Evaluating Biocidal Potential of Electrolyzed Solution against Targeted Microbial Species in Water Distribution Network of NUST

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CERTIFICATE

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

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I certify that research work titled ***"Evaluating biocidal potential of electrolyzed solution against targeted microbial species in water distribution network of NUST"*** is my own work. The work has not been presented elsewhere for assessment. Where material has been used from other sources, it has been properly acknowledged / referred.

Sabtain Ali
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DEDICATION

This work is dedicated to my beloved Parents!

For their endless love and support

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LIST OF ABBREVIATIONS

Abbreviation	Description
BGLB	Brilliant Green Bile Broth
BLAST	Basic Local Alignment Search Tool
CFU	Colony Forming Unit
COD	Chemical Oxygen Demand
DBPs	Disinfection byproducts
DO	Dissolved Oxygen
DOM	Dissolved Organic Matter
EC	Escherichia Coli
ECAS	Electrochemically Activated Solutions
ECAW	Electrochemically Activated Water
EOW	Electrolyzed Oxidized Water
ESOL	Electrolyzed Solution
EW	Electrolyzed Water
FRWA	Florida Rural Water Association
GAC	Granular Activated Carbon
GI	Galvanized Iron

HAAs	Halo Acetic Acids
HANs	Halo Acetonitriles
HAs	Halo Aldehydes
HDPE	High Density Polyethylene
HGAAS	Hydride Generation Atomic Absorption Spectroscopy
HIV	Human Immunodeficiency Virus
HKs	Halo ketones
HNMs	Halo Nitromethanes
HPC	Heterotrophic Plate Count
I-THMs	Iodo-Tri Halo Methanes
LFH	Laminar Flow Hood
LTB	Lauryl Tryptose Broth
MF	Membrane Filtration
MGD	Million Gallons per day
MIOX	Mixed Oxidant
MPN	Most Probable Number
Mv	Millivolt
NCBI	National Center for Biotechnology Information

ND	Not Detected
NSW	New South Whales
OD	Optical Density
OHT	Overhead Tank
OM	Organic Matter
ORP	Oxidation Reduction Potential
PCRWR	Pakistan Council Research in Water Resources
PP	Polypropylene
RO	Reverse Osmosis
SPC	Spread Plate Count
T.W	Tube well
TOC	Total Organic Carbon
UPVC	Un-Plasticized Polyvinyl Chloride
UV	Ultraviolet
WHO	World Health Organization
XLD	Xylose Lysine Deoxycholate

ABSTRACT

The biocidal efficiency of electrochemically activated solution (ECAS) against isolated bacterial species from the water distribution network of study area was analyzed. Active microbial consortia (identified using 16s rRNA gene sequencing as *Aeromonas* sp., *Enterobacter* sp., and *Escherichia*. *coli*) with a mean count of 1.15×10^6 CFU/mL was inoculated in 100 liters tap water in a prototype network to simulate real conditions. The physicochemical quality of tap water of subsurface origin was assessed before experimentation. The contact times of 10 sec, 10 mins and 1 hr. were selected against the ECAS dosages of 1, 5 and 10 % (v/v) respectively. Each test organism showed an independent response at varying dosages. A 10% disinfectant dosage corresponding to a contact time of 1 hour showed the highest log reduction as 4.11 >3.50 > 2.74 for *E. Enterobacter sp. > E. coli > Aeromonas sp.* respectively. *The* results indicated that high organic carbon altered the inactivation kinetics and reduced the oxidative potential of ECAS which hindered complete inhibition of test organisms. The study concludes that adequate pretreatment could enhance the removal efficacy and reduce the minimum inhibitory concentration of ECAS.

Chapter 1

INTRODUCTION

1.1. Background

Water is essential to sustain life on earth. An adequate, safe and accessible water supply must be available to all. Accelerated population growth coupled with impoverished socioeconomic development, limited water resources and poor sanitation leads to an increase in diseases associated with poor living conditions among which water related and water borne diseases play a major role (Lippmann, 2000). Around 844 million people of the world lack access to safe drinking water sources (WHO and UNICEF, 2017). Consumption of unsafe water causes around 1.7 to 2 million deaths annually from diseases like diarrhea (Clayton et al., 2017) from developing or underdeveloped countries due to poor sanitary conditions and water distribution systems (Pruss-Ustun et al., 2008). Improving access to safe drinking water results in tangible benefits to the health of living beings. The greatest risk to public health from microbes in water is associated with consumption of drinking-water that is contaminated with human and animal excreta, although other sources and routes of exposure may also be significant. Waterborne outbreaks have been associated with inadequate treatment of water supplies and unsatisfactory management of drinking water distribution. For example, in distribution systems, such outbreaks have been linked to cross-connections, contamination during storage, low water pressure and intermittent supply. Water borne outbreaks have been associated with inadequate treatment of water supplies (WHO, 2017). The problem may significantly be reduced through adequate disinfection (Stalter et al., 2016). Conventional

disinfection methods have been majorly divided between chemical and physical processes. Chemical disinfection of water supplies bring significant public health hazards in addition to its benefits. Ozone, chlorine, sodium hypochlorite or chlorine dioxide are mostly added to the water to be treated in chemical disinfection. The process may however, result in the formation of toxic compounds through interactions between disinfectants and organic material in the source water known as disinfection byproducts (DBPs) which has raised concerns due to their potential adverse health effects (Komaki et al., 2014). Chlorination is ineffective in achieving the reduction below the permissible limits against various waterborne pathogens, such as *Legionella, Cryptosporidium, Giardia Noroviruses and Hepatitis A Virus (HAV)* due to their resistant nature and ability to persist in water (WHO, 2017). According to Razzolini et al. (2010), chlorination is ineffective against resistant microorganisms that produce toxins and colonize biofilms such as *Aeromonas sp.* The residual chlorine concentration required to inactivate each type of microbe is specific (Martínez-Hernández et al., 2013). Chlorine-based disinfectants also poses a risk associated with the handling and storage of chemical disinfectants (Ghebremichael et al., 2011; Kraft, 2008). These limitations have instigated the search of an alternative disinfection method. Electrochemically activated solution (ECAS) is considered to be a more environmentally friendly disinfectant (Ashraf et al., 2014) with a higher disinfection potential compared to chlorine due to the presence of multiple oxidants in it (Martfnez-Huitle and Brillas, 2008). It is referred as “green biocide” with no need to transport and store potentially hazardous chemicals (Hsu, 2005). ECAS has been reported to have a wide range of antimicrobial activity against *Salmonella, Listeria monocytogenes, Escherichia coli O157:H7, hepatitis B virus, herpes*

simplex viruses, *human norovirus surrogates*, *avian influenza* and other pathogens (Tamaki et al., 2014). Due to its rapid mode of action and fast kill kinetics (Thorn et al., 2013; Robinson et al., 2011) it is found to have beneficial application in drinking water disinfection (Clayton et al. 2017; Ghebremichael et al., 2011; Martfnez-Huitle and Brillas ,2008).

ECAS is known by various terms, most commonly electrolyzed water (EW), electrochemically activated water (ECAW), electrolyzed oxidizing water (EOW), electrolyzed solution (ESOL), super oxidized water and mixed oxidant (MIOX) solutions. It is produced by using water, salt and electricity. Electrolysis of a salt solution through ion exchange membrane of electrochemical cell produces an anolyte and catholyte (Fang et al., 2016). The anolyte being ECAS has a very low pH i.e. 2.3 - 2.7 and high oxidation reduction potential (ORP) i.e. 1000 – 1200 mV (Hsu, 2005). ECAS is usually composed of multiple oxidizing agents i.e. chlorine species, hydrogen peroxide, oxygen species such as ozone, and hydroxyl radicals (Ghebremichael et al., 2011; Kunigk et al, 2008). The oxidants such as ozone, hydroxyl radicals and ClO_2 are transient in nature and short lived (Clayton et al., 2017; Gherbrimichael et al., 2011). ECAS rapidly degrades and reverts to a weak salt solution during the time of chemical relaxation (Thorn et al., 2012). High ORP generates strong oxidative stress which causes damage to the cell membrane (Huang et al., 2008) and creates an environment outside the working domain of major microbial processes such as energy generating mechanisms. The powerful oxidants sequester electrons from microbial structure compounds with high efficiency which results in rupturing of the biochemical bonds and loss of function subsequently. The

generated anolyte solution (ECAS) is dosed to the water to be disinfected (Thorn et al., 2012)

1.2. Problem Statement

Chlorination is widely adopted method for drinking water disinfection in Pakistan. But there are significant implications to the public health from chlorination. DBPs production potential, inefficient against resistant species, irritant odor, low stability, ecological risk, toxic residues, and specific residual concentration for each microbe demands a substitute disinfectant to ensure the microbially safe drinking water at user end point (Diao et al., 2004; Zeng et al., 2011). ECAS is widely adopted as a water disinfectant for primary or secondary disinfection in because of it broad range antimicrobial activity, ease of production, onsite generation, rapid kill kinetics, low propensity of DBPs production, residual kill of pathogens and rapid degradability. The antimicrobial efficacy of ECAS has been analyzed either in its pure form or in demineralized water. However, limited work has been done to analyze the disinfection potential of ECAS in tap water. Ghebremichael et al. (2011) found that the biocidal efficacy of disinfectants is low in tap water than demineralized water which may be associated with the influence of dissolved substances in addition to organic carbon in it. Nowadays, disinfection of tap water systems is mainly carried out using superheat and flush, copper or silver ionization, ultraviolet light UV, heating and hyper chlorination (Kim et al., 2002) which are more expensive and less convenient (Diao et al., 2004). By maintaining an ORP value of 650mV through ECAS dosing, drinking water can adequately disinfected (NSW Gov, 2016).

Therefore, the current study investigated the efficacy of ECAS in tap water of ground water origin inoculated with high density enteric bacteria (isolated from water distribution network of the study area), employing prototype distribution network.

1.3. Objectives of the Study

Based on the problem statement, study was based on the following objectives:

i. Characterizing water quality of the Zone 1 of NUST.

ii. Analyzing the biocidal potential of ECAS in prototype distribution network.

iii. Determining the ECAS potential for IESE tap water.

Chapter 2

LITERATURE REVIEW

Safe and readily available water is imperative for drinking, domestic use, food production or recreational purposes. The highest risk to public health from microbes in water is associated with intake of drinking water that is contaminated with human and animal excreta, although other sources and routes of exposure may also be significant. Water-related diseases continue to be one of the major health problems globally. Around 2 billion people globally use drinking water source containing fecal contamination. Water borne diseases caused by contaminated water consumption includes diarrhea, cholera, dysentery, typhoid and polio. The diarrheal death toll due to the consumption of contaminated drinking water is estimated to be up to 502,000 each year. In low- and middle-income countries, 38% of health care facilities lack an improved water source, 19% do not have improved sanitation, and 35% lack water and soap for hand washing (WHO and UNICEF, 2017).

In Pakistan, water at the source, in the distribution network, and at the user end is heavily polluted with coliforms and fecal coliforms all over the country which has detrimental repercussions for the environment and human health (Daud et al., 2017). A study conducted by Nabeela et al. (2014) revealed that over 7,000 water samples tested, an average of over 71 and 58% samples in the country was contaminated with total coliforms and fecal coliforms, respectively. Drinking water contamination accounts for 20 to 40% of all diseases in the country, which results in national income losses of PKR 25-58 billion per annum i.e., approximately 0.60-1.44 % of the country's GDP. In

Pakistan only 72% of water supply schemes were found to be functional, and 84% of those had supplied water that was unfit for consumption (Khan, 2016).

Waterborne outbreaks have been associated with inadequate treatment of water supplies and unsatisfactory management of drinking water distribution. In distribution systems, such outbreaks have been linked to cross-connections, contamination during storage, low water pressure and intermittent supply. Waterborne outbreaks are preventable if an integrated risk management framework based on a multiple-barrier approach from catchment to consumer is applied. Implementing an integrated risk management framework to keep the water safe from contamination in distribution systems includes the protection of water sources, the proper selection and operation of drinking-water treatment processes, and the correct management of risks within the distribution systems.

Drinking water pollution is a critical issue which is aggravating the stress arising as a result of unprecedented population growth, escalating urbanization, and industrialization since 1990s (Velea et al., 2009).

2.1. Water Distribution Network

Water distribution network comprises of an intricate system of hydraulic and hydrologic components i.e., pipelines, storage tanks and treatment plants that are utilized to convey water to consumers. A positive pressure is maintained throughout in the supply network to ensure the conveyance of water to all parts. Moreover, high pressure is also necessary to avoid the infiltration of sewage or contaminated water into the pipelines through leakages and cracks. The water is typically pressurized by high pressure pumps that pump water into surface reservoir or overhead tanks, usually constructed at the

highest local point in the network. The uprightness of these systems is indispensable in supplying clean water to end users. Along with releases and blasts, bacterial regrowth in water supply networks is a critical issue that can adversely impact water supply utilities. Regrowth occurs when disinfected water that enters the distribution network with a few microscopic organisms is found to have high measure of microorganisms which makes water flimsy in a distribution network (Whittle et al., 2013).

The potential for the water in the distribution network to transport microbial pathogens is found in different countries (Shakya *et al*., 2012). According to WHO, the water that enters the distribution network ought to be microbiologically safe and organically stable (WHO, 2017). For this purpose, a specific dose of disinfectant residual is usually sustained in the distribution system (Lautenshclager *et al.,* 2013). However, different elements during distribution, for example, temperature variation, pipe material, biofilms, interruption of untreated water and septic zones due to stagnation can affect the nature and quality of water.

2.1.1. Biofilm Formation in Distribution System

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

1989). Biofilms grow virtually everywhere including inside pipes and microbes start attaching, growing and releasing on a man-made surface. Each year, biofilms cause billions of dollars in damage to filters, tanks and pipes by promoting microbial growth that corrodes pipes and promotes scaling. Internal surfaces of drinking water distribution systems are always colonized by microorganism, mostly in the form of single cell or microcolonies, but sometimes a dense multiple cell-layer biofilm is also formed with the passage of time. It is estimated that 95% of the overall biomass in a distribution system is located on drinking water pipe walls, while only 5% occurs in the water phase (Flemming et al., 2002). Thus, biofilms on pipe surfaces, may provide a microbiological contamination potential, resulting in deterioration of hygienic drinking water quality. There are indications that microorganisms with pathogenic properties can persist, and in some cases, multiply in biofilms, these organisms include bacteria, enteric viruses and protozoan parasites (Percival et al., 2011)

2.1.2. Deficiencies in Distribution System

Distribution systems are susceptible to performance inadequacies that results in recontamination of disinfected water and potentially lead to increased risk of waterborne diseases (Ercumen et al., 2015). Contamination mainly occurs as a result of both hydraulic and physical breaches. Lack of water pressure during hydraulic breaches allows external contamination to enter pipelines commonly known as infiltration through the portals created by physical breaches. Entry of pathogens may be in the form of backflow from cross-connections or intrusion through leaks and cracks. The aging effect of water infrastructure is also a major contributor that makes distribution network vulnerable to pathogen intrusion through pipe breaks and other types of age-related deterioration as

pipelines approach end of their service life. In addition to the aforementioned failures, loss of adequate disinfectant residual, low water pressure, intermittent service and ageing of infrastructure may also result in the deteriorating quality of water supply. Pathogen intrusion may happen in such circumstances in case of poor sanitary conditions because of improper sewage collection and leakages in the network. Subsequently, these deficiencies may result in the cross-contamination due to infiltration or exfiltration of a clean water supply which consequently leads to outbreaks of waterborne and water related illness. The World Health Organization (WHO), in this regard has recommended maintaining a chlorine residual of 0.2–0.5 mg/L in the distribution system to ensure protection against pathogen intervention in the event of breaches of physical or hydraulic pipe integrity (WHO, 2011).

