator in the medium to turn from forest green to royal blue. Simmon's citrate agar slants were prepared and inoculated with 24 hour fresh culture of suspected colonies and incubated at 37^{o}C for 24 hrs.

3.2.2.3. Motility test

Freshly grown culture (24 hr old) was used for preparing the wet mount. The growth was transferred to a drop of water or saline solution on a microscope slide, mixed, and a cover slip was placed (Jarrell and McBride, 2008). Motility was interpreted using high dry magnification. Motility test is positive if bacteria swim randomly and if bacteria seem to be buffeted around and all moving in the same direction then there is no motility.

3.2.2.4. Mannitol fermentation test

An inoculum from fresh bacterial culture was transferred aseptically to a sterile test tube of phenol red mannitol broth. The inoculated tube was incubated at 37^{o}C for 24 hours. A positive mannitol test indicates a color change from red to yellow (Baron *et al.,* 1994).

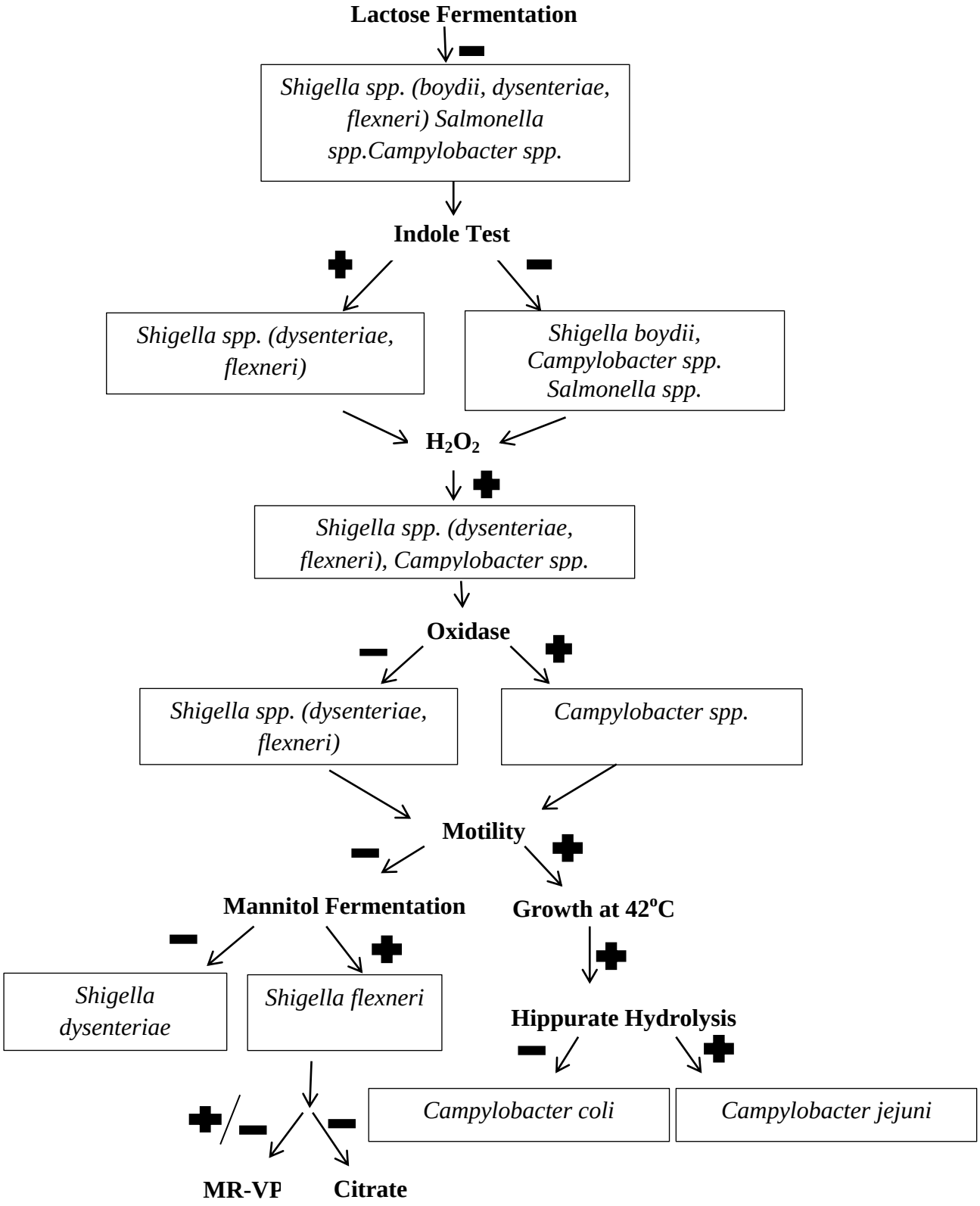


Figure 3.2 Schematic diagram of biochemical identification of *Shigella flexneri* and *Campylobacter jejuni*

Table 3.2: API E20 Interpretation

TESTS	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	tryptophan	indole production	yellow	red
VP	Na pyruvate	acetoin production	colorless	pink/red
GEL	charcoal gelatin	gelatinase	Black no 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	No color/ yellow	violet

3.2.2.5. Hippurate hydrolysis test

A loopful of the suspected colonies isolated on sheep blood agar was added to 0.5 ml of a 1% sodium hippurate solution and mixed by shaking, followed by 2 h incubation at 37°C in a water bath. Then, 0.2 ml of 3.5 % ninhydrin solution in a mixture of acetone and butanol (1:1) was added in each tube. For color development, further incubation was carried out at 37°C for 10 min. A deep purple color, crystal violet-like, was an indication of positive result, indicating the presence of glycine resulted from the hydrolysis of hippurate (Forbes and Sahm, 2002).

3.2.2.6. Analytical Profile Index-20E

API-20E test strip (BioMerieux, Canada) was used to identify the enteric gram negative rods. There are 20 separate small capules on the strip in dehydrated form each responding to a biochemical test. A bacterial suspension is used to rehydrate each of the wells. Single colony of the SM1 was inoculated into the 0.85 % NaCl solution, ensuring that the suspension was homogenous and without clumps of floating bacteria. Capules of LDC, ODC, ADH, H_2S, and URE are half filled. These capules were then filled up to the top with sterile mineral oil. CIT, VP, and GEL capules were filled upto the top of the well. Strip was labeled and kept in an incubator at 37^oC for 24 hours. After 24 hours reagents were added in IND, VP and TDA chambers. The positive and negative results of API E20 translated into numerical profiles and interpreted with the API web software.

3.3. CHLORINE DISINFECTION STUDIES

Chlorine disinfection studies using *Shigella flexneri* and *Campylobacter jejuni* were carried out in two phases;

1. **Phase-I: Bench scale disinfection studies**
2. **Phase-II: Lab scale disinfection studies**

3.3.1. Phase-I: Bench scale disinfection studies

Pure *Shigella flexneri* and *Campylobacter jejuni* colonies were taken from nutrient and charcoal based selective medium agar plates and streaked on particular agar slants. These slants were incubated at 37^{o}C for 24 hrs.

The culture was washed twice with phosphate buffer (pH 7) and transferred to a centrifugation tube and centrifuged at 4000 rpm for 15 minutes until a pellet was formed at the bottom, which was then re-suspended in 10 ml of phosphate buffer. Approximately 2 ml of this bacterial (*Shigella* and *Campylobacter*) suspension was added to 1000 mL reaction vessel containing autoclaved and de-chlorinated tap water. This procedure provided the desired number of bacteria, approximately 10^{7} CFU/mL in the reaction vessel for chlorine disinfection studies. After inoculating the culture into the reaction vessel, serial dilutions for spread plate count were made before disinfection. This gave the required number of bacteria in the sample. Chlorine stock solution was prepared by using sodium hypochlorite tablets (20 mg free chlorine) and added to the reaction vessel to get final concentrations of 0.5, 1 and 1.25 mg/L with continuous stirring with magnetic stirrer.

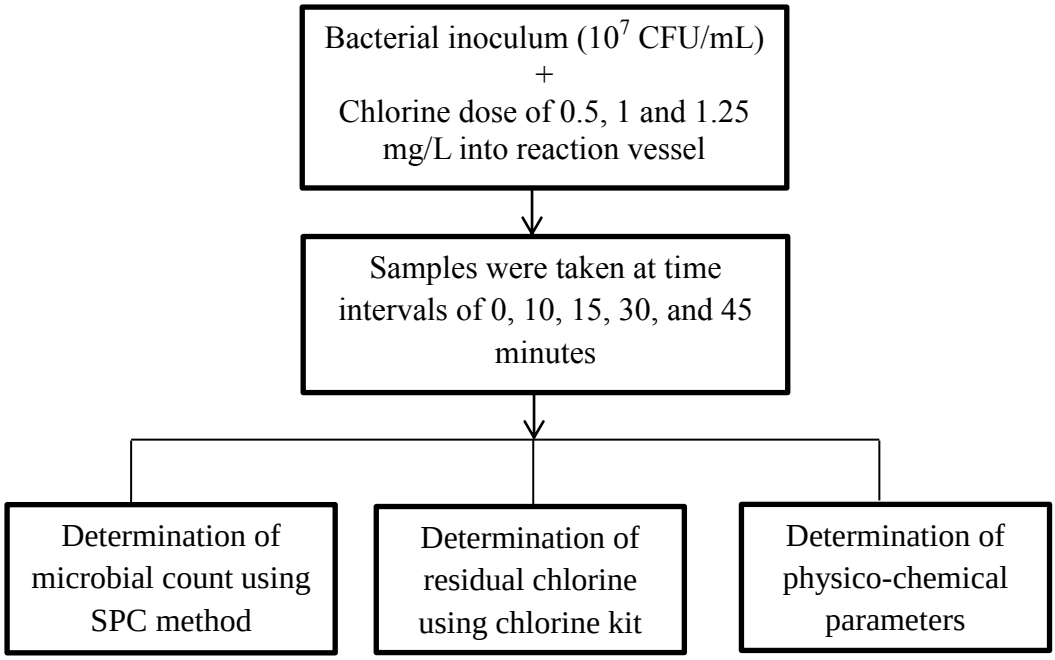


Figure 3.3 Experimental design for bench scale disinfection studies with chlorine using *Shigella* and *Campylobacter* as model organisms

Samples were periodically taken at 0, 10, 15, 30, 45 minutes and stored at 4^oC in two sets of test tubes, with one set of test tubes containing 0.1 mL of sodium thiosulphate ($Na_2S_2O_3$) for determination of bacterial counts and second set of test tubes for residual chlorine determination. Sodium thiosulphate ($Na_2S_2O_3$) fixes the surplus chlorine and retards its action on bacteria so that it does not interfere with the particular spread plate count (SPC).

3.3.2. Phase-II: Lab scale disinfection studies

Selected pathogens (*Shigella flexneri* and *Campylobacter jejuni*) were cultured, grown, and suspended in chlorine free demand buffer as per standard method. Bacterial colonies were separated from liquid nutrient broth prior to all experimentation by

aseptically transmitting the cultures to sterile plastic tubes and then centrifuged at 6000 rpm for 5 minutes. The resulting pellet was re-suspended in 0.1 M chlorine demand free phosphate buffer. Centrifuging, washing and re-suspension were done thrice to make certain that all potential media were removed from the suspension (Berman *et al*., 1988). Final microbial concentration was adjusted at 10^7 CFU/mL.

Table 3.3: Sampling points and their distance from reservoir

Sampling Ids	Sampling points	Distance from reservoir (m)
R	Reservoir	0
SP1	Tap 1	22
SP2	Tap 5	88
SP3	Tap 10	110

Total length of reservoir is 0.22 Km (220 m) with 10 sampling sites as shown in Figure 3.4. The capacity of reservoir is 588 L but it was filled up to 100 L for experimentation purpose. The reservoir of prototype distribution network was filled with de-chlorinated tap water at the start of each experiment. The flow rate was adjusted at 1.2 L/M. Chlorine stock solutions were prepared by using sodium hypochlorite tablets (20 mg free chlorine). Chlorine was given 30 minutes of contact time in the reservoir before disinfected water was pumped through the distribution network according to standard method.

Samples were periodically taken from reservoir at 0 and 30 minutes time interval and from three sampling points SP1, SP2, and SP3 at 45, 60, 75 and 90 minutes

respectively and stored at 4°C in one set of sterile test tubes containing 0.1 mL sodium thiosulphate ($Na_2S_2O_3$) for spread plate count and second set of sterile test tubes for chlorine determination. Also samples were taken to determine physico-chemical parameters, organic load and trihalomethanes.

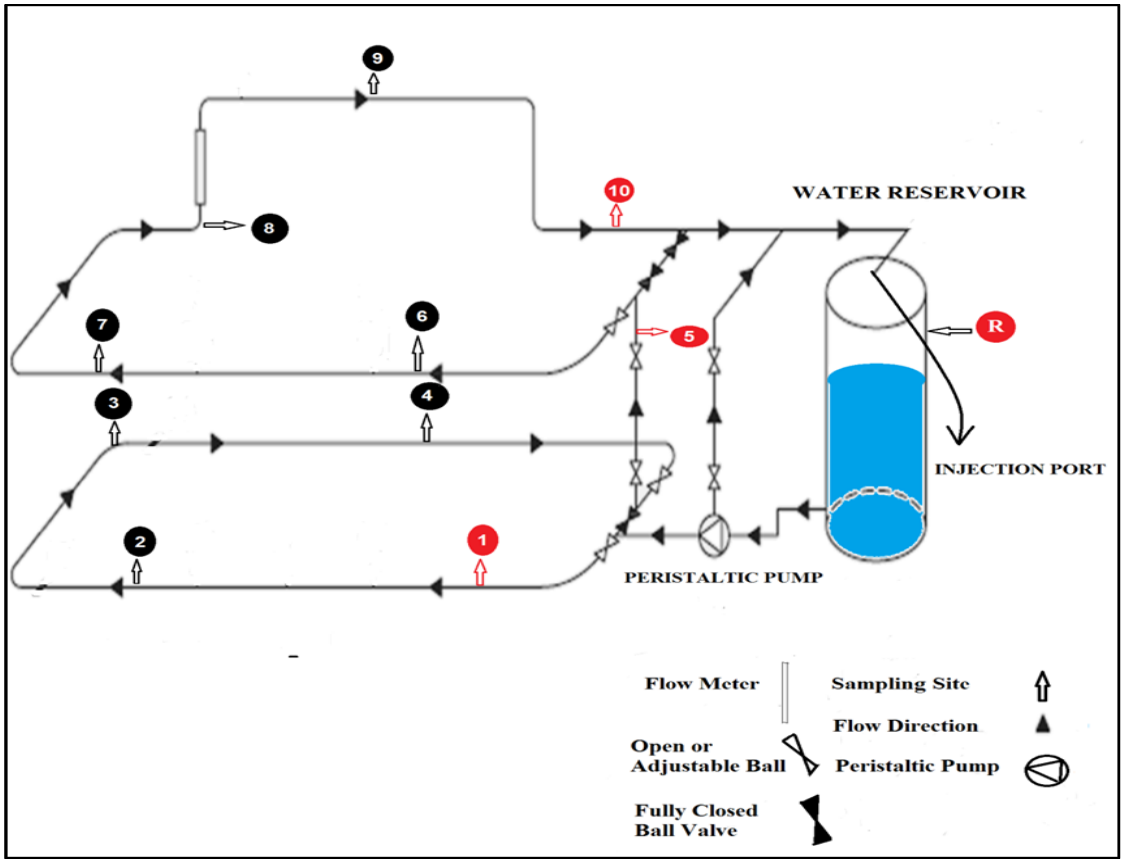


Figure 3.4 Lay-out of prototype distribution network

3.3.2.1. Preparation of humic acid

1 g of commercially available humic acid was dissolved into 1 L of distilled water. pH of resulting solution was maintained to pH 10 by adding 0.1N NaOH. The bottle was placed on shaker for 24 hrs. After being mixed for 24 hours, humic acid was filtered using whatman filter paper of 0.45µm size.

3.3.2.2. Preparation of bromide ion solution

1.48 g of potassium bromide (PBr) was added to 1 L of distilled water to form 1000 mg/L bromide ion solution.

