6.1. Introduction

Water quality deterioration due to the microbial biofilm formation in pipelines is a major issue which is unable to be monitored properly through routine assessment. The ubiquitous biofilms are very common in water distribution pipelines and have attracted the attention of researchers worldwide (Boe-Hansen 2001; Meckes et al. 2007; Srinivasan and Harrington 2007; Srinivasan 2008; Srinivasan et al. 2008; Zhou et al. 2009; Zingg and Pittet 2012; Mulamattathil et al. 2014; Moore et al. 2015). The problem is not only limited to municipal or household systems, but also other environments like hospitals. In the hospital associated drinking water distribution systems (DWDS’s) the knobs of tap can be seriously contaminated due to which many hospitals introduced non touch sensor taps to reduce the risk (Zingg and Pittet 2012). *Legionella* and potentially pathogenic enterobacterial species were reported to be detected from the distribution systems of hospitals (Stojek et al. 2008).
Chapter 6

Survival and Risk Assessment of Biofilm Associated Bacteria in Drinking Water Microcosms and Distribution Systems

DWDS’s are recognized to be a successful haven for many pathogenic bacterial contaminants those exists viable and being protected from the harsh conditions including shear stress, temperature and chlorine. When the biofilm chunks slough off, these pathogens enter the bulk water and there by the intestinal track of the consumers which is of serious risk (Chaves Simões and Simões 2013). Most of the disinfection methods diminish the bacteria in the water phase but unfortunately there is minimal effect when it comes to the biofilm associated bacteria (BAB) due to their profoundly rooted and immersed existence. Moreover the penetration of disinfectants is much lower as the extracellular polymeric substance (EPS) matrix acts as a diffusion barrier lessening their concentration and efficiency (Chaves Simões and Simões 2013).

The attached life in DWDS’s can be contributed to the nutrients present in the water flowing through municipal / house hold system as research revealed that very low levels of organic carbon is sufficient for the heterotrophic bacterial life (Meckes et al. 2007). Some other factors responsible for the biofilm associated colonization of DWDS’s apart from the concentration of nitrogen, phosphorus and carbon, includes the decontamination methods, and the pipe age and materials in the real world (Moore et al. 2015).

Bacterial biofilms in DWDS’s are receiving attention as never before, indicating the increasing awareness of their impact on human health. Both the biofilms and the bulk bacteria in the DWDS’s can be loaded with bacterial pathogens causing the deterioration problems. Most of the drinking water treatment plants routinely analyze the bulk water for bacterial analysis especially the indicator organism, *Escherichia coli*, completely neglecting the
Survival of *Escherichia coli* and *Salmonella enterica* in the biofilms and bulk water of model drinking water distribution systems

Biofilms. The present chapter was proposed considering the fact that pathogens and indicators act differently in a drinking water system. Most of the normal aqueous bacteria are oligotrophic while pathogenic ones are highly nutrient demanding. Possibly if they find shelter in the highly protective cozy biofilms, they can survive for longer periods, which is probably high risk to the public. Inadequate or improper treatment of different types of drinking water like municipal, well and tank were reported to be sources for the outbreak of pathogens like *Salmonella* serovars (Levantesi *et al.* 2012). *Salmonella* was the leading etiological agent capable of causing intestinal diseases, typhoid and paratyphoid fevers across the world (Levantesi *et al.* 2012).

The present study attempts to expand the knowledge of the complex bacterial growth attached to the biofilms of DWDs’s. For this purpose an indicator and pathogenic bacteria were inoculated separately in the laboratory scale model drinking water distribution systems (MDWDS’s). The experiment was run for a period of seven weeks and the biofilm formation was closely monitored through assessing the survival of the bacteria in the system through heterotrophic plate counts (HPC) using selective media.

### 6.2. Specific objectives

1. To determine the biofilm formation capability and survival of *Escherichia coli* isolated from the biofilm of the PDW microcosm in MDWDS’s.
2. To determine the biofilm formation capability and survival of *Salmonella enterica* subspecies enterica isolated from the biofilm of PDW microcosm in MDWDS’s.

*Survival and Risk Assessment of Biofilm Associated Bacteria in Drinking Water Microcosms and Distribution Systems*
3. To analyze the relative survival capabilities of *Escherichia coli* in the biofilms and bulk water.

4. To assess the relative survival capacity of *E. coli* in non-chlorinated and chlorinated conditions.

5. To analyze the relative survival capacity of *S. enterica* in the biofilms and bulk water.

6. To compare the survival of *S. enterica* in non-chlorinated and chlorinated conditions.

7. To compare the survival of *E. coli* and *S. enterica* with respect to the mediums and conditions in the MDWDS’s.

### 6.3. Materials and methods

#### 6.3.1. Experimental design

Five different MDWDS’s were set up using glass tanks, poly vinyl chloride (PVC) pipes and biofilm test plug coupons (Plate VIII to IX). Each model distribution system consisted of identical pipe loops connected in series. The water in the MDWDS’s was continuously circulated for a period of seven weeks, with the help of a motor with a flow rate of maximum 2500 liter per hour. The loops of each tank contained four biofilm test plugs. Each PVC coupon contained a string of seven continuous 1 cm$^2$ surface, attached to a rubber cork. These biofilm test plug coupons were plugged into the pipe loops intermittently inserted in a rubber cork. The biofilm test plugs coupons were made from PVC pipes of an Indian brand. In total, 25 biofilm test plug coupons were inserted tightly in to the pipelines of the five glass tanks of 60L capacity. All the MDWDS’s were filled with 20 liters of autoclaved tap
Survival of *Escherichia coli* and *Salmonella enterica* in the biofilms and bulk water of model drinking water distribution systems

water. The MDWDS’s were thoroughly washed with 90% ethanol before filling the autoclaved tap water. Two tanks were meant to be chlorinated while two were meant to be non-chlorinated and the remaining one was the control tank.