2.1.3. Inadequate Secondary Disinfection

Secondary disinfection is the second disinfection step followed by primary disinfection to protect water in transmission pipes and water reservoir of the distribution system. There is a potential relationship between the presence of microorganisms and chlorine residual in distribution network. Chlorine residuals in drinking water is recognized as an adequate indicator for analyzing quality of water in the distribution system (Lee et al., 2004). An adequate level of residual chlorine at tap water is effective in improving sanitary conditions and inhibiting the regrowth of microorganisms and hence the formation of biofilms on the internal surface of pipelines.

The microbial growth will be recovered in the absence of a proper disinfectant residual in the distribution system. The presence of any disinfectant residual alleviates the microbial contamination in addition to frequency of occurrence at the user end

(Kitazawa, 2006). According to WHO (2005) free residual chlorine should be between 0.2-0.5 mg/L whereas, the maximum allowablE value for chlorine residual is 5 mg/L to prevent biofilm growth and protect water quality deterioration. The retention or contact time in chlorination is also a crucial parameter as a long and adequate interaction between chlorine and the microorganism's results in an effective secondary disinfection. The contact time varies with residual chlorine concentration, the nature of pathogens present, pH and temperature of the water.

Inadequate secondary disinfections can pose serious harm to public health as this disinfection has a significance role to stop waterborne diseases and other outbreaks that has been occurring in both developed and less developed countries in the global water supply system (Craun et al. 2006). Disinfection residues play a very important role especially in developing countries where water has high contaminants during water distribution and possess poor sanitary conditions. This is important mainly where water has to travel a long distance to fulfill the needs of consumers and the residual chlorine level reduces when water travels over long distance from where it is stored (Egorov et al., 2003).

2.1.4. Water Pressure in the Distribution System

To ensure the quality of water it is imperative to maintain and normalize the pressure of water throughout the whole water supply network. According to "Florida Rural Water Association (FRWA)" the minimum permissible water pressure in distribution system under all conditions of flow should be up to 20 psi whereas, pressures less than this is unacceptable as they produce conditions for back-siphon age and potential contamination of the water supply system. Increase in water pressure is mainly caused by the leakages in

the distribution system as a result of damages, defects in pipes and lack of maintenance (Nejjari et al., 2015). Pressures above 100 psi should be evaded to alleviate leakages, minimize water consumption and prevent excessive wear and tear in the system. Pressure reducing valves could be installed on individual homes or on an isolated portion of the distribution system to manipulate the high pressures to less than 100 psi. Studies reveal that most of the contamination in the water distribution system is the outcome of less flow of water due to low pressure and back washing or reverse flow (Trussell et al., 1998). To cope up with the scarcity of water, water pressure in the distribution system is reduced throughout the transmission lines so each household use minimum water. When the water level is decreased, it reflects to move the water towards the less pressure or result in reverse flow. This back flow of water causes unwanted water to flow back into the drinking water supply system. Two types of back flow may occur, the leading one is back siphonage that happens when the pressure level reduces so much to create the vacuum influence in the pipes, which causes addition of contaminants from pipes at the point of leakages (Kelkar *et al*., 2001).

2.1.5. Mode of Water Supply in the Distribution System

Deficiencies in the piped distribution systems in most of the developing countries is linked to the contamination of water at user end and outbreaks of water-borne diseases (Lee and Schwab, 2005). One prevalent deficiency is the practice of intermittent water delivery in the distribution system. Approximately one-third of water supplies in Latin America and Africa and more than half in Asia delivers water through intermittent mode of supply (Kumpel et al., 2013). Intermittent supply of water is a common practice throughout the globe that enhances the risk of microbial contamination through numerous

mechanisms. Upgrading an intermittent supply to a continuous supply has the potential to enhance the quality of water supplied to ultimate users. Studies show that intermittently supplied systems convey impaired water quality at consumer taps. Contaminants can infiltrate the pipes by backflow or infiltration when pipes are empty or during low pressure conditions. Moreover, intermittent supply of water necessitates household collection and storage, a practice associated with recontamination (Eshcol et al., 2009). It compared water quality at reservoirs, taps, and in drinking water in homes in intermittent and continuously operated distribution systems in the same cities in India. Andey and Kelkar (2007) evaluated the performance of water distribution systems during intermittent versus continuous water supply and concluded the benefits of upgrading an intermittent to a continuous supply in terms of increase access to safe water in terms of water quality at reservoirs, transmission lines and consumer end.

2.1.6. Aging Infrastructure of Distribution Network

Ageing, outdated or poorly maintained distribution systems can result in deteriorated quality of piped drinking water and poses serious health threats. A number of deficiencies in the distribution system i.e., inadequate disinfectant residual, low water pressure, intermittent supply of water and ageing of infrastructure can result in the declining quality of the water in the distribution network (WHO and UNICEF, 2000). Poor sanitary conditions can exist because of improper wastewater collection and leakages in the network. Consequently, these various deficiencies can result in the cross-contamination of a clean water supply which, in some cases, has led to outbreaks of waterborne and water related diseases. As demonstrated above, it must be stated that it is often not a

single flaw, but the combination of a number of failures in the system that result in poor water quality.

2.2. Drinking Water Disinfection

The presence of pathogens in drinking water has become an emerging concern, especially as some of them are able to proliferate in the water distribution networks (Delaedt et al., 2008). The purpose of drinking water treatment is to remove pathogenic microorganisms, toxic chemicals and aesthetic contaminants from untreated water. For surface water sources i.e., rivers, lakes, streams and reservoirs a conventional treatment in commonly used. The process mainly includes coagulation, flocculation, sedimentation, filtration and disinfection. Disinfection is a common unit process and generally constitutes the final step in the water treatment (Diao et al., 2004). The primary goal of disinfection of public water supplies is the elimination of the pathogens that are responsible for waterborne diseases. The secondary purpose is to provide a disinfectant residual in the finished water and prevent microbial regrowth in the distribution system (Xie, 2016). Elimination of microbial pathogens is achieved through the oxidation of the microorganism by a disinfectant which results in termination of their growth and reproduction. The transmission of diseases such as typhoid and paratyphoid fevers, cholera, salmonellosis, and shigellosis can be controlled with treatments that substantially reduce the total number of viable microorganisms in the water. Since disinfection represents the last barrier against pathogen microorganisms, its effectiveness is a crucial point in ensuring public health. It is the most crucial and decisive treatment step in the production of safe drinking water. Water-borne diseases contribute considerably to the morbidity and mortality rate in developing countries. Effective elimination of pathogenic

microorganisms and other contaminants in the drinking water supply and the provision of a residual in the distribution network is imperative. (Martínez-Huitle et al., 2008)

Disinfection of water is a well-established and effective means of preventing pathogenic organisms from being transmitted through public water supplies. Oxidants are added to drinking water for several reasons, but the most important is disinfection. There are usually two points where disinfectants are added. The first is made early in treatment process, with an intent of inactivating all pathogenic microbes and viruses that might contaminate the source water. This is referred to as primary disinfection or peroxidation. In most water supplies there is a second addition of disinfectant as the water leaves the treatment plant and occasionally at distal sites in the distribution system. This addition is referred as secondary or post disinfection. The intent of secondary disinfection is to reduce microbial colonization in the water distribution system and to minimize the impact of sources of contamination in the system i.e., cross contamination, infiltration, exfiltration and leakages (Lippimann, 2000). In addition to the pathogen kill or inactivation, disinfectants also react with batural organic matter i.e., humic and fulvic substances or bromide in water to produce various organic and inorganic byproducts coomonly known as disinfection byproducts (DBPs).The potential health risk associated with the precence of DBPs requires their production to be controlled. Xie (2016) has higlighted the association between bladder and rectal cancer with water chlorination and its byproducts.

Disinfection commonly takes place as a result of cell wall corrosion in the cells of microorganisms, changes in cell permeability, protoplasm or enzyme activity and a structural change in enzymes. These disturbances in cell activity cause microorganisms to

no longer be able to multiply. This will cause the microorganisms to die out. On the basis of step wise application there are two kinds of disinfection i.e., primary and secondary disinfection. Primary disinfection achieves the desired level of microorganism kill or inactivation, while secondary disinfection maintains a disinfectant residual in the finished water that prevents the regrowth of microorganisms.

Disinfection can be attained by means of various methods depending upon the mode of action and the disinfecting agent used. Depending upon the raw water quality, availability of resources, degree of disinfection, stringency of regulations, means of application, concentration and potency of disinfectants, and chemical factors, various disinfection techniques exists

2.2.1. Physical Disinfection

The physical disinfection methods are based on the use of various physical fields for disinfection. These reagents-less techniques are of great interest. Among them are thermal treatment, ultraviolet irradiation, ultrasonication, pulsed electric fields, irradiation, magnetic-enhanced disinfection, and microwave systems (Martínez-Huitle, 2008). Filtration via filters capable of retaining bacteria and reverse osmosis also accounts for physical disinfection (Patermarakis et al., 1990). An important advantage of physical techniques is their ability to act directly on microorganisms, leaving the properties and composition of water virtually intact. Among all Ultraviolet irradiation have gained acceptance as the viable and economical water disinfection technique. Most of the physical alternatives at present do not fulfill the requirements of residual drinking water disinfection (Kerwick et al., 2005). Mercury-vapor lamps possess a high efficiency of conversion of electric energy to radiation with a wavelength of about 254 nm. The

absorption band of nucleic acids peaks at 260 nm. Because of this, the doses needed to kill the microorganisms are insignificant. However, radiation should hit the microorganism directly (Biryukov, 2005). In the case where the water contains suspension particles, they can shield the bacteria from radiation and reduce the disinfection efficiency. Moreover, many microorganisms attach to the surface of suspension particles or penetrate into their pores. Thus, to provide efficient disinfection of water by ultraviolet radiation, one has to remove both the particles suspended in it down to a permissible concentration below 3 mg/liter and the organic compounds that absorb radiation with a wavelength of about 254 nm. Hence physical methods are suitable only for primary disinfection and locations where residual disinfection is not required. (Kerwick et al., 2005)

2.2.2. Chemical Disinfection

Chemical inactivation of microbiological contamination in natural or untreated water is usually one of the final steps to reduce pathogenic microorganisms in drinking water. Combinations of water purification steps i.e., oxidation, coagulation, settling, disinfection and filtration renders the water safe after production. As an extra measure many countries apply a secondary disinfection step at the end of the water purification process, in order to protect the water from microbiological contamination in the water distribution system. Usually one uses a different kind of disinfectant from the one earlier in the process, during this disinfection process. The secondary disinfection makes sure that bacteria will not multiply in the water during distribution. Bacteria can remain in the water after the first disinfection step or can end up in the water during back flushing of contaminated water which may contain groundwater bacteria as a result of cracks in the plumbing.

Chemical disinfection of drinking-water includes any chlorine-based technology, such as chlorine dioxide, ozone, numerous oxidants and some strong acids and bases. Except for ozone, proper dosing of chemical disinfectants is intended to maintain a residual concentration in the water to provide some protection from post-treatment contamination during storage. Disinfection of drinking-water for municipalities in developing countries is achieved primarily with free chlorine, either in liquid form as hypochlorous acid i.e., bleach or sodium hypochlorite or in dry form as calcium hypochlorite or sodium dichloroisocyanurate. The reason being that these forms of free chlorine are suitable, relatively safe to handle, inexpensive and easy to dose. However, sodium trichloroisocyanurate and chlorine dioxide are also used in some municipality-based water treatment technologies. Proper dosing of chlorine for household water treatment is critical in order to provide enough free chlorine to maintain a residual during storage and use. Recommendations are to dose with free chlorine at about 2 mg/L to clear water with turbidity less than 10 NTU, and about 4 mg/L to water with turbidity greater than 10 NTU. Although these free chlorine doses may lead to chlorine residuals that exceed the recommended chlorine residual of 0.2 – 0.5 mg/L at user end for centrally treated water, these doses are considered suitable for household water treatment to maintain a free chlorine residual of 0.2 mg/L in stored household water treated by chlorination.