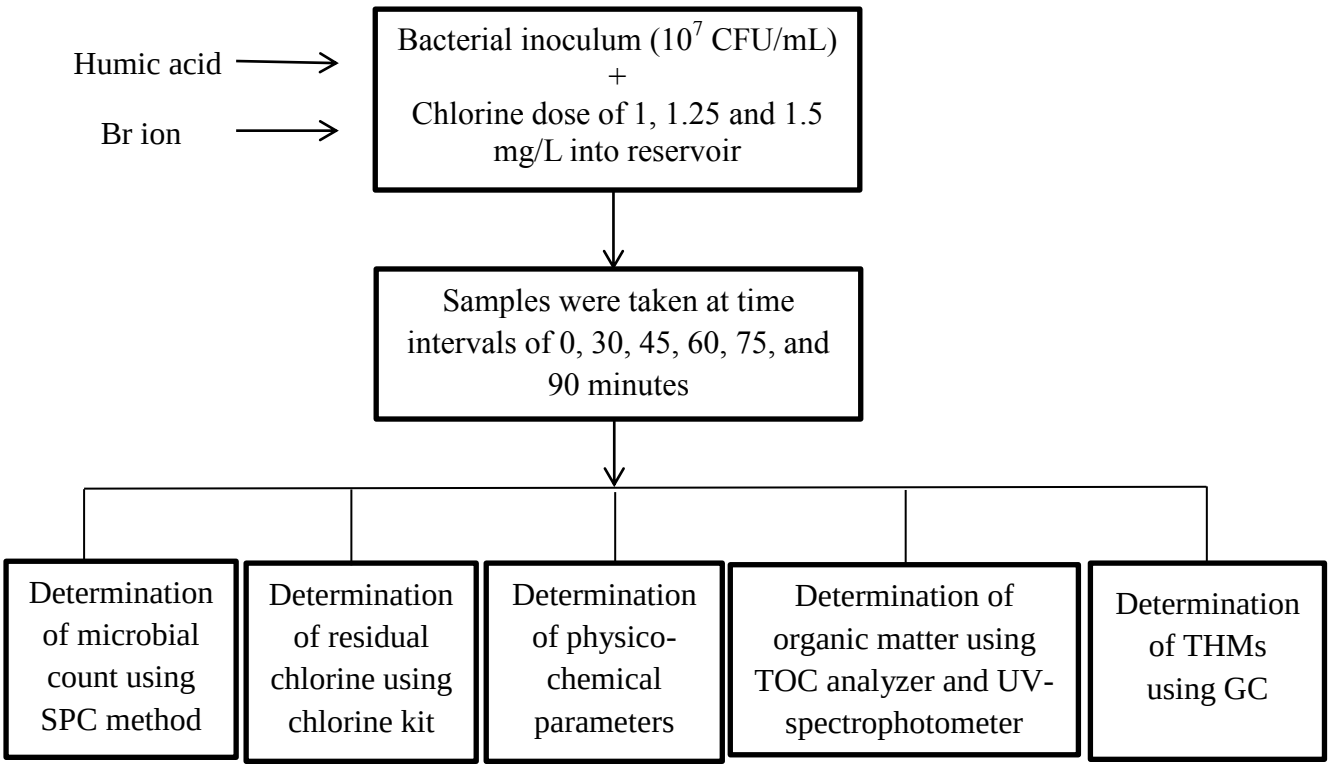


Figure 3.5 Experimental design for lab scale disinfection studies with chlorine using *Shigella* and *Campylobacter* as model organisms

3.4. SPREAD PLATE COUNT (SPC)

Spread Plate Count is a technique that is used to determine the total number of aerobic bacteria present at mesophylic temperatures (30^oC-35^oC) (Nazmul Alam *et al.*, 2003). For standard plate count method, agar plates were prepared by pouring approximately

20 mL of molten nutrient agar or CSM agar (45^{o}C) into sterilized petri plates, evenly distributed and incubated at 37^{o}C for 24 hours. For getting countable range of microbial colonies i-e 30-300 colonies, serial dilutions were made. Each dilution was spread with an L-shaped bent loop on sterile petri plate containing agar by pipetting out 0.5 mL of dilution (Cappuccino and Sherman, 1996; Winn *et al*., 2006). After incubation for 24 hrs, colonies obtained were counted using illuminated colony counter.

3.5. RESIDUAL CHLORINE MEASUREMENT

Residual chlorine measurement was determined using Spectroquant picco chlorine kit (Merck SN 059008) according to standard method (APHA, 2012).

3.6. TOTAL ORGANIC CARBON (TOC)

Total Organic Carbon was estimated by collecting 10 mL of sample at 0, 30, 45 and 60, 75 and 90 minutes respectively. Samples were preserved by adding few drops of 3% H_2SO_4. Each sample was then analyzed by TOC analyzer (Multi N/C 3100, Analytikjena).

3.7. GAS CHROMATOGRAPHY (GC)

Trihalomethanes analysis was performed using a Shimadzu 2010 series gas chromatograph coupled to ECD detector. The column used was fused silica capillary column whose length is 30 cm, inner diameter is 0.53 mm, thickness is 0.88 µm and the filling material is 5 % diphenyl, 95 % dimethyl-polysixolane. In injector, split

injection mode was used and the split ratio was 90. Helium and nitrogen were used as carrier or makeup gases respectively.

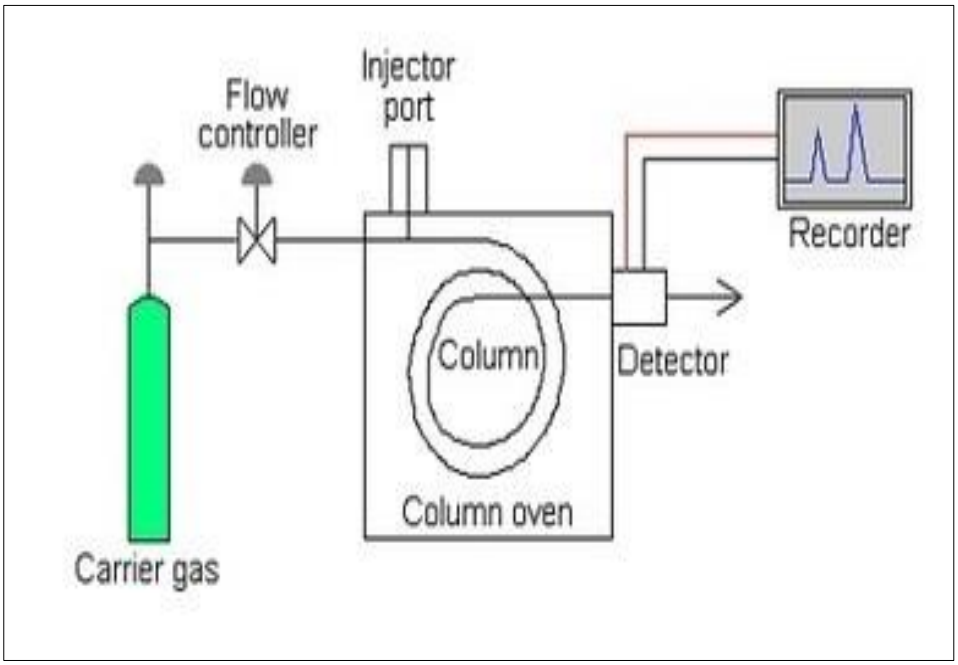


Figure 3.6 Schematic diagram of gas chromatography

Standard stock solutions of chloroform, bromodichloromethane and dibromochloromethane were prepared in 10 ml of GC grade methanol and several dilutions were prepared to formulate standard calibration curves (See Annexure V). For example chloroform stock solution (67µL/10mL) was diluted to 20, 5, 1 and 0.5 mg/L to obtain the reproducible peaks. A stock mixture of trihalomethanes was obtained by mixing individual stock solutions of standard analytes in a specific ratio.

3.7.1. Sample extraction and preparation

Liquid-liquid extraction technique was used for sample extraction. 1 ml of GC grade ethyl acetate solvent was added to 5 ml of sample in a test tube. The sample and ethyl

acetate was mixed vigorously using vortex for 2 min. After that, sample was left undisturbed for 5-10 minutes for organic and inorganic layers to get separated. Upper layer (organic) was collected. This step was repeated twice to ensure complete extraction. The extracted sample was kept in GC vials in refrigerator at 4^{o}C.

3.7.2. Gas chromatographic conditions

Table 3.4: Gas Chromatographic Conditions

Parameters	Conditions
Injection port	
Temperature	220^{o}C
Injection mode	Split
Column	
Initial temperature	50^{o}C
Final temperature	200^{o}C
Column length	30 m
Electron capture detector (ECD)	
Temperature	220^{o}C
Carrier gas	Helium
Make-up gas	Nitrogen

3.8. UV-VIS SPECTROPHOTOMETER

UV-Vis spectrophotometry analysis was performed by collecting 10 mL of water sample at 0, 30, 45, 60, 75 and 90 minutes respectively. Samples were first filtered

using 0.45 µm filter paper and then analyzed at specific UV range of 200-300 nm by UV-Vis spectrophotometer (UVS-2800).

3.9. PHYSICAL PARAMETERS

Water samples were analyzed for pH, temperature, total dissolved solids, turbidity, and conductivity using standard methods as described in Table 3.5.

Table 3.5: Methods & instruments used for physical parameters

Parameters	Analyzed by
pH	pH meter (HACH Sens ion 1 b)
Temperature	Thermometer (HACH Sens ion 1 b)
Total dissolved solids	TDS meter (HACH Sens ion 5)
Turbidity	UV visible spectrophotometer (Spectronic Genesys 5)
Conductivity	Conductivity meter (HACH Sens ion 5)

(APHA, 2012)

3.10. WATER QUALITY INDEX

Water quality index (WQI) is primarily a mathematical mean of calculating a single value to express overall water quality using multiple physico-chemical and microbiological parameters (Rao *et al.*, 2010).

Table 3.6: Water quality classification based on WQI value

WQI value range	Water quality
0-25	**Very Bad**
26-50	**Bad**
51-70	**Medium**
71-90	**Good**
91-100	**Excellent**

(Srivastava *et al.,* 2013)

Water quality index was calculated using standard formula (Srivastava *et al.,* 2013). Q-values (Q_Y) of available water quality parameters (Y) were calculated and multiplied with their respective weighing factors (W_Y) as mentioned in Annexure IV.

$$\mathbf{WQI} = \sum \mathbf{W_Y Q_Y} / \sum \mathbf{W_Y}$$

$$\mathbf{W_Y Q_Y} = \mathbf{W_{pH} Q_{pH}} + \mathbf{W_{F.C} Q_{F.C}} + \mathbf{W_{TDS} Q_{TDS}} + \mathbf{W_{Temp} Q_{Temp}} + \mathbf{W_{Turbidity} Q_{Turbidity}}$$

CHAPTER 4

RESULTS AND DISCUSSION

The present study aimed to determine the optimized chlorine dosages at optimum conditions for maximum inactivation of *Shigella flexneri* and *Campylobacter jejuni* as model organisms.

4.1. ISOLATION AND PURIFICATION OF *SHIGELLA FLEXNERI* AND *CAMPYLOBACTER JEJUNI*

For the experimental work, indigenous strains of selected enteric pathogens were isolated from contaminated drinking water with the help of selective enrichment technique. Colony morphology, gram staining and different biochemical tests (including API E20) was conducted to identify the bacterial species. On the basis of observations of results mentioned in Table 4.1, 4.2 and 4.3 with pictorial representation in Annexure I, the strains were identified as *Shigella flexneri* and *Campylobacter jejuni*.

The results of API E20 were also noted and converted into numerical code which was then run on API web software. The results of APIE20 identified the strains as *Shigella flexneri* and *Campylobacter jejuni*. There morphological and biochemical characteristics were further compared with standard ATCC cultures of *Shigella flexneri* ATCC12022 and *Campylobacter jejuni* ATCC33291.

Table 4.1: Characteristic colony appearance on various culture media for *Shigella* and *Campylobacter*

Sr. No	Microorganisms	Medium	Observations	Results
1.	***Shigella flexneri***	XLD	Red translucent colonies	*Shigella* colonies confirmed
		MLA	Colorless convex colonies	*Shigella* colonies confirmed
		DCA	Colorless colonies	*Shigella* colonies confirmed
2.	***Campylobacter jejuni***	CSM	Small, raised, gray-white and mucoid colonies	*Campylobacter* colonies confirmed
		Skirrow's	Small, mucoid, flat, slightly raised, translucent and gray	*Campylobacter* colonies confirmed

Table 4.2: Gram-staining response of *Shigella* and *Campylobacter*

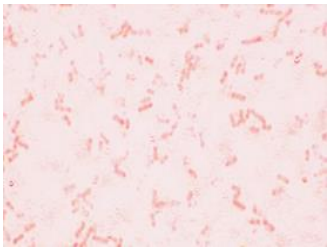
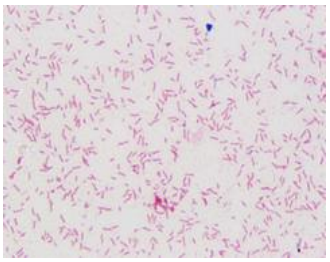
Sr. No	Gram-staining	Observations	Results
1.	***Shigella flexneri***		Gram-negative light pink short rods
2.	***Campylobacter jejuni***		Gram-negative dark pink short curved or spiral shaped rods

Table 4.3: Confirmatory tests for *Shigella* and *Campylobacter*

Sr. No	Biochemical Tests	Observations	Results
1.	**Indole test**	Pink to red color ring appeared at the top of the medium (+)	*Shigella*
		No color change (-)	*Campylobacter*
2.	**Methyl Red test**	Methyl red turns red (+)	*Shigella*
3.	**Voges-Proskauer test**	Yellow-brown color appeared at the top of the broth (-)	*Shigella*
4.	**Citrate test**	No growth or color change was observed on citrate agar (-)	*Shigella*
5.	**Motility test**	Negative (-)	*Shigella*
		Positive (+)	*Campylobacter*
6.	**Oxidase test**	No color change (-)	*Shigella*
		Filter paper turns dark purple (+)	*Campylobacter*
7.	**Catalase test**	O_2 produced as bubbles (+)	*Shigella*
		O_2 produced as bubbles (+)	*Campylobacter*
8.	**Mannitol fermentation test**	Broth color changes from red to yellow (+)	*Shigella flexneri*
9.	**Hippurate hydrolysis test**	Colorless solution color changes to dark deep purple (+)	*Campylobacter jejuni*

4.2. PHASE-I: BENCH SCALE DISINFECTION STUDIES

4.2.1. Survival of *Shigella flexneri* against chlorine at bench scale

Disinfection studies of *Shigella flexneri* with chlorine were carried out using de-chlorinated tap water as medium at optimum conditions. pH, temperature, electrical conductivity, turbidity and total dissolved solids of all the samples were measured at each time interval and their ranges are mentioned in Table 4.4. CT values were calculated by multiplying chlorine dose with time. The initial *S. flexneri* count, after inoculation of pure culture, was 2.20 x 10^7 CFU/mL.

Table 4.4: Physicochemical analysis of water samples at different chlorine doses

Parameters	Chlorine dose (mg/L)		
	0.5	**1**	**1.25**
pH	6.2-6.4	6.5-6.8	6.9-7
Temperature (^{o}C)	21.5-22	19-21.5	17-19.5
Electrical Conductivity (µS/cm)	372-398	298-315	275-280
Turbidity (NTU)	0.60-1.25	0.47-0.88	0.36-0.67
TDS (mg/L)	223-238	178-189	165-168

(APHA, 2012)

After first 10 minutes of exposure, the inactivation was not evident and the viable counts decreased to 1.82 x 10^7 CFU/mL at CT value of 5 mg-min/L as shown in Figure 4.1. The respective chlorine residual at this time was 0.35 mg/L as shown in Figure 4.4. The results are contrary to the results shown by LeChevallier *et al.*, (1988) showing that 99 percent of viable bacterial counts decreased on exposure to 0.08 mg/L

of hypochlorous acid at pH 7 for 1 min. In the next 5 minutes of exposure, i.e. after 15 minutes, there was inconspicuous inactivation of the *S. flexneri* counts.

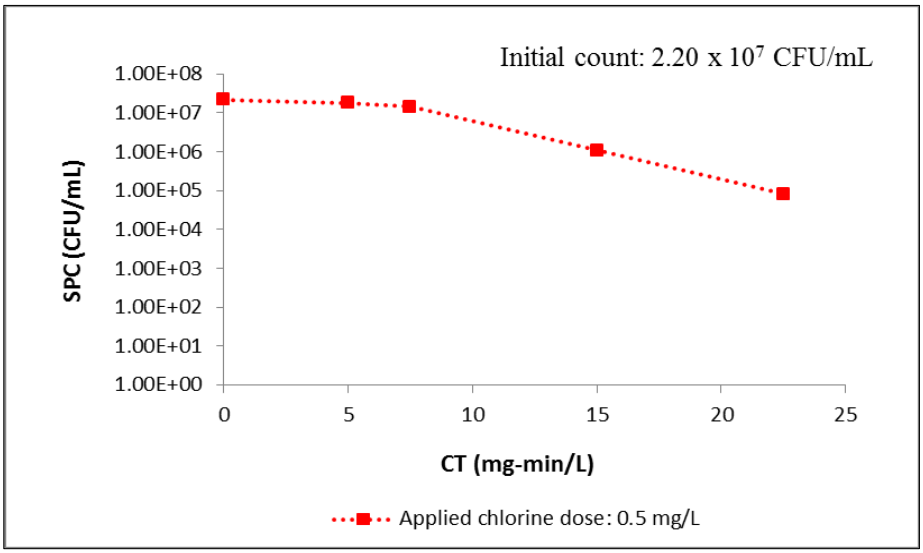


Figure 4.1 Survival of *S. flexneri* at 0.5 mg/L chlorine dose

Applied chlorine dosage of 0.5 mg/L required 15 more minutes to yield a noticeable inactivation and 1 log removal was accomplished at CT value of 15 mg-min/L. The overall removal after 45 minutes of exposure was 3-log at CT value of 22.5 mg-min/L. Residual chlorine measurement at 30 and 45 minutes for 0.5 mg/L of chlorine dose was 0.25 and 0.16 mg/L respectively.