Plate VIII. Model Drinking Water Distribution Systems

*Survival and Risk Assessment of Biofilm Associated Bacteria in Drinking Water Microcosms and Distribution Systems*
Plate IX. Pictures illustrating water flow and biofilm sampling surfaces (PVC coupons)

a: Water circulating with the help of a motor
b and c: Biofilm Sampling surfaces which was inserted to the cork
Initially, the first two MDWDS’s were inoculated with approximately \(10^7-10^8\) cfu/mL cells of \textit{Escherichia coli}. The second and third MDWDS’s were inoculated with approximately \(10^9\) cfu/mL cells of the \textit{Salmonella enterica} sub spp. enterica. The organisms were inoculated after the water was being circulated for 24 hours, confirming them to be leak free. The first sampling was taken after 48 hours for the biofilm to be established. The sampling was continued at every seven days interval for a period of seven weeks. The second and fourth DWDS’s were chlorinated using sodium hypochlorite tablets, after 24 hours of the inoculation. Chlorination was done twice a week and the residual chlorine was measured before every sampling. The fifth circulating tank was used as the control, filled with 20L autoclaved tap water without organisms and without chlorine.

6.3.2. Sequence deposition

The organisms used for the study are \textit{Escherichia coli} (NCBI Accession number- KR935233.1; Gene-info Identifier: 943876036) and \textit{Salmonella enterica} sub spp. enterica (NCBI Accession No: KR935232.1; Gene-Info Identifier: 943876035). The bacteria selected for the study were resistant to more than six antibiotics, producing multiple enzymes and were haemolytic. The 16S rRNA sequences of these potential strains with virulence features screened out in the previous chapter, were submitted to Genbank.

6.3.3. Preparation of the inoculum of the test organisms:

\textit{E. coli} and \textit{S. enterica} were inoculated separately into 10mL sterile nutrient broth in triplicate, and incubated at 37\(^\circ\)C for 18-24 hours. After
incubation the cells were concentrated by centrifugation at 3000 rpm for 15 minutes and washed thrice with sterile isotonic saline. After the final wash the cells were suspended in 10mL sterile isotonic saline. The final suspension of 10mL of both the strains was inoculated into the corresponding glass tanks (chlorinated and non-chlorinated) containing 20mL of the autoclaved tap water. The remaining suspensions were taken for the initial inoculum density analysis. The initial inoculum density was estimated using spread plate technique after serial dilutions in sterile isotonic saline (Harley and Prescott 2007). The initial inoculum density estimated was $10^7 - 10^8$ cfu/mL and $10^9$ cfu/mL respectively for *E. coli* and *S. enterica* respectively.

### 6.3.4. Estimation of free chlorine concentration/residual chlorine concentration measure

Sodium hypochlorite tablets (NICE, India) were used to chlorinate two of the tanks to analyze the survival of the organisms in the presence of chlorine, which is the main disinfectant used in the municipal distribution systems. The residual chlorine was analyzed using a chlorine test kit which contains ampules and ortho-toluene. Residual chlorine was measured as per the DPD method, following the manufacturer’s instructions (Clesceri *et al.* 1998).

### 6.3.5. Sampling of biofilms from the biofilm test plug coupons and bulk water samples

Samples were taken from the biofilm test plug coupons and bulk water at every seven days continuously for a period of seven weeks. The initial enumeration after 48 hours was taken as the count for week one, followed by the sampling at every seven day interval. Every seven days 4
samples from the coupon and bulk water were taken from each tank and the results were presented as mean log cfu/cm$^2$ and mean log cfu/mL for the biofilms and bulk water respectively.

The biofilm in the coupons were sampled by removing the test plugs and subsequently swabbing a one square centimeter surface from the four coupons inserted at different parts of the MDWDS, with a sterile autoclaved cotton bud, after the water flow was temporarily stopped. The bud was then transferred to 20mL test tubes containing 10mL autoclaved distilled water. Immediately after the sampling, the test tubes with samples were vortexed vigorously for a minute to release the bacteria. The bulk bacteria in the water phase were also sampled with sterile pipette tips. Four samples of biofilm and bulk water were taken from each system every seven days for up to a period of seven weeks. Appropriate dilutions were made using sterile isotonic saline and the samples were plated on selective medium by spread plate method and incubated at 37$^\circ$C for 24 hours. The medium used for *E. coli* was Eosine Methylene Blue (EMB) agar while for *S. enterica* it was Xylose Lysine Deoxycholate (XLD) agar.

### 6.3.6. Nutrient analysis

The samples were analyzed for anions such as Fl, Cl, NO$_3$, PO$_4$ and SO$_4$ using Ion Chromatography system (Dionex ICS-1100). The system is equipped with IonPac AG12A Guard Column (4 x 50 mm), IonPac AS12A analytical column (4 x 200 mm), ASRS 300 suppressor and DS6 heated conductivity cell. The eluent used was 2.7mM Na$_2$CO$_3$/0.3mM NaHCO$_3$ at a flow rate of 1mL/min. The samples were introduced into the 25μl sample loop via 1mL syringe. Calibration was carried out daily using a series of
standards prepared from a stock solution. Deionized water with a specific resistance of 18.2 MΩ cm$^{-1}$ was used for the preparation of all reagents and standards.

Total organic carbon (TOC) was analyzed using Thermo Hyper TOC analyzer. Here, total carbon was calculated using UV and high temperature method whereas the TOC was estimated using 10% phosphoric acid, as per the instructions of the manufacturer.

Physical parameters like temperature, pH total dissolved solids (TDS), salinity and conductivity were measured as per the methods of American Public Health Association (Eaton et al. 2005).

