1.1. Waterborne diseases and drinking water

Drinking water is the only vehicle of transmission for pathogens spread by the faecal to oral route. As per the definitions in the guidelines of World Health Organization (WHO), safe drinking water does not signify any substantial risk to health over a lifetime of ingestion, including different sensitivities that may occur between life stages. Bacteriological water quality may change quickly, that even the short term peaks in the concentration of pathogens may increase disease risks significantly and may also activate outbreaks of waterborne infection (WHO 2011). Apart from a fundamental human right, drinking water is also indispensable for health even though many parts of developing world is deprived of safe drinking water (UNICEF 2008).
Waterborne diseases, the most important water related health problem, are caused by the consumption of water contaminated by pathogens from human and animal faeces or urine (UNICEF 2008). Any disease causing bacteria, virus, helminthes or protozoa that are transmitted to humans through consumption of improperly treated water can be called as waterborne pathogens. Among the pathogenic group, bacteria is the most sensitive to any kind of disinfection (Simões and Simões 2013).

Waterborne outbreaks are defined as a condition in which a minimum of two people encounters similar illness after exposure to water, where the epidemiological evidence suggests the water as a probable source (Andersson and Bohan 2001; Hunter et al. 2003; Simões and Simões 2013). Waterborne disease outbreaks are not only limited to developing nations, but to affluent countries as well (Craun et al. 2006; Beaudreau et al. 2008; Blasi et al. 2008; Simões and Simões 2013).

1.2. Opportunistic, obligate and emergent bacterial pathogens

The effects of exposure to pathogenic organisms are not the same for all populations. Frequent exposure to a pathogen may be related with a lower possibility of illness due to the effects of acquired immunity. Hygienically relevant water borne pathogens can be associated with water related illness and disease outbreaks. World Health Organization (WHO 2008) had reported index organisms (pathogenic organisms of faecal origin) in drinking water distribution systems, which can be also classified as the hygienically relevant groups, which in turn indicates the integrity of the system (Wingender and Flemming 2011). Pathogens can be generally classified into three groups:
a. Obligate water related pathogens: These are usually faecally derived which causes illness independent of the health status of humans. *Salmonella enterica*, *Shigella* spp., *Vibrio cholerae*, Pathogenic *Escherichia coli* variants and *Yersinia enterocolitica* are all relevant waterborne bacterial pathogens that infect gastrointestinal tracts of humans. They are all normally transmitted to the environment through faeces and thereby enter the human gut by ingesting faecally contaminated water (Wingender and Flemming 2011). All of them may live successfully as potential members of biofilm which increases the risk of drinking contaminated water stored under prolonged conditions.

b. Opportunistic pathogens: These affect the immunocompromised sensitive population like the elderly, children, pregnant women, patients undergoing chemotherapy, AIDS patients etc. with impaired immunity (Wingender and Flemming 2011). These include very common typical drinking water related bacteria, frequently recovered from oligotrophic environmental conditions. They include *Staphylococcus epidermidis*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Streptococcus pneumoniae* and *Streptococcus pyogenes*.

c. Emergent pathogens: Numerous pathogenic bacteria such as *Aeromonas* spp., (Hiransuthikul et al. 2005), *Campylobacter* spp., *Helicobacter pylori*, *Legionella* spp., and more have been currently recognized as emerging pathogens either as variants of already known species or as newly discovered pathogens (Szewzyk et al. 2000; Nel et al. 2004; Nel and Weyer 2004).
1.3. Definitions and historical perspectives of biofilms

In nature, microorganisms never live as pure cultures of dispersed single cells, instead they accumulate at interface as poly-microbial aggregates like mats or biofilms (Flemming and Wingender 2010). In literature, definition for biofilm exists in multitude (Denkhaus et al. 2007) ever since Antonie Van Leeuwenhoek examined the plaque on his teeth in the seventeenth century (Leeuwenhoek 1684; Percival et al. 2011). Most of the definitions commonly states that biofilm is an accumulation of different types of microbial population at interfaces including bacteria, algae and fungi (Donlan and Costerton 2002; Hall-Stoodley et al. 2004), surrounded by extracellular polymeric substances (Percival et al. 2000). Afterwards, the bottle effect in marine microbes was identified (Heukelekian and Heller 1940) and revealed that the bacterial growth was considerably increased when they were attached to surfaces. Added advancements in the awareness of biofilms were made by another researcher when he found that bacteria on surfaces were more when compared with the surrounding seawater and thus postulated that the bacterial adhesion consists of both reversible and irreversible stages (Zobell 1943). But there were not many studies until the late 1960s and the early 1970s, when a few researchers investigated the prevalence of bacterial biofilms (Jones et al. 1969; Characklis 1973; Costerton et al. 1978). The work of a researcher (Jones et al. 1969), revealed the diversity of microbes in the polymeric matrix of biofilm using scanning and transmission electron microscopy. High antimicrobial resistance of biofilms to chlorine was revealed in 1973 (Characklis 1973), who explored microbial slimes in industrial water. Father of Biofilology, Dr Bill Costerton, hypothesized the mechanisms by which microbes adhered to
surfaces and showed that majority of bacterial life is sessile through his studies in dental plaque and alpine streams (Costerton et al. 1978). The basic concept of a biofilm model (Costerton et al. 1995) was formed through the biophysical, structural and chemical studies conducted. As per this model microbes form micro-colonies bounded by abundant exopolysaccharide. It has been proposed that the water filled channels in between these micro-colonies support the entry of nutrients and the removal of unwanted products. Even though scientists are focused on pure cultures, most bacteria lives in this polymeric multi-bacterial matrix of ecosystems called biofilms, in a sessile mode attached to the surfaces (Percival et al. 2011).