Disinfection of drinking-water with iodine, which is also a simple, effective, cost effective and strong oxidant, is generally not recommended for extended use unless the residual concentrations are controlled, because of serious health concerns pertaining the adverse effects of surplus intake on the thyroid gland.

i. Elemental Chlorine Disinfection

Chlorine is supplied as liquid in high pressure cylinders. At normal pressures, it is a toxic yellow-green gas and is liquid at high pressures. It is released from a liquid chlorine cylinder by a pressure reducing and flow control valve operating at a pressure less than atmospheric pressure. Chlorine gas reacts to form hypochlorous acid in water which dissociates to hypochlorite and hydrogen ions with a pKa of 7.5. At equivalent pH in the finished water, these processes yield the same reactive chemical species where the efficiency of (OCl^-) is less than HOCl. The gas is led to an injector in the water supply system where highly pressurized water is passed through a venture orifice creating a vacuum that draws the chlorine into the water stream. Adequate mixing and contact time must be provided after injection to ensure complete disinfection of pathogens. It may be necessary to control the pH of the water. This method of disinfection is very effective for removing almost all microbial pathogens and is appropriate as both a primary and secondary disinfectant but the major limitation of the method is the harmful nature of gas as it is lethal at concentrations as low as 0.1 % air by volume.

ii. Sodium Hypochlorite Disinfection

Chlorination can be achieved by using sodium hypochlorite solution also known as household bleach. Sodium hypochlorite solution is readily available and is generated onsite by electrolysis of sodium chloride solution in specialized proprietary equipment. The only supplies required are common salt and electricity. Hydrogen is given off as a by-product and must be safely dispersed. Sodium hypochlorite solution is dosed using a positive-displacement electric dosing pump or gravity feed system. Chlorine in the form

of sodium hypochlorite or calcium hypochlorite, dissolves in water yielding hypochlorous acid (HOCl) and hypochlorite ion (OCl^-) and the compound that dominates depends upon the pH of water. Sodium hypochlorite is easier to handle than gaseous chlorine or calcium hypochlorite. The major limitation of the disinfectant is that sodium hypochlorite is very corrosive and should be stored with care and kept away from equipment that can be damaged by corrosion. Hypochlorite solutions decompose and should not be stored for more than one month. It must be stored in a cool, dark and dry place.

iii. Calcium Hypochlorite Disinfection

Calcium hypochlorite or bleaching powder/chlorinated lime is a white solid that contains 65% available chlorine and dissolves easily in water. When packaged, calcium hypochlorite is very stable, allowing a year's supply to be bought at one time. Calcium hypochlorite is a corrosive material with a strong odor that requires proper handling. It must be kept away from organic materials such as wood, cloth, and petroleum products. Reactions between calcium hypochlorite and organic material can generate enough heat to cause a fire or explosion. Calcium hypochlorite readily absorbs moisture, forming chlorine gas. Therefore, shipping containers must be emptied completely or carefully resealed. Calcium hypochlorite may be dissolved in a mixing or holding tank and injected in the same manner as sodium hypochlorite. The equipment used to mix the solution and inject it into the water is the same as that for sodium hypochlorite. Calcium hypochlorite can be purchased in granular, powdered, or tablet form. Calcium hypochlorite has to be dissolved in water, then mixed with the main supply. Chlorine, whether in the form of

chlorine gas from a cylinder, sodium hypochlorite or calcium hypochlorite, dissolves in water to form hypochlorous acid (HOCl) and hypochlorite ion (OCl^-).

iv. Chloramine Disinfection

Chloramines i.e. monochloramines, dichloramines, trichloramines or nitrogen trichloride are formed when water containing ammonia is chlorinated or when ammonia is added to water containing chlorine either simultaneously or sequentially. The type of chloramine formed depends on the relative ratios of chlorine to ammonia and the pH. Among the three forms of chloramine, monochloramine is preferred due to the fact that it creates less taste and odor problems than dichloramine and trichloramine (Amy *et al.,* 2000). Chloramine is generally considered to be an effective disinfectant with overall lesser DBPs formation using mono chloramine then using chlorine and is capable of being generated onsite (Yang et al., 2012). The most commonly found DBPs in chlorinated water are Halo acetic acids (HAAs), Halo nitromethanes (HNMs), Halo acetonitriles (HANs), Halo aldehydes (HAs), Haloketones (HKs) and Iodo-THMs (I-THMs). The type of disinfectant used has a major influence on the final DBPs formation in treated water. Bougeard *et al.* (2010) reported lower concentrations of DBPs formation associated with chloramines as compared to chlorine with the exception of 1, 1-dichloropropanone and confirmed that higher concentration of brominated DBPs results from higher bromide levels in source water. The total Halo aldehydes (HAs) were decreased by 90% by the use of monochloramine while HANs reduced by 81%.

Usually, chloramine-forming reactions are 99 % complete within a few minutes which represents its fast kinetics. However, chloramine is a less effective against viruses or protozoa than free chlorine. Chloramine is appropriate for use as a secondary disinfectant

to prevent bacterial regrowth in a distribution system. Nitrogen trichloride appears to be the only detrimental reaction. It may be harmful to humans and imparts a disagreeable taste and odor to the water. The use of the proper amounts of each chemical reactant will avoid its production.

The generation of chloramines requires the hypo chlorination and equipment for adding ammonia. Chemicals used to generate chloramine from ammonia and chlorine gas depend on the ammonia-based chemical used. Anhydrous ammonia is the least expensive, while ammonium sulfate is the most expensive. Despite monochloramine addition as a disinfectant, it may also be formed in chlorinated water in the presence of ammonia (Pozzi et al., 2011).

v. Ozone Disinfection

The application of ozone as a disinfectant in drinking water is widespread throughout the globe. The main reasons for the use of ozone are disinfection and oxidation resulting in taste and odor control, discoloration, elimination of micropollutants, etc. Ozone is a strong oxidizing agent and a powerful disinfectant. It is recognized as one of the most effective disinfectants for drinking water, as an alternative to chlorination (Xu et al., 2002). It can be used as a primary disinfectant. Ozone gas (O_3) is formed by passing dry air or oxygen through a high-voltage electric field. The resultant ozone enriched air is dosed directly into the water by means of porous diffusers at the base of baffled contactor tanks. Requiring shorter contact time and dosage than chlorine, ozone is widely used as a primary disinfectant worldwide. It does not directly produce halogenated organic materials unless a bromide ion is present. A major shortcoming of Ozone gas as a

disinfectant is its unstable nature and limitation to be generated onsite. Moreover, a secondary disinfectant, usually chlorine, is required because ozone does not maintain an adequate residual in water.

The performance of ozonation relies on achieving the desired concentration after a given contact period. For oxidation of organic chemicals, such as some oxidizable pesticides, a residual of about 0.5 mg/l after a contact time of up to 20 minutes is typically used. The doses required to achieve this vary with the type of water but are typically in the range 2–5 mg/l (WHO, 2017). Higher doses are needed for untreated waters, because of the ozone demand of the natural background organics. Sigmon et al. (2015), investigated inactivation efficiencies of ozone on four pathogenic viruses and found that the viruses are easily inactivated at the Ct (concentration × time) values lower than 0.1 mg-min/L. Ozone reacts with natural organics to increase their biodegradability, measured as assimilable organic carbon. To avoid undesirable bacterial growth in distribution SYSTEM, ozonation is normally used with subsequent treatment, such as biological filtration or granular activated carbon (GAC), to remove biodegradable organics, followed by a chlorine residual, as ozone does not provide a disinfectant residual. Ozone is effective for the degradation of a wide range of pesticides and other organic chemicals.

2.3. Electrochemical Disinfection

2.3.1. General Overview

Electrochemical disinfection is the elimination of microorganisms by means of an electric current applied through the water under treatment suing suitable electrodes. At the phase boundary between the electrodes and the water, the electric current leads to the

electrochemical production of disinfecting species from the water itself e.g., Ozone or from species dissolved in the water (Kraft et al., 2008). It has emerged as one of the more feasible alternatives to chlorination being effective against a range of pathogens. It is a convenient and highly efficient way to produce disinfected water (Thorn et al., 2012). The technique works without the addition of chemical compounds to the water to be treated, but is nevertheless based on the biocidal action of various chemical substances. The EC disinfection process has the potential to be developed as a robust, cost-effective and environmentally friendly alternative of disinfection. The effectiveness of EC disinfection has been demonstrated in tests for water, wastewater, seawater and other liquid media (Diao et al., 2004). Multiple terms are used to refer this type of water disinfection process or the water produced by this process, such as electrolytic disinfection, electrochemical disinfection, anodic oxidation, functional water, electrochemically activated water and mixed oxidant solution (Fang et al., 2016; Kraft et al., 2008).

Electrochemical disinfection devices can be separated into two categories, direct electrolyzers and mixed oxidant generators. Direct electrolysers interact directly with contaminated water, whilst mixed oxidant generators utilize a concentrated solution of brine to generate anolyte with a strong biocidal potential (Cho et al., 2001).

2.3.2. Generation of ECAS

Transformation of low mineral salt solution into an activated metastable state by electrochemical unipolar action generates ECAS (Thorn et al., 2012). Electrolysis of a low mineral salt solution through ion exchange membrane of electrochemical cell produces an anolyte and catholyte (Fang et al., 2016). Catholyte being produced at the

cathode end referred to as catholyte with a weak ORP whereas anolyte being produced at the anodic end with a strong biocidal potential is referred to as ECAS. ECAS is usually composed of multiple oxidizing agents i.e. chlorine species, hydrogen peroxide, oxygen species such as ozone, and hydroxyl radicals (Ashraf et al., 2014; Ghebremichael et al., 2011; Kunigk et al, 2008). It is rapidly degradable and reverts to a weak salt solution during the time of chemical relaxation (Thorn et al., 2012). The oxidizing species such as chlorine dioxide, ozone, hydrogen peroxide are short-lived radicals are transient in nature and degrades rapidly. (Clayton et al., 2017; Gibrimechaeal et al 2011). ECAS can be obtained using salts other than NaCl such as KCl and $MgCl_2$. Bromine which has been extensively recognized as an efficient biocidal agent could also be used as a salt for ECAS generation and can substitute toxic chemical biocides for the control of microorganisms in cooling towers.

2.3.3. Properties of ECAS

The majorly reported properties of ECAS are its oxidation reduction potential (ORP), pH and free chlorine. ECAS exhibits an ORP value in the range of 800 and 1200 mV with a low pH range from 2 to 5 and a high concentration of free chlorine (Ashraf *et al.,* 2014, Thorn *et al.,* 2012; Cloete *et al.,* 2009).

ORP is a measurement of the tendency for a solution to either gain or lose electrons. It is the potential of a disinfectant to do its work of inactivating micro-organisms and oxidizing organic materials. The higher the millivolt (mV) value, the more powerfully the water is able to oxidize and disinfect. Oxidizers cause the millivolt value to increase and therefore increase disinfection. Typical oxidizers are hypochlorous acid (a component of free chlorine), ozone, and hydrogen peroxide. ECAS exhibits a strongly positive ORP

due to the concentration of oxidizers and their activity or strength. ECAS will therefore tend to gain electrons from less positive solutions or cells. The ORP value of solutions has been proven to be the most meaningful way of establishing the killing time and effectiveness of a disinfecting product. It can also be used in conjunction with a free chlorine measurement. Reductants cause the millivolt value to decrease and therefore decrease disinfection. Typical reductants are the hypochlorite ion (a component of free chlorine), chloramines, cyanuric acid, organic matter, urine, perspiration, sputum, micro-organisms, and fecal material.

ORP measurement correlates weakly with free chlorine measurement because they measure two different entities. ORP measures oxidative disinfection power but not the concentration of free residual chlorine. Free chlorine measures the concentrations of hypochlorous acid (HOCl) and the hypochlorite ion (OCl^-) not the oxidation disinfection power. Free chlorine is a variable component of ORP. Oxidative disinfection does not correlate well to free chlorine .The ORP will be low if free chlorine exists as the hypochlorite ion (OCl^-). This will occur when the pH is high. Therefore, free chlorine could be high and the ORP low at a pH greater than 7.6.

2.3.4. Disinfection Potential of ECAS

It has been reported that anaerobic bacteria are more prone and sensitive towards oxidants than the aerobic bacteria, and thus ECAS are strong anti-anaerobes. Bacterial spores are highly resistant to traditional biocides, while ECAS has been reported to be effective against a wide range of pathogens i.e., *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Enterococcus faecalis*, *Clostridium difficile*, *Bacillus atrophaeus*, *Bacillus cerus*, *Bacillus anthracis*,human norovirus, hepatitis B virus, HIV, *Aspergillus flavus*,

Candida albicans, *Cryptosporidium parvum* oocysts and staphylococcal enterotoxin. (Zeng at al., 2011; Xiong et al., 2010). Kerwick et al. (2005) found out that ECAS is capable of successfully inactivating Clostridium perfringens, Coliphage MS2 (>2.5 log), Escherichia coli (>2.5 log), and Cryptosporidium parvum oocysts (2.6 log) (Casteel et al., 2000).

Venkitanarayanan et al. (1999) found that ECAS achieved 7 log reduction of *Escherichia coli* 0157H:H7, *Saltnonella enteritis* and *Listeria monocytogenes after* 5 min contact time, while chlorine, with the same concentration, had similar efficacy only for *E. coli* 0157H:H7 and *L monocytogenes.* Gonzalez (2002) also reported that ECAS has superior inactivation of biofilms and long-lasting residual chlorine.

The biocidal potential of ECAS is significantly influenced by the change in concentration, temperature and storage period (Robinson et al., 2010). In a study, ECAS was stored at two varying temperatures, i.e. 4 and 20 ◦C, for 398 days to analyze the changes in the free Chlorine content, pH, oxidation reduction potential (ORP) and antimicrobial efficacy. The results demonstrated a rapid degradation at higher temperature as compare to lower temperature, with a concomitant decrease in bactericidal efficacy, whereas the pH remained almost unchanged (Gareth et al., 2013).

2.3.5. Areas of Application

ECAS with chlorine as its primary oxidant, has been extensively used in many industries, including drinking-water treatment plants, water supply lines, food industries, wastewater treatment facilities and hospital and health care centers (Ashraf et al., 2014; Thorn et al., 2012).

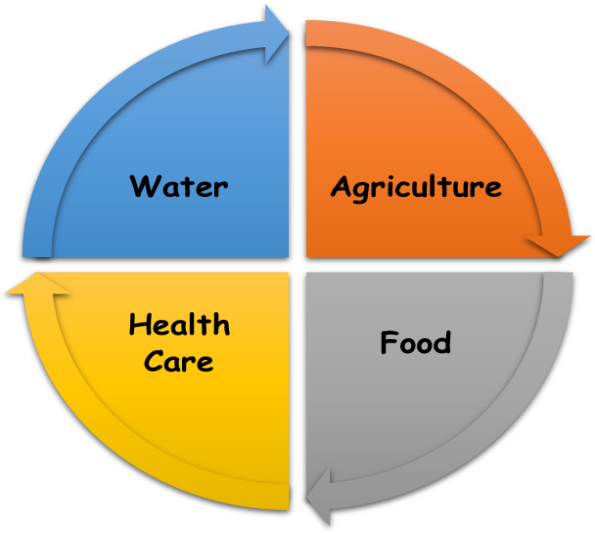


Figure 1: ECAS areas of Application

2.3.5. Disinfection Mechanism of ECAS

Biocides eliminated microorganisms through targeting numerous structures, macromolecules or biochemical pathways (Denyer and Stewart 1998; McDonnell and Russell 1999; Russell 1999). The high ORP of ECAS may inhibit microbial growth through the oxidation of sulfhydryl compounds on cell surfaces and other important metabolites. ECAS has the ability to destroy bacterial endospores (Rogers et al. 2006). Since they are capable of reducing endospores, they possibly have the potential to affect the bacterial spore coat and inactivate the spore. The sport coat, is composed of specific proteins (Rogers et al. 2006). Thus, if ECAS influence the spore proteins, then possibly, inactivation of vegetative cells may be due to the contact between the ECAS and cellular proteins. Zinkevich et al. (2000) analyzed the mechanism action of ECAS for E. coli. They found complete destruction of and proteins nucleic acids within 5 min of exposure time. Numerous studies demonstrated that high ORP generates strong oxidative stress which causes damage to the cell membrane (Huang et al., 2008) and creates an

environment outside the working domain of major microbial processes such as energy generating mechanisms. The powerful oxidants sequester electrons from microbial structure compounds with high efficiency which results in rupturing of the biochemical bonds and loss of function subsequently. The generated anolyte solution (ECAS) is dosed to the water to be disinfected (Thorn et al., 2012)

2.3.6. Advantages of ECAS

ECAS has multiple advantages over conventional disinfection i.e., chlorination, mainly in terms of its inactivation efficacy and potential to form disinfection by-products. It is generated on-site due to which the need for the storage and transportation of biocide is eliminated and provide a basis for building new control programs (Kiuru et al., 2010). These electrochemically generated biocides are environmentally friendly, non-toxic, hypoallergenic, chemical residue-free, fast-acting powerful biocide agents that do not require special handling and can be safely disposed of in municipal sewage systems, which can be used during all stages of disinfection and cleaning, applied in liquid, aerosol or frozen forms, generated on-site or in concentrated amounts for imminent use from municipal tap water and salt (Clayton et al., 2017; Ashraf et al., 2014). The rapid kill kinetics of ECAS render it a novel disinfectant with a potential to eliminate even the resistant and persistent microbial species (Cloete et al., 2009)

2.3.6. THMs Production potential of ECAS

The formation and distribution of DBPs depend upon a number of factors such as type of disinfectant, dosage, Organic Matter (OM), Dissolved Organic Nitrogen (DON), pH, contact time, ammonia levels, temperature, bromide/iodide concentration and molecular

weights in raw water. The production of disinfection by-products (THMs) in natural water treated with electrochemically generated mixed oxidants has reported more than 50% reduction in total THMs (Kerwick et al., 2005). Ghebremichael et al., (2011) reported 50% reduction in the formation of trihalomethanes (THMs) and halo acetic acids (HAAs) compared with NaOCl. The lower of THMs formation was attributed to the presence of chloro-oxygen species, despite of their transient nature and short life.