6.3.7. Scanning electron microscopic (SEM) observations

SEM images of the detached coupons were taken after the experimental period. Prior to the SEM observations, the coupons detached from the biofilm test plug was washed and dried with ethanol. After sputter coating with gold the surface examination was performed using JEOL/ EO (version 1.0) to study the morphological nature of the biofilm.

6.3.8. Statistical analysis

The data was evaluated with the Statistical Program for Social Sciences (SPSS) version 20. All data were subjected to one-way analysis of variance (ANOVA) and the means were separated using Duncan’s multiple range test (DMRT) when the weekly difference of the biofilm and bulk water samples were done individually for each DWDS. The values bearing the same lower case letters are not significantly different at 5% level. The log transformed cfu values per square centimeter (log cfu/cm$^2$) of BAB entered in
the graph is the average of the four coupons of one square centimeter from the biofilm test plug surfaces from each DWDS. The log transformed cfu values per mL (log cfu/mL) of planktonic bacteria entered in the graph is the average of the four samples from the bulk water in each DWDS. The error bars represent the standard deviation values at 1% level of significance. Data was also subjected to repeated measure ANOVA for the comparison of the media (biofilm and bulk water), conditions (non-chlorinated and chlorinated) and organisms (*E. coli* and *S. enterica*). In all the cases F values are stated. All the statistical calculations were based on a 95% confidence interval.

6.4. Results

6.4.1. Physico-chemical parameters and nutrient analysis

The physico-chemical parameters and nutrients in the bulk water samples were tested before conducting the experiments. All the parameters tested were within the limit (WHO 2011; Bureau of Indian Standards (BIS) 2013). The average temperature of the water was 30.1°C, pH was 8.29, conductivity was 187.9 µS, TDS was 133 ppm and salinity was 88.3 ppm. Fluoride, chloride, nitrate, phosphate, sulfate and TOC were 0.275, 4.441, 0.3305, 0.0, 3.305 and 1.952 in mg/L respectively.

6.4.2. Survival of *E. coli* in the biofilms of non-chlorinated MDWS’s

Discretely presenting the results, Figure 6.1 shows the survival of *E. coli* in the biofilms of non-chlorinated MDWDS. The HPC of biofilm associated *E. coli* in the first week was 6.020 log cfu/cm². Though
significantly superior (p<0.01) and successfully high growth was displayed in
the second week (6.178 log cfu/cm²), the next three weeks witnessed a
reduction in the bacterial count (5.320 log cm²). However the significantly
highest (p<0.01) growth statistically similar to the second week as per
DMRT, was again witnessed in the sixth week with a count of 6.213 log
cfu/cm². It was then followed by a statistically significant reduction (p<0.01)
in the final week (5.155 log cfu/cm²).

F value=842.566**
** The difference in growth is significant at 0.01 level
The error bars represent the standard deviation of three experiments at 1 % level of significance
Means followed by a common letter(s) above the graph are not significantly different at the 5 %
level by DMRT.

Figure 6.1 Survival of *E. coli* in the biofilm formed in non-chlorinated
MDWDS

6.4.3. Survival of *E. coli* in the bulk water of non-chlorinated
MDWS’s

At the same time Figure 6.2 shows the survival of *E. coli* in the bulk
water of the non-chlorinated MDWDS. The count was extremely high and
statistically highest (p<0.01) in the first week with 10.553 log cfu/mL, as per
the DMRT. However, the experiment witnessed a drastic reduction (5.690 log
Survival of *Escherichia coli* and *Salmonella enterica* in the biofilms and bulk water of model drinking water distribution systems

cfu/mL) in the second week. The reduction continued up to week four. Statistically significant increase (p<0.01) was observed in the remaining weeks until it reached 5.603 log cfu/mL in the final week.

![Graph showing survival of *E. coli* in bulk water of non-chlorinated MDWDS](image)

**Figure 6.2 Survival of *E. coli* in bulk water of non-chlorinated MDWDS**

### 6.4.4. Comparison of the growth of *E. coli* in the biofilms and bulk water of the non-chlorinated MDWS’s

The results show that though the count of *E. coli* in the water phase of non-chlorinated system was dangerously extreme in the initial week, which was almost double the count of the attached counterparts, most of them joined the biofilm matrix by the second week (Figure 6.3). This ultimately decreased their number in the bulk water in the following weeks compared to the biofilm. However in the final week, the number in bulk water increased significantly and outweighed the biofilm counterparts, which can be attributed to the slough off phenomena. However, the difference between the
load of *E. coli* in biofilms and bulk water was statistically significant (p<0.01).

**Figure 6.3 Relative survival of *E. coli* in the biofilm and bulk water of non-chlorinated MDWDS**

![Graph showing relative survival of *E. coli* in biofilms and bulk water of non-chlorinated MDWDS](image)

4.00 5.00 6.00 7.00 8.00 9.00 10.00 11.00 12.00
Week 1 Week 2 Week 3 Week 4 Week 5 Week 6 Week 7

**F value = 1080.553**

**The difference in growth is significant at 0.01 level.**

The error bars represent the standard deviation of three experiments at 1 % level of significance

6.4.5. **Survival of *E. coli* in the biofilms of chlorinated MDWS’s**

The survival of *E. coli* in the biofilms of chlorinated DWDS’s is illustrated in **Figure 6.4**. The initial mean load of BAB in the coupons was 3.518 log cfu/cm² which was statistically in par with the final load (p>0.01) in the seventh week. The number steadily increased to a statistically superior (p<0.01) count towards the second week (5.565 log cfu/cm²). Then the count gradually reduced towards the next two weeks, and gradually increased achieving the statistically highest count (p<0.01) in the experimental period (6.410 log cfu/cm²) in week six. But the seventh week witnessed the largest decline to almost half the log which was significantly lower (p<0.01) and in par with the initial count as per the DMRT (3.680 log cfu/cm²).
Survival and Risk Assessment of Biofilm Associated Bacteria in Drinking Water Microcosms and Distribution Systems