The initial *S. flexneri* count, after inoculation of pure culture, was 2.51 x 10^7 CFU/mL. After first 10 minutes of exposure, the viable count decreased from 2.51 x 10^7 to 1.94 x 10^6 at CT value of 10 mg-min/L as shown in Figure 4.2. The respective chlorine residual at this time was 0.57 mg/L as shown in Figure 4.4.

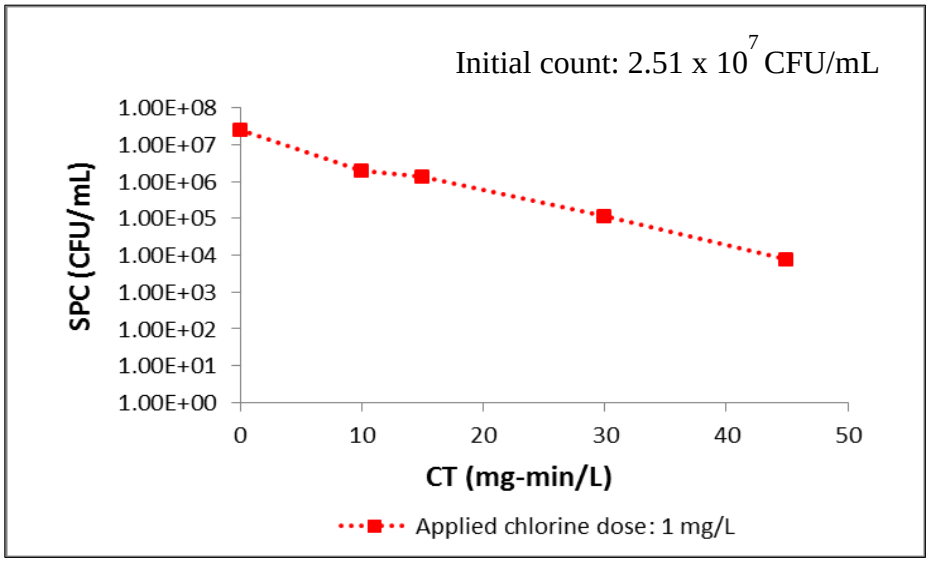


Figure 4.2 Survival of *S. flexneri* at 1 mg/L chlorine dose

A 2-log removal of gram negative bacteria was reported by Koseki *et al.*, (2002). No significant bacterial count decreased after further 15 minutes of exposure displaying resistant nature of *S. flexneri* as compared to its closely related enterobacteriaceae family members *E. coli* and *Salmonella*. Similar result was reported by LeChevallier *et al.*, (1985) that higher chlorine doses (0.9 to 1.5 mg/L) were necessary to produce injured *Shigella spp.* and *Yersinia enterocolitica* than to produce injured *Escherichia coli* or *coliform* bacteria (0.25 to 0.5 mg/L). Applied chlorine dosage of 1 mg/L resulted in further 1-log removal at CT value of 30 mg-min/L. Maximum of 2-log bacterial reduction was achieved after 45 minutes of exposure to 1 mg/L of chlorine with a residual concentration of 0.23 mg/L.

The initial *S. flexneri* count, after inoculation of pure culture, was 2.73×10^7 CFU/mL. Viable bacterial count decreased from 2.73×10^7 to 1.61×10^5 at CT value of 12.5 mg-min/L as shown in Figure 4.3.

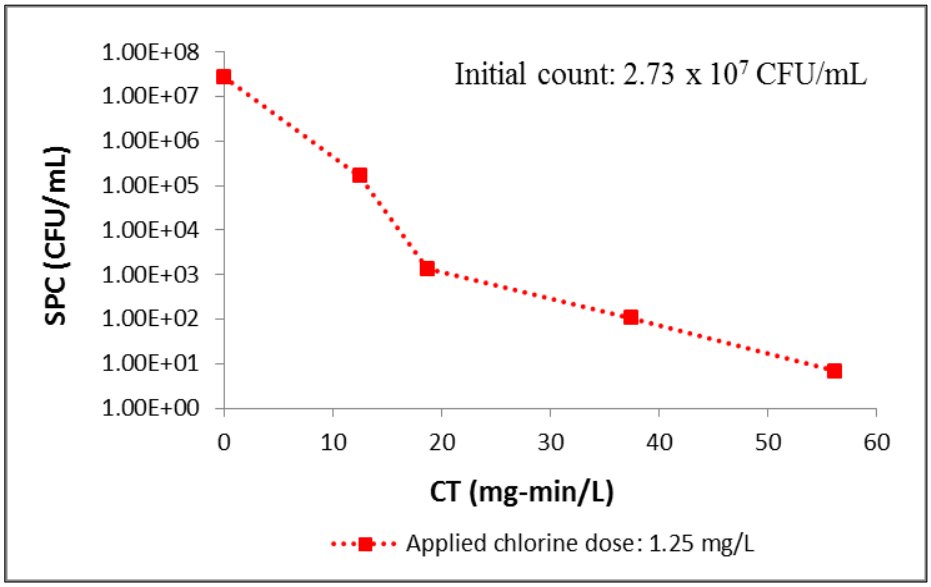


Figure 4.3 Survival of *S. flexneri* at 1.25 mg/L chlorine dose

The respective chlorine residual at this time was 0.75 mg/L as shown in Figure 4.4. Koseki *et al.*, (2002) reported similar 2-log reduction of bacterial counts. *S. flexneri* counts showed additional 2-log reduction on being exposed to 15 more minutes. CT value of 56.25 mg-min/L resulted in 6-log reduction of *S. flexneri* counts thus showing complete reduction at 1.25 mg/L of chlorine dose as shown in Figure 4.3. Allwood *et al.*, (2005) stated rapid decline of gram negative *coliforms* in highly chlorinated water and *coliforms* were undetected for 24 hours irrespective of the temperature. Similar results were also reported by (Johnson *et al.*, 1997) that 1.1 mg/L of chlorine is adequate to achieve maximum disinfection against *Helicobacter pylori*. Residual chlorine measurement ranged from 1.25, 1, 0.5 mg/L to 0.5, 0.23 and 0.16 mg/L after 45 minutes of exposure time as shown in Figure 4.4.

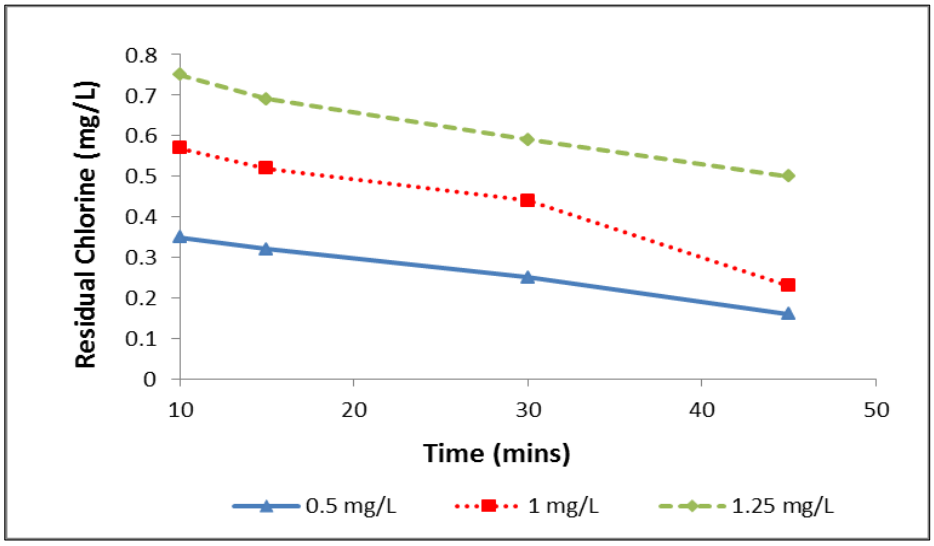


Figure 4.4 Variation in residual chlorine with time in chlorinated water

Inactivation rate of *S. flexneri* was rapid at 1.25 mg/L of chlorine dosage because chlorine works efficiently at pH 7 as hypochlorous acid (HOCl) predominates at lower pH values. Research showed that at pH 6.5, 90% of the free chlorine exists as HOCl which is 70 to 80 times more effective than OCl^- (Gorchev, 1996). Similarly, temperature range (17-19.5) was found to be the most effective against *S. flexneri* because microbial growth is slower at lower temperatures i.e. pathogen inactivation increases predominantly as water temperature lowers (US-EPA, 1999). Turbidity is another factor that significantly affects microbiological quality of drinking water. Turbidity values as shown in Table 4.4 were found to be within the Highest Desirable Level (HDL) values of WHO i.e. < 1 NTU.

The percent survival of *S. flexneri* was 0.58, 7.72 and 82.72 percent after 10 minutes of chlorine exposure to 1.25, 1 and 0.5 mg/L respectively. After 30 minutes of exposure to the above chlorine dosages, 0.0003, 0.45, and 4.95 percent of viable counts were able to survive as shown in Figure 4.5. Survival percentage of *S. flexneri*

after 45 minutes of contact time was 0.00002, 0.02, and 0.36 for 1.25, 1 and 0.5 mg/L of chlorine respectively. Figure 4.5 shows decrease in survival of *S. flexneri* with increase in time.

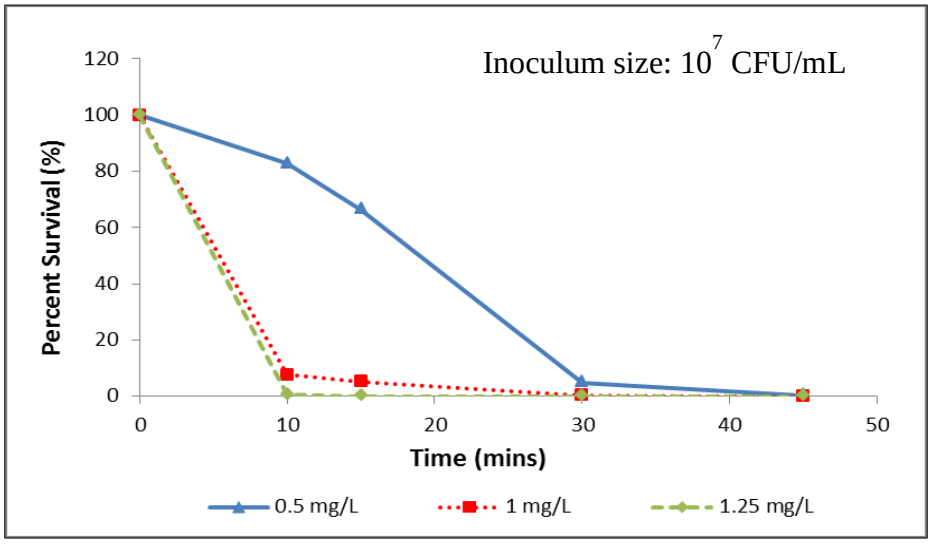


Figure 4.5 Percent survival of *S. flexneri* at various applied chlorine dosages

4.2.2. Survival of *Campylobacter jejuni* against chlorine at bench scale

Bench-scale studies were conducted to optimize chlorine dosage against *C. jejuni* at optimum conditions; pH (6.75-6.8) and temperature (17.5-18^{o}C). The initial bacterial inoculum introduced into reaction vessel was 2.44 x 10^7 CFU/mL. After first 10 minutes of chlorination exposure to 0.5 mg/L of chlorine dose the counts decreased to 1.23 x 10^6 CFU/mL showing insignificant reduction. Even after 5 more minutes there was no log-removal achieved. One-log removal was achieved after 30 minutes at CT value of 15 mg-min/L. These results are contrary to the findings reported by Lund, (1996) that 0.02-0.04 mg/L of free chlorine resulted in 3-log reduction of *C. jejuni* in 12 min at 4°C and 2 min at 10°C. Overall 3-log reduction occurred after 45 minutes at

CT value of 22.5 mg-min/L with a chlorine residual of 0.15 mg/L as shown in Figure 4.6 and 4.8. Dupuy *et al.*, (2011) reported 3-log inactivation of *Legionella pneumophila* at CT 5 mg-min/L. *Campylobacter jejuni* showed increased resistance to low chlorine dose.

Table 4.5: Physicochemical analysis of water samples at 0.5 and 1 mg/L of chlorine dose

Parameters	Chlorine dose (mg/L)	
	0.5	1
pH	6.75-6.8	6.95-7.2
Temperature (^{o}C)	17.5-18	15.8-18.5
Electrical Conductivity (µS/cm)	397-401	387-399
Turbidity (NTU)	0.78-1.27	0.41-0.66
TDS (mg/L)	238-240	233-240

(APHA, 2012)

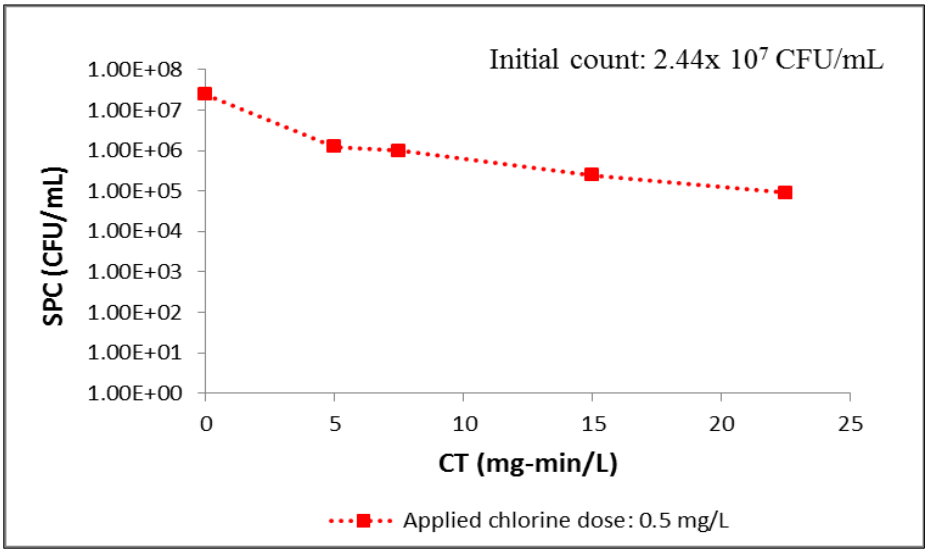


Figure 4.6 Survival of *C. jejuni* at 0.5 mg/L of chlorine dose

Chlorine dose was increased to 1 mg/L with initial bacterial inoculum introduced into reaction vessel was 2.46 x 10^7 CFU/mL at pH (6.95-7.2) and temperature (15.8-18.5^{o}C). After 10 minutes of exposure, there was 4-log reduction at CT value of 10 mg-min/L with residual chlorine of 0.55 mg/L. No counts were observed after 10 minutes as shown in Figure 4.7. The residual chlorine measurement at 15, 30 and 45 minutes was 0.5, 0.43, and 0.38 respectively.