1.4. Various stages of the bacterial biofilm formation

From the literature available (Donlan 2002; Hall-Stoodley et al. 2004; Parsek and Fuqua 2004; Lindsay and von Holy 2006; Marić and Vraneš 2007; Clontz 2008; Annous et al. 2009; Gautam et al. 2013), it is commonly established that biofilm growth occurs in the following phases.

a. Surface conditioning: Conditioning the surface takes place by adsorption of inorganic and organic nutrients (Lindsay and von Holy 2006).

b. Reversible attachment of the microbes: Reversible adhesion occurs with the help of various elements including Brownian motion of microbial cells, convection currents within the water which transports bacteria to the surface, microbial appendages like flagella which promote motility and interactions like Van der Waals, physical and electrostatic connections between bacterial cell surface and substratum (Lindsay and von Holy 2006; Marić and Vraneš 2007; Annous et al. 2009; Gautam et al. 2013).
c. Irreversible attachment of the microbes: As time passes, the reversibly attached microbes produces extracellular polymeric substances (EPS) that binds the microbes to the surface, and make channels and bridges in between, which in turn results in the permanent attachment (Lindsay and von Holy 2006; Marić and Vraneš 2007; Annous et al. 2009; Gautam et al. 2013).

d. Cell division and surface colonization: Micro colonies which are considered to be the basic structural units of a biofilm are formed by attached bacteria by continuous growth and division. Planktonic cells also the join EPS, resulting in the formation of a biofilm. Biofilm maturation occurs thereby forming three dimensional structures with networks and channels to transport nutrients and water, along with the removal of wastes. The finalized biofilm has a complex design, consisting of biofilm bacteria in EPS enclosed micro colonies intermingled with less compact sections of the matrix that contain highly absorptive water channels transporting nutrients and waste products (Lindsay and von Holy 2006; Marić and Vraneš 2007; Annous et al. 2009; Gautam et al. 2013).

e. Dispersion of cells: When the concentration of the cell increases or maximizes, dispersion occurs in the peripheral cells to the surroundings by shedding (separation of daughter cells from matured biofilm), detachment or dispersion (shearing of top layers) by physical forces like sloughing (rapid removal of large portion of cell mass when there is nutrient depletion) and abrasion (when high number of particulate matter is present). Detachment is the result of cell signalling (quorum sensing) when there is high cell concentrations followed by stationary phase. When death phase comes, this is marked by the breakdown of exo poly
saccharides that hold the matrix. Dispersed cells colonize new surfaces maintaining their biofilm phenotype including resistance to antimicrobials, thereby posing high risk to product contamination (Donlan 2002; Hall-Stoodley et al. 2004; Parsek and Fuqua 2004; Lindsay and von Holy 2006; Marić and Vraneš 2007; Clontz 2008; Annous et al. 2009; Chaves Simões and Simões 2013; Gautam et al. 2013).

Figure 1.1 Stages of biofilm formation
Adapted from Kim et al. (2012)

1.5. How do these bacteria communicate in the biofilm?

Bacteria have developed the skill to direct the behavior by using various modes of communication. Numerous bacteria use a key mechanism called quorum sensing, where bacteria communicate each other via releasing, sensing and thereby responding to minute diffusable signal particles (Li and Tian 2012). Production of small biochemical signal molecules by the
bacterial cell, releasing the same to the adjacent environment and recognition
of the signal particles by specific receptors after exceeding a threshold
concentration and finally the resultant changes in gene regulation are the
basic steps in quorum sensing (Sifri 2008).

Quorum sensing, which is considered as the best communication
mechanisms in bacteria is based on the production of low molecular mass
signalling molecules. The local environment created by the large cell
densities existing in biofilms is suitable for cell density dependent bacterial
communication (Van Houdt and Michiels 2010). The signalling molecules
can be diverse in nature. N-acyl-homoserine lactones are the signalling
molecules used by Gram-negative bacteria while amino acids and short post-
translationally processed peptides are used by Gram-positive bacteria
(Sturme et al. 2002; Lazdunski et al. 2004). Other identified bacterial
signalling molecule for both Gram-negative and Gram-positive bacteria is
Auto inducer- 2 (Schauder and Bassler 2001; Xavier and Bassler 2003; Van
Houdt and Michiels 2010).