6.4.6. Survival of *E. coli* in the bulk water of chlorinated MDWS’s

The survival of *E. coli* in the bulk water of chlorinated MDWS is depicted in Figure 6.5. The initial count after 48 hours of inoculation was 2.278 log cfu/mL. The result revealed that towards the third week the planktonic numbers were increasing significantly (p<0.01) towards 4.825 log cfu/mL as if the bacteria has attained the resistance. Through a significant (p<0.01) drop (4.503 log cfu/mL) in the fourth week, the planktonic counterparts attained its significantly highest peak 5.340 (log cfu/mL) in the fifth week. However, then the count declined and was in par (p>0.01) in the last two weeks (4.608 log cfu/mL). Nevertheless by the end of the experimental period (from third week) the count of floating bacteria in the
chlorinated tank increased exponentially and in the final week it was almost more than double the initial count after 48 hours in the first week.

** F value =1504.954**

** The difference in growth is significant at 0.01 level

The error bars represent the standard deviation of three experiments at 1 % level of significance

Means followed by a common letter(s) above the graph are not significantly different at the 5 % level by DMRT.

** Figure 6.5 Survival of *E. coli* in bulk water of chlorinated MDWDS**

6.4.7. **Comparison of the growth of *E. coli* in the biofilm and bulk water of the chlorinated MDWS’s**

The growth of *E. coli* in the biofilm and bulk water of chlorinated MDWDS is being compared in the **Figure 6.6**. The graph shows that, although the initial bacterial count in the biofilm is almost double the chlorinated tank, by the end of the experiment the value drops steadily. As in the previous cases, here also the decreasing biofilm count and increasing bulk water values in the final period can be attributed to the dispersed biofilm chunks.
Survival of Escherichia coli and Salmonella enterica in the biofilms and bulk water of model drinking water distribution systems

F value = 426.276**
** The difference in growth is significant at 0.01 level.
The error bars represent the standard deviation of three experiments at 1% level of significance

Figure 6.6 Relative survival of E. coli in the biofilm and bulk water of chlorinated MDWDS

6.4.8. Comparison of the growth of E. coli in the biofilms of the non-chlorinated and chlorinated MDWS’s

The comparison of the survival of E. coli in the biofilms of non-chlorinated and chlorinated MDWDS’s depicted in the Figure 6.7 shows that the bacterial count in the biofilms of chlorinated system were significantly lower (p<0.01) than the non-chlorinated one. The study points out that though biofilm protects the E. coli from disinfection stress, the number is almost reduced to half during chlorination. The figure shows that the apart from the fifth week and sixth week where counts in both the system reached their peak, other sampling periods witnessed the highly succeeded growth of bacteria in the attached phase of non-chlorinated system. The most important finding was that, in the fifth and sixth week the biofilm counts in the chlorinated tank outweighed the non-chlorinated MDWDS. However, the general trend remains the same in all the cases, i.e., after a reaching the peak
of the maturation phase, the biofilm sloughs off to the bulk water phase which in turn increases the count of cells in the water phase.

\[ F \text{ value} = 265.458^{**} \]

** The difference in growth is significant at 0.01 level.

The error bars represent the standard deviation of three experiments at 1 % level of significance.

**Figure 6.7** Relative survival of *E. coli* in the biofilms of non-chlorinated and chlorinated MDWDS

### 6.4.9. Comparison of the growth of *E. coli* in the bulk water of the non-chlorinated and chlorinated MDWS’s

The significant difference \((p<0.01)\) in the bacterial counts of *E. coli* in the bulk water of non-chlorinated and chlorinated MDWS’s is illustrated in the **Figure 6.8**. The graph clearly depicts that in the initial weeks of the experiment, the bacterial count in the bulk water phase of the chlorinated tank was almost 5 times lower compared to the non-chlorinated system. But gradually the difference narrowed, towards the increased count of bacteria in the fifth week, compared to the non-chlorinated system. However, the count was significantly less in chlorinated tank except in the fifth week.

It is noteworthy to mention that when there was drastic reduction in the number of *E. coli* cells in the bulk water of non-chlorinated system to
almost half in the second week, the cells in the chlorinated system was gradually adapting to the stress, thereby increasing the counts to double the log by third week. Surprisingly afterwards, the *E. coli* counts not only goes hand in hand with the values in non-chlorinated system, but also exceeds the non-chlorinated system in the fifth week. Though the *E. coli* counts in the chlorinated system reduced in the final weeks, the reduction was never drastic like the initial decline of the non-chlorinated system, instead much balanced and stable, letting the counts of final weeks to be in par, without a steep trough.

\[ F \text{ value}=2347.282^{**} \]

\*\* The difference in growth is significant at 0.01 level.

The error bars represent the standard deviation of three experiments at 1 % level of significance

**Figure 6.8 Relative survival of *E. coli* in the bulk water of non-chlorinated and chlorinated MDWDS**

6.4.10. Survival of *S. enterica* in the biofilms of non-chlorinated MDWS’s

The survival of *S. enterica* in the biofilms of non-chlorinated MDWDS is illustrated on **Figure 6.9**. The biofilm count in the first week, after 48 hours of inoculation was 4.398 log cfu/cm². The second week
displayed significantly highest growth (p<0.01) as per the DMRT (5.488 log cfu/cm\(^2\)). Towards the third week the bacteria showed a steep decline and reduced to 3.428 log cfu/cm\(^2\). However, the fifth week witnessed a peak of the bacterial growth (4.990 log cfu/cm\(^2\)) in the biofilms. This is followed by a steep decline in the survival of the organism to 1.935 log cfu/cm\(^2\) in the final week.