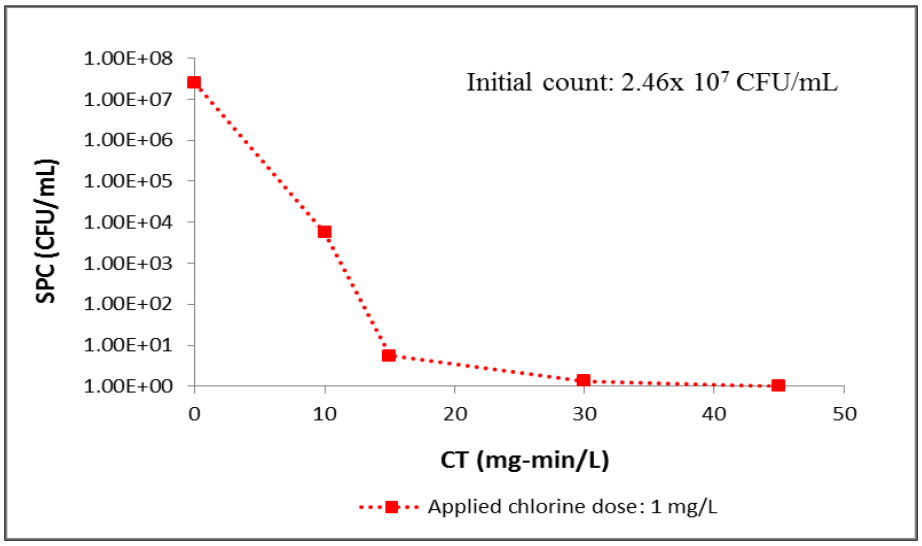


Figure 4.7 Survival of *C. jejuni* at 1 mg/L of chlorine dose

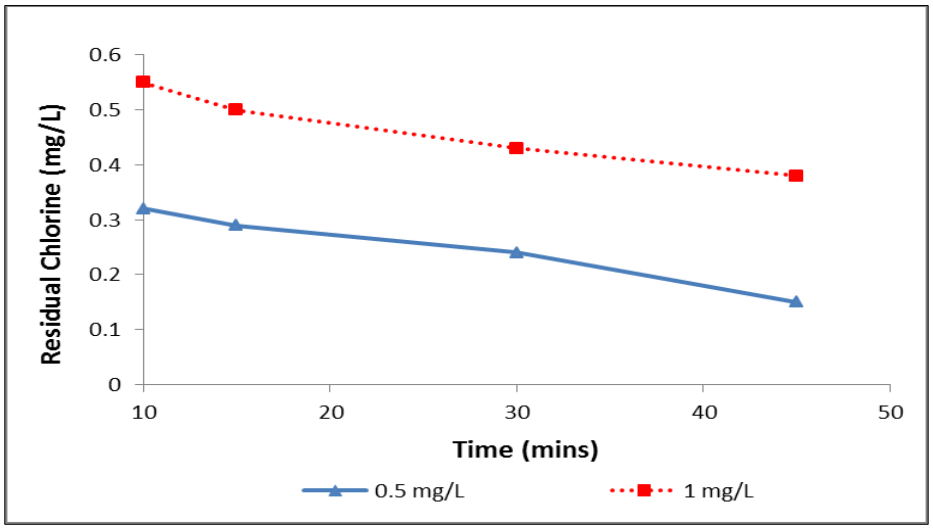


Figure 4.8 Variation in residual chlorine with time in chlorinated water

The percent survival of *C. jejuni* at 1 mg/L of chlorine dose was insignificant as compared to 0.5 mg/L of chlorine dose as shown in Figure 4.9. At 1 mg/L of chlorine dose more than 99% microbial die-off occurred just after 10 minutes.

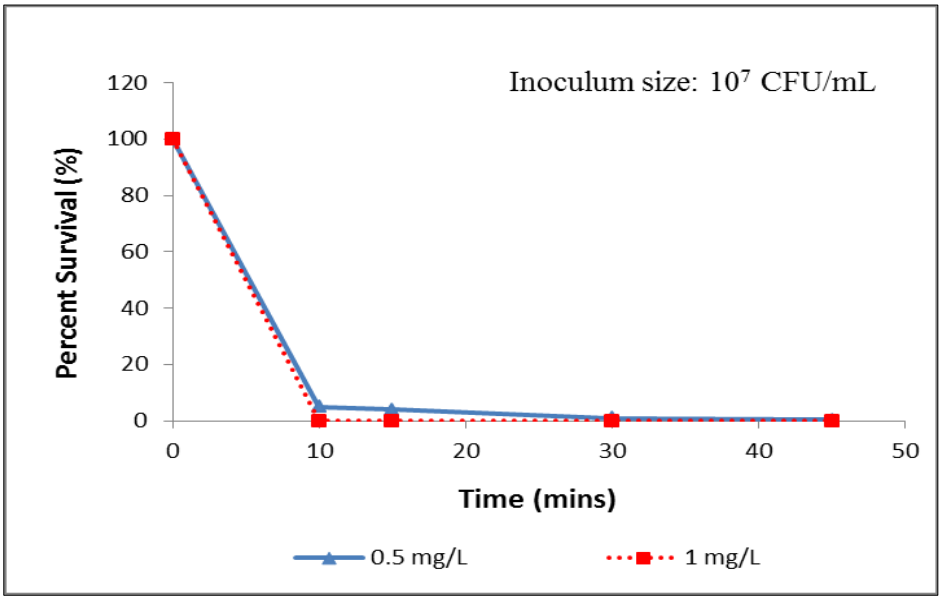


Figure 4.9 Percent survival of *C. jejuni* at various chlorine dosages

4.3. PHASE-II: LAB SCALE DISINFECTION STUDIES

4.3.1. Survival of *Shigella flexneri* against chlorine at lab scale

Observing the results of previous bench scale experiments for optimizing disinfection conditions for *S. flexneri*, it is evident that 1.25 mg/L of applied chlorine dosage at optimum conditions is adequate for maximum disinfection. Considering these results, same optimized conditions along with humic matter and bromide ion solution were applied at lab scale distribution network.

Table 4.6: Physicochemical analysis of water samples at 1.25 and 1.5 mg/L of chlorine dose

Parameters	Chlorine dose (mg/L)	
	1.25	**1.5**
pH	6.6-6.8	6.9-7.2
Temperature (^{o}C)	19.5-21.5	16-18.5
Electrical Conductivity (µS/cm)	445-455	419-460
Turbidity (NTU)	2.95-3.2	2.85-3
TDS (mg/L)	267-273	251-276

(APHA, 2012)

The initial bacterial count applied for inactivation was 2.01 x 10^7 CFU/mL. After 30 minutes of exposure to applied chlorine dosage, initial bacterial count decreased to 8.60 x 10^4 CFU/mL at CT value of 37.5 mg-min/L. Chlorine effectively resulted in 3-log bacterial die-off within the reservoir of lab scale distribution network. Chlorine was given an additional contact time of 15 minutes with bacteria within the pipeline network. At CT value of 56.25 mg-min/L, further reduction in bacterial counts occurred with 2.93 x10^3, 1.71x10^2, and 1.13 x10^2 CFU/mL at sampling points (taps) SP1, SP2 and SP3 respectively. At SP1, there is only 1-log reduction but as distance increases, residual chlorine present within the pipeline network interacts with *S. flexneri* and resulted in 2-log reduction at sampling points SP2 and SP3. CT value of 75 mg-min/L resulted in further 1-log reduction at sampling sites SP1, SP2 and SP3 with 1.67 x 10^2, 9.40 x 10^1, and 5.60 x 10^1 CFU/mL.

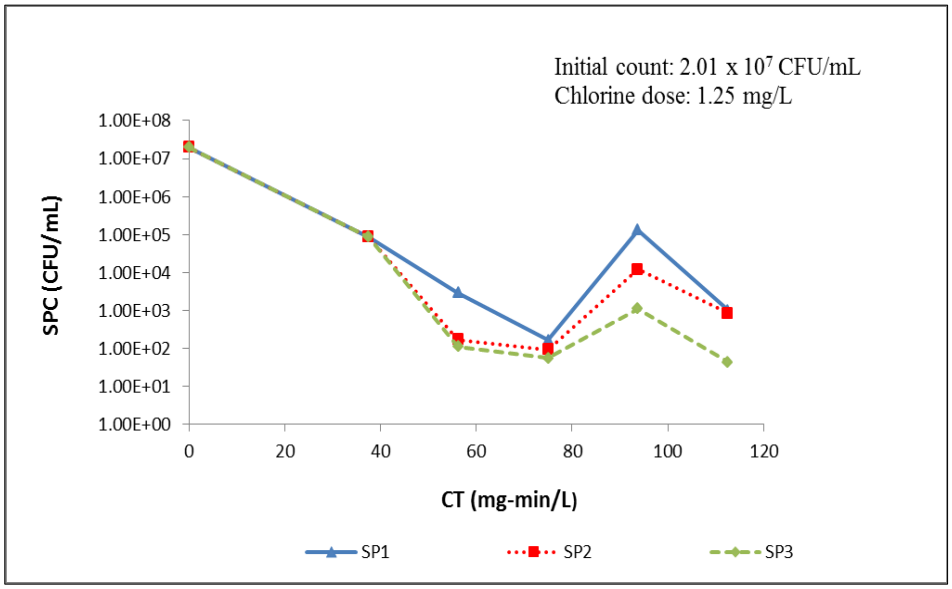


Figure 4.10 Survival of *S. flexneri* at lab scale distribution network

Inoculum size of 10^7 CFU/mL was re-inoculated at 60 minutes as shown in Figure 4.10 in order to check the efficacy of already present residual chlorine. After 15 minutes of contact time, at CT value of 93.75 mg-min/L, 2, 3 and 4-log reduction occurred at sampling sites SP1, SP2, and SP3 respectively. Bacterial die-off achieved after 15 more minutes was 2-log for all the three sampling sites at CT value of 112.5 mg-min/L with a residual chlorine content of 0.26, 0.21, and 0.19 respectively as shown in Figure 4.10 and 4.11. The survival of *S. flexneri* varied with contact time, distance and residual chlorine content.

Due to presence of organic matter in tap water as shown in Figure 4.12 the chlorine demand was higher, resulted in slower and inadequate inactivation of *S. flexneri*. Water with high turbidity from organic matter may give rise to a substantial chlorine demand and so is unavailable to kill pathogens (Crump *et al.*, 2004).

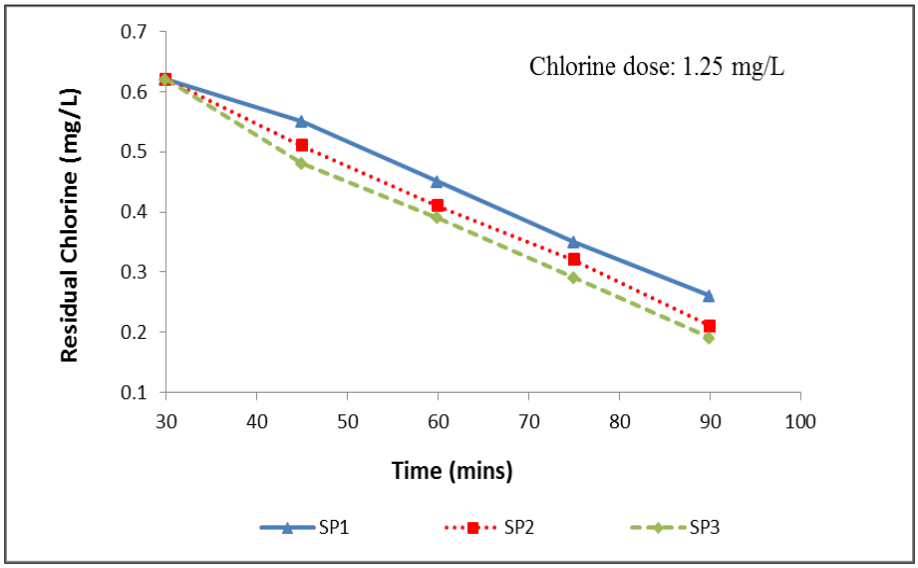


Figure 4.11 Variation in residual chlorine with time at lab scale distribution network

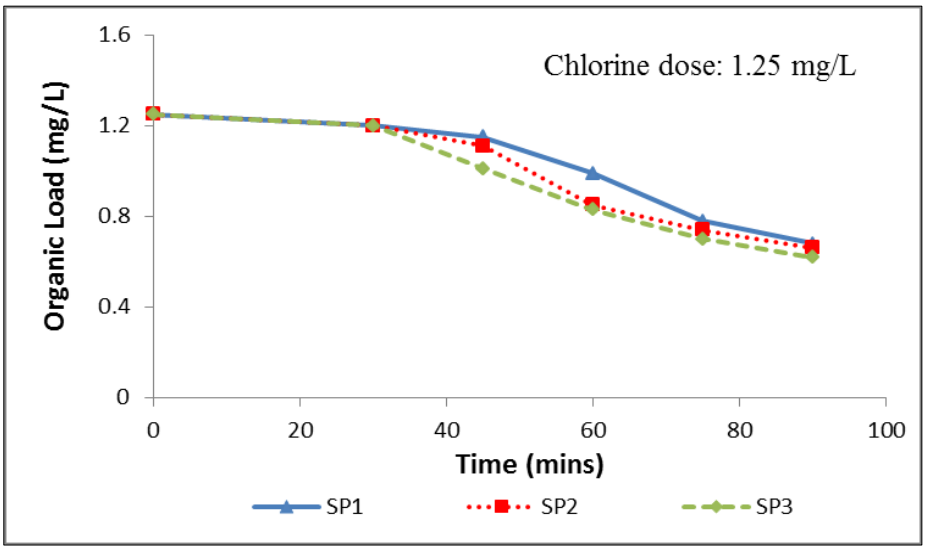


Figure 4.12 Variation in organic load with time at lab scale distribution network

In order to meet up the chlorine demand of water, chlorine dosage was increased to 1.5 mg/L to fasten the process of chlorination with effective chlorine residual. Di Giovanni and LeChevallier, (2000) found that particulate material did not interfere with disinfection process once the increase in oxidant demand had been satisfied.

Higher chlorine dose also ensured higher residual chlorine within water that would persisted for longer period of time.

Initial count introduced was 2.75 x 10^7 CFU/mL to observe the effect of chlorination. After 30 minutes of contact time to 1.5 mg/L of chlorine dose, there was significant reduction in the counts. *S. flexneri* counts decreased from 2.75 x 10^7 CFU/mL to 1.27 x 10^2 CFU/mL with residual chlorine of 0.75 mg/L. WHO, (1993) recommended residual chlorine concentration for flowing tap water is greater than or equal to 0.5 mg/L after at least 30 minutes of contact time at pH less than 8. At CT value of 67.5 mg/min/L further 1-log reduction occurred at sampling taps SP1 and SP2, and 2-log reduction occurred at sampling SP3. This is due to the reason that as residual chlorine moves within the network it kept on interacting with microbial population resulting in their destruction. No counts were observed after additional 15 more minutes of contact time.

Bacterial inoculum of 10^7 CFU/mL was re-inoculated into the distribution network in order to check the efficacy of residual chlorine against accidental contamination. Only after 15 minutes of contact time there was significant reduction in counts from 10^7 to 10^3 and 10^2 at the respective sampling sites. Residual chlorine measured at 75 minutes was found to be ranged between 0.47-0.4 mg/L. At CT value of 135 mg-min/L, there were no counts observed. 1.5 mg/L under tested conditions pH (6.9-7.2) and temperature (16-18.5^{o}C) were found to be very effective against the destruction of *Shigella flexneri* within lab scale distribution network.

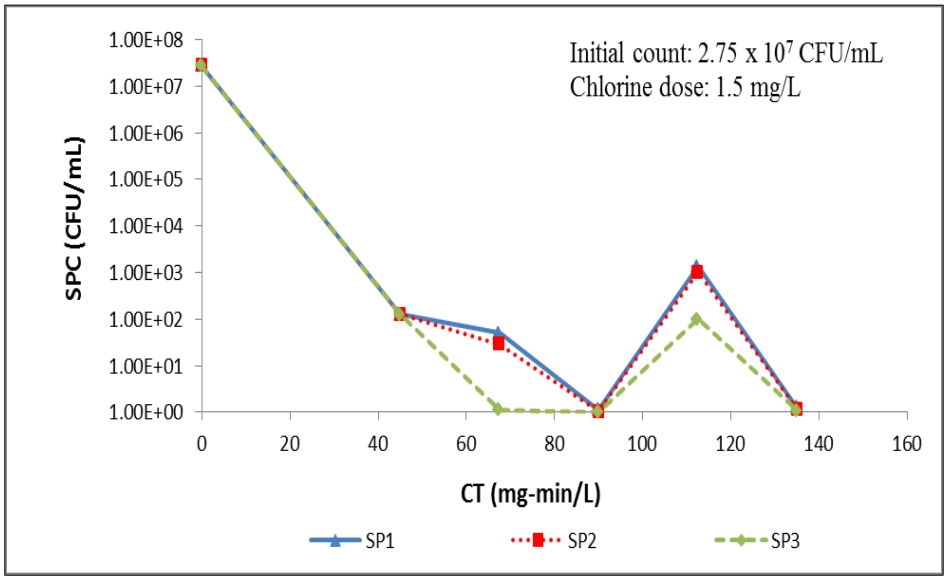


Figure 4.13 Survival of *S. flexneri* at lab scale distribution network

Organic load at selected sampling taps showed decreasing trend with respect to time as shown in Figure 4.15 this is because bacteria present within water consumed organic matter and also free chlorine combines with it.