According to (Moons et al. 2006), the biofilm development, virulence
and the antimicrobial compounds production and secondary metabolite
formations are all controlled by the above said communication systems. A
study (Nadell and Bassler 2011) reported quorum sensing being used by
bacterial populations to control biofilm formation, which helps to access
nutrients and thereby enabling the members of the group to outcompete non-
biofilm producing ones. The food spoilage capability, stress survival,
boluminescence, competence, antimicrobial peptide synthesis, symbiosis,
sporulation, conjugative plasmid transfer, surface appendages formation and
motility are other factors that can be controlled by this quorum sensing (Van
Houdt et al. 2006; Moons et al. 2006; Van Houdt et al. 2007a; Van Houdt et al. 2007b; Ammor et al. 2008; Wevers et al. 2009; Annous et al. 2009; Van Houdt and Michiels 2010; Rutherford and Bassler 2012).

Additional investigation is required to apprehend the role of quorum sensing in influencing the virulence and antimicrobial resistance of biofilm communities (Annous et al. 2009).

1.6. **The advantages of biofilm life style, which makes the bacteria strong**

Discovery of 3.2 to 3.4 billion years old biofilms is an evidence that biofilm existed since pre historic times (Hall-Stoodley et al. 2004). There are innumerable benefits for a bacterium in a biofilm when compared to their counterparts in water (Lindsay and von Holy 2006; Paraje 2011), which warns us to be cautious. Some of them are described below:

a. Protection of the cells from antimicrobial agents by EPS: Long before in 1981, this was proposed by the father of Biofilmology - J W Costerton. It can be by various ways like creating a diffusion barrier, neutralizing antimicrobial or by chemical reactions (Costerton et al. 1981).

b. Great resistance to metal toxicity, ultra violet radiations, desiccation, harsh environmental conditions like unstable temperature, pH, starved conditions and phagocytosis (Hall-Stoodley et al. 2004; Hall-Stoodley and Stoodley 2005; Lindsay and von Holy 2006).

c. Exhibition of the resistant biofilm phenotype tolerant to antimicrobials, once microorganisms attach to a surface (Lindsay and von Holy 2006; Percival et al. 2011)
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1.7. The role of extracellular polysaccharides in a biofilm

EPS, the major component of a biofilm matrix, is composed of diverse biopolymers, including polysaccharide, protein and extracellular DNA (e DNA) in which the microbial matrix is attached together like an adhesive. A single bacterium produces numerous types of biofilm matrix components at different conditions. Exopolysaccharides play important roles...
in maintaining the biofilm structure and activity (Sutherland 2001a; Flemming et al. 2007; Meliani and Bensoltane 2015) including the transport of chemicals and ions. The EPS matrix effectively blocks permeation of antibiotics and antimicrobial agents (Meliani and Bensoltane 2015). This polymeric matrix normally comprises of up to 97% water, 2–5% microbial cells, 3–6% EPS and ions (Sutherland 2001a; Clontz 2008; Percival et al. 2011; Meliani and Bensoltane 2015). Most of the total organic carbon (TOC) of biofilm is contributed by the matrix which is primarily composed of extracellular proteins and enzymes, DNA and RNA, which is less than 2% of the total matrix while alginate, cellulose and poly-N-acetyl glucosamine are the commonly found exopolysaccharides (Flemming et al. 2007; Meliani and Bensoltane 2015).

Figure 1.2 Bacteria surrounded by the EPS
Adapted from Dreeszen (2003)
1.8. Bacterial pathogens in drinking water associated with biofilms

Waterborne disease is not a myth. Most of the bacterial pathogens are transmitted by water and food, many of which are discharged from the excreta of infected humans and other animals. Nevertheless some opportunistic bacteria which is naturally present in the environment enter the host by inhalation or direct contact and cause episodes of infection. Presented below are the details of some commonly isolated obligate, opportunistic and emergent strains in drinking water and their documented reports on biofilm association.

a) *Salmonella* spp.

The most frequently isolated serovar from humans is *Salmonella enterica* subsp. *enterica* serovar Enteritidis. Omnipresent *Salmonella* serovars such as Typhimurium frequently causes foodborne *Salmonella* gastroenteritis. Typhoid and paratyphoid fever and gastroenteritis are the two types of salmonellosis that are pathogenic that can cause clinical symptoms, even with very low infectious doses of less than 1000 cells (Cabral 2010).