*S. enterica* is a fastidious pathogen capable of causing salmonellosis. The result revealed that though they are highly demanding, it survived well in the biofilms attaining peak growth twice between the study periods. However with a maturation peak of biofilms in the sixth week, the biofilm tend to have dispersed.

**Figure 6.9 Survival of *S. enterica* in the biofilm formed in non-chlorinated MDWDS**

F value =337.247**
** The difference in growth is significant at 0.01 level
The error bars represent the standard deviation of three experiments at 1 % level of significance
Means followed by a common letter(s) above the graph are not significantly different at the 5 % level by DMRT.
6.4.11. Survival of *S. enterica* in the bulk water of non-chlorinated MDWS’s

The condition of *Salmonella enterica* in the bulk water of the non-chlorinated system was much different (Figure 6.10). The first week of the experiment witnessed the significantly highest (p<0.01) bacterial count with a value of 5.548 log cfu/mL. Followed by the initial and successful survival in the first week, the graph depicts a drastic reduction gradually towards the end of the experiment and reduced to 1.965 log cfu/mL. The failure of the pathogen to flourish in the bulk water can be contributed to its fastidious nature.

**Figure 6.10 Survival of *S. enterica* in bulk water of non-chlorinated MDWDS**
6.4.12. Comparison of the survival of *S. enterica* in the biofilms and bulk water of non-chlorinated MDWS’s

The growth of *S. enterica* in biofilm and bulk water of the non-chlorinated MDWDS had been compared in the Figure 6.11. Initially the survival was higher in the bulk water (5.55 log cfu/mL) compared to the biofilm (4.40 log cfu/cm²). But the second week witnessed the gradual but drastic reduction of the organism towards the end of the experiment in a decreasing order. On the other hand as described earlier, biofilm displayed its significantly highest (p<0.01) growth in the second week. Twice in the experimental period did the organism in biofilm exhibited peak growth, though ended up reduced to several logs in the final week similar to the bulk water phase. The growth in the biofilm was statistically superior to the counterparts in water phase. The figure also revealed that in the first week, the bacterial count was higher in the bulk water. Then the trend changed with the decreasing count in water, cells increased in the biofilms. *S. enterica* was more dominant in biofilms and they preferred the biofilm phenotype, gradually migrating. However the final count was almost similar in both the phase, indicating the sensitivity of the pathogen towards oligotrophic conditions.
**Survival of Escherichia coli and Salmonella enterica in the biofilms and bulk water of model drinking water distribution systems**

The survival of *S. enterica* in the biofilms of chlorinated DWDS is illustrated in Figure 6.12. Initially the count was 3.008 log cfu/cm\(^2\). Up to three weeks the growth exhibited an exponential phase. Third and fifth week witnessed the peak counts, though the most significant was shown at week 5 as per the DMRT. However in between these peaks, there was a steep decline which again repeated with an increased magnitude in the final two weeks. The fourth and seventh week exhibited the significantly lowest survival of the organisms according to the DMRT (2.775 log cfu/cm\(^2\) and 2.765 log cfu/cm\(^2\) respectively). However, the fastidious nature of the organism possibly has affected the flourished survival in the biofilms. However, even in the chlorinated system the fastidious pathogen survived in the biofilm attaining...
the peaks twice, during the study period. The *Salmonella* showed an adaptive response towards chlorination.

Figure 6.12 Survival of *S. enterica* in the biofilm formed in chlorinated MDWDS

6.4.14. Survival of *S. enterica* in the bulk water of chlorinated MDWS’s

The survival of *S. enterica* in the bulk water of the chlorinated MDWDS is plotted in Figure 6.13. Initially the count was 1.465 log cfu/mL but gradually survived the disinfectant by attaining a peak growth of 3.003 log cfu/mL in the third week. The most significant growth was attained in the third and fifth week, as per the DMRT. However, here also a steep decline was observed between the peaks in the fourth week with 2.425 log cfu/mL. After the peak growth at week 5, suddenly the bacteria seized its growth, and failed to survive any longer in water, with absolutely no trace of the bacteria in bulk water.
Survival of *Escherichia coli* and *Salmonella enterica* in the biofilms and bulk water of model drinking water distribution systems

6.4.15. **Comparison of the survival of *S. enterica* in the biofilms and bulk water of chlorinated MDWS’s**

Interestingly the survival of *S. enterica* in bulk water followed the same pattern with that of biofilm, though significantly lower (p<0.01) in the counts (Figure 6.14). In both the biofilm and bulk water phase, peaks were observed in the third and fifth weeks. However the study revealed that nutrient limitation and stress induced by chlorination limits the longer survival of *S. enterica* in the water phase. It implicates the nature of this strict biofilm producer to transform into the biofilm phenotype, migrating completely without leaving a trace in the water phase. Nevertheless biofilm is an important threat which protects and keep the bacteria vital which poses a serious threat to the consumers.
**The difference in growth is significant at 0.01 level.** The error bars represent the standard deviation of three experiments at 1% level of significance.