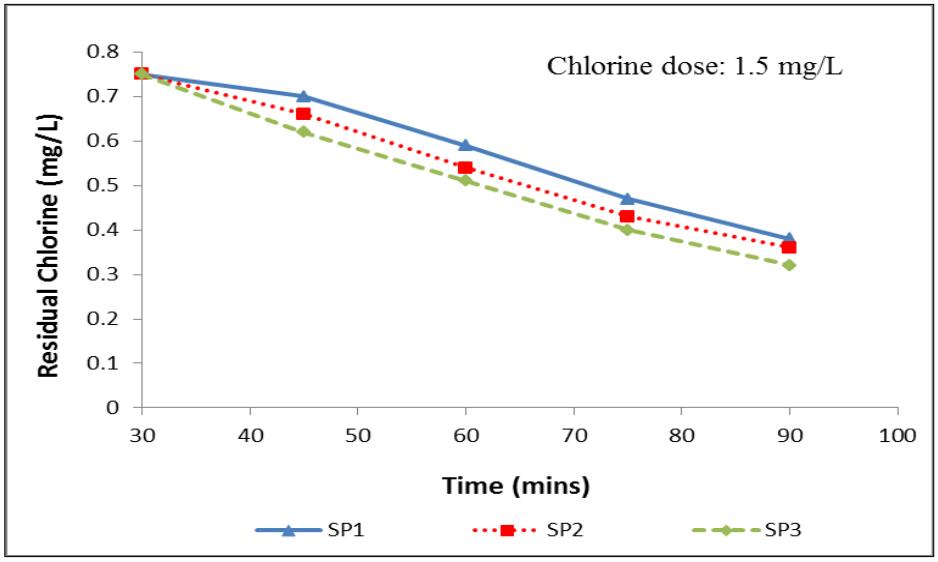


Figure 4.14 Variation in residual chlorine with time at lab scale distribution network

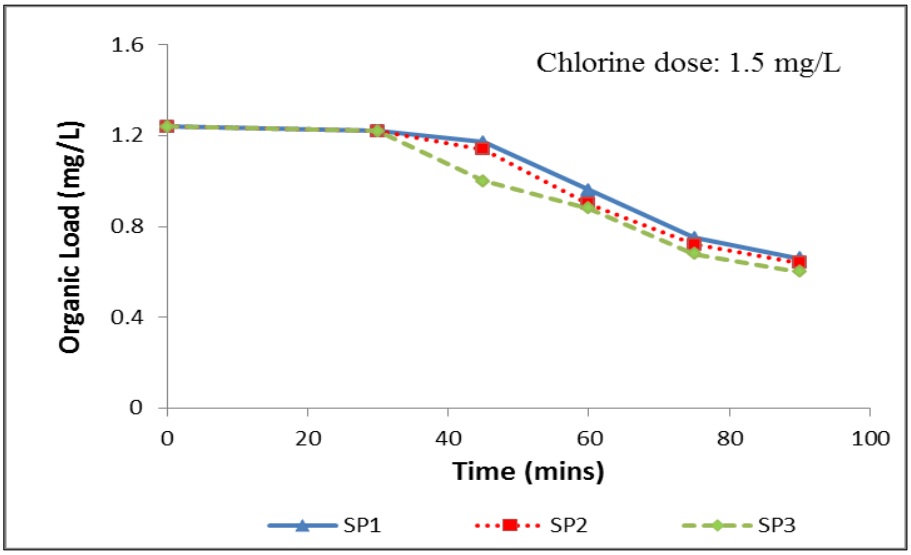


Figure 4.15 Variation in organic load with time at lab scale distribution network

4.3.2. Survival of C*ampylobacter jejuni* against chlorine at lab scale

Optimized bench-scale disinfection conditions for *C. jejuni* showed that 1 mg/L of chlorine dose is necessary for maximum inactivation. Similar conditions along with humic matter and bromide ion solution were introduced at lab scale distribution network in order to observe the survival of *C. jejuni*. All experiments were conducted in triplicates to validate the results.

Bacterial inoculum of 2.84 x 10^7 CFU/mL was injected into the reservoir. After 30 minutes of chlorination, the counts decreased to 1.53 x 10^2 CFU/mL at CT value of 30 mg-min/L. Due to high chlorine demand of tap water because of the presence of organic matter as shown in Figure 4.18 *C. jejuni* took longer time to inactivate at lab-scale distribution network.

Table 4.7: Physicochemical analysis of water samples at 1 mg/L of chlorine dose

Parameters	Chlorine dose
	1 mg/L
pH	7-7.25
Temperature (^{o}C)	17.5-19
Electrical Conductivity (µS/cm)	480-495
Turbidity (NTU)	2.70-2.98
TDS (mg/L)	288-297

(APHA, 2012)

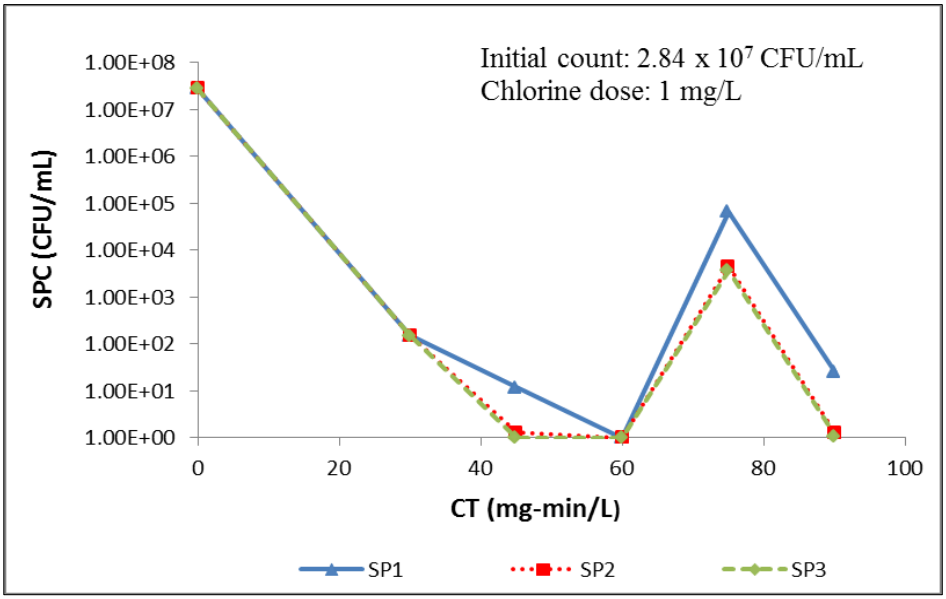


Figure 4.16 Survival of *C. jejuni* at lab scale distribution network

After 15 more minutes of exposure the counts decreased to 1.18 x 10^{1} at sampling point SP1, and no counts were observed at sampling points SP2 and SP3. At CT value of 60 mg-min/L, no counts were observed in all the selected sampling taps. *C. jejuni*

inoculum size of 10^7 CFU/mL was re-inoculated at 60 minutes and sample was taken after 15 minutes of contact time from sampling tap SP1, SP2 and SP3 respectively. Log reduction of 4, 3, and 3 was observed at taps SP1, SP2 and SP3 respectively. At CT value of 90 mg-min/L there is further reduction of 3-log at all the sampling taps.

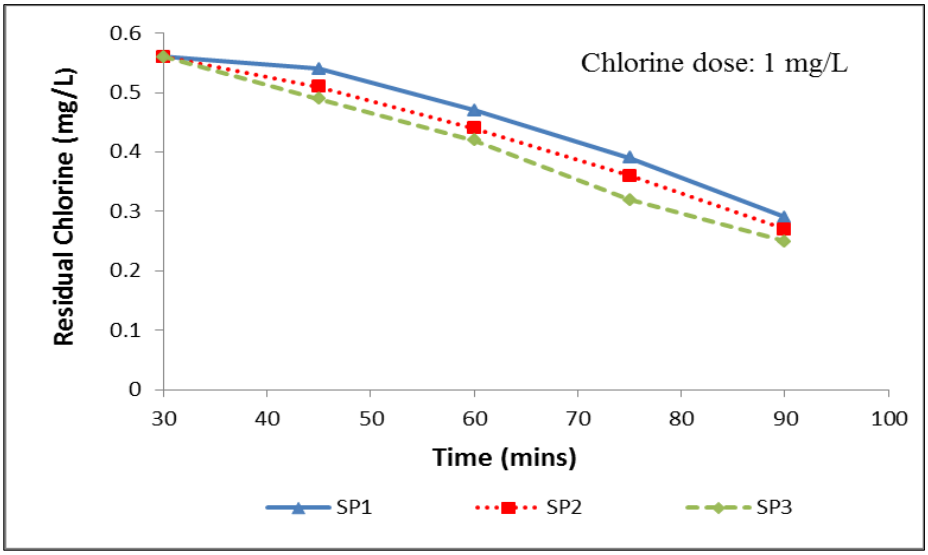


Figure 4.17 Variation in residual chlorine with time at lab scale distribution network

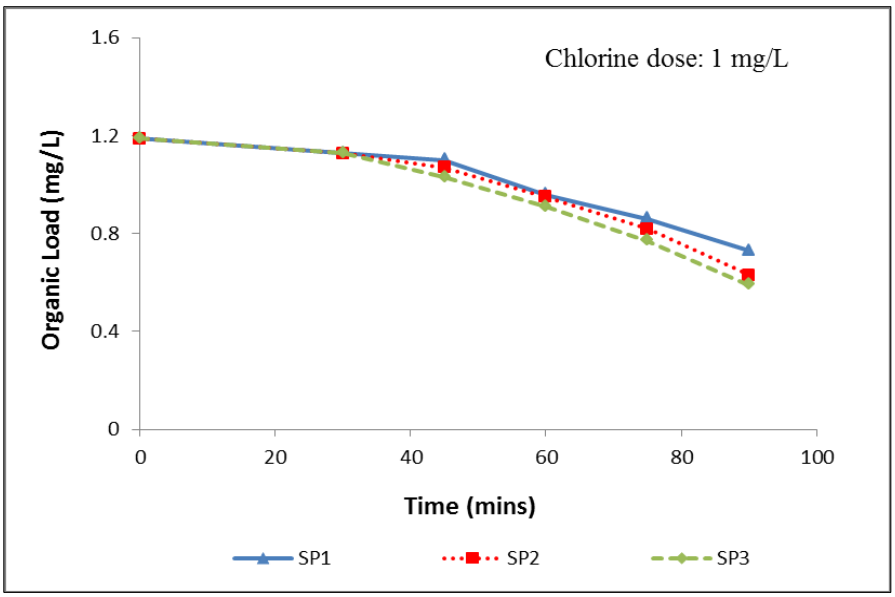


Figure 4.18 Variation in organic load with time at lab scale distribution network

4.4. DISINFECTION STUDIES WITH MIX CULTURE AT LAB SCALE DISTRIBUTION NETWORK

Table 4.8: Physicochemical analysis of water samples at 1.5 mg/L of chlorine dose

Parameters	Chlorine dose
	1.5 mg/L
pH	6.78-7.3
Temperature (^{o}C)	19.2-19.8
Electrical Conductivity (µS/cm)	429-475
Turbidity (NTU)	0.97-1.13
TDS (mg/L)	257-285

(APHA, 2012)

The effect of chlorination was observed on *S. flexneri* and *C. jejuni* in mix culture of *S. flexneri* and *C. jejuni* using 1.5 mg/L of chlorine concentration. The initial count of *S. flexneri* and *C. jejuni* after inoculation of pure culture was 1.43 x 10^7 and 1.21 x 10^7 CFU/mL, respectively. After 30 minutes of contact time, at CT value of 45 mg-min/L the survival of *S.flexneri* was higher as compared to *C. jejuni* in mix culture. There was 2-log reduction observed for *S. flexneri* and 4-log reduction observed for *C. jejuni*. At Ct value of 67.5 mg-min/L there was reduction in *S. flexneri* counts from 7.20 x 10^5 to 4.50 x10^3, 3.20 x 10^2 and 1.60 x 10^2 at sampling points SP1, SP2 and SP3 respectively.

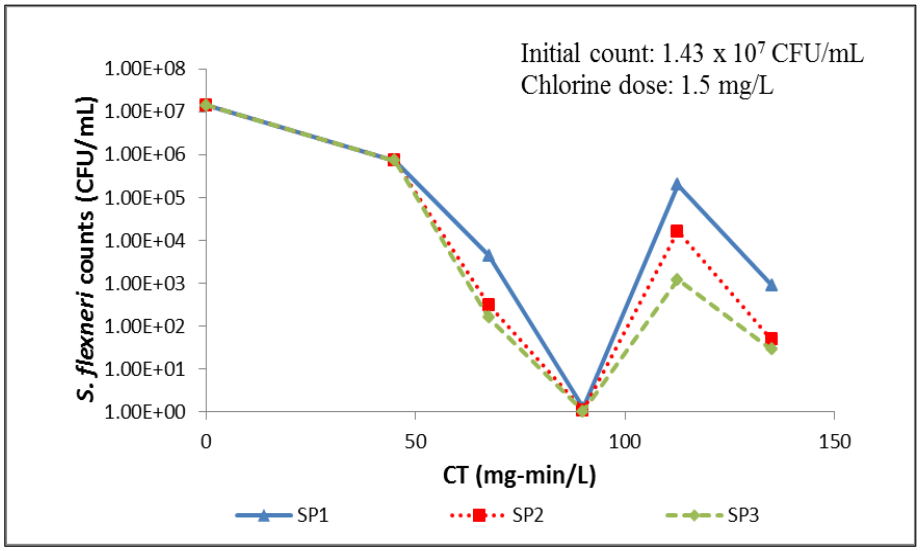


Figure 4.19 Survival of *S. flexneri* in mix culture at lab scale distribution network

For same exposure time and CT value, *C. jejuni* counts decreased from 8.40 x 10^3 to 5.50 x 10^2 at SP1 and no counts were observed at SP2 and SP3. After 60 minutes *S. flexneri* and *C. jejuni* showed complete inactivation in mix culture at sampling sites SP1, SP2 and SP3. Mix culture of *S. flexneri* and *C. jejuni* was re-inoculated at 60 minutes and after 15 minutes of contact time there were 2, 3 and 4-log reductions in *S. flexneri* counts at sites SP1, SP2 and SP3 respectively.

However, *C. jejuni* counts showed 3, 4 and 5-log reductions at taps SP1, SP2 and SP3. At CT value of 135 mg-min/L there was again log-removal of 3, 3 and 2-log for *S. flexneri* and no counts were observed for *C. jejuni*. *Campylobacter jejuni* showed increased susceptibility to chlorine in single or mix cultures as compared to *S. flexneri*.

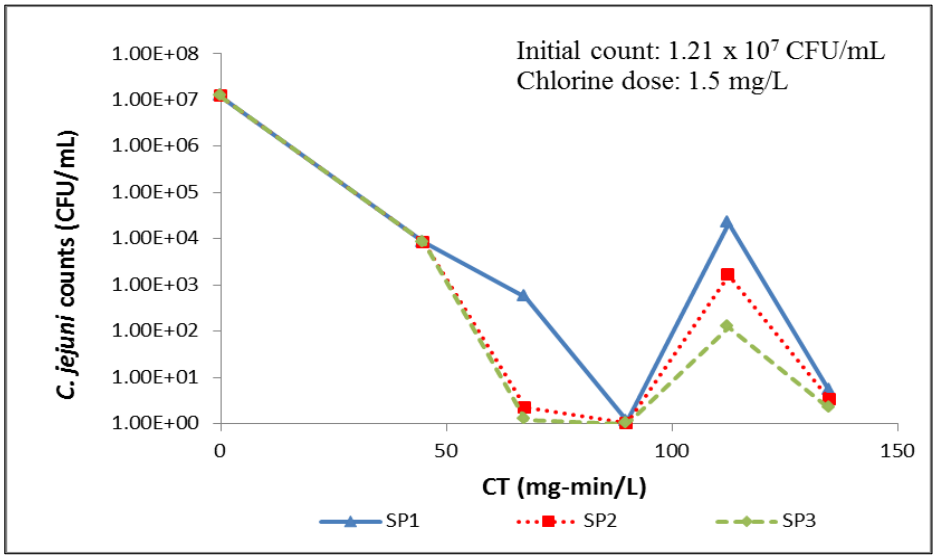


Figure 4.20 Survival of *C. jejuni* in mix culture at lab scale distribution network

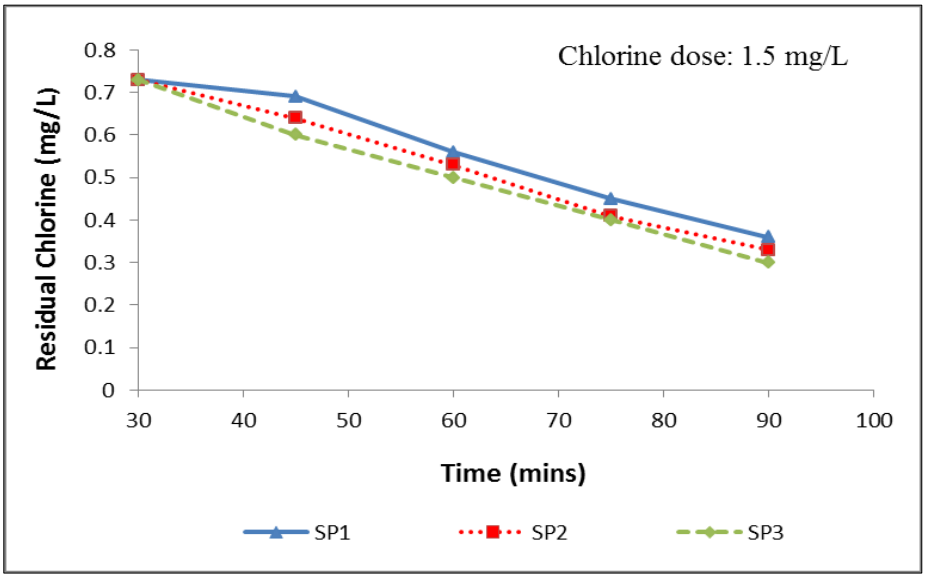


Figure 4.21 Variation in chlorine residual with time at lab scale distribution network

4.5. WATER QUALITY INDEX AT APPLIED CHLORINE DOSES

Water quality indices were calculated to assess water quality at applied chlorine doses of 1, 1.25 and 1.5 mg/L. WQI for optimized chlorine doses of 1 and 1.5 mg/L against

Campylobacter jejuni and *Shigella flexneri*, respectively, showed that water quality falls in good category according to water quality classification stated in Table 3.6. WQI values obtained at applied chlorine doses for both bacteria are mentioned in Table 4.9 with calculations mentioned in Annexure IV.