Chapter 3

MATERIALS AND METHODS

3.1. Phase 1: Water Quality Characterization

Water quality has an impact on the treatment needed and ultimately on the quality of the produced drinking water. Source water characterization and assessment is a key component of the planning and design of water disinfection methods. The type and detection level of the measured source water pollutants are mainly determined by the applicable product water quality regulations and regulatory requirements.

Drinking water treatment operation typically consists of a series of processes, including aeration, coagulation-flocculation-sedimentation, activated carbon treatment, and chlorination. Each operation targets specific impurities or contaminants and has some economical or technical limitations. Disinfection with various oxidants not only disinfects water, but also react with substances other than pathogens present in the source water, i.e., natural organic matter (NOM) or dissolved organic carbon (DOC), which leads to the formation of numerous disinfection byproducts.

3.1.1. Description of Study Area

NUST is a multi-campus university spread all over Pakistan. The main in H-12 Islamabad was established in 2008 to cater for the fast-growing requirements of a technologically advanced campus of science and technology. The main campus covers an area of about 707 acres (2.68 km^2). It has more than 15 schools and institutes, faculty residence as well as male and female residence hostels. Water distribution system of the university is served with ground water sources. Water is pumped through 9 tube wells from the

catchment area and transferred to 3 underground storage reservoiors followed by overhead tanks.These storage locations are either supplied direclty or stored in overhead reservoirs overnight with storage time extending upto 2-3 days. Water is supplied to the overhead tanks which further surve to their designated areas. Tube wells having a pumping capacity of 0.2 million gallons per day (MGD) serve a population of around 11,400. The distribution lines of the system comprises of Galvanized iron (GI) which turn to unplasticized Polyvinyl chloride (uPVC), Polypropylene (PPR) and Galvanized iron service pipelines.University has a total of 6 water filtration plants located at various locations throughout the campus.

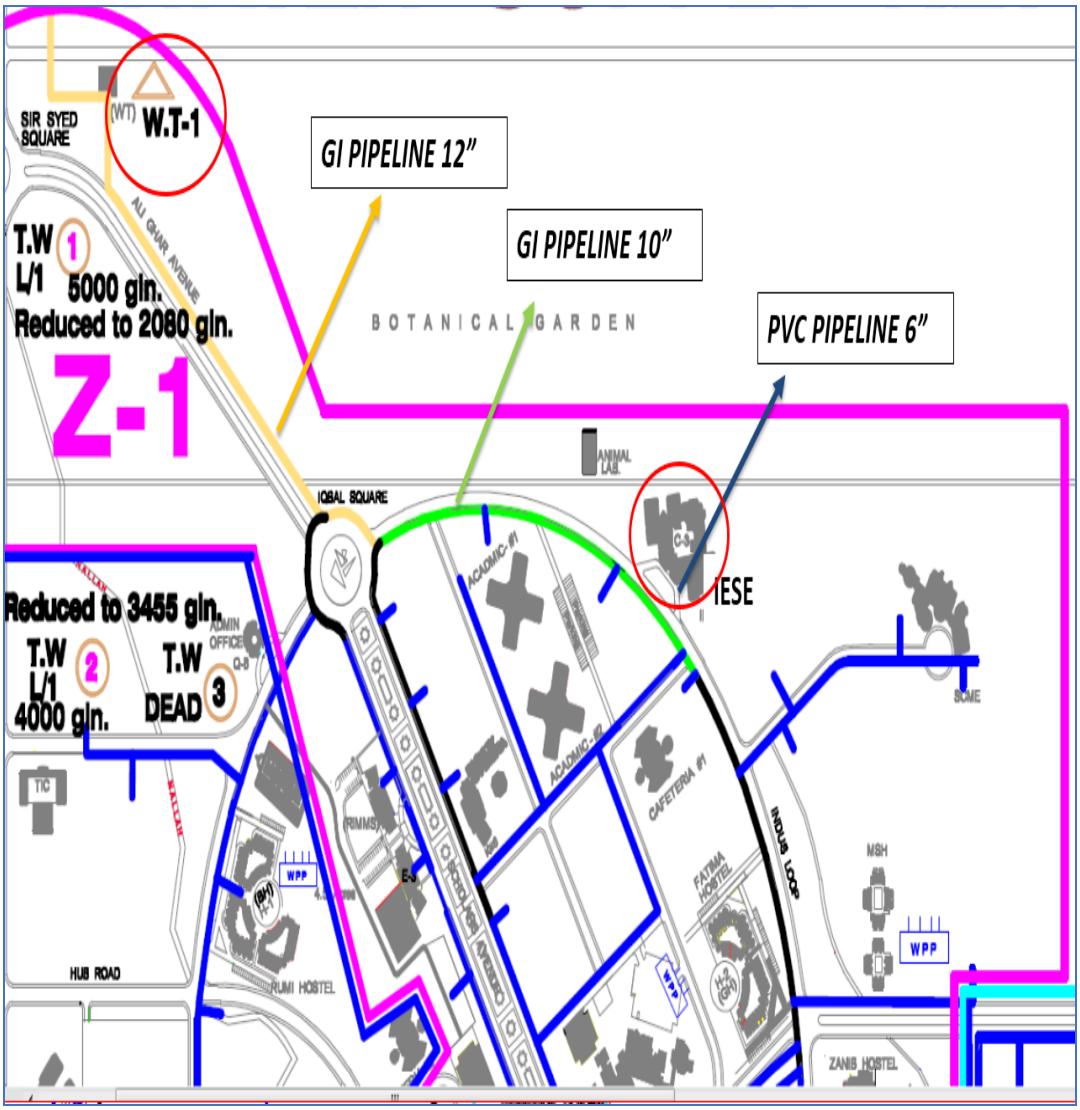


Figure 2: Water Distribution Network Layout

This research was focused on water distribution network of NUST Islamabad, based on ground water resource. Water distribution network of the campus is distributed into 3 Zones.With in these zones water is pumped through 9 tube wells from the catchment area and transferred to 3 underground storage tanks followed by 3 overhead tanks.These storage locations are either supplied direclty or in overhead reservoirs overnight.The targeted zone of the study is Zone- 1 demarcated in pink borders as shown in Figure 2

which serves up to 8,500 population including residents and nonresidents. Ground water from four various boreholes i.e., (T.W 1, T.W 2, T.W 5 & T.W 8) is pumped in the surface storage reservoir (W.T-1) located near Gate 2 of the campus. The water is pumped to the overhead tank with a storage capacity of 150,000 Gallons, through the pumping station with a disinfectant dose of I ppm through flow paced dosing pump. The targeted water transmission line initiates near the OHT and ends at IESE building. The distribution lines of the system comprises of Galvanized iron (GI) which turn to unplasticized Polyvinyl chloride (PVC). The configuration of the distribution network is an integration of branched and grid-based system and water is pumped by means of gravitational flow in addition to pumping.

3.1.2. Targeted Sampling Sites

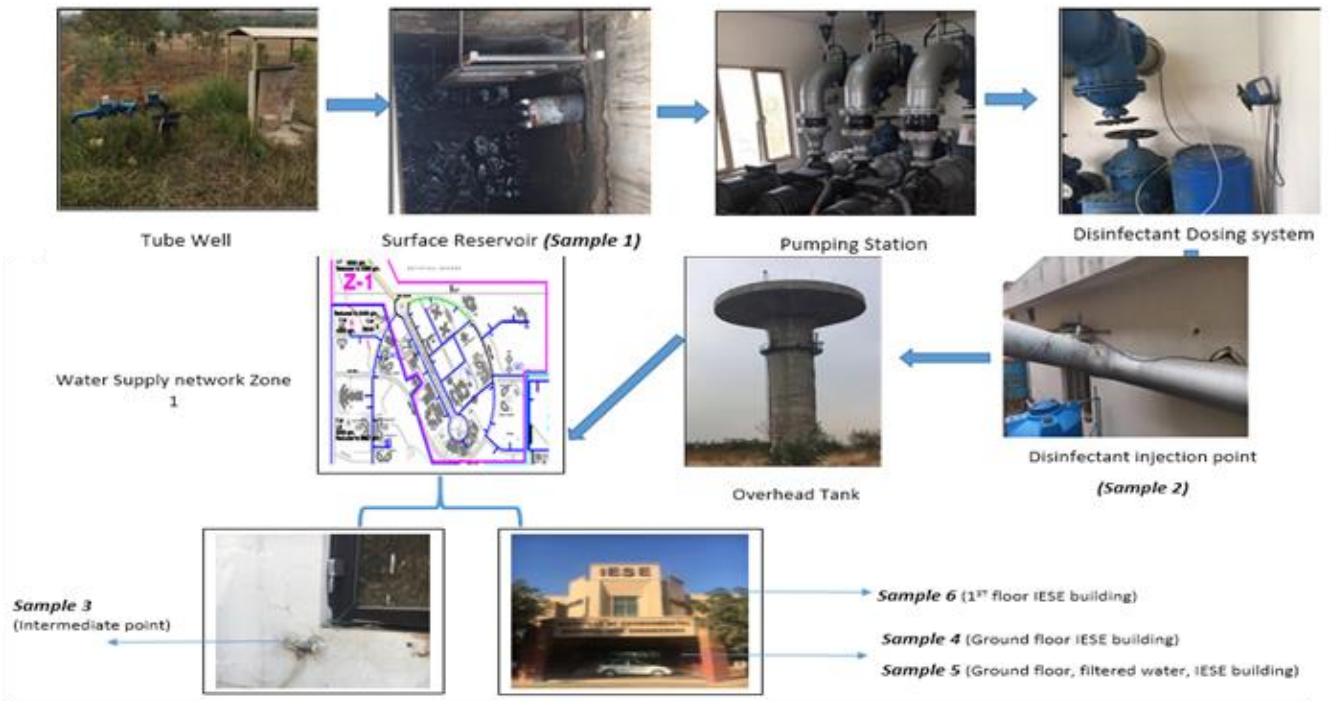


Figure 3: Targeted Water Sampling sites in Zone 1

To analyze the ground water variation and contaminants a total of six sampling sites were targeted in Zone- 1 of the distribution network. As shown in the Figure 3 Ground water from the respective tube well is pumped to the surface storage reservoir and is subsequently pumped to the overhead tank (OHT) after a flow paced disinfectant dosing. The OHT distributes the water to respective zone under the action of gravity. The targeted transmission line is distributed into 3 stages as upstream, intermediate and downstream point with respective sampling locations to analyze the spatial and temporal variation of water quality in terms of microbial and physiochemical parameters.

Sample 1 illustrates the upstream sampling location, Sample 3 illustrated the intermediate sampling location and the sample 4, 5, 6 being the downstream or end point sampling locations.

3.1.3. Water Sampling and Analysis

To assess the variation in water quality and disinfection efficacy, a total of 18 water samples were collected from targeted sampling sites of the respective zones in the month of May (Pre-monsoon) and October (Post monsoon), 2017 and were subjected to chemical analysis. The samples were stored in autoclaved water sampling bottles with screw caps. Prior to sample collection, all the plastic bottles were thoroughly washed and oven dried and before sample collection, the bottles were rinsed with the same collected water samples. The bottles were then labeled with the respective sampling locations. The collected samples were subjected to elemental, physicochemical and microbial water analysis.

3.1.4. Elemental Water Analysis

Inorganic elemental contamination of ground water majorly occurs as a result of natural deposits due to geogenic sources. The trace elements and minerals in water at 3 out of 6 sampling sites were sent for analysis to Central Analytical Facility Division, PINSTECH Nilore. The parameters of analysis included Manganese, Zinc, Chromium, Lead, Cadmium, Arsenic, Mercury, Nickel, Selenium and Tin. The technique used for analysis was either Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES) or Hydride Generation Atomic Absorption Spectrometry (HGAAS).

3.1.5. Physicochemical Water Analysis

The collected water samples were analyzed for major water quality parameters including pH, temperature, conductivity, Hardness, TDS, TSS, phosphate, nitrate and nitrite and COD following Standard Methods for the Examination of Water and Wastewater (APHA et al., 2012) at Water and Wastewater Lab, IESE, NUST. Total Organic Carbon (TOC) was analyzed using TOC analyzer at Central Analytical Facility Division, PINSTECH Nilore.

3.1.6. Microbial Water Analysis

Most Probable Number Technique

The bacteriological analysis of water samples was performed using MPN technique to enumerate the bacteria present in drinking water. Measured volume of sample was added in a series of tube containing a liquid indicator growth medium.

Preparation of Growth Medium

A total of 10 test tubes of Laural Tryptose Broth (Oxoid Ltd, Hampshire, England), Brilliant Green Bile Broth (Oxoid Ltd, Hampshire, England) and E.coli broth (Oxoid Ltd, Hampshire, England) broth each were prepared with a fix volume of respective media in it corresponding to each sample collected. For the preparation of first day media i.e., LTB 36.5 g of media was mixed in 1L distilled water. 10 mL of the liquid media was added in the respected tubes each containing an inverted Durham tube. The tubes were then autoclaved at 121°C and 15 psi for 15 minutes and placed in an incubator at 37°C for 24 hours to ensure sterility. Second day media (BGLB) and third day media (EC broth) were prepared following the similar procedure by dissolving 40 g BGLB in 1L of distilled water and 37 g EC broth in 1L of distilled water respectively.

Enumeration of Total Coliform and E. coliform

Total coliforms and E. coli were enumerated using 3 days (MPN) test. The first day test being the presumptive phase for detection and estimation of coliform in water. 10 test tubes each containing 10 mL LTB and an inverted Durham's tube were used. Gentle shaking was done to ensure the removal of bubble from Durham tube, 1 mL sample was added to each tube and the tubes were kept at 37°C for 24 hours in incubator. Production of gas and cloudiness of growth medium in the tubes showed a positive presumptive reaction and gave an indication of presence of total coliforms.

