Understanding the Effect of Intermittent Water Supply on Drinking Water Quality

By

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B.S. Mechanical Engineering
Massachusetts Institute of Technology, 2016

Submitted to the Institute of Data, Systems and Society in partial fulfillment of the requirements for the degree of Master of Science in Technology and Policy at the MASSACHUSETTS INSTITUTE OF TECHNOLOGY

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Abstract

There are nearly 1 billion people who obtain their drinking water through piped distribution networks that operate intermittently. Intermittent Water Supply (IWS) operations allow for periods of stagnation and depressurization that create conditions favorable for biofilm growth on pipe surface. Biofilms are complex microbial communities that are likely sources of opportunistic waterborne pathogens and can cause disease outbreaks. Flushing of the water pipes and re-pressurization, which occur at the start of each IWS supply cycle, cause the erosion of the biofilm and its transport into the bulk water, which can potentially contaminate the drinking water. This thesis describes the development and proof-testing of an experimental pipe testbed installed on the Nanyang Technological University (NTU) campus in Singapore. The testbed comprises an array of 100 mm diameter PVC pipes, supplied from a water tank with flow paths controlled through a set of valves, and water samples obtained at up to 7 pipe outlets. Data are presented from an initial program of tests that compare the effects of priming for two pipe sections: 1) with continuous laminar flow (Continuous Water Supply, CWS) under pressure; and 2) IWS, where the pipe section is flushed during re-pressurization (supply period) and then allowed to drain and stagnate for the remainder of the daily cycle. The change of the water quality from both the inlet and outlet was evaluated by determining the microbial load using flow cytometry (with Live/Dead staining), together with physical and chemical water parameters measured on a time series of water samples. The data compare the response of the CWS pipe section for steady laminar flow, with the transient response following a step-change in flow rate (turbulent conditions) with transient behavior during flushing of the IWS section. Initial filling of the IWS section cause a significant increase in total and live cell counts, confirming that erosion of biofilm can contribute to biomass transported in the bulk water. Further tests are in progress to validate and replicate these results.

Thesis Supervisor: Andrew Whittle
Title: Edward K. Turner Professor of Civil and Environmental Engineering
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Contents

CHAPTER 1: INTRODUCTION ................................................................................................................... 9

CHAPTER 2: BACKGROUND ..................................................................................................................... 12
  2.1 Water-borne Diseases and IWS Systems ....................................................................................... 12
  2.2 Microbial Communities within DWDS .......................................................................................... 13
    2.2.1 Biofilms .................................................................................................................................. 16
    2.2.2 Laboratory Experiments .......................................................................................................... 18
    2.2.3 In situ Studies .......................................................................................................................... 21
    2.2.4 Summary ................................................................................................................................ 25

CHAPTER 3: EXPERIMENTAL TEST PROGRAM ................................................................................... 28
  3.1 Overview of the Testbed ................................................