Table 4.9: WQI at applied chlorine doses

Chlorine dose (mg/L)	$\sum W_X Q_X$	$\sum W_X$	**WQI**
1	39.47	0.52	75.90
1.25	27.45	0.52	52.7
1.5	39.6	0.52	76.15

4.6. GAS CHROMATOGRAPHIC ANALYSIS

4.6.1. Chromatogram of THMs analytes mixture

Trihalomethanes mixture stock solution was prepared by mixing three analytes chloroform, dibromochloromethane and bromodichloromethane in such a composition that the mixture exhibits reproducible peak signals to be compared with chromatograms obtained from chlorinated water samples. The composition of THMs mixture is given in Table 4.10.

Table 4.10: THMs mixture

Analytes (stock solution)	Volume (mL)
Chloroform	8.0
Bromodichloromethane	1.0
Dibromochloromethane	1.0

Mixture of THMs was introduced into GC under the conditions mentioned in Table 3.4. In this chromatogram as shown in Figure 4.22 all peaks are well resolved and reproducible. This chromatogram is now comparable with chlorinated water samples. Samples collected from lab scale distribution network at optimized chlorine dosages of 1.5 and 1 mg/L at different time intervals of 30, 45, 60, 75 and 90 minutes were than run on gas chromatography to investigate THMs formation.

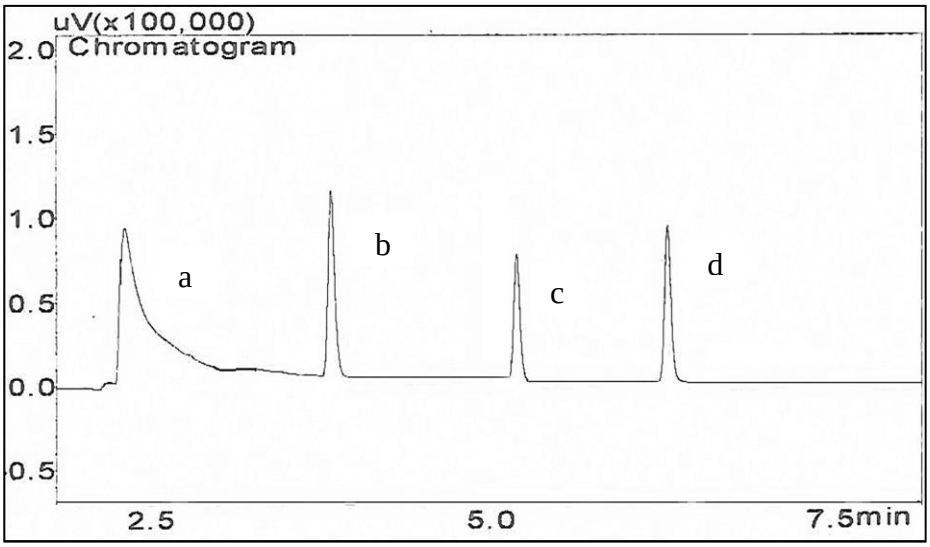


Figure 4.22 Chromatogram representing the retention time of stock mixture of trihalomethanes (a) methanol (b) chloroform (c) bromodichloromethane (d) dibromochloromethane

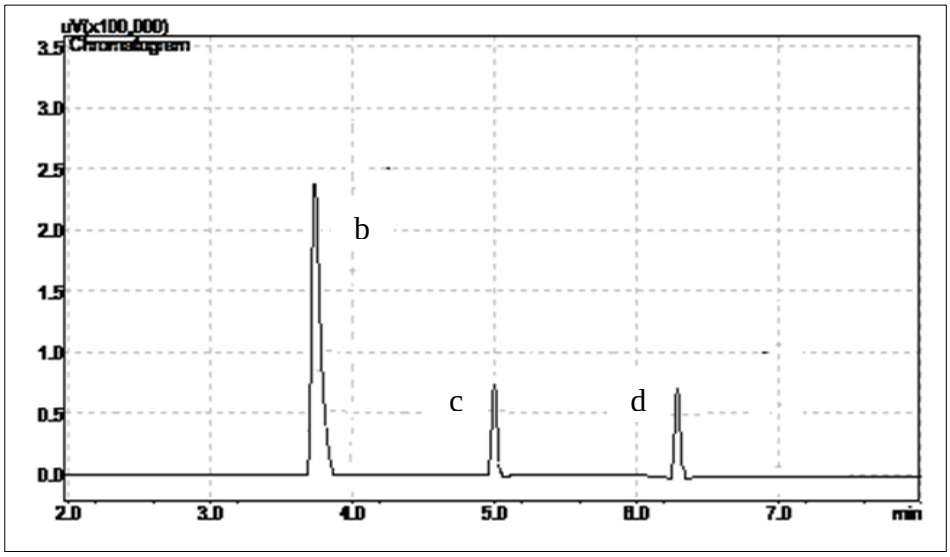


Figure 4.23 Chromatogram representing (a) Ethyl acetate (solvent)-peak not detected (b) Chloroform (c) BDCM (d) DBCM peaks at 30 minutes and 1.5 mg/L of chlorine dose

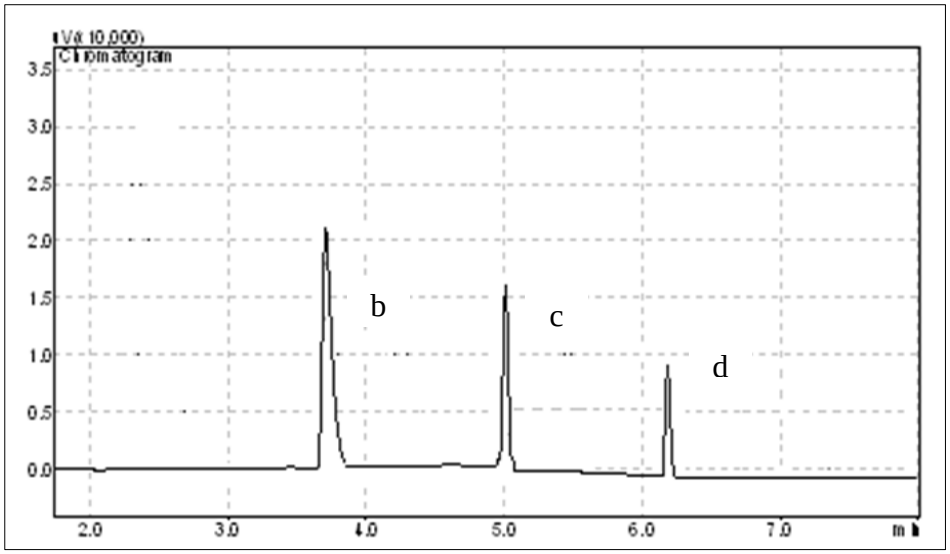


Figure 4.24 Chromatogram representing (a) Ethyl acetate (solvent)-peak not detected (b) Chloroform (c) BDCM (d) DBCM peaks at 60 minutes and 1.5 mg/L of chlorine dose

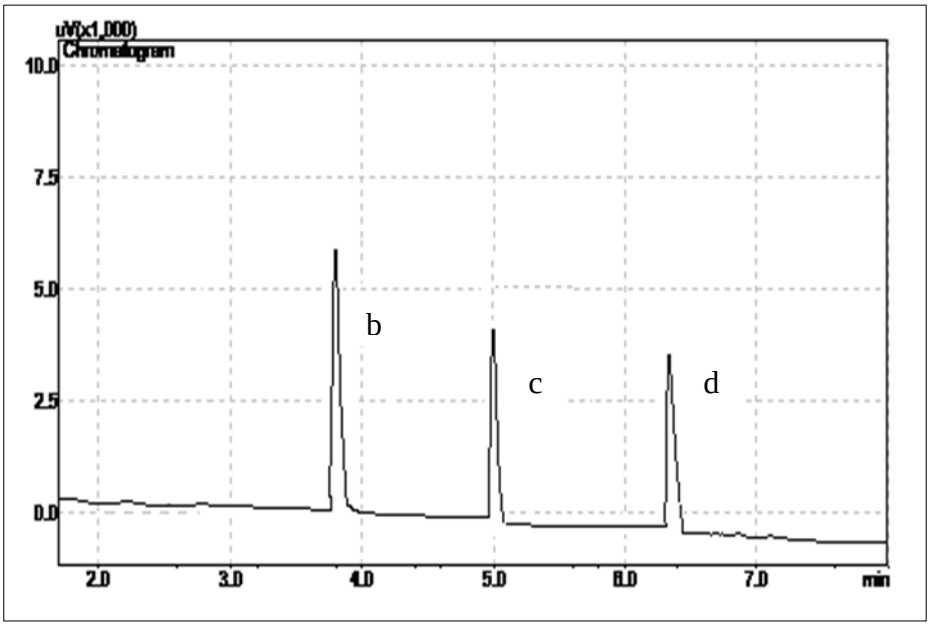


Figure 4.25 Chromatogram representing (a) Ethyl acetate (solvent)-peak not detected (b) Chloroform (c) BDCM (d) DBCM peaks at 60 minutes and 1 mg/L of chlorine dose

4.6.2. Effect of contact time on trihalomethanes formation

Contact time has a significant impact on the formation of trihalomethanes as shown in Figure 4.26 and 4.27. Trihalomethanes formation increases with time at both optimized dosages of 1.5 and 1 mg/L. Similar results were reported by (Xanthopoulou *et al.,* 2005) that longer the water contacts with free chlorine the more abundant the THMs are produced. Chloroform was abundant among all trihalomethanes formed and this shows that chlorine ion react rapidly with organic matter as compared to brominated ion.

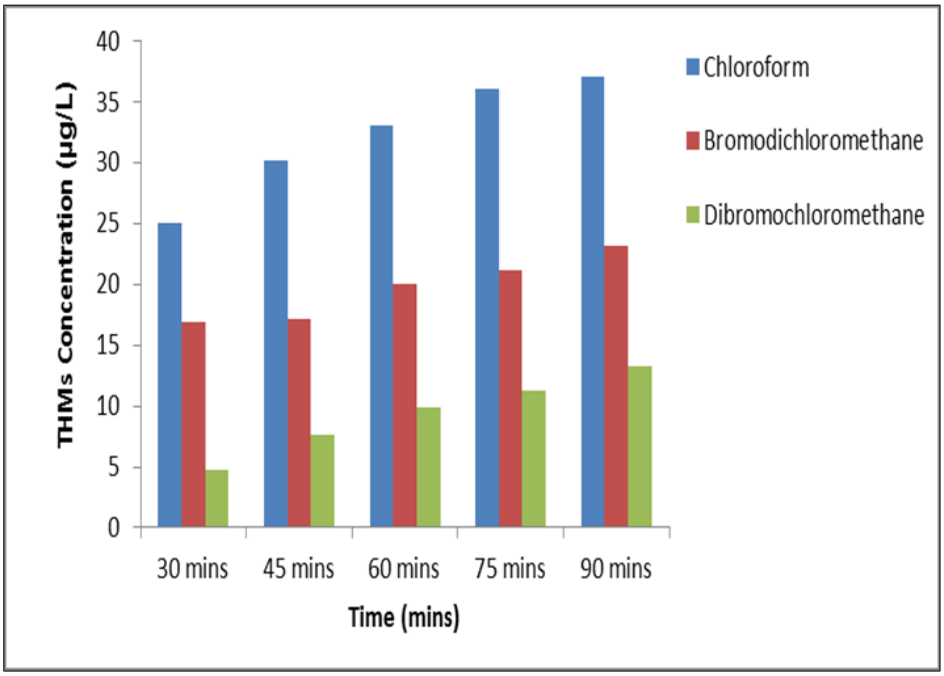


Figure 4.26 Effect of contact time on THMs formation at 1.5 mg/L chlorine dose

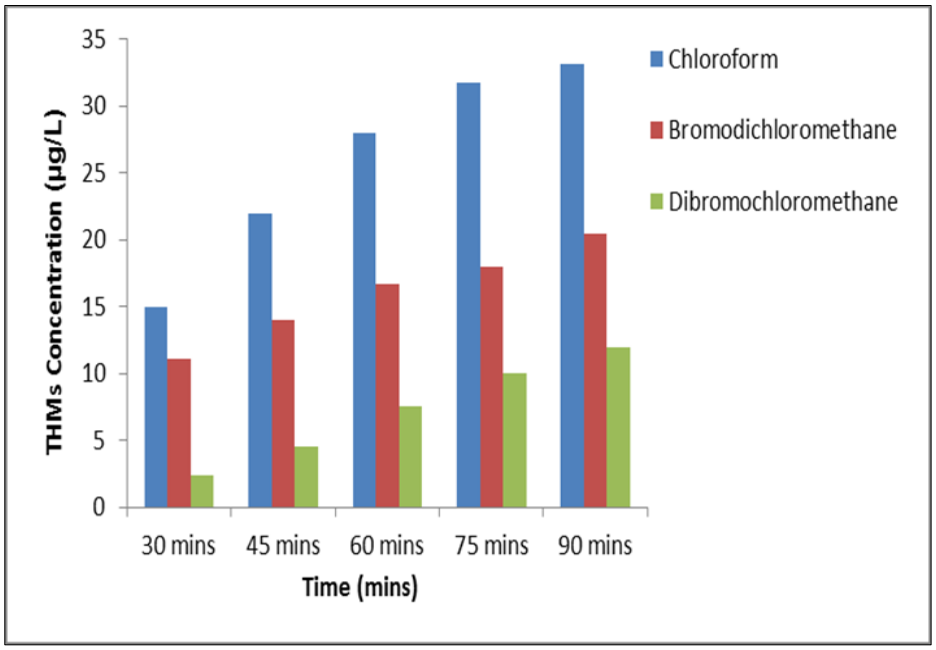


Figure 4.27 Effect of contact time on THMs formation at 1 mg/L chlorine dose

4.6.3. Effect of dissolved organic carbon on trihalomethanes formation

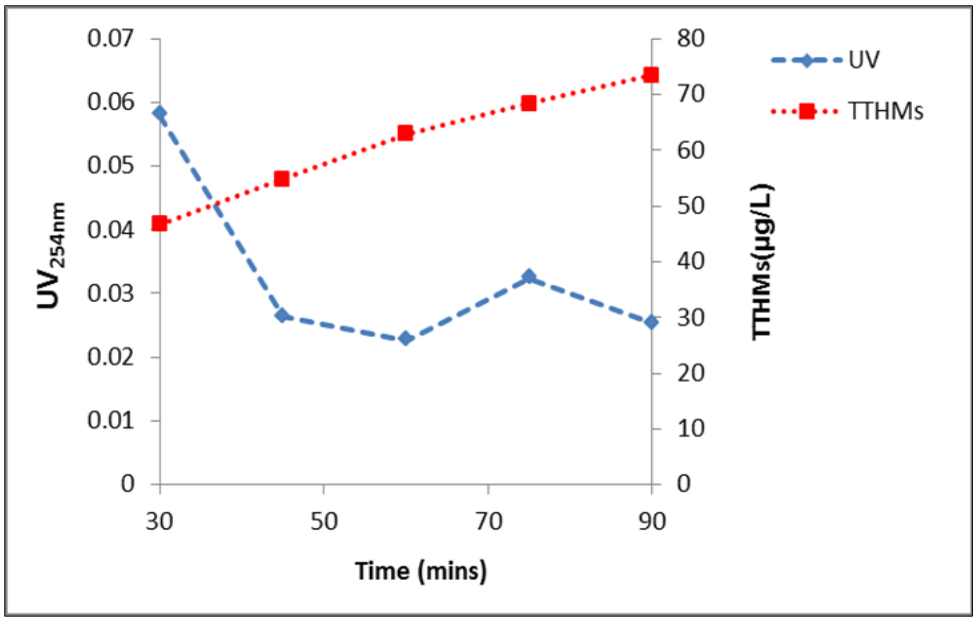


Figure 4.28 THMs relationship with dissolved organic carbon at 1.5 mg/L chlorine dose

THMs are formed when the disinfectant reacts with natural organic matter. UV_{254} nm has been widely used to predict natural dissolved organic carbon (DOC) in water or its reactivity in forming disinfection by-products during chlorination process (Ye *et al.*, 2009).