Positive tubes were further subjected to confirmation phase. Positive LTB tubes were shaken slightly and inoculum using sterile wire loop was transferred to BGLB tubes.

BGLB tubes were then placed in an incubator at 37°C for 24 hours. Production of gas and cloudiness of the growth medium after 24 hours in BGLB tubes confirmed presence of

total coliforms. Positive tubes from previous phase were subjected to 3rd day test. Inoculum from positive tubes taken and after gently shaking a small amount using sterile wire loop was added to EC broth tubes and incubated at 37°C for 24 hours. Production of gas confirmed the occurrence of fecal coliforms (E. coli) (APHA et al., 2012). Some spore forming bacteria give false positive test in presumptive test, confirmatory test is done to ensure whether coliforms are of fecal origin or not.

3.1.7. Isolation of Bacteria

Stenotrophomonas sp.

Stenotrophomonas sp. grows on many of the selective media intended for the Enterobacteriaceae such as MacConkey, Brilliant green and XLD agars (Markey et al., 2013). Water samples taken from the targeted sampling sites were spread on XLD agar. All the glassware was washed with distilled water, autoclaved and oven dried prior to use. XLD agar (Oxoid Ltd, Hampshire, England) poured plates were checked for sterility 24 hours prior to sample spreading. Sample seeded plates were incubated at 37°C for 24 hours to observe the growth of colonies. *Stenotrophomoas sp.* showed characteristic red colonies on XLD agar. Red colonies appearing on the media were picked and re streaked for multiple times to obtain pure colonies.

Aeromonas sp.

Water sample taken form the targeted sampling sites was spread on xylose lysine deoxycholate (XLD) agar (Oxoid Ltd, Hampshire, England). *Aeromonas sp.* colonies appeared on XLD media which is regarded as highly *Salmonella* and *Shigella sp.* isolation media. All the glassware was washed with distilled water, autoclaved and oven

dried prior to use. Agar poured plates were checked for sterility 24 hours prior to sample spreading. Sample seeded plates were incubated at 37°C for 24 hours to observe growth. *Aeromonas Eucernophilia* being enteric pathogen ferments xylose under acid production forms yellow colonies surrounded by yellow zones on XLD agar.

Sphingomonas sp.

Water sample taken form the targeted sampling sites was spread on buffered charcoal yeast (BCYE) agar (Oxoid Ltd, Hampshire, England). All the glassware was washed with distilled water, autoclaved and oven dried prior to use. Agar poured plates were checked for sterility 24 hours prior to sample spreading. Agar plates cultured with water samples were incubated at 37°C for 24 hours to observe growth. Bacteria belonging to *Sphingomonas sp.* was grown on BCYE agar plates and was identified through biochemical screening and molecular characterization. Greenish brown colonies appearing on the media were randomly picked and purified using sub culturing.

Enterobacter sp.

Enterobacter sp. was isolated using the BCYE growth agar. Water sample taken form the targeted sampling sites was spread on buffered charcoal yeast (BCYE) (Oxoid Ltd, Hampshire, England) agar. All the glassware was washed with distilled water, autoclaved and oven dried prior to use. Agar poured plates were checked for sterility 24 hours prior to sample spreading. The media was prepared according to the manufacturers' instructions. Agar plates cultured with water samples were incubated at 37°C for 24 hours to observe growth. Enterobacter sp. produced a characteristic greyish white

pigment on BCYE agar. Colonies were purified by twice sub culturing using the streaking plate method to obtain the pure colonies.

Escherichia. coli

Eosin Methylene Blue (EMB) agar (Oxoid Ltd, Hampshire, England) was used for the isolation of E coli. Prior to isolation, all the glassware was washed with distilled water and autoclaved at 15 psi at 121°C for 15 minutes. The glassware was oven dried at 150°C for 3 hours. Eosin Growth medium was prepared as per the manufacturer's instructions. Agar plates were prepared and tested for sterility 24 hours prior to streaking. *E. coli* exhibited green sheen colonies on the EMB agar plate.

3.1.8. Identification of Bacteria

3.1.8.1. Morphological characteristics

The shape, size, color, surface appearance and consistency of a colony for a given incubation time was used in the identification of bacterial strains. Colony morphology of strains being isolated was analyzed. Different types of bacteria produced different looking colonies. Table 1 outlines the morphological characteristics determined for isolated strains.

Table 1: Morphological Characteristics for isolated bacteria

Morphological Characteristics	Description
Colony Size	Punctiform, Small, Large
Margins	Entire, Undulate, Filiform, Curled
Forms	Circular, Irregular, Filamentous, Rhizoid
Texture	Creamy, Mucoid, Dry
Elevation	Raised, Convex, Flat, Umbonate
Color	Yellow, Orange, Pale yellow, Off-white

3.1.8.2. Gram staining

Gram staining test differentiated gram negative and gram-positive strains to be used for inactivation experiment based on different cell wall constituents. Gram positive bacteria stain violet due to the presence of a thick layer of peptidoglycan in their cell walls, which retains the crystal violet these cells are stained with. Alternatively, Gram negative bacteria stain red, which is attributed to a thinner peptidoglycan wall, which does not retain the crystal violet during the decoloring process.

A smear of each 24 hours fresh culture was prepared on clean grease-free slides, fixed by passing over flame. The heat-fixed smear was then stained by the addition of a few drops of crystal violet solution for 1 min and rinsed with water afterwards. The smear was then flooded with iodine for 30 sec and rinsed with water, decolorized with almost 70% alcohol for around 15 sec and were rinsed with water. They were then counter stained with 2 drops of Safranin for 60 sec and finally rinsed with water, then allowed to air dry.

The smears were mounted on a microscope and observed under oil immersion objective lens. Gram negative cells appeared pink or red while gram positive organisms appeared purple (Abiola et al., 2016)

3.1.8.3. Biochemical characterization

Following tests performed to support bacterial specie confirmation;

Motility Test: A sterile needle was used to pick a loop of a 24 hrs old culture and was stabbed onto nutrient agar in glass vials. The vials were incubated at 37°C for 24-48 hrs. Non-motile bacteria had growth confined to the stab line with definite margins without spreading to surroundings area while motile bacteria exhibited diffused growth extending from the surface (Olutiola et al., 2000)

Catalase Test: A small quantity of 24 hrs fresh culture was transferred into a drop of 3% Hydrogen peroxide solution on a clean slide with the aid of sterile inoculating loop. Gas seen as white froth indicates the presence of catalase enzyme (Cheesbrough, 2006).

Citrate Test: This test detects the ability of an organism to use citrate as a sole source of carbon and energy. About 2.4 g of citrate agar was dissolve in 100 mL of distilled water. About (10 mL) of citrate medium was dispensed into each tube and covered, then sterilized and allowed to cool in a slanted position. The tubes were inoculated by streaking the organisms once across the surface. A change from green to blue indicates utilization of the citrate.

Oxidase test: A piece of filter paper was soaked with few drops of oxidase reagent. Sterile inoculating loop was used to pick a colony of the test organism and smeared on

the filter paper. If the organism is oxidase producing, the phenylenediamine in the reagent will be oxidized to a deep purple color (Cheesbrough, 2006).

3.1.8.4. Analytical Profile Index-20E

API 20E kit (BioMerieux) was used to identify the enteric gram-negative rods. It is a standardized identification system for *Enterobacteriaceae* and other non-fastidious, gram negative bacteria which uses 21 miniaturized biochemical tests and a database. The strip consists of 20 micro tubes containing dehydrated substrates. These tests are inoculated with a bacterial suspension that reconstitutes the media. During incubation, metabolism produces color changes that are either spontaneous or revealed by the addition of reagents. The reactions are read according to the reading table and the identification is obtained by referring to the Analytical Profile Index or using the identification software. A bacterial suspension is used to rehydrate each of the wells. Single colony of each strain (18-24 hrs. old) was inoculated into 5ml of sterile distilled water, carefully emulsified to achieve a homogeneous bacterial suspension. Using a pipette, tube and cupule were filled with bacterial suspension for the tests CIT, VP and GEL. While for remaining tests only tubes were filled. These cupules marked ADH, LDC, ODC, H_2S and URE were then filled up to the top with mineral oil to create anaerobiosis. Strip was labelled and closed in the incubation box. Subsequently the incubation box with strip in it was incubated at 36°C ± 2°C for 18-24 hrs. After 24 hrs of incubation, reagents were added in IND, VP and TDA chambers. The positive and negative results of API 20E translated into numerical profiles and interpreted with the API web software.

Table 2: API 20E Interpretation Table

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	Blue-green
H2S	Na thiosulfate	H2S 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
GLU	glucose	Fermentation/oxidation	blue/blue-green	yellow
MAN	mannitol	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
ARA	arabinose	Fermentation/oxidation	blue/blue-green	yellow
OX	oxidase	oxidase	Colorless/Yellow	violet

3.1.8. Partial gene sequencing and Phylogenetic analysis

Glycerol stocks of pure colonies of isolated strains were prepared for identification, storage and further molecular characterization. For the preparation of stocks, 0.5 mL LB broth in cryovial tubes was autoclaved for 15 minutes at temperature 121°C. The tubes were cooled to room temperature in laminar flow hood (LFH) to attain sterile environment for stock preparation. The tubes were assigned identification codes and

inoculated with pure colonies. After incubation for 24 hours at 37°C, 0.4 mL of (50%) glycerol was added to the tubes containing cultures. After proper mixing, stocks were stored at -20°C. The glycerol stocks were sent for 16SrRNA partial gene sequencing by third party (Macrogen, Korea). The sequence data were aligned using alignment editor and compared with representative 16S rRNA gene sequences of most closely related organisms. Basic Local Alignment Search Tool (BLAST) search was carried out for sequence similarity in National Center for Biotechnology Information (NCBI) database (Iqbal et al., 2018). Phylogenetic tree was constructed using MEGA7 software to represent phylogenetic relationship among isolated strains (Kumar et al., 2016).

3.2. Phase 2: Prototype Distribution Network Study

To analyze the potential of disinfectant (ESOL) dose and contact time on isolated bacterial strains inactivation, a range of values were selected as input. Multiple Dosages and contact times were consulted from literature i.e. dose of 1, 5 and 10% and contact time of 10 sec, 10 min and 1 hr. to run the model. Dechlorinated tap water of the IESE (Toxicology Lab) was used to simulate the real conditions of water distribution network. The bacterial count of the targeted strains was initially determined through spread plate count (SPC) and optical density (OD). A specific OD at 600 nm of each bacterial strain was analyzed corresponding to the desired count using SPC technique. Before each experimental run, the predetermined OD at 600 nm corresponding to the desired CFU was achieved and bacterial inoculum was added in the protype distribution network maintaining sterile conditions. 24 hours freshly cultured inoculum of *Aeromonas spp., Enterobacter sp., and E. coli in* nutrient broth was centrifuged at 4000 rpm, suspended twice in phosphate buffer and set at 10^6 CFU/mL. Sampling was performed using

autoclaved sampling bottles at dosages and contact times as selected. All experiments and sampling were conducted in replicates. Bacterial count before and after the addition of disinfectant was determined by Spread Plate Count. Control was left without any disinfectant. All experiments were conducted in replicates.

3.2.1. Specifications of Prototype Water Distribution Network

A schematic of prototype distribution network is shown in Figure 4. The system comprises of high-density polyethylene (HDPE) water reservoir, with a working volume of 100 liters and transmission lines, made up of 1-inch HDPE piping, fittings, and valves. The transmission line comprises of 10 sampling ports with each port located at an equal distance from adjacent port. A high-pressure monophasic pump (PKm 60®, Pedrollo, Italy) was used for continuous water flow from reservoir to the transmission line. Sampling ports labelled (1), (5), and (10) were used to collect samples before and after disinfection for real time monitoring (pH, ORP, DO, Free chlorine, Conductivity and Temperature).

Table 3: Prototype Distribution Network Specifications

Characteristics	Specifications
Water Storage Tank	
Volume of tank	588 Liters
Working Volume of tank	100 Liters
Height of tank	37 inches
Diameter of tank	36 inches
Prototype distribution network	
Piping Material	HDPE
Length of pipe	220 m
No. of sampling ports	10
Targeted sampling ports	1, 5, 10
Distance in adjacent points	22 m
Electrical Components	
High Pressure Pump	PKm 60®, Pedrollo,

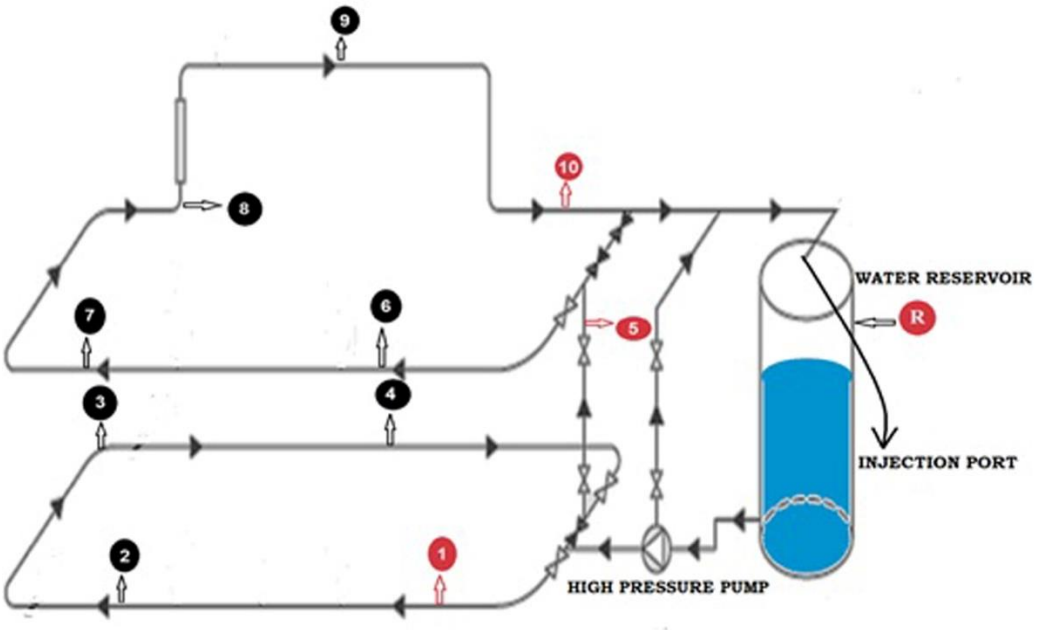


Figure 4: Schematic of the Prototype Distribution Network

3.2.2. Electrolyzed Solution (ESOL) System

ESOL also referred as ECAS is a unique disinfectant produced by a **Bridge Biotechnology ESOL System** using only water, salt and electricity. There are three inputs involved in the process of producing ESOL with the Bridge Biotechnology ESOL System, potable water saline solution and electricity (240V). In its pure form, ESOL has an Oxidation Reduction Potential (ORP) of between +1,100mV and +1,200 mV. This creates an environment outside the working range of important microbial processes, including energy-generating mechanisms. If immersed in ESOL, pathogens will come into contact with powerful oxidants which will sequester electrons with high efficiency from microbial structural compounds, causing the rupturing of biochemical bonds and subsequent loss of function. This is often described as an “oxidative stress”.