The more the hygienic conditions, the less the incidence of typhoid fever since the probability of fecal contamination of water and food remains low. Intestinal tract of humans and animals being the main habitat of *Salmonella*, they reach the environment through the faeces. Though they can persist numerous weeks in water and in soil under favorable temperature, humidity and pH they cannot multiply in natural environment (Arvanitidou et al. 2005; WHO 2008). Non-typhi or paratyphi serovars constitute those from environmental sources while *Salmonella enterica* subsp. *enterica* serovars Anatum, Enteritidis and Corvallis are the common ones sequestered from
foodstuff (Aissa et al. 2007). *Salmonellae* infected people can be a vehicle carrying the bacterium in gut, for a long period even without any symptoms of the sickness, thereby constituting a major reservoir of the pathogen in the environment. Uncooked sea food also contributes to the sickness (Popoff and Le Minor 2005; Cabral 2010).

These facultative anaerobic Gram-negative rods are well documented for their biofilm production (Niemira and Solomon 2005; Solomon et al. 2005; Ngwai et al. 2006; Giaouris and Nychas 2006; Kim and Wei 2007; Annous et al. 2009; Giaouris et al. 2012; Corcoran 2013) in coupons made of stainless steel at the air liquid interfaces, poly vinyl chloride and polystyrene surfaces, glass slides, food contact surfaces and food environments.

**b) *Escherichia coli***

Infantile gastroenteritis, which commonly occurs in developing nations, is caused by Enterotoxigenic *Escherichia coli* (ETEC) serotypes. Consumption of polluted food or water contributes to “traveller’s diarrhoea” which is caused by ETEC strains (Bettelheim 2003; Scheutz and Strockbine 2005). Enterohemorrhagic *Escherichia coli* (EHEC) strains, which causes acute renal failure, are transmitted through parboiled meat and milk (Bettelheim 2003). Faecally contaminated fruits, vegetables and water has also been documented to be involved in disease outbreaks (Caprioli et al. 2005). Enteroinvasive *Escherichia coli* (EIEC) strains epidemics have been connected with hamburger meat and unpasteurized milk (Bettelheim 2003; Scheutz and Strockbine 2005). *Escherichia coli* (*E. coli*) was reported to be the best indicator for water quality comparing with others (Edberg et al. 2000).
The production of biofilm by *E. coli* is well documented by various researchers (Faille *et al.* 2002; Ryu and Beuchat 2005; Hancock and Klemm 2007; Movassagh and Karami 2010; Van Houdt and Michiels 2010; Sara *et al.* 2011; Wang *et al.* 2012; Nakao *et al.* 2012; Adetunji *et al.* 2014). The above cited literature vividly reported on various types of biofilm formation by *E. coli*, including food contact glass surfaces, medical related catheter associated biofilms, food environment and food industry related, biofilms formed in the bladder epithelium causing urinary tract infections, poly styrene plates and stainless steels.

e) *Vibrio spp.*

*Vibrio’s* capable of causing diarrhoea and other gastro intestinal infections are *Vibrio parahaemolyticus, Vibrio cholerae, Vibrio fluvialis, Vibrio hollisae, Vibrio mimicus* and *Vibrio furnissii* (Sack *et al.* 2004; Farmer *et al.* 2005; WHO 2008; Cabral 2010). The biofilm formation in *V. cholerae* is much studied since it occupies most of its life in the water environment, outside the host (Teschler *et al.* 2015). The *V. cholerae* associated with phytoplankton is capable to produce biofilm in the water environment on abiotic surfaces like plexi-glass discs; and thus acts as microenvironment (Islam *et al.* 2007). A results of a study (Faruque *et al.* 2006) demonstrated that, accidental consumption of metabolically dormant *V. cholerae* cells initiate biofilm formation inside which agitates and become highly infectious when excreted and thereby persisting in the environment. There are also other well documented studies on the *V. cholerae* biofilm formation including the pathogens ability to persist and proliferate in street vended food (Pallaval *et al.* 2014). Clinically relevant *V. cholerae* are likely to ingested by humans (Hall-Stoodley and Stoodley 2005; Seper *et al.* 2014), in infective doses that
b) **Shigella spp.**

Improper hygiene and sanitation accounts to *Shigella dysenteriae* serotype 1 risk while *Shigella flexneri* is more connected to the environment. There are 163.2 million cases of *Shigella* episodes in developing countries every year and 61% of child death accounts to shigellosis (Emch et al. 2008). *Shigella dysenteriae* serotype 1 is predominant in India, Malaysia and Guatemala; whereas western nations has the dominance of *Shigella sonnei* (Emch et al. 2008). Several food borne outbreaks caused by *Shigella* were reported earlier (Agle and Blaschek 2006). Literature regarding the biofilm formation of *Shigella* spp. suggests stress related triggers as responsible factors (Agle and Blaschek 2006; Xu et al. 2010).