**Figure 6.14 Relative survival of *S. enterica* in the biofilm and bulk water of chlorinated MDWDS**

### 6.4.16. Comparison of the survival of *S. enterica* in the biofilms of non-chlorinated and chlorinated MDWS’s

The comparison of *S. enterica* in the biofilms of chlorinated and non-chlorinated system is illustrated in **Figure 6.15**. The statistical difference was significant at 1% level. Though the bacterial count was higher in the non-chlorinated tank for the first two weeks, the count in the chlorinated tank dominated in the last two weeks, outweighing the other. While the final count of biofilm associated bacteria in the non-chlorinated system was 1.94 log cfu/cm², it was 2.77 log cfu/cm² in the chlorinated MDWDS, which was almost double. The study clearly confirmed the high survival rate of biofilm associated bacterial pathogen in a chlorinated tank than the non-chlorinated system.
Survival of *Escherichia coli* and *Salmonella enterica* in the biofilms and bulk water of model drinking water distribution systems

6.4.17. Comparison of the survival of *S. enterica* in the bulk water of non-chlorinated and chlorinated MDWS’s

There was significant difference (p<0.01) between the number of *S. enterica* in the bulk water of non-chlorinated and chlorinated MDWS’s (Figure 6.16). Although the survival graph showed a decreasing trend towards the end of the experiment in the non-chlorinated system, it never exhibited a complete absence of the pathogen unlike chlorinated system though at times the counts crossed the other. Chlorination induced stress together with the nutrient limitation eradicated the pathogen from the water phase of the chlorinated MDWDS. Here also chlorine induced high biofilm migration due to the stress of chlorine in the bulk water thereby leaving no trace, as biofilms are well known survival strategy for these pathogenic bacteria.
**The difference in growth is significant at 0.01 level.
The error bars represent the standard deviation of three experiments at 1 % level of significance

**Figure 6.16 Relative survival of *S. enterica* in the bulk water of non-
chlorinated and chlorinated MDWDS**

### 6.4.18. Comparison of the survival of the *E. coli* and *S. enterica* in the MDWS’s

The two organisms used in the experiment, *E. coli* and *S. enterica* are being compared from **Figure 6.17 - 6.20**. In all the conditions (non-chlorinated and chlorinated) and mediums (biofilm and bulk water), the survival of *E. coli* is found to be significantly higher (p<0.01) than the *S. enterica*. *E. coli* attained high resistance to chlorine in the study period. *E. coli* thrived well in all the MDWDS’s while in the water phase of chlorinated system, *S. enterica* seemed to be completely eradicated. Comparing to the survival of *E. coli*, the growth was much lower, especially in the final days which can be attributed to the nutrient limitation in the drinking water environment. Unlike *E. coli* which adapted to chlorine stress and thrived well in the water phase, *S. enterica* migrated to biofilm niche completely leaving no single cell in the bulk water phase.
Survival of *Escherichia coli* and *Salmonella enterica* in the biofilms and bulk water of model drinking water distribution systems

**Survival and Risk Assessment of Biofilm Associated Bacteria in Drinking Water Microcosms and Distribution Systems**

**Figure 6.17** Relative survival of *E. coli* and *S. enterica* in the biofilms formed in the non-chlorinated MDWDS

**Figure 6.18** Relative survival of *E. coli* and *S. enterica* in the bulk water of non-chlorinated MDWDS

F value = 282.996**
** The difference in growth is significant at 0.01 level.
The error bars represent the standard deviation of three experiments at 1% level of significance

F value = 337.344**
** The difference in growth is significant at 0.01 level.
The error bars represent the standard deviation of three experiments at 1% level of significance
**F value = 129.921**
**The difference in growth is significant at 0.01 level.
The error bars represent the standard deviation of three experiments at 1% level of significance**

**Figure 6.19** Relative survival of *E. coli* and *S. enterica* in the biofilms formed in chlorinated MDWDS

**F value = 1248.050**
**The difference in growth is significant at 0.01 level.
The error bars represent the standard deviation of three experiments at 1% level of significance**

**Figure 6.20** Relative survival of *E. coli* and *S. enterica* in the bulk water of chlorinated MDWDS

---

*Survival and Risk Assessment of Biofilm Associated Bacteria in Drinking Water Microcosms and Distribution Systems*
6.4.19. Concentration of residual chlorine in the distribution systems

The residual chlorine was always above 0.4 ppm. In the initial week it ranged from 1.6 to 1.8 ppm, in both the systems. However, afterwards, free chlorine was detected between 1.2 to 0.8 ppm in the second and third weeks, followed by 0.8 to 0.4 ppm towards the remaining weeks.

6.4.20. Scanning electron microscopic observations

The SEM images, showing the growth of *E. coli* and *S. enterica* are given in Plate X - XIII. The micrographs clearly depict the organisms attached in the surface of the coupons. The SEM images reveal that biofilms are comparatively more organized in the chlorinated systems.
a: Biofilms of *E. coli* in the coupons of chlorinated DWDS  
b: Biofilms of *E. coli* in the coupons of chlorinated DWDS  

Plate X. SEM observations
Survival and Risk Assessment of Biofilm Associated Bacteria in Drinking Water Microcosms and Distribution Systems

Plate XI. SEM observations

a: Biofilms of *E. coli* in the coupons of non-chlorinated DWDS
b: Biofilms of *E. coli* in the coupons of non-chlorinated DWDS
Plate XII. SEM observations

a: Biofilms of *S. enterica* in the coupons of chlorinated DWDS
b: Biofilms of *S. enterica* in the coupons of chlorinated DWDS
Survival of Escherichia coli and Salmonella enterica in the biofilms and bulk water of model drinking water distribution systems

Plate XIII. SEM observations

- a: Biofilms of S. enterica in the coupons of non-chlorinated DWDS
- b: Biofilms of S. enterica in the coupons of non-chlorinated DWDS
6.5. Discussion

The results of the present study proved that both the indicator and pathogenic bacteria survives in oligotrophic DWDS’s for a long period and attains peak growth at least twice in its lifetime in the system. Biofilms associated with DWDS’s and long been known to be a successful harbor to bacteria. Biofilm formation potential of HPC bacteria in plasticized polyvinyl chloride (PVC), cross-linked polyethylene (PEX) and high density polyethylene (HDPE) pipes have been reported recently (Rożej et al. 2015). There are well documented researches which support the view that water is of inferior quality once it reaches the customers tap (Juhna et al. 2007). *E. coli* is considered to be the best biological indicator for monitoring the drinking water (Edberg et al. 2000), especially today when the outbreaks related to DWDS’s are increasing (Juhna et al. 2007).