The feed water required for the production of ESOL must comply with WHO guidelines. If water supply hardness exceeds 50 ppm the water must first be softened otherwise damage will occur to the fabric of the cell. The potable water for the feed may be generated by employing RO system or three stage filters. Saline solution is fed into the ESOL generating unit using a different mechanism to the water input. A separate saline bin must be established and the saline solution drawn down to the unit by providing a potential under the action of gravity by external tubing under no pressure.

According to the manufacturer’s instructions the saline bin must contains enough salt and water. The saline solution must be fully saturated saline (35.89g NaCl /100g water), contained in the saline bin as recommended. The ESOL unit must be kept within three meters of the saline bin when in operation. The power input requires a 240V mains connection, which can also be modified on manufacture to accommodate 110V mains.

The program logic controller (PLC) located on the front of the Control Module will turn on and text will be visible.

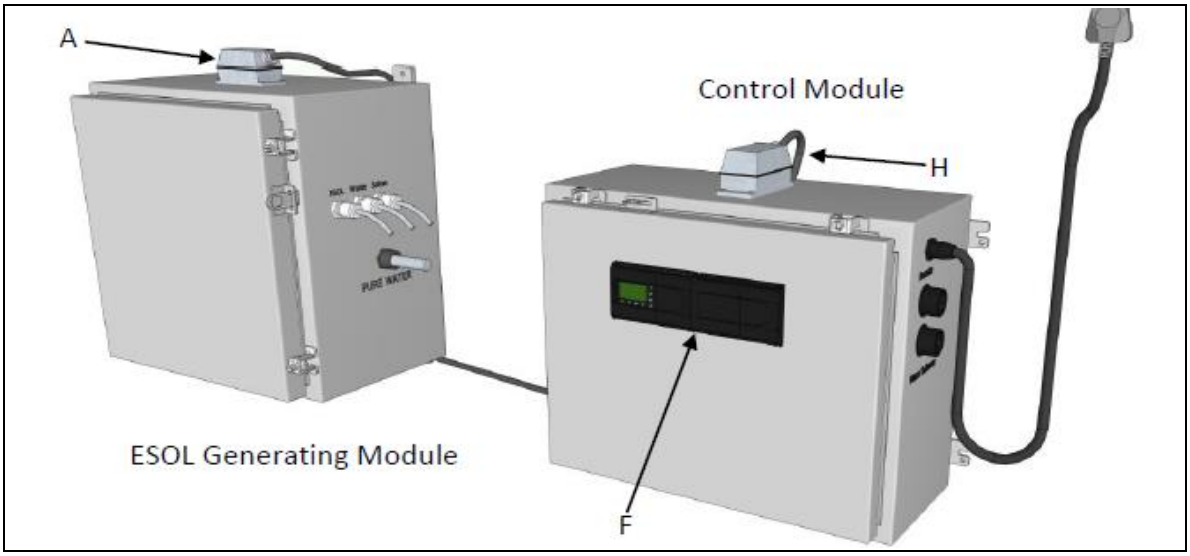
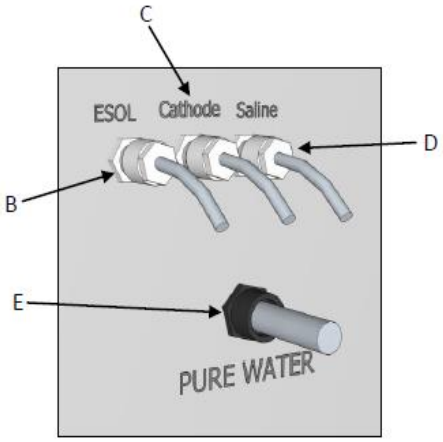
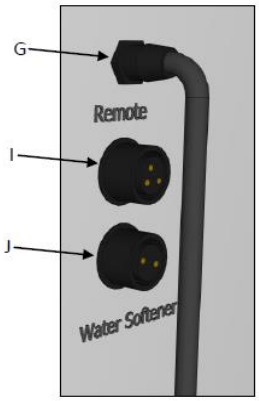


Figure 5: ESOL System Components





ESOL Generating Module	Control Module
A. Low Voltage Cable	F. Programmable Logic Controller
B. ESOL Output	G. Power Module Voltage Input
C. Cathode Output	H. Low Voltage Output
D. Saline Input	I. Remote Start/Stop
E. Water Input	J. Water Softener /Aux. Remote

3.2.3. Generation of ECAS

ECAS was produced by a Bridge Biotechnology (Dunfermline, Fife, Scotland) ESOL system (60 L/hour) using water, salt and electricity as inputs. The system comprises of two major components i.e. ESOL generating module coupled with a control module. The electrolysis of a saline solution in the generating module under a direct current produces anodic and cathodic solution. The anodic solution (ECAS) produced with a high ORP ranging between 1100 - 1200 mV and a pH value of 2.3 - 3.0. ECAS generated was subsequently stored under controlled temperature.

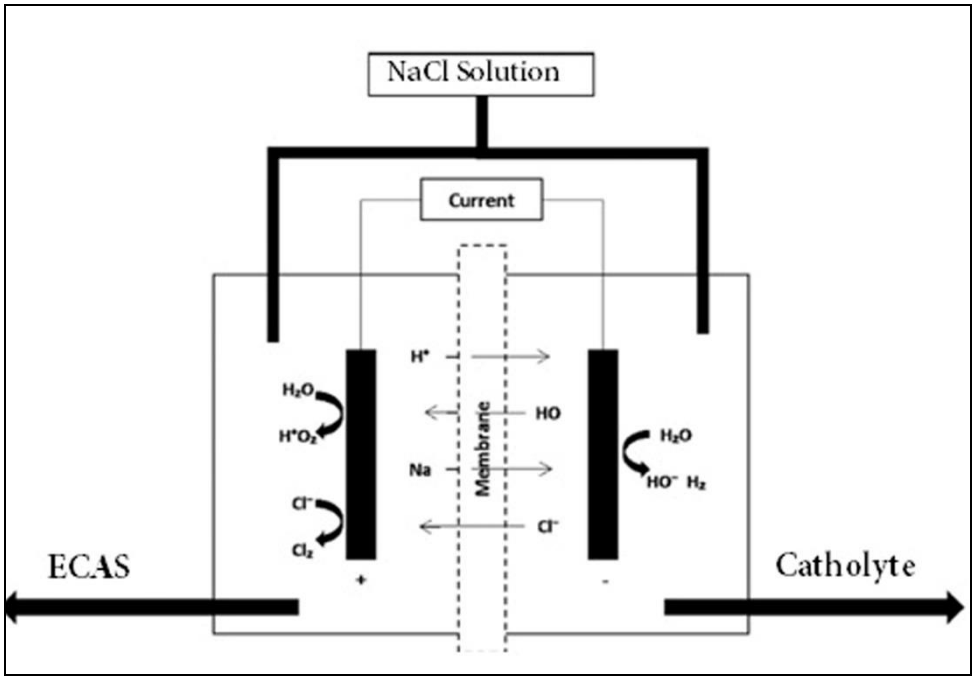


Figure 6: Schematic of ECAS generator (Electrolysis of NaCl solution generates ECAS)

3.2.4. Preparation of Bacterial Stock Solutions

Three isolated bacterial strains analyzed through 16S rRNA gene sequencing belonged to *Aeromonas sp*., *Enterobacter sp*. and *E. coli* genera as shown in Figure 7. Representative colonies of *Aeromonas sp*. from xylose lysine deoxycholate (XLD) medium (Oxoid Ltd,

Hampshire, England), Enterobacter *sp*. from buffered charcoal yeast extract (BCYE) agar (Oxoid Ltd, Hampshire, England) and *E. coli* from eosin methylene blue (EMB) agar (Oxoid Ltd, Hampshire, England) plates were collected and inoculated in 5 mL nutrient broth (Oxoid Ltd, Hampshire, England). These inoculated broth tubes were incubated overnight at 37°C with shaking at 200 rev/min. The optical density (OD) of bacterial culture after incubation was measured at 600 nm using spectrophotometer to adjust bacterial concentration of desired CFU/mL (Amiri et al., 2010). Centrifugation of each bacterial culture was done at 6000 rcf using centrifuge (K3 Series, Centurian Scientific,UK) for 5 min at 4°C to isolate cells from broth and form a pellet at the bottom. The supernatant was discarded and pellet was rinsed and resuspended with phosphate buffer (pH 7) thrice in order to prevent the addition of non-cellular components in the system to minimize disinfectant demand (Virto et al., 2005). After washing the resultant pellet was suspended in phosphate buffer to form a suspension.

3.2.4. Culture Inoculation and Treatment

Freshly prepared stock suspensions of test organisms were inoculated with a mean bacterial count of 1.15×10^6 CFU/mL to the prototype reservoir with 100 liters tap water and well mixed to ensure uniform dispersal. The control sample was collected immediately after the inoculation of bacterial stock to analyze the change in bacterial growth during the experimental time. Freshly generated ECAS with known properties (ORP, pH and free chlorine) was dosed as per targeted dosages. Samples were collected at 10 sec, 1 min and 1 hour in sterile screw caped tubes with 0.1 mL quenching agent ($Na_2S_2O_3$) to inhibit the residual effect of disinfectant and estimate an accurate count of surviving organisms (Qureshi, 2018). The surviving bacterial population (CFU/mL) of

each strain was quantified by plating 0.1 mL portion of respective sample on selective agar plates. The colonies of each strain were confirmed by API-20E test kit (Biomerieux, Canada) (Venkitanarayanan et al., 1999). Experiments were performed in duplicate at each sampling time and the results were replicated twice. The pH, ORP, free chlorine, Conductivity and temperature of the samples were measured in triplicate immediately after collection.

3.3. Phase 3: ECAS Dose Optimization for IESE Tap Water

Tap water sample of IESE ground floor was collected in a sterile screw capped bottle and was analyzed for bacteriological contamination through MF and MPN technique. The water sample was subsequently subjected to the varying dosages and contact times to estimate the optimum combination.

Chapter 4

RESULTS AND DISCUSSION

This chapter discusses results attained from experiments conducted in three different phases of research. Isolation and characterization of selected bacterial species, followed by Spread Plate Count (SPC) technique; subsequent analysis of antimicrobial potential of ECAS followed by dose and contact time optimization for IESE Tap Water.

4.1. Phase 1: Water Quality Characterization

Table 1 demonstrates the physicochemical analysis of tap water samples collected before each experimental run. The analysis depict that all the parameters were within range and meet the WHO limits except high total organic carbon (TOC) load which may be due to natural organic matter (NOM) in groundwater of study area. Organic matter which constitutes around 58% organic carbon (Martínez et al., 2018) significantly impacts the antimicrobial potential of ECAS (Thorn et al., 2012; Fang et al., 2016) as microorganism proliferate using organic carbon as major food source. The heterotrophic plate count (HPC) of the tap water for each experimentation was found to be <30 CFU/mL. Spread plate method was used for enumeration of heterotrophic bacteria at 37^{o}C on nutrient agar. The maximum acceptable count may range from 20 to 1000 CFU/mL (Allen et al., 2004)

Table 4: Physicochemical and Elemental Analysis of Sampling Sites

Parameter	Unit	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6
pH	---	7.08± 0.18	8.02± 0.21	8.22± 0.33	8.25± 0.26	7.88± 0.14	7.13± 0.12
Conductivity	µs/cm	866±5.30	828±5.56	796±7.54	823±6.55	824±6.24	822±6.16
TDS	mg/L	477±2.91	455.4±3.06	437.8±4.15	411.5±3.60	412±3.43	452± 3.11
TSS	mg/L	ND	ND	ND	ND	ND	ND
Hardness	mg/L	365±3.60	440±2	330± 2.64	340± 2.65	330± 2	345±3
Nitrate	mg/L	ND	ND	ND	ND	ND	ND
Nitrite	mg/L	ND	ND	ND	ND	ND	ND
Phosphate	mg/L	0.55±0.02	0.084±0.01	1.082±0.11	1.142±0.04	1.082±0.02	0.78± 0.09
DO	mg/L	5.76±0.32	6.22±0.56	6.06±0.34	6.56±0.22	6.78±0.26	6.45±0.97
Temp	˚C	10	13	20	22	20	23
Total Coliform	MPN Index/ 100 mL	>1.1	<23	12	<23	>1.1	<23
Fecal Coliform		>1.1	<23	12	<23	>1.1	<23
Free Chlorine	mg/L	0	0.6	0	0	0	0
TOC	mg/L	-	-	-	1.75	1.75	1.75
Mg	µg/mL	ND	ND	ND	ND	ND	ND
Zn	µg/mL	0.04	-	0.03	0.05	-	-
Cr	µg/mL	ND	ND	ND	ND	ND	ND
Pb	µg/mL	ND	ND	ND	ND	ND	ND
Cd	µg/mL	ND	ND	ND	ND	ND	ND
As	ng/mL	ND	ND	ND	ND	ND	ND
Hg	µg/mL	ND	ND	ND	ND	ND	ND
Ni	µg/mL	ND	ND	ND	ND	ND	ND
Se	µg/mL	ND	ND	ND	ND	ND	ND
Sn	µg/mL	ND	ND	ND	ND	ND	ND

*Results shown are the mean value (n= 3±SD), ND: Not Detectable

4.2. Isolation and purification of bacteria

Drinking water microbes identified as *Aeromonas sp*., *E. coli*, *Enterobacter* sp., *Stentotrophomanas sp*., *Sphingomonas sp*., were isolated from targeted sampling sites by SPC method. Gram staining analysis showed that all the species were gram negative producing pink rods of varying size as represented in Table 6. Biochemical tests such as oxidase, catalase, agar test and API 20E were performed for all three bacteria and results are mentioned in Table 5.

Table 5: Isolation and confirmatory tests of bacterial Species

Bacterial Strains	**Sampling Location**	**Gram Staining**	**Oxidase Test**	**Catalase Test**
Aeromonas sp.	Sample 3 and 5	Gram -ve	- ve	+ve
E. coli	Sample 3,4 and 6	Gram -ve	-ve	+ve
Stenotrophomanas sp.	Sample 1 and 2	Gram -ve	-ve	+ve
Sphingomonas sp.	Sample 1 and 3	Gram -ve	-ve	+ve
Enterobacter sp.	Sample 2 and 3	Gram -ve	-ve	+ve

Bacterial colonies grew on selective solid media i.e., agar with distinct features and morphology. A characteristic colony is a visible mass of microorganisms originating from a single mother cell. Key features of these bacterial colonies were followed as important aspect for their identification as highlighted in Table 6.