c) **Helicobacter pylori**

This Gram-negative, helical, micro aerophilic, *Helicobacter pylori* (*H. pylori*) is associated with peptic ulcer and gastric carcinoma. This etiologic microbe for gastritis quickly loses cultivability, entering a viable but non-culturable (VBNC) state. But Polymerase chain reaction successfully detected the bacterium in drinking water distribution system associated biofilms (Hulten et al. 1996; Giao et al. 2008; Annous et al. 2009; Cabral 2010). Though water and food transmission is of minor significance as the transmission routes of *H. pylori* are yet to be confirmed, once entered this bacteria survives in a biofilm for more than a month (Giao et al. 2008).

d) **Aeromonas hydrophila**

*Aeromonas hydrophila*, (*A. hydrophila*) frequently occurring in water, food and other aquatic environments, is a potential gastroenteritis agent
causing septicemia, meningitis, and wound infections. It is substantially involved in intestinal complaints in young children, the elderly, and the immunocompromised people (Handfield et al. 1996; Daskalov 2006; Cabral 2010). Many reports associated with its biofilm in drinking water distribution systems are reported (Chauret et al. 2001; Mulamattathil et al. 2014). Resistance to chlorine in \textit{A. hydrophila} aids its survival in biofilms (Fernández et al. 2000; Daskalov 2006; Cabral 2010). Natural mineral water also contributes to a possible source of contamination for humans (Fernández et al. 2000; Daskalov 2006; Cabral 2010).

\textbf{g) Mycobacterium avium complex}

The \textit{Mycobacterium avium} complex (MAC) is well-thought-out to be opportunistic human pathogens since it affects the immunocompromised individuals like HIV carriers. Being chlorine and other disinfectants resistant, they can survive a wide range of environment including biofilms of distribution systems. Higher densities of biofilm associated MAC creates potentially high exposure (World Health Organization 2004). Apart from the availability of nutrients like phosphorus, the survival of these slow growers in biofilms is much depended on temperature (Torvinen et al. 2007). The presence of non-tuberculous \textit{Mycobacteria} in household water and biofilm samples across the United States have been documented recently (Falkinham III 2011; Falkinham et al. 2015).

\textbf{h) Pseudomonas aeruginosa}

\textit{Pseudomonas aeruginosa} (\textit{P. aeruginosa}) is a serious opportunistic pathogenic bacterium causing acute and chronic infections. They are aerobic and polarly flagellated, Gram-negative rod, belonging to the family \textit{Pseudomonadaceae} (WHO 2008). With its ability to form biofilm, it may be
found in low nutrient or high nutrient environments capable of causing a wide range of infections while being the principal source of infection in immunocompromised individuals. Its existence in drinking water is undoubtedly related more to its capability to colonize biofilms in plumbing fittings (Mena and Gerba 2009). Bressler and team (Bressler et al. 2009) reported drinking water biofilms in distribution systems to be a pool for *P. aeruginosa* which is considered as a source of contamination in water. Numerous epidemics have been recognized to be due to tap water associated *P. aeruginosa* biofilms (Ferroni et al. 1998; Durojaiye et al. 2011; Moore et al. 2015).

**i) Bacillus spp.**

*Bacillus* spp. are Gram-positive, strictly aerobic, or facultative anaerobic encapsulated bacilli (WHO 2008). The members of the *Bacillus* genus cause significant biofilm related food product contamination, especially dairy products (Simões et al. 2010; Oknin et al. 2015). It has been already reported that binary biofilms of dairy associated *Bacillus cereus* DL5, prevalent in food processing industries can survive hygiene regimes and may represent pools of product contamination and consequently spoiling the food (Lindsay et al. 2002). The confirmation of the presence of high number of cytotoxic *Bacillus* spp. in Norwegian surface waters; and the spores isolated from the tap water of most common drinking water in Norway (Østensvik et al. 2004), revealed that filtration and chlorination could not remove the spores of this *Bacillus*, which is of high risk including poisoning. The interest in *Bacillus cereus* is growing in scientific world since this Gram-positive spore producer (closely related to the pathogen *Bacillus anthracis*) is repeatedly recognized as the contributing agent of foodborne diseases (Nicholson et al. 2000). The dormancy and resistance of *Bacillus* to high
temperature and radiations and its importance to food spoilage and disease linkage is well documented (Nicholson et al. 2000; Sanchez-Salas et al. 2011).