The nutrients in the bulk water of the present study were within the limits set by WHO (WHO 2011). However, studies reported the migration of organic compounds like monobutyltin, dibutyltin, tributyltin, lead and volatile organic compounds like xylene, styrene, phenols, and ethylmethylbenzene from plastic pipes (Kowalska et al. 2011). These organic compounds can be utilized by the BAB as a possible source of nutrients to thrive in the oligotrophic conditions of the DWDS’s.

The study proved that *E. coli* survives in the DWDS’s for longer periods and flourishes well under the protection of biofilms. According to a researcher *E. coli* are brought into the DWDS due to failure in the treatment procedure or invasion through pipes (Juhna et al. 2007). However, the results of the present study are in contrary to the aforementioned one, as they reported *E. coli* to be not multiplying in the DWDS, as they never detected
Survival of *Escherichia coli* and *Salmonella enterica* in the biofilms and bulk water of model drinking water distribution systems

micro colonies, other than individual cells in the networks through fluorescence in situ hybridization (FISH) technique.

As mentioned in the results, suddenly after every peak in both the organisms, especially *E. coli*, the biofilm collapses and disperses the chunks to water. This may be due to the loosely structured monoculture biofilms of the organisms. Contrary to the common notion that majority of the bacteria remain in the biofilm which is extra ordinarily strong, the present study observed that the drinking water associated biofilms were delicate and cannot hold much, thereby collapsing easily and dispersing the chunks with bacteria to the bulk water, which adds to the risk. The condition may be different in aged pipes with strong thick films. The results of the present work are in agreement with an investigation on the fate and persistence of non-typhoidal *Salmonella* in laboratory scale DWDS’s (Schaefer *et al.* 2013). The aforementioned study witnessed the loosely structured biofilms of the *S. typhimurium* in monocultures formed. However Schaefer and team confirmed the established colonies of *Salmonella* in the multi species drinking water biofilms, releasing high levels to the bulk water in the system. This is an indication of potential and persistent health risk, since original biofilms in the DWDS’s consist of diverse bacteria, which helps to produce more complex and integrated biofilms.

Hikes in the bulk water of chlorinated tanks of *E. coli* urged the bacteria to migrate to the protective film and thereby expanding the biofilm niche. The study proved that chlorination stress induces more bacteria to join the biofilm matrix resulting in bacterial peaks in the biofilm. Though biofilm protects the *E. coli* cells from disinfection stress, chlorination significantly reduced the count. A research from Singapore (Park and Kim 2008) reported
that the maintenance of very high monochloramine residual as high as 2 mg/L in oligotrophic DWDS’s reduces the metabolic activity of biofilm cells. However we are not in agreement with the abovementioned report since a study from Portugal proved the continuous exposure of bacteria to disinfectants develops more virulent and tolerant strains through adaptive resistance acquisition via phenotypic adaptation (Machado et al. 2012).

The present work is consistent with the results of an investigation (Srinivasan et al. 2008), which revealed that increasing chlorine concentration decreases the bacteria in bulk water as they migrate to the biofilms, and whenever the chlorine residual decreases the bacteria increases in the water phase. The aforementioned study reported that with increased chlorine dosages 70% bacteria migrates to the biofilm.

The result of the present study revealed that even maintaining the disinfectant residual above 0.4 ppm on the chlorinated systems, *E. coli* survived in the system attaining its peak twice, which is a clean sign of the following possibilities: a) inefficiency of the disinfectant to penetrate the biofilm matrix and b) the adaptive response and resistance to chlorination acquired by the *E. coli* as mentioned by a research team (Machado et al. 2012). However, the final week displayed a sudden decline, to almost half the number which can be again attributed to the matured biofilm chunks being dispersed to the water phase. It is evident from the figures that the planktonic count of *E. coli* in the chlorinated tank was almost double in the final period, which can be due to the added, dispersed cell. It is worth mentioning that though the survival of *E. coli* in the bulk water and the biofilms in the chlorinated system, is significantly lower (p<0.01) than the non-chlorinated system, the study revealed that generally chlorination initiated the adaptive
response of \textit{E. coli} and makes it more stable and competent than the counterparts in chlorinated system.

Both the organisms witnessed their highest growth in the initial bulk water phase of the non-chlorinated system for both the organisms. However, \textit{Salmonella} never went beyond 5.5 log cfu in both the mediums and even its highest count was almost half the number of \textit{E. coli} in the same condition. In all the mediums (biofilm and bulk water) and conditions (chlorinated and non-chlorinated), the growth of the \textit{Salmonella enterica} was observed to be significantly lower than \textit{E. coli} in the DWDS’s.

However, \textit{S. enterica} in the chlorinated DWDS showed its peak growth twice in the same trend in both biofilms and bulk water, though the later was significantly lower than the former. It is understood from the results that whether chlorinated or non-chlorinated, \textit{S. enterica} never attempts to survive in bulk water rather they migrate to biofilms, either decreasing throughout, or completely vanishing from the water phase. However, the performance of \textit{S. enterica} in chlorinated condition proves the organism to be a strict biofilm producer as well as a competitor with intelligent adaptive response towards chlorination. While \textit{E. coli} thoroughly survives and resists chlorine even in the water phase, \textit{S. enterica} completely transforms to biofilm phenotype migrating gradually.

While addressing this issue it is worth mentioning that attached cells of \textit{S. enterica} serovars Enteritidis have been reported to be early adaptive and efficient response to benzalkonium chloride than the planktonic cells (Mangalappalli-Illathu \textit{et al.} 2008). We are in complete agreement with this research. However, the biofilm formation capability of \textit{S. enterica} in the
oligotrophic systems like drinking water distribution systems is of high risk to the public, unless proper measures are to be taken to prevent this.