Table 6: Characteristic colony appearance on various culture media of bacterial species

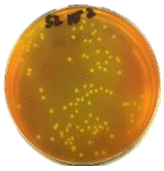
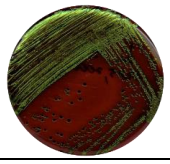
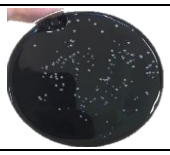
Bacterial Strain	Medium	Results	Observation
Aeromonas sp.	XLD Agar	Pale Yellow	
E. coli	EMB Agar	Green Sheen	
Stenotrophomanas sp.	XLD Agar	Pink Mucoid	
Sphingomonas sp.	BCYE Agar	Brownish Yellow	
Enterobacter sp.	BCYE Agar	Greyish White	

Table 7: API 20E Analysis interpretation for isolated bacterial species

Tests	**Substrate**	***Sphingomonas sp.***	***E. coli***	***Aeromonas sp.***	***Stenotrophomonas sp.***	***Enterobacter sp.***
ONPG	ONPG	+ve	+ve	+ve	-ve	-ve
ADH	arginine	+ve	+ve	+ve	+ve	+ve
LDC	lysine	-ve	+ve	-ve	+ve	+ve
ODC	ornithine	-ve	-ve	-ve	-ve	+ve
CIT	citrate	-ve	-ve	-ve	-ve	+ve
H2S	Na thiosulfate	-ve	-ve	-ve	-ve	-ve
URE	urea	-ve	-ve	-ve	-ve	-ve
TDA	tryptophan	-ve	-ve	-ve	-ve	+ve
IND	tryptophan	-ve	+ve	+ve	-ve	-ve
VP	Na pyruvate	+ve	-ve	-ve	-ve	-ve
GEL	Charcoal	-ve	-ve	-ve	+ve	+ve
GLU	glucose	-ve	+ve	+ve	-ve	+ve
MAN	mannitol	-ve	+ve	+ve	-ve	-ve
INO	inositol	-ve	-ve	-ve	-ve	-ve
SOR	sorbitol	-ve	+ve	-ve	-ve	-ve
RHA	rhamnose	-ve	+ve	+ve	-ve	-ve
SAC	sucrose	+ve	-ve	+ve	-ve	-ve
MEL	melibiose	+ve	+ve	-ve	-ve	+ve
AMY	amygdalin	+ve	-ve	-ve	-ve	-ve
ARA	arabinose	+ve	+ve	+ve	-ve	+ve
OX	oxidase	+ve	-ve	+ve	-ve	+ve

Characteristic colony appearance on various culture media further confirmed the morphological characteristics of isolated bacteria

Table 8: Morphological characteristics of isolated bacterial strains

Morphology	***Aeromonas sp.***	***Stenotrophomanas sp.***	***E. coli***	***Enterobacter sp.***	***Sphingomanas sp.***
Colony size	Small	Small to medium	Punctiform	Small medium	Small
Margins	Flat	Entire	Fixed	Filamentous	Entire
Forms	Round	Circular	Circular	Irregular	Circular
Texture	Smooth,Dry	Smooth, Glistening	Smooth	Gummy	Mucoid
Elevation	Convex	Raised	Raised	Pulvinate	Convex
Color	Pale Yellow	Red	GreenSheen	Greyish White	Green Brown

4.3. Identification of Isolated Strains

The gene sequence analysis (16S rRNA) showed that the isolated bacterial strains MH245227, MH250046 and MH250051 belong to *Aeromonas, Enterobacter* and *E. coli* genera, respectively. Strain MH245227 showed 99.9% sequence similarity to (NZCDDF01000007) *Aeromonas Eucrenophila*. Strain MH250046 showed maximum sequence similarity to (NC002695) *E. coli O157:H7* whereas, strain MH250051 showed maximum similarity to (NZCPO17179) *Enterobacter Homaechei*. All the three strains belong to the phylum proteobacteria. Based on the 16S ribosomal RNA gene sequencing,

phylogenetic tree of the isolated bacterial strains was constructed as shown in the Figure 7.

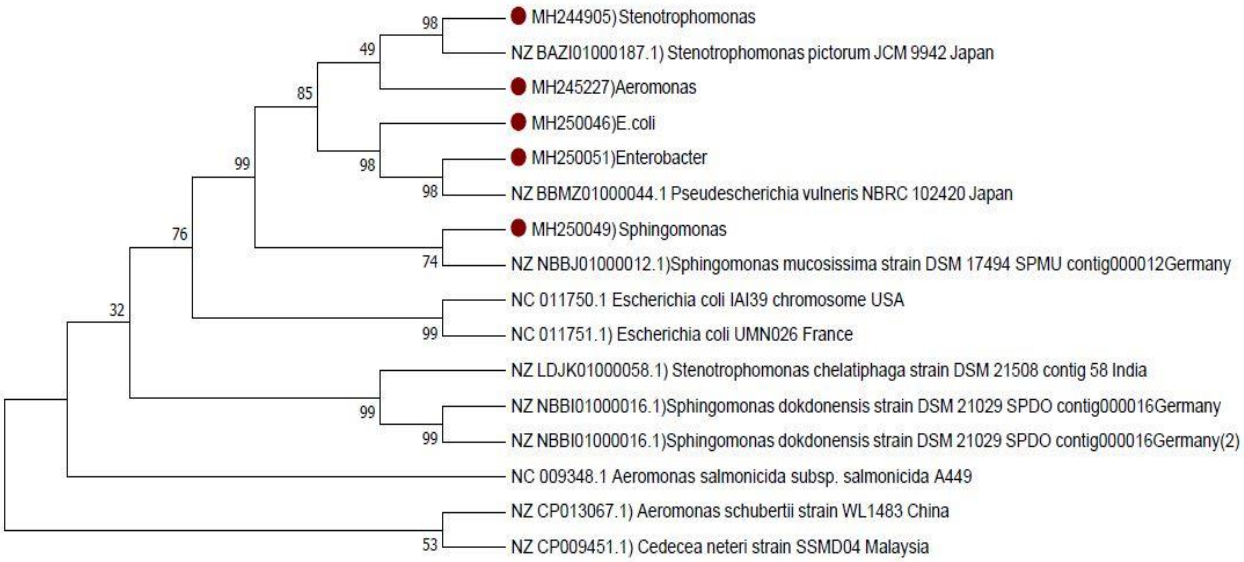


Figure 7: Phylogenetic tree based on 16S rRNA sequences

4.4. Phase 2: Prototype Distribution Network Study

4.4.1. Bacterial Inactivation at 1% ECAS Dosing

Microbial consortia (comprised of *E. coli, Enterobacter* sp. and *Aeromonas* sp.) with a mean bacterial count of 1.15×10^6 CFU/mL (6.06 log CFU/mL) at 1 % ECAS dosing showed variable extent of disinfection, at respective contact times. *Aeromonas sp.*, and *E. coli* exhibited an initial reduction to 4.80 and 4.05 log CFU/mL at 10 sec exposure time and subsequent increase in bacterial count due to the regrowth recovery. Aeromonas being resistant to disinfection (Razzolini et al., 2011) with an ability to recover and regrow (WHO, 2011) has shown least reduction at 10 sec, followed by regrowth till 1 hour exposure time. The overall recovery potential of *E. coli* was higher than *Aeromonas sp*.

due to its persistent nature (Warburton et al., 1998) and ability to thrive in water rich environment (Delaedt et al., 2008).*Enterobacter sp.* underwent an overall hike in log reduction throughout the experimental run with maximum of 2.85 at 1 hour due to its sensitivity and low resistance to disinfectants (WHO, 2017). However, no significant change in bacterial counts of the control samples were observed. Since a high ORP of ECAS i.e. 800- 1100 mV, in combination with low pH i.e., 2-5 and free chlorine plays a significant role in killing of microorganisms (Ashraf et al., 2014), the bacterial regrowth is also associated with insufficient disinfectant residual supplemented by high concentration of assimilable organic carbon (WHO, 2017) . The potential oxidizing capacity of the disinfectant at low dosage causes the microorganisms to undergo sub lethal injury instead of being inactivated (Wesche et al., 2009). These injured cells, under suitable conditions recover the cellular damage and outgrow (Manas and Pagan, 2005). The exposure of bacterial cells to adequate free chlorine (one of the antimicrobial components of ECAS) causes extensive permeabilization of the cytoplasmic membrane and disruption of protein synthesis (Huang et al., 2008) which results in cellular collapse. The high organic matter stabilizes the cytoplasmic membrane against permeabilization which results in inadequate degree of disinfection (Virto et al., 2005).The adhesion of bacteria on surfaces also affects the degree of disinfection (Lechevallier et al., 1988; Fang et al., 2016). At 1 hour the ORP and free chlorine of treated water was reduced to 196 mV and 0 mg/L respectively.

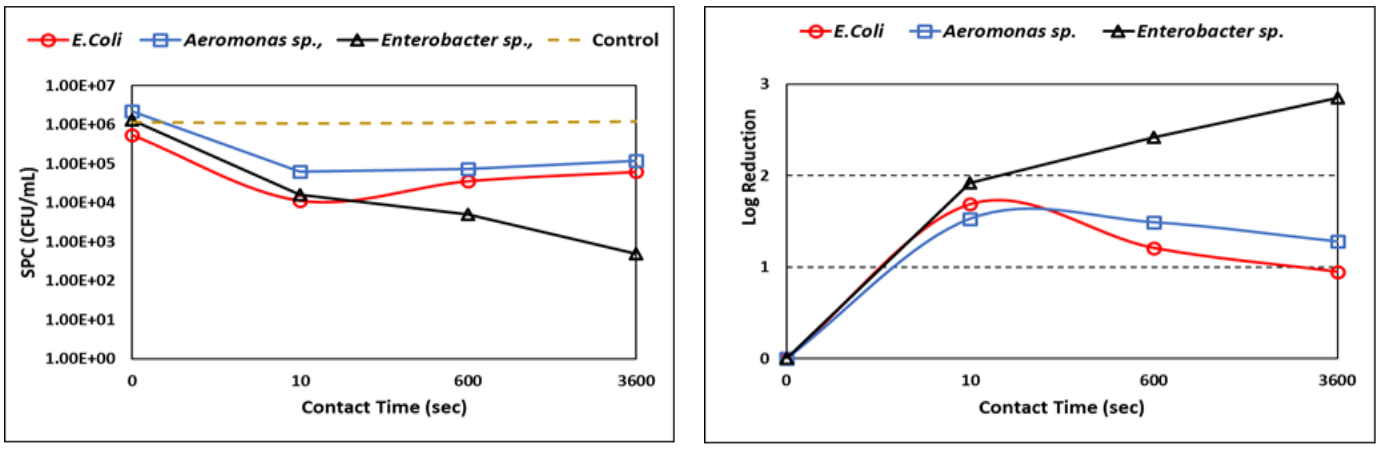


Figure 8: Bacterial Inactivation of test organisms at 1% ECAS and varying Contact time. Log Reduction of test organisms at 1% ECAS and varying Contact time

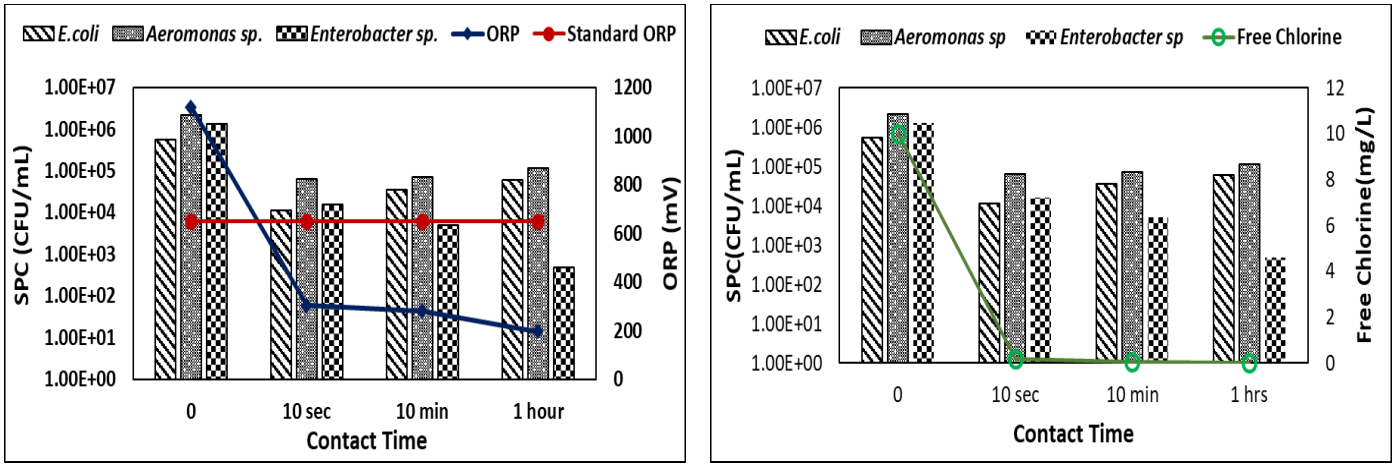
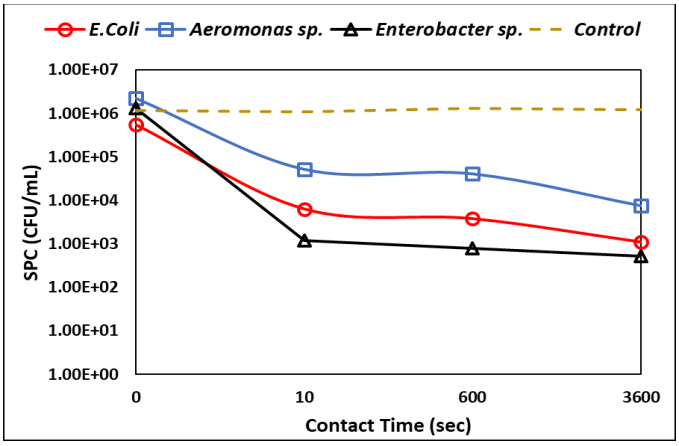


Figure 9: Bacterial Inactivation vs ORP at 1% ECAS. Bacterial Inactivation vs. Free Chlorine at 1% ECAS

4.4.2. Bacterial Inactivation at 5 % ECAS Dosing

ECAS has shown prominent antibacterial activity at 5% (v/v) dosage at targeted contact times as shown in Figure 11. Mean bacterial count of all the test organisms progressively reduced to 3.21 Log CFU/mL after 1hour exposure time. In control sample any significant change in bacterial count was not observed. Table 2 shows that the population

of *Enterobacter sp*. and *E. coli* were reduced from 6.11 to 2.72 log CFU/mL and 5.74 to 3.04 log CFU/mL respectively. This corresponds to a retardation in bacterial activity of > 99% for *Aeromonas* sp. and *E. coli* whereas > 99.9% for *Enterobacter sp.* Maximum resistance exhibited by *Aeromonas sp.* is associated with its strong potential to colonize biofilms (Scoaris Dde et al., 2008) and was reduced from 6.34 log CFU/mL to 3.87 log CFU/mL. The varying degree of bacterial kill for each strain is associated with level of stress response to the ECAS oxidative stress (Kochhar and Kochhar 2005). After 1 hr. of exposure, the highest log reduction of bacterial populations were observed as 3.39 > 2.7 > 2.47 for *Enterobacter sp*., > *E. coli* > *Aeromonas sp*. The Figure 5 (b) indicates that with time the rate of log reduction impedes which may be due to decrease in antimicrobial efficacy as a result of loss of short-lived oxidative species of ECAS (Clayton et al., 2017). Ghebremichael et al. (2011) determined that freshly generated ECAS with ozone concentration of 5.3 mg/L decayed to 0 mg/L within 30 min influencing the disinfection potential of ECAS. This ORP reduced to 332 mV corresponding to 1 hour which corresponds to the concomitant decrease in the oxidative stress (Kelly, 2004; Park et al., 2004). Oxidative stress at higher OPR causes a significant reduction in the number of protein bands and hence the cellular protein of bacteria whereas a low ORP value causes partial degradation of protein due to weak oxidative stress(Cloete et al., 2009). The substantial decrease in residual chlorine to 0.12 mg/L at 1hour contact time is associated with increased chlorine demand of water due to high organic loading (Martinez-Hernandez et al., 2013). The diminishing chlorine from the system may indicates conversion to disinfection by products DBPs as concluded by Gougoutsa et al. (2016).