1.9. Coliforms and its relevance to biofilm in drinking water

Important faecal indicators comprise total coliform (TC), faecal coliform (FC), *E. coli*, and faecal streptococci (FS). Since non-faecal coliform representatives may indicate faecal pollution, *E. coli* are the ideal parameter for monitoring the quality of water. But the presence of coliforms that are of non faecal origin can be taken as an indicator of treatment deficiencies; moreover some index organisms can be pathogenic (WHO 2008; Wingender and Flemming 2011). There are many reports regarding the growth of coliforms in biofilm associated water systems. Recently a study conducted in rural coastal Ecuador questioned the suitability of present water quality indicators in health research in the settings of tropical developing country (Levy et al. 2012). On the contrary, an investigation (Gruber et al. 2014), reviewed the suitability based on the available literature, concluding that *E. coli* in household drinking water is associated with diarrhea. Since it is not reasonable to analyze the presence of every recognized waterborne pathogen for the quality of drinking water, fecal indicator organisms is used for the purpose (Reynolds et al. 2008; Gruber et al. 2014). Recently the latest edition of WHO recommended *E. coli* and FC as index organisms for the possible occurrence of fecal pollution and waterborne pathogens, as well the treatment efficiency of the system (WHO 2011; Gruber et al. 2014).
A study conducted on drinking water distribution systems (DWDS’s) (Kilb et al. 2003) points out the fact that coliforms which were detected in the drinking water samples were actually harbored by the same species of coliforms attached to the biofilm in rubber coated valves which appeared to be the real culprit and the source of contamination, even when substantial biofilm growth was not observed in pipe surfaces. This finding was supported by research (Wingender and Flemming 2004) confirming biofilms to be a suitable habitat for coliforms in contaminating the drinking water consequently. The conclusions drawn from an experiment (Camper et al. 1991) revealed that coliforms in the environment mainly *Klebsiella pneumoniae*, *E. coli*, *Enterobacter aerogenes*, and *Enterobacter cloacae*, has more potential to grow in biofilms of DWDS’s, when compared with clinical isolates, even at low concentration of nutrients, which indicate a comfortable life style of coliforms in household and municipal drinking water systems. Transitory establishment of the drinking water system by artificially injected *E. coli* strains fundamentally occupied with an autochthonous population was revealed previously (Fass et al. 1996). Persistence of exceeded coliform levels than those set by the Environmental Protection Agency (EPA) for drinking water in the drinking water pools of a National Park in New Mexico, in the presence of biofilms was reported by a researcher (Hunter et al. 2004). The biofilms of *E. coli* (Williams and Braun-Howland 2003) isolated from a model drinking water distribution system (DWDS) exhibited not only disinfectant resistance but also high metabolic activity showing much persistence in the system.
1.10. Impacts of the bacterial biofilm in drinking water, food and medical environments

The potential to act as a persistent source of microbial contamination in water and food environments and disease transmitting gives biofilm special importance among the food microbiologists and other health professionals. According to a researcher, biofilms are underestimated and lesser known hidden reservoirs of infection since they conceal pathogens that have entered into VBNC (Bloomfield et al. 2015).

Water distribution systems can be totally affected by microbially influenced corrosion caused by biofilms (Farkas et al. 2012). The establishment of biofilms in DWDS’s has been noticed even in nations with pioneering water treatment and health care conveniences (Kilb et al. 2003). Potential pathogens may enter water treatment or distribution systems by evading prevention methods and form colonies, leading to biofilm formation and adhesion to surfaces. Further they magnify in number by microbial regrowth and aftergrowth processes (Van der Kooij 2003; Simões and Simões 2013). Legionella pneumophila, Mycobacterium spp., P. aeruginosa, Klebsiella and coliforms have been reported in biofilms related to distribution systems (Ashbolt 2004; Karanis et al. 2007; September et al. 2007; WHO 2011; Simões and Simões 2013). This type of accumulation pose a great risk, as the biofilm can act as pathogen reserve which can deteriorate the quality of the water distributed at a later time (Wingender and Flemming 2011). Contrastingly, a literature (Srinivasan 2008) stated that in some cases, bulk bacteria may dominate the attached counter parts in distribution systems, when the residual chlorine is lower than 0.1mg/L for more than 12 hours. More studies in this area are required to specifically explain this
phenomenon. Biofilms dynamically respond to the surface they are attached to. These type of microfouling by biofilms can cause serious damage to water distribution systems as well as the quality of the water distributed (Coetser and Cloete 2005; Farkas et al. 2012). Exotoxins such as cytolytic enterotoxin, haemolysin/aerolysin, lipase, protease and other cell-associated factors links to the pathogenicity of *Aeromonas* (Yogananth et al. 2009; Pablos et al. 2009; Mulamattathil et al. 2014). Detection of virulent genes *exoA*, *exoT* and *hylH* in *Pseudomonas* and *Aeromonas* spp. in raw and drinking water biofilm samples in a recent work (Mulamattathil et al. 2014) is another example for the cause of concern.