Because of easy measurement, UV_{254} nm offers potentially simple and reliable method to quantify the contribution of organic carbon in water leading to formation of THMs during chlorination. Figure 4.28 and 4.29 illustrates the relation between THMs and UV_{254} nm in all water samples tested at optimized doses of 1.5 and 1 mg/L.

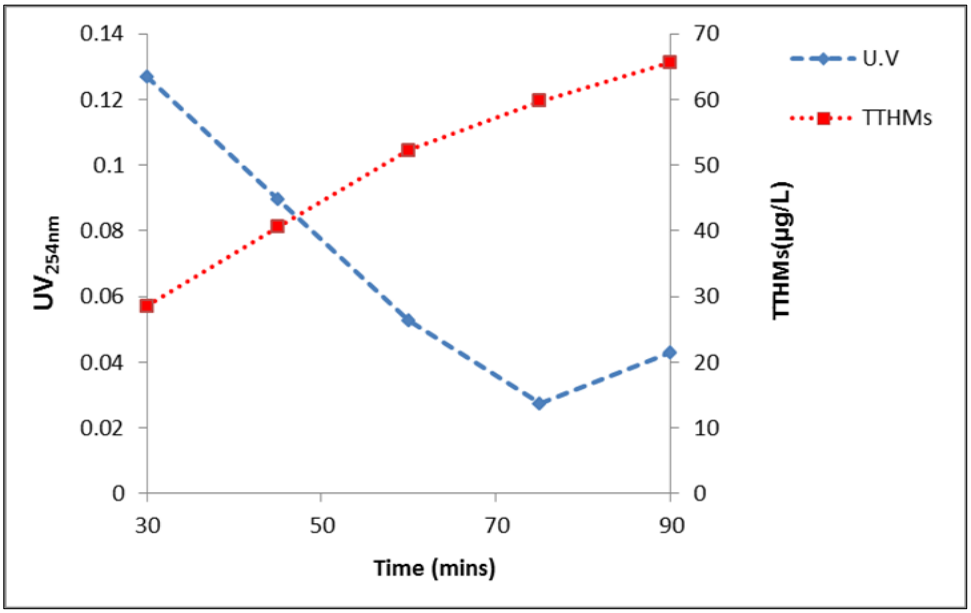


Figure 4.29 THMs relationship with dissolved organic carbon at 1 mg/L chlorine dose

Maximum loss of UV absorbance for the chlorinated water samples was observed at 254 nm that depicts the breakdown of NOM into dissolved organic carbon. THMs formation at different time intervals increases with decreasing UV absorbance (increase dissolved organic carbon content) as function of UV_{254} nm in chlorinated water. Similar results were reported by (Adin *et al.*, 1991) that the rate of THMs formation is equal to that of the organic matter consumption.

CHAPTER 5

CONCLUSIONS AND RECOMMENDATIONS

5.1. CONCLUSIONS

The main aim of this study was to optimize chlorine dosage and contact time against survival of *S. flexneri* and *C. jejuni* at lab scale distribution network. Selected enteric pathogens are known to cause adverse water borne diseases especially in developing countries. The outcomes of the study are listed below:

1. **Identified Isolates:** Selected pathogenic bacteria were isolated from contaminated water supply through enrichment technique. On the basis of cultural and biochemical characterization bacterial isolates were identified as *Shigella flexneri* and *Campylobacter jejuni*.

2. **Comparison of bench-scale and lab-scale chlorination studies revealed:**

    - *S. flexneri* and *C. jejuni* showed maximum inactivation at CT values of 56.25 and 15 mg-min/L at bench scale.
    - Maximum inactivation of *S. flexneri* and *C. jejuni* occurred at CT values of 90 and 45 mg-min/L at lab scale.
    - Residual chlorine was sufficient to combat microbial regrowth.

3. **THMs analysis for optimized chlorine doses indicated:**

    - Chloroform concentration was highest among TTHMs formed at different time intervals and were within range of (47-73 μg/L) for 1.5 mg/L and (29-66 μg/L) for 1 mg/L chlorine, respectively.
    - TTHMs increases with increase in water contact with residual chlorine and increasing soluble humic material content (UV_{254nm}).

5.2. RECOMMENDATIONS

Disinfection is very crucial step during drinking water treatment because it acts as a frontline of defense against microbial contamination. Chlorine's wide array of benefits may not be provided by any other single disinfectant.

1. Chlorine effectiveness against different pathogens varies so there is a need to conduct studies against other emerging water borne pathogens like *Helicobacter pylori*, Vibrio, *Giardia*, and *Cryptosporidium* etc.
2. Chlorination studies against selected pathogens need to be conducted at different pH and temperature.
3. Study should be extended to real distribution network.
4. Further studies on surface water starting from coagulation and flocculation to tertiary treatment of chlorine to validate study results.

CHAPTER 6

REFERENCES

APHA (2012). Standard Methods for the Examination of Water and Wastewater. 22nd ed. American Public Health Association. Washington, D.C.

Azizullah, A., Khattak, M. N. K., Richter, P., Hader. and D. P. (2011). Water pollution in Pakistan and its impact on public health — A review. Environment International, 37: 479-497.

Allwood, P. B., Malik, Y. S., Maherchandani, S., Hedberg, C.W. and Goyal, S. M. (2005). Effect of temperature on the survival of F-specific RNA coliphage, *Feline Calicivirus* and *E. coli* in chlorinated water. Int. J. Environ. Res. Public Health, 2(3): 442-446.

Adin, A., Katzhendler, J., Alkaslassy, D. and Rav, C. A. (1991). Trihalomethanes formation in chlorinated drinking water: a kinetic model. Water Res, 25: 797–805.

ADS (2005). Application Data Sheet: ADS 4950-22/rev. A. Free chlorine measurement in drinking water treatment. Emerson process management. Water and Wastewater industry.

Abdel-Wahab, A., Khodary, A. and Bensalah, N. (2010). Formation of Trihalomethanes during Seawater Chlorination. Journal of Environmental Protection, 1: 456-465.

Blaser, M. J., Smith, P. F., Wang, W. L. and Hoff, J. C. (1986). Inactivation of *Campylobacter jejuni* by chlorine and monochloramine. Appl Environ Microbiol, 51(2): 307-11.

Bishankha, S., Joshi, P. T., Joshi, R. D. and Bhatta, R. D. (2012). Assessment of microbial quality of chlorinated drinking tap water and susceptibility of gram negative bacterial isolates towards chlorine. Nepal Journal of Science and Technology, 13 (1): 173-178.

Beletshachew, S. S., Shallow, R., Marcus, S. S., Soderlund, D., Hardnett, F. P. and Gilder, V. T. (2004). Trends in Population-Based Active Surveillance for *Shigellosis* and Demographic Variability in Food Net Sites, Clinical Infectious Diseases, 38: 175-180.

Beveridge, T. J. (1990). Mechanism of Gram Variability in Selected Bacteria. J. Bacteriol., 172(3): 1609- 1620.

Berman, D. R., Eugene, W. and Hoff, J. C. (1988). Inactivation of particle-associated *coliforms* by chlorine and monochloramine. Appl. Environ. Microbiol., 54(2): 507-512

Baron, E. J., Peterson, L. R. and Finegold, S. M. (1994). Bailey and Scott's diagnonostic microbiology, 9th Ed. Mosby-Year Book, Inc., St. Louis, Mo.

Chen, Y. Q., Duan, X. D., Lu, P. P., Wang, Q., Zhang, X. J. and Chen, C. (2012). Inactivation of the chlorine-resistant bacteria isolated from the drinking water distribution system. Water Research, 33(1):104-109.

Cedergren, M. I., Selbing, A. J., Lofman, O. and Kallen, B.A. (2002). Chlorination byproducts and nitrates in drinking water and risk for congenital cardiac defects. Environ. Res, 89(2):124-130.

Calomiris, J. J. and Christman, K. A. (1998). How does chlorine added to drinking water kill bacteria and other harmful organisms? Why does not it harm us? Scientific American Inc: Chlorine Chemistry Council. U.S.A.

CDC and WHO (2003). Manual for the laboratory identification and antimicrobial susceptibility testing of bacterial pathogens of public health importance in the developing world. CDS Information Resource Centre, Atlanta, Georgia; USA.

Crump, J. A., Okoth, G.O., Slutsker, L., Ogaja, D. O., Keswick, B. H. and Luby, S. P. (2004). Effect of point-of-use disinfection, flocculation and combined flocculation-disinfection on drinking water quality in western Kenya. Journal of Applied Microbiology, 97(1): 225–231.

Cappuccino, J. G. and Sherman, N. (1996). Microbiology: A Laboratory Manual. 4th Ed. The Benjamin/Cummings Publishing Company, Inc.

Dupuy, M., Mazoua, S., Berne, F., Bodet, C., Garrec, N., Herbelin, P., Oberti, S., Soreau, S., France, W. and Hechard, Y. (2011). Efficiency of water disinfectants against *Legionella pneumophila* and *Acanthamoeba*. Water Research, 45, 1087-1094.

Di Giovanni and LeChevallier. (2000). Factors Limiting Microbial Growth in Distribution Systems: Laboratory and Pilot-Scale Experiments. J. American Water Works Association, 67: 579-595.

Farooq, S., Hashmi, I., Qazi, I. A., Qaiser, S. and Rasheed, S. (2008). Monitoring of coliform and chlorine residual in water distribution network of Rawalpindi. Environmental Monitoring and Assessment, 140: 1-3.

Forbes, A. B. and Sahm, F. D. (2002). Bailey and Scott's Diagnostic Microbiology, 11th Ed., The C.V. Mosby Co., St. Louis.

Friedman, C. R., Neimann, J., Wegener, H. C. and Tauxe, R. V. (2000). Epidemiology of *Campylobacter jejuni* infections in the United States and other industrialized nations. In: Nachamkin, I., Blaser, M. J., editors. *Campylobacter*. 2d ed. Washington, DC: ASM Press, pp: 121-38.

Gorchev, H.G. (1996). Chlorine in water disinfection. Pure and Appl. Chem., 68(9): 1731-1735.

Gagnon, G., O'Leary, K., Volk, C., Chauret, C., Stover, L. and Andrews, R. (2004). Comparative Analysis of Chlorine Dioxide, Free Chlorine and Chloramines on Bacterial Water Quality in Model Distribution Systems. J. Environ. Eng., 130(11): 1269–1279.

Guanghui, H. and David, A. R. (2007). Comparison of disinfection byproduct formation from chlorine and alternative disinfectants. Water Research, 41(8): 1667–1678.

Hong, H. C., Lianga, Y., Hanb, B. P., Mazumderc, A. and Wonga, M. H. (2007). Modeling of trihalomethane (THM) formation via chlorination of the water from Dongjiang River (source water for Hong Kong's drinking water). Science of the Total Environment, 385(1–3): 48–54.

Holt, J. G., Krieg, N. R., Sneathm, P. H. A., Staley, J. T. and Williams, S.T. (1994). Bergey's Manual of Determinative Bacteriology, 9th edn. Baltimore, MD: Williams and Williams.

Health Canada (2009). Drinking water chlorination: It's your health. [http://www.he-sc.gc.ca/hl-vs/iyh-vsv/environ/chlor-eng.php].

Health Canada (1994). Canadian Environmental Protection Act. Human health risk assessment for priority substances. Catalogue No. En40-215/41E. Minister of Supply and Services Canada, Ottawa, Ontario.

Hafeez, A. (2008). Proposal for clean water supply in Pakistan. United Kingdom Association for medical aid to Pakistan. Registered charity number 1123929.

Haydar, S., Arshad, M. and Aziz. J. A. (2009). Evaluation of Drinking Water Quality in Urban Areas of Pakistan: A Case Study of Southern Lahore. Pak. J. Engg. & Appl. Sci, 5:16-23.

IARC (1991). International Agency for Research on Cancer (IARC) – Summaries & Evaluations. Chlorinated Drinking Water, 52(45).

Johnson, C. H., Rice, E. W. and Reasoner, D. J. (1997). Inactivation of *Helicobacter pylori* by Chlorination. Applied and Environmental Microbiology, 63(12): 4969–4970.

Jarrell, K. F. and McBride, M. J. (2008). The surprisingly diverse ways that prokaryotes move. Nature reviews. Microbiology 6: 466–476.

Karim, Z., Mumtaz, M. and Kamal, T. (2011). Evaluation of trihalomethanes in tap water samples of Karachi city (Pakistan). International Journal of Environmental Research and Public health, 7(1): 105-108.

Krasner, W. S. (2009). The formation and control of emerging disinfection by-products of health concern. Phil. Trans. R. Soc. A, 3(67): 4077–4095.

Khanzadi, S., Jamshidi, A., Soltaninejad, V. and Khajenasiri, S. (2010). Isolation and Identification of *Campylobacter jejuni* from Bulk Tank milk in Mashhad-Iran. World Applied Sciences Journal, 9(6): 638-643.

Koseki, S., Yoshida, K., Isobe, S. and Itoh, K. (2002). Decontamination of lettuce using acidic electrolyzed water. J. Food Protection. 64(5): 652-658.

Lee, J., Kim, E. S., Roh, B. S., Eom, S. W. and Zoh, K.D. (2013). Occurrence of disinfection by-products in tap water distribution systems and their associated health risk. Journal of Environmental Monitoring and Assessment, 185 (9): 7675-7691.

Lew, J. F., Swerdlow, D. L., Dance, M. E., Griffin, P. M., Bopp, C. A., Gillenwater, M. J., Mercatante, T. and Glass, R. I. (1991). An outbreak of *Shigellosis* aboard a cruise ship caused by a multiple-antibiotic-resistant strain of *Shigella flexneri*. American Journal of Epidemiology. 134(4): 413-420.

LeChevallier, M. W., Cawthon, C. D. and Lee, R. G. (1988). Factors promoting survival of bacteria in chlorinated water supplies. Appl. Environ. Microbiol, 54: 649–654.

LeChevallier, M. W. (1985). *Coliform* regrowth in drinking water: a review. J. Am. Water Works Assoc., 82: 74–86.

Lund, V. (1996). Evaluation of *E. coli* as an indicator for the presence of *Campylobacter jejuni* and *Yersinia enterocolitica* in chlorinated and untreated oligotrophic lake water. Water Research, 30(6): 1528–1534.

Liu, F., Chen, Z., Chang, S. and Shen, J. (2013). Inactivation of *Bacillus subtilis* spores in water with chlorine. J. CIESC, 64(8): 2982-2988.

Nazmul Alam, S. M., Mostafa, G. and Bhuiyan, D. H. (2003). Prevalence of bacteria in the muscle of shrimp in processing plant. Internat. J. Food Safety, 5: 21-23.

National Cancer Institute (1976). Report on the carcinogenesis bioassay of chloroform. NTIS PB-264-018. Bethesda, MD: National Cancer Institute.

National Toxicology Program (1986). Toxicology and carcinogenesis of bromodichloromethane in F344/N rats and B6C3F1 mice. Technical Report Series No. 321. NIH Publication No. 88-2537, CAS No. 75-27-4. Washington, DC: US Department of Public Health and Human Services.

National Toxicology Program (1989). Toxicology and carcinogenesis of tribromomethane (bromoform) in F344/N rats and B6C3F1 mice. Technical Report Series No. 350. NIH Publication No. 89-2805, CAS No. 75-25-2. Washington, DC: US Department of Public Health and Human Services.

Ozdemir, K., Toroz, E. and Uyak, V. (2013). Assessment of trihalomethanes formation in chlorinated raw waters with differential UV spectroscopy approach. The Scientific World Journal, 20(13): 890-898.

Payal, S. and Charles, P. G. (2012). Inactivation of *Naegleria Fowleri* by chlorine and ultraviolet light. J. AWWA, 104(3): 173-180.