_E. coli_ was found to be superior to _S. enterica_ in terms of survival in DWDS’s and resistance to chlorine in both the sessile and planktonic forms. However, _S. enterica_ being a strict pathogen, though limited in number than _E. coli_ in the DWDS’s, poses severe threat to the community. Many a times during the experiment _S. enterica_ attained peak growth, even in the chlorinated systems, which is to be seriously considered. The results of the present work are consistent with a study (Wong et al. 2010) which reported that three day old biofilm cells of _Salmonella_ are less susceptible to disinfectants than the free floating counterparts, and therefore elimination of bacteria involved in the biofilms is a tedious task.

It was earlier reported that the disinfectants including sodium hypochlorite, sodium hydroxide, and benzalkonium chloride were unable to prevent the mature biofilm formed by _S. enterica_ in food contact surfaces (Corcoran et al. 2014). We do agree with the results of the aforementioned study as the sodium hypochlorite failed to eradicate the biofilm associated survival of bacteria in the present study. Likewise, a similar work also reported that the levels of chlorine and chloramines generally employed were inadequate to eradicate the microbial biofilms attached in the DWDS’s (Zhou et al. 2009). However we disagree with the opinion of maintaining high residual/free chlorine to reduce the density of biofilm formation compared with low residual chlorine, since it increases the adaptive response of the pathogen and they tend to acquire more virulence.

_S. enterica_ serovars, capable of causing salmonellosis, has been already reported to form biofilms rapidly on intact and cut lettuce surfaces
Survival of Escherichia coli and Salmonella enterica in the biofilms and bulk water of model drinking water distribution systems

(Patel and Sharma 2010), tomato surfaces in green houses (Iturriaga et al. 2007) food, spices and water samples (Xia et al. 2009). Investigations reported that S. enterica serovars Enteritidis is a severe biofilm forming pathogen in food industry (Xu et al. 2010). Though the potential biofilm formation capability of this pathogen have been investigated by various researchers in food industry (Sheffield and Crippen 2012), there is a scarcity of work done in the area of drinking water contamination of this pathogen. Conversely food contact surfaces are different from the oligotrophic drinking water environment in terms of rich nutrients. Pathogens like Salmonella enterica are highly demanding, fastidious, which may have resulted in the complete absence of the same in the water phase at the last two weeks of the experiment. However, the study revealed its long term survival for approximately five weeks, in the bulk water of DWDS’s and throughout in the biofilm phase, which is of high risk to the consumer.

A study conducted in USA revealed that the density of biofilm in the low nutrient, reverse osmosis (RO) treated water with reduced organic and inorganic substances in the DWDS was lower than the same in a municipal system without treatment (Meckes et al. 2007). The present work is in agreement with the aforementioned work, though the nutrients in the present study were within the limit. Further investigations in this regard are needed to decrease the nutrient availability in the public DWDS’s so as to reduce the bacterial regrowth.

The study suggests that, through proper application of the protocols of municipal, household and public DWDS, pathogens could be kept under control. According to a research waterborne outbreaks are normally associated with intermittent/interrupted or inadequate treatments (Larsson et
al. 2009). The present study completely agrees with the abovementioned work since adequate and uninterrupted disinfection methods, even before the entrance of pathogen, prevents the accumulation of vital cells in the biofilm niche. Repeated recovery of other opportunistic pathogens like *Pseudomonas aeruginosa* from the outlets from an experimental water distribution system (WDS) designed for the biofilm development with the organism being injected to the tap assemblies, over a period of 2 years was reported recently, even when the tap was flushed twice daily (Moore *et al*. 2015).

It is indispensable to supply superior quality drinking water from the treatment plants to the consumers tap to prevent water related illness. Preventing the biofilm formation of pathogenic bacteria is easier than eliminating the once established pathogenic biofilm matrix. For that adequate chlorination uninterruptedly even before the bacteria get established is important. However maintaining too high chlorine residual is not advisable, as bacteria attains adaptive resistance against disinfection. Despite the water being chlorinated or non-chlorinated, it is advised to the public, especially the immunocompromised population including the children and elderly, in a developing country like India, to consume water only after boiling since the chlorination process employed cannot be ensured for the safety of pathogen free water.

### 6.6. Conclusions

*E. coli* is found to be the most successful survivor in the DWDS’s compared to *S. enterica* in the biofilms of MDWDS’s being highly resistant to chlorination and the well flourished establishment in the system. However,
Survival of Escherichia coli and Salmonella enterica in the biofilms and bulk water of model drinking water distribution systems

S. enterica is a potential threat to DWDS’s as they are highly inclined to the adaptive response to chlorine by shifting to the biofilm phenotype. Although chlorination in the miniature MDWDS’s could eradicate S. enterica from the bulk water, it couldn’t eradicate the biofilm phenotypes developed in the biofilm test plugs coupons inserted. However, measures in eradicating pathogens should be taken considering the ability of biofilm formation of the pathogen. Routine monitoring techniques analyzed needs a thorough revision expanding the analysis to the attached reservoirs of pathogens. The study suggests that proper management of the DWDS’s with regular/uninterrupted and adequate chlorination from the initial stages preventing the entrance of a pathogen may help with the problem. Moreover, distribution pipelines and associated attachments are to be replaced once in a while through proper monitoring. Though the work utilized and proved the biofilm formation potential of E. coli and S. enterica, other opportunistic pathogens like Mycobacterium avium, Acinetobacter baumannii and Pseudomonas aeruginosa with potentially virulent features can be associated with the biofilms in DWDS’s which can be a threat to the public. However maintaining high residual chlorine is not a wise choice as the bacteria acquires adaptive resistance to chlorine through phenotypic adaptation. Further research in this area is an utmost need of the time to prevent water related disease outbreaks.