In medical field and hospitals, several diseases linked to biofilm associated microbes are reported nowadays (Rao et al. 2005; Kokare et al. 2009) like native valve endocarditis, otitis media (a chronic ear infection), chronic bacterial prostititis, cystic fibrosis, periodontitis etc.. Considering the wide role of biofilm in the spread and persistence of human infection, bacterial biofilms has huge importance in the present day research. The change of opportunistic strains to virulent form of the bacteria is also reported (Huq et al. 2008; Hänsch 2012). The biofilm of cholera germs are well protected from the gastric juices, pH and antimicrobials in the gastro intestinal tract (Huq et al. 2008). Medical devices like intra uterine devices and catheters also gets microbially polluted (Kokare et al. 2009). According to some researchers (Kokare et al. 2009; Zubair et al. 2014), *Klebsiella*, *P. aeruginosa*, *Staphylococcus aureus* etc. are few among the biofilm associated strains related to medical device inhibitors (Zubair et al. 2014). Medical relevance of bacterial biofilms are widely documented across the world (Costerton et al. 1981; Ferroni et al. 1998; Checkley et al. 2000; Keren et al.)
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Contamination of food products due to food spoilage by pathogenic bacteria in biofilms, usually lower the shelf life period of the product and results in diseases (Lindsay and von Holy 2006). (Corcoran et al. 2014) reported that it was ineffective to decontaminate an initial (48 h) and comparatively matured (168 h) Salmonella biofilm even at the 90-min contact time, using sodium hypochlorite, sodium hydroxide, and benzalkonium chloride; which implies a high risk of salmonellosis, the second most common cause of foodborne illness worldwide. In actual food processing environments, biofilm populations could be occupied by diverse species of bacterial neighbors that are closely connected (Carpentier and Chassaing 2004; Sanders et al. 2007; Giaouris et al. 2014). Numerous research documented the capability of food borne pathogens to produce biofilms (Lindsay and von Holy 2006; Ammor et al. 2008; Shi and Zhu 2009; Elias and Banin 2012; Giaouris et al. 2012; Giaouris et al. 2014; Meliani and Bensoltane 2015).

1.11. Packaged drinking water and heterotrophic plate count

People prefer packaged drinking water (PDW) due to the concerns raised on the microbiological quality of tap water. There is a strong misconception on the fact about taste and safe microbial level in PDW. There are numerous literature from various parts of the world on the quality of bottled drinking water (Jeena et al. 2006; Ajayi et al. 2008; Zeenat et al. 2009; Duranceau et al. 2012; Akpoborie and Ehwarimo 2012; Venkatesan et
General introduction, review and organization of the thesis

al. 2014; Igbeneghu and Lamikanra 2014; Igbeneghu and Lamikanra 2014). Unless the bottle is sterile after the water being bottled, chances of quantitative changes in microbial population are high. Even though the organic content of the water is low, biofilm formation is a strategy under starved conditions (Prakash et al. 2003; Rosenberg 2003). All the types of containers regardless of material is proven to act as substrate for the formation of biofilms, whether it is plastic or glass (Ryu and Beuchat 2005; Jyothy et al. 2014). The nutrient release from the plastic bottles was reported to be the one among the source of nutrients to the bacterial folks (Schmid et al. 2008; Coelho et al. 2011). The expiration date placed by the bottlers is normally beyond six months, which makes no sense when the biofilm formation and bacterial multiplication is taken into consideration. However, at present, there are no reports available on the survey of biofilm associated bacteria (BAB) attached to the surfaces of bottled water in the markets of any region.

The comprehensive word “heterotrophic bacteria” comprises all bacteria that utilize organic nutrients for growth. This includes all the bacteria in the environment, water and air from normal to pathogenic. But “Heterotrophic Plate Count” (HPC) bacteria signify the bacteria isolated on an agar based medium under defined incubation temperature and time. (Allen et al. 2004). Bacteria isolated through HPC methods mostly consist of a fraction of the total population. However its presence indicates the efficiency of water treatment procedures, thus ultimately signifying and ensuring the pathogen removal (Bartram et al. 2003; Allen et al. 2004). Throughout the years many methods including pour plate, spread plate, membrane filtration method, the Live/Dead Bac Light Bacterial Viability Kit test, 5-cyano-2, 3-ditotyl tetrazolium chloride (CTC) and impedance methods to enumerate the
HPC (Reasoner and Geldreich 1985; Ramalho et al. 2001; Reasoner 2004; Harley and Prescott 2007). Earlier, it was proved that (Ramalho et al. 2001) HPC method was capable of detecting the large percentage of viable bacteria and there was good correlations with Bac Light and CTC method used. Another work (Lepeuple et al. 2004) also reported that the results yield by rapid method for the detection of Total Viable Count (TVC) using direct fluorescent labeling was equivalent to those of R2A method (Reasoner and Geldreich 1985), regardless of the fact that CTC and TVC methods require short time frame when compared to R2A method which require an incubation time of a week or more.

Few literature addressed the potential pathogenecity of HPC bacteria from treated drinking water (Edberg et al. 1996; Pavlov et al. 2004). However, there are literally no works available on a quantitative and qualitative analysis, neither of biofilm associated bacterial counts nor its virulence in the bottled water marketed globally.

1.12. Research gaps and conclusion

Since there is no advanced technology to completely eliminate the biofilms in drinking water systems, avoiding initial contamination and replacing the storage systems and pipes are the strategies for control. Preventing the development of biofilms is the rational approach than treating them, since the present technology is not accessible to completely control it; even hot water sanitation is not sufficient to eliminate the biofilms of P. aeruginosa and Pseudomonas stutzeri (Clontz 2008; Kiskó et al. 2011). According to few reports (Dreeszen 2003; Clontz 2008) physical methods
General introduction, review and organization of the thesis

like heat sanitizing; mechanical methods like scraping, scrubbing and high pressure spraying; chemical methods like sanitizing with oxidizing agents (chlorine, chlorine dioxide, ozone and hydrogen peroxide) and non-oxidizing biocides (quaternary ammonium compounds and formaldehyde) could be utilized in the case of storage vessels and tanks to control biofilms. But these methods cannot be fully and safely utilized in ready to drink PDW, which is of high risk to the consumer. This area remains untouched, and the gap needs to be mended at the earliest.

Biofilm dispersal in drinking water sources is an important factor, which threatens the consumers risking an encounter with BAB into their system. Microbial contamination of drinking water is well addressed in several platforms, but the potential characteristics and threats hidden with the pathogens encapsulated under the protection of biofilms were not addressed widely. Despite the immense publications of biofilm lifestyles and advantages to the bacteria, no work has reported a technology to tackle it effectively. In the current scenario, it is high time to conduct more research in this area, particularly concentrating on bottled water microcosms, since this is the only commodity which is being consumed without boiling or any kind of other treatment. The PDW is considered to be a boon to the travellers and tourists across the world, but the microbiological quality reported on this commodity is completely restricted to the planktonic folks floating in the water, while the main culprit is left behind, which is definitely a threat to the public health.
1.13. Significance of the study

The relevance of bacterial biofilms in medicine, environment and public hygiene has gained attention over the past two decades. But the significance of the present work lies in the fact that the risk associated with PDW biofilms has not gained enough attention in many parts of the world especially in India and other developing nations. Since 95% of bacteria in drinking water system are located at the surface, the indicators and pathogens detected from the tested water samples is from the remaining 5% of the bacteria found in the water phase during sampling. And here is where the risk resides when this attached film protects the inhabitants from disinfectants and from being noticed, which accounts to the periodical shedding of the microbes to the system. This can be more serious when it comes to PDW, which is a ready to drink commodity, and when the public believes it to be of superior quality.

The study attempts to reveal the pathogenic properties of BAB in PDW which is a public risk. Moreover it also determined the survival of a biofilm associated pathogen and indicator bacteria in the simulated DWDS with and without disinfectant. The research made clear evidence for the risk associated with biofilm mode of life in drinking water environments, especially PDW and distribution systems. The thesis will be an eye opener for the authority to include the detection of BAB in the routine drinking water samples being tested for quality, thus minimizing the disease burden of the human population from stored drinking water.
1.14. Objectives of the study:

Based on the light of the existing knowledge, the present work has mainly five specific objectives:

1. To analyze the occurrence and extended survival of BAB in different types of drinking water microcosms under prolonged storage conditions. The objective was mainly set to select the high risk category of drinking water available to the public.

2. To investigate the bacteriological quality of PDW commercialized in the markets of Kerala, with special reference to bacterial biofilm.

3. Testing the potential pathogenicity/virulence of BAB isolated from the PDW microcosms and thus to screen out the bacteria with pathogenic properties.

4. Analysis of biofilm production and quantification of the BAB with pathogenic features and the molecular identification of the screened bacteria with features of virulence.

5. To evaluate the survival of *Salmonella enterica* (*S. enterica*) and *E. coli* in the bulk water and biofilms developed in model DWDS’s with and without chlorination.

1.15. Road map to the thesis

The structure of this thesis broadly corresponds to the chronological order that was adopted throughout the research, which also relates to the objectives listed previously.

- **Chapter 1** deals with general introduction, review and organization of thesis.
Chapter 1

- **Chapter 2** focuses on the occurrence, extended survival and risk assessment of health significant BAB in different types of drinking water microcosms under prolonged storage conditions. This was intended to identify the type of drinking water microcosms with high risk, associated with BAB. After the completion of this preliminary chapter, the next three chapters are concentrated on the biofilm aspects of PDW which is found to be the high risk category.

- **Chapter 3** deals with the bacteriological quality monitoring of PDW available in the markets of Kerala, South India. The chapter also attempts to reveal the biofilm associated life in PDW commercialized in the markets of Kerala.

- **Chapter 4** deals with the potential pathogenic features of BAB isolated from PDW samples.

- **Chapter 5** deals with the biofilm production, quantification and molecular identification of selected BAB with virulent properties.

- **Chapter 6** analyses the survival of indicator (*E. coli*: NCBI Accession number- KR935233.1; Gene-info Identifier: 943876036) and pathogenic bacteria (*S. enterica*: NCBI Accession No: KR935232.1; Gene Info Identifier: 943876035) in simulated DWDS’s.

- The thesis concludes with **Chapter 7**, a summary based on the findings of the whole thesis.

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