Parkhill, J., Wren, B. W. and Mungall, K. (2000). The genome sequence of the foodborne pathogen *Campylobacter jejuni* reveals hypervariable sequences. Nature, 403:66-8.

Park, H. J., Hung, Y. C. and Chung, D. (2004). Effects of chlorine and pH on efficacy of electrolyzed water for inactivating *Escherichia coli* O157:H7 and *Listeria monocytogenes*. Int. J. Food Microbiology, 91(1): 13-18.

Ram, S., Vajpayee, P., Singh, R. L. and Shanker, R. (2009). Surface water of a perennial river exhibits multi-antimicrobial resistant shiga toxin and enterotoxin producing *Escherichia coli*. Ecotoxicology and Environmental Safety, 72(2): 490-495.

Rice, E. W., Clark, R. M. and Johnson, C. M. (1999). Chlorine inactivation of *Escherichia coli O157:H7*. Emerging infectious diseases. 5(3): 461-3.

Rao, C. S., Rao, B. S., Hariharan, A. V. L. N. S. H. and Bharathi, N. M. (2010). Determination of water quality index of some areas in guntur district Andhra Pradesh. International Journal of Applied Biology and Pharmaceutical Technology, 1(1): 79-86.

Shehzad, I. (2006). Pakistan debates new water and sanitation policies. Asia Water Wire (END/AWW/IPSAP/EK/BB/06).

Sadia, J., Mahmood, Q., Tariq, S., Nawab, B. and Elahi, N. (2011). Health impact caused by poor water and sanitation in district Abbottabad. Journal of Ayub Med Coll Abbottabad, 23(1): 432-440.

Schaechter, M., Engleberg, N. C., Eisenstein, B. I. and Medoff, G. (1999). Mechanisms of Microbial Disease (3rd edition). Lippincott Williams and Wilkins. Philadelphia, PA. pp. 178.

Srivastava, G. and Kumar, P. (2013). Water quality index with missing parameters. International Journal of Engineering Research and Technology, 2(4): 609-614.

Theresa, L. C., Amy, M. K. and Vincent, R. Hill. (2010). Inactivation of *Adenoviruses*, *Enteroviruses*, and *Murine Norovirus* in Water by Free Chlorine and Monochloramine. Appl. Environ. Microbiol, 78(4): 1028-1033.

Taylor, D. N. and Blaser, M. J. (1991). The epidemiology of *Helicobacter pylori* infection. Epidemiol. Rev, 13: 42–58.

Uyanik, H. M., Yazgi, H. and Ayyildiz, A. (2008). Survival of *Salmonella typhi* and *Shigella flexneri* in different water samples and at different temperatures. Turk Journal of Med Sci., 38 (4): 307-310.

U.S. Environmental Protection Agency (1998). National Primary Drinking Water Regulations: Disinfectants and Disinfection Byproducts; Final Rule, 63 (157).

U.S. Environmental Protection Agency (1999). EPA Office of Water: Alternative Disinfectants and Oxidants Guidance Manual. (EPA 815-R-99-014). Washington, D.C.

U.S. Environmental Protection Agency (2002). Final draft for the addendum to drinking water criteria document for monochloroacetic acid and trichloroacetic acid. Prepared for Health and Ecological Criteria Division, Office of Science and Technology, Office of Water, US Environmental Protection Agency, Washington, DC (EPA-822-R-02-036).

UNICEF (2005). Chlorination: Guidelines on chlorination practices. Trinchomalee, UNICEF Srilanka.

Uyak, V. and Toroz, I. (2007). Investigation of bromide ion effects on disinfection by-products formation and speciation in an Istanbul water supply. Journal of Hazard Materials, 149(2):445-51.

Wullings, B. A., Bakker, G., Kooij. and Dick, V. D. (2011). Concentration and diversity of uncultured *Legionella spp*. in two unchlorinated drinking water supplies with different concentrations of natural organic matter. Appl. Environ. Microbiol., 77:634–641.

Wielen, V. D. and Medema, G. (2010). Unsuitability of quantitative Bacteroidales 16S rRNA gene assays for discerning fecal contamination of drinking water. Appl. Environ. Microbiol., 76:4876–4881.

WWF (2007). World Wildlife Federation. Pakistan's water at risk. Water and health related issues in Pakistan and key recommendations. Fresh water & toxics programme, World Wildlife Federation-Pakistan.

WHO (1993). Guidelines for drinking water quality. Geneva, Switzerland; Office of Publication, WHO.

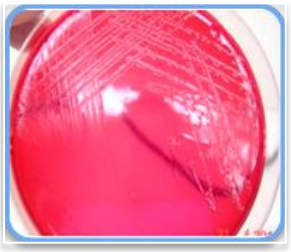
Wang, W. L., Powers, B. W., Leuchtefeld, N. W. and Blaser, M. J. (1983). Effects of disinfectants on *Campylobacter jejuni*. Appl Environ Microbiol, 45(4):1202–1205.

Winn, W., Allen, S., Janda, W., Koneman, E., Procop, G., Schreckenberger, P. and Woods. G. (2006). Koneman's Color Atlas and Textbook of Diagnostic Microbiology, 6th edition. Curved Gram negative bacilli and oxidase positive fermenter: *Campylobacteriaceae* and *Vibrionaceae*. Chapter 8, pp.393-428, Lippincott Williams & Wilkins, Baltimore, USA.

Xanthopoulou, J. N., Papagianni, V. M., Papaioannou, A. and Haralambidou, A. (2005).Volatile organic compounds in the finished water of the water treatment plant in Thessaloniki, Greece. Global NEST Journal, 7(1): 119-127.

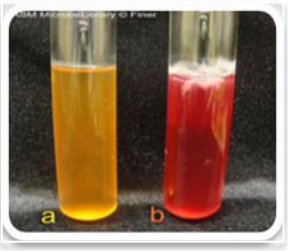
Ye, B., Wang, W., Yang, L., Wei, J. and Xueli, E. (2009). Factors influencing disinfection by-products formation in drinking water of six cities in China. Journal of Hazardous Materials, 171: 147–152.

ANNEXURE-I



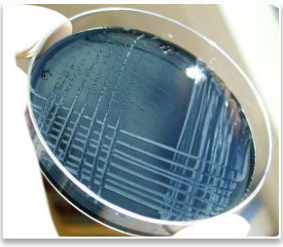
XLD agar

Catalase Test



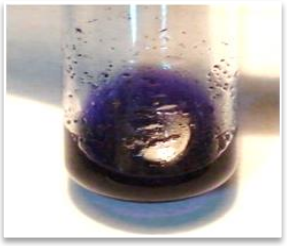
Mannitol Fermentation

Biochemical Identification of *Shigella flexneri*



CSM agar

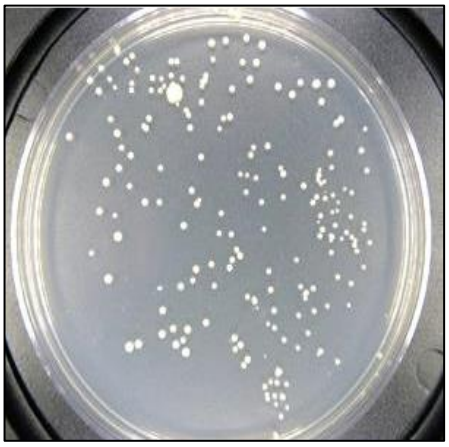
Oxidase Test



Hippurate Hydrolysis Test

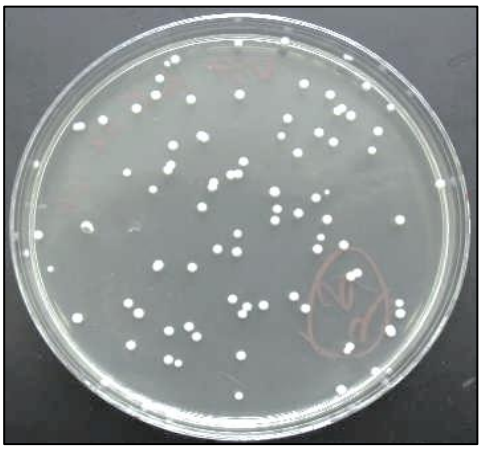
Biochemical Identification of *Campylobacter jejuni*

ANNEXURE-II



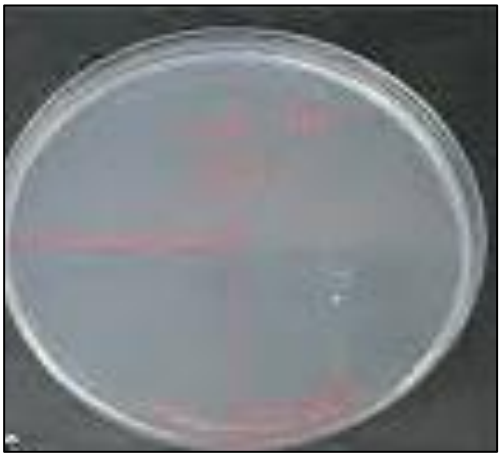
0 hr (Reservoir)

Before chlorine treatment



30 minute (Reservoir)

After chlorine treatment

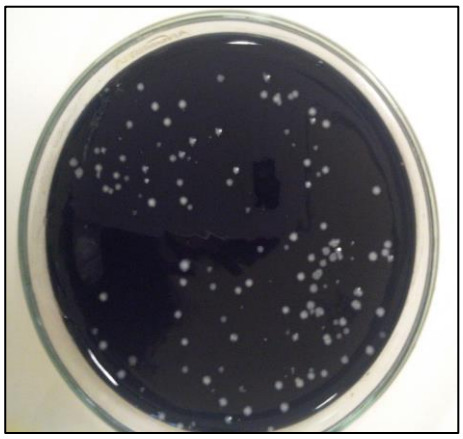


60 minutes (Tap 10)

After chlorine treatment

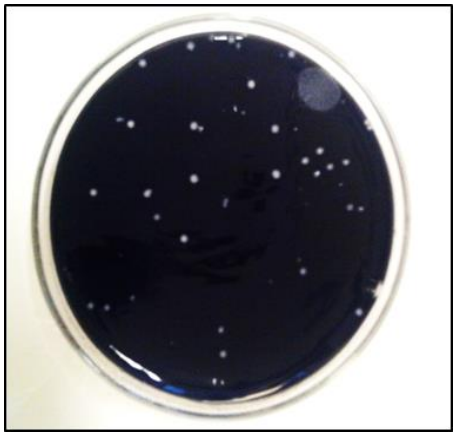
Survival of *S. flexneri* at lab scale distribution network (1.5 mg/L)

ANNEXURE-III



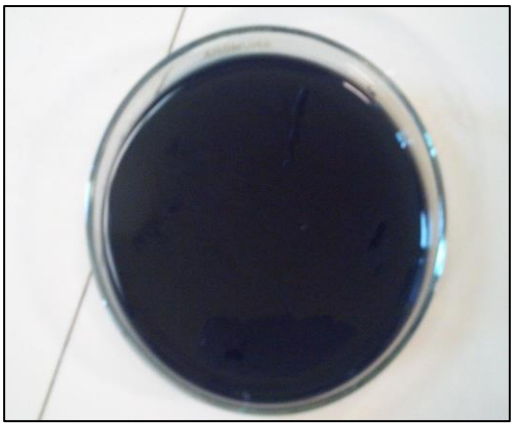
0 minute (Reservoir)

Before chlorine treatment



30 minutes (Reservoir)

After chlorine treatment



45 minutes (Tap 10)

After chlorine treatment

Survival of *C. jejuni* at lab scale (1 mg/L)

ANNEXURE-IV

Table: Weighing factors of water quality parameters

Parameters	Units	Weight factors
Fecal coliform	CFU/mL	0.16
pH	-	0.11
Temperature	^{o}C	0.10
Turbidity	NTU	0.08
Total dissolved solids	mg/L	0.07

Tables: Q-values

Chlorine dose: 1 mg/L

Parameters	Concentrations	Q-values
Fecal coliform	0 CFU/mL	98
pH	7.1	91
Temperature	18.25^{o}C	22
Turbidity	2.84 NTU	90.5
Total dissolved solids	292.5 mg/L	62.5

Chlorine dose: 1.25 mg/L

Parameters	Concentrations	Q-values
Fecal coliform	106 CFU/mL	37
pH	6.7	72.5
Temperature	20.5^{o}C	19.5
Turbidity	3.07 NTU	90.5
Total dissolved solids	270 mg/L	62.5

Chlorine dose: 1.5 mg/L

Parameters	Concentrations	Q-values
Fecal coliform	0 CFU/mL	98
pH	7.01	91
Temperature	17.25^{o}C	23
Turbidity	2.92 NTU	90.5
Total dissolved solids	263.5 mg/L	62.5

WQI Calculations:

$$\mathbf{WQI} = \sum \mathbf{W_Y Q_Y} / \sum \mathbf{W_Y}$$

$$\mathbf{W_Y Q_Y} = \mathbf{W_{pH} Q_{pH}} + \mathbf{W_{F.C} Q_{F.C}} + \mathbf{W_{TDS} Q_{TDS}} + \mathbf{W_{Temp} Q_{Temp}} + \mathbf{W_{Turbidity} Q_{Turbidity}}$$

Chlorine dose: 1 mg/L

$$W_Y Q_Y = 0.11 \times 91 + 0.16 \times 98 + 0.07 \times 62.5 + 0.10 \times 22 + 0.08 \times 90.5$$

$$\sum W_Y Q_Y = 39.47$$

$$\sum W_Y = 0.52$$

$$WQI = 75.90$$

Chlorine dose: 1.25 mg/L

$$W_Y Q_Y = 0.11 \times 72.5 + 0.16 \times 37 + 0.07 \times 62.5 + 0.10 \times 19.5 + 0.08 \times 90.5$$

$$\sum W_Y Q_Y = 27.45$$

$$\sum W_Y = 0.52$$

$$WQI = 52.7$$

Chlorine dose: 1.5 mg/L

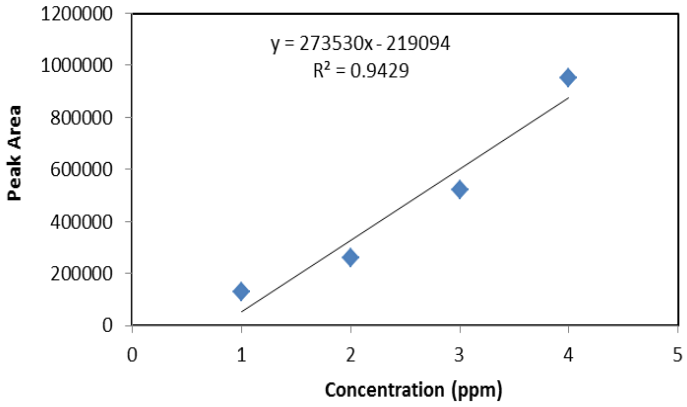
$$W_Y Q_Y = 0.11 \times 91 + 0.16 \times 98 + 0.07 \times 62.5 + 0.10 \times 23 + 0.08 \times 90.5$$

$$\sum W_Y Q_Y = 39.6$$

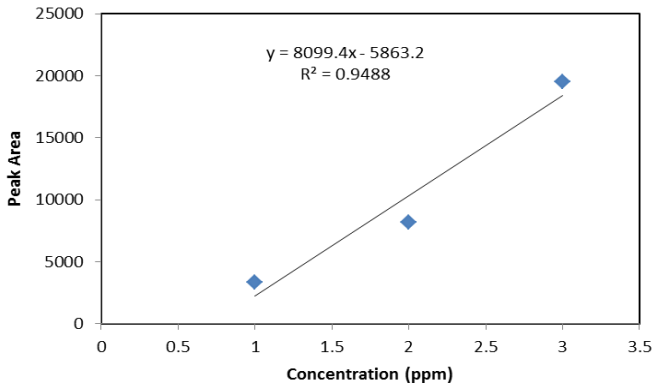
$$\sum W_Y = 0.52$$

$$WQI = 76.15$$

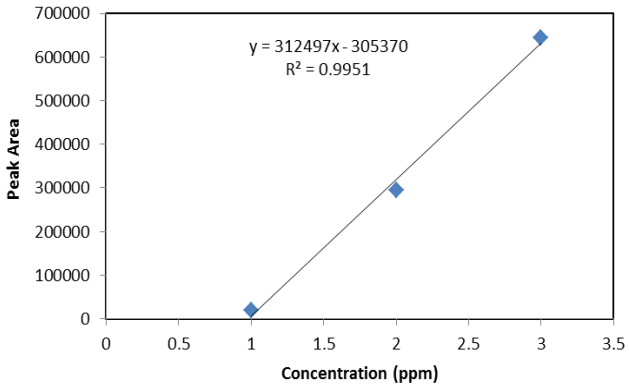
ANNEXURE-V



Chloroform Standard Calibration Curve



Bromodichloromethane Standard Calibration Curve



Dibromochloromethane Standard Calibration Curve