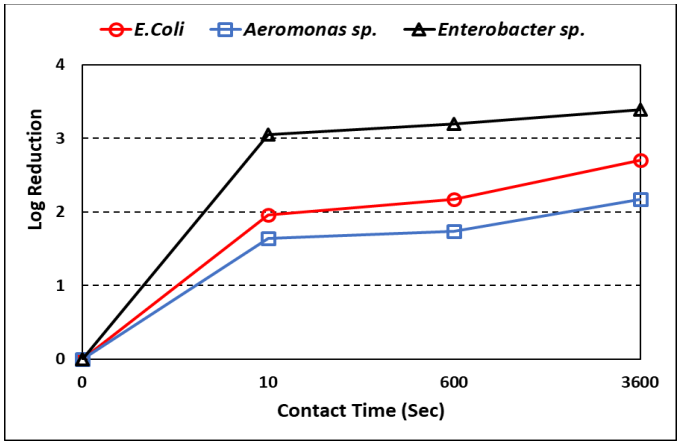
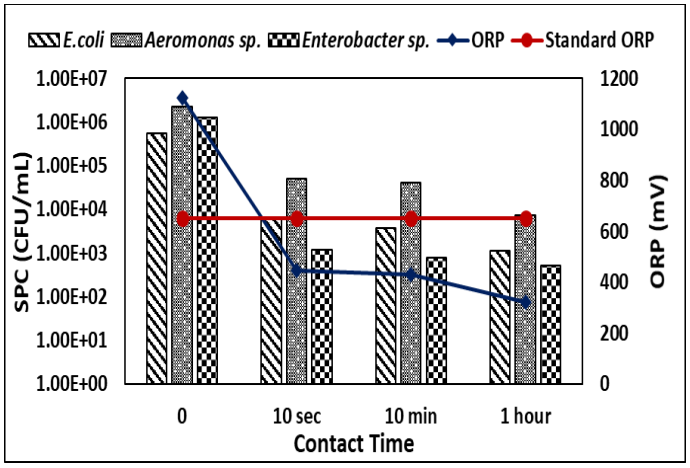


Figure 10: Bacterial Inactivation of test organisms at 1% ECAS and varying Contact time. Log Reduction of test organisms at 1% ECAS and varying Contact time



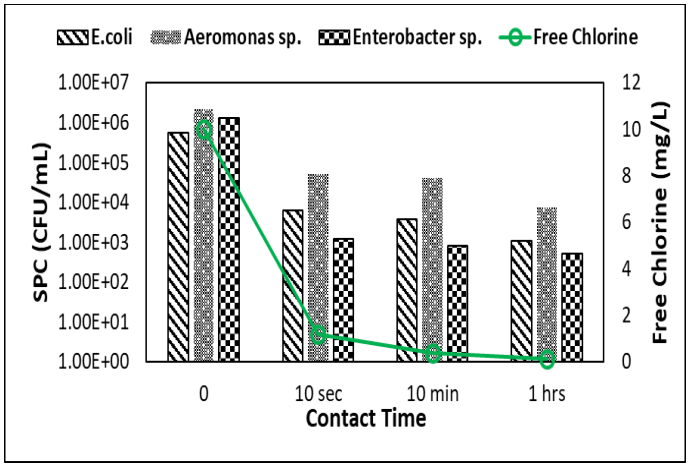


Figure 11: Bacterial Inactivation vs ORP at 1% ECAS. Bacterial Inactivation vs Free Chlorine at 5% ECAS Dosing

4.4.3. Bacterial Inactivation at 10 % ECAS Dosing

Active microbial consortium of test organisms showed a significant mean log reduction of 3.45 upon adding 10% (v/v) freshly generated ECAS at 1 hour exposure time suggesting the immediate effect of ECAS in retarding the bacterial activity to > 99.9 %. The mean log count of the consortia was reduced from 6.05 Log CFU/mL to 4.20 Log

CFU/mL. The surviving potential of *Aeromonas sp.* was found to be highest out of the three species with maximum log reduction of 2.74 at exposure time of 1 hour due to its resistant nature and re-growth potential in even low-nutrient environment (Razzolini et al., 2011). *Enterobacter sp*., turned out to be the least resistant and showed the maximum log reduction of 4.11 whereas *E. coli* showed a maximum log reduction of 3.50. The log reduction trend demonstrates that each strain has a varying inactivation response. Analysis reflects an efficient biocidal effect of ECAS but none of the test organisms were completely eliminated. The findings of Ghebremichael et al. (2011) justifies the trend as inactivation potential of ECAS for *E.coli* was found to be 57% lower in tap water than demineralized water. Warburton et al. (1998) also quoted that bacterial mortality rate is lower in tap water due to varying chemical composition and carbon content of water. The drop in ORP value to 336 mV at 1 hour denotes deteriorating oxidative stress. This may be associated with formation of reductants which significantly lowers the ORP by donating electrons and hence influencing the log reduction (NSW Gov 2016). The degradation of transient oxidative functional groups (Ozone, ClO_2, Hydrogen peroxide) of ECAS with time also weakens the oxidative stress (Cloete et al., 2009; Clayton et al., 2017). Free chorine was found to be 0.2 mg/L at 1 hour exposure time.

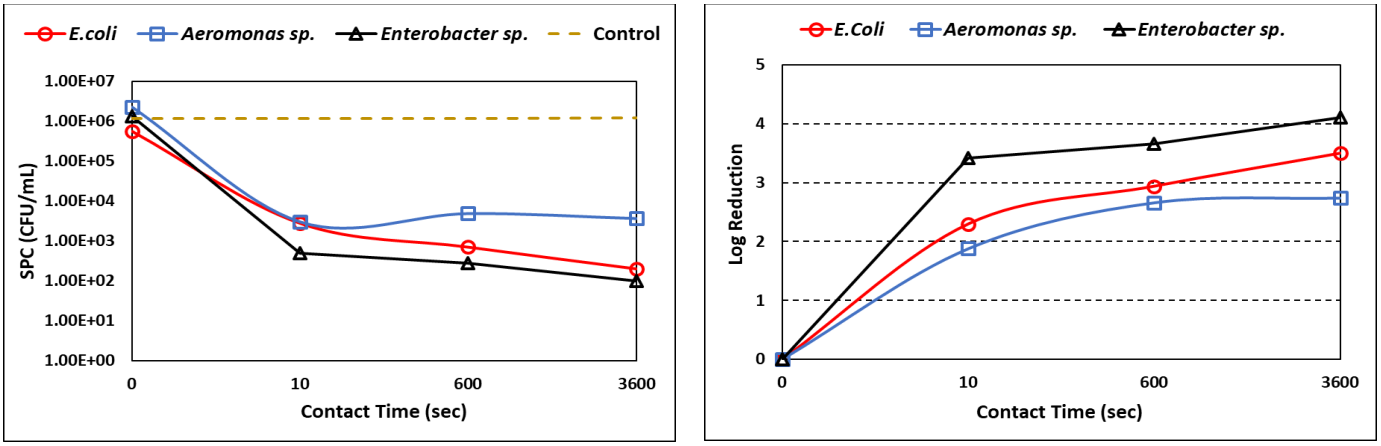


Figure 12: Bacterial Inactivation of test organism at 10% ECAS and varying Contact time Log Reduction of test organisms at 10% ECAS and varying Contact time

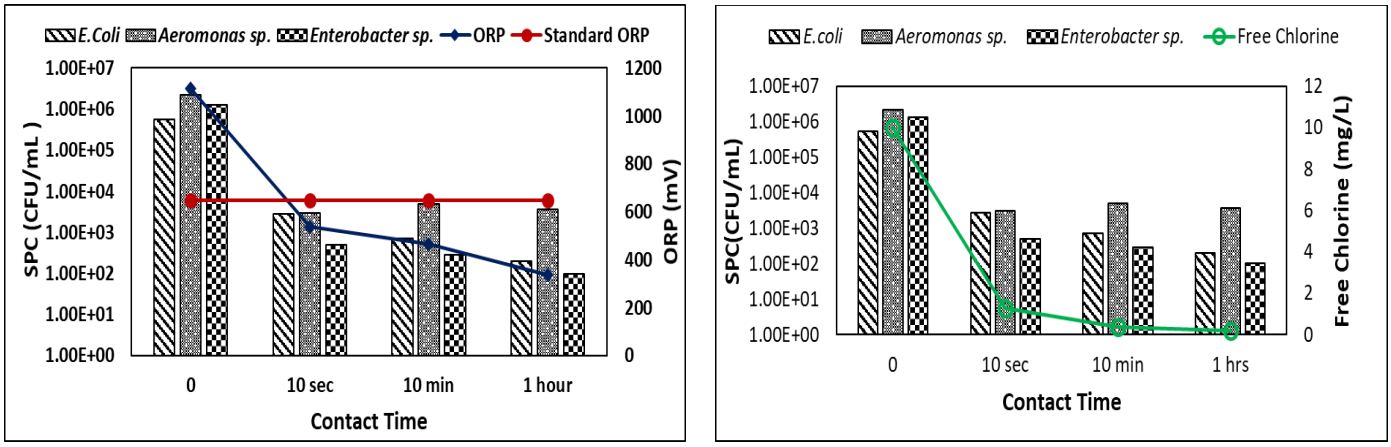


Figure 13: Bacterial Inactivation vs. Free Chlorine at 10% ECAS

4.4.4. Phase 3: ECAS Dose Optimization for IESE Tap Water

Tap water of IESE was analyzed for bacterial contamination through MPN and MF technique. The MPN index for total coliform and fecal coliform was found to be >23. Whereas, MF analysis showed 175 CFU/100mL of water sample.

The tap water sample was dosed with 1, 5 and 10% ECAS for 10 mins and 1 hour contact time. It was concluded that 1% ECAS dosing for 10 mins exposure time is suitable for adequate disinfection. The MPN index was found to < 1.1 and no colony was grown on EMB agar during MF analysis.

Table 9: ECAS dose and contact time optimization for IESE tap water

Contact Time	**Most Probable Number**		**Membrane Filtration**
min/hr	Total coliform	Fecal coliform	Cfu/100 mL
Tap water			
Tap water	>23	>23	175
1% ECAS Dosage			
10 min	<1.1	<1.1	ND
1 hour	<1.1	<1.1	ND
5% ECAS Dosage			
10 min	<1.1	<1.1	ND
1 hour	<1.1	<1.1	ND
10 % ECAS Dosage			
10 min	<1.1	<1.1	ND
1 hour	<1.1	<1.1	ND

Chapter 5

Conclusions and Recommendations

Provision of drinking water at user end is crucially important. Potable water released into the distribution system becomes altered during its passage through pipes due to the hydrological, physical, chemical and biological processes occurring within the distribution system. Water quality is affected by varying parameters, most importantly the treatment technique applied. Antimicrobial potential, kill kinetics and residual potential of the disinfectant are the core parameters to ensure provision of water at user end with immaculate disinfection. The current study intended to characterize the water quality at targeted sampling sites followed by analyzing the biocidal potential of ECAS in tap water inoculated with indigenously isolated enteric bacteria at varying dosages and contact times. Subsequently in the final phase of study the tap water of IESE was analyzed at varying dosages and contact times of disinfectant for its further up scaling. The conclusions drawn from this study and recommendations for future studies are discussed below.

5.1. Conclusions

Phase 1: Water Quality Characterization

1. All the physicochemical parameters at targeted sampling sites were within WHO limits. The excess hardness of the samples i.e., between 330 to 440 mg/L was probably due to calcium and magnesium ions.

2. All the elemental parameters were within WHO limits. Whereas, the TOC load at the downstream sampling sites was found to be 1.75 mg/L.

3. High TOC load could be associated to NOM in ground water. It also provides the extent of ground water contamination by organic compounds.

4. The MPN analysis revealed that sampling location 2 (Disinfection Injection point), 3 (Intermediate point), 4 (Ground floor IESE building) and 6 (1^{st} floor IESE building) were microbially unsafe for drinking with MPN index < 1.1.

Phase 2: Prototype Network Study

1. *Enterobacter sp.* and *E. coli* was found to be more vulnerable than *Aeromonas sp.* in microbial consortium at all the respective dosages and contact times.

2. There exists a negative correlation between bacterial inactivation and Oxidative stress i.e., with decreasing oxidative stress the extent of bacterial inactivation recedes.

3. A 10% disinfectant dosage corresponding to a contact time of 1 hour showed the highest log reduction as 4.11 >3.50 > 2.74 for *Enterobacter sp*. > *E. coli* > *Aeromonas sp*. which corresponds to a mean log reduction of 3.45

4. Complete inactivation was not achieved as kinetics of inactivation varies in tap water

5. pH, chlorides and conductivity of ECAS disinfected water increases within WHO limit.

Phase 3: Dose and Contact Time Optimization for IESE Tap Water

1. The tap water of IESE showed complete inactivation at 1%, 5% and 10% disinfectant dosage.

2. The optimum disinfectant dosage and contact time was found to be 1% ECAS for 10 mins.

3. The MPN index for Total and fecal coliforms of the tap water reduced from >23 which was reduced to <1 at 1% ECAS dosing for 10 mins.

4. The MF analysis showed the tap water with 157 CFU/100 mL was reduced to 0 CFU/100mL at 1% ECAS dosing for 10 mins.

5.2. Recommendations

1. Employing activated Carbon as a prefilter at source of distribution network to efficiently reduce the organic carbon load resulting in an increase in disinfection efficiency and better disinfection kinetics

2. Analyzing the potential of THMs formation using ECAS for tap water disinfection

3. Upscaling and developing ECAS based micro water treatment plants for semi urban or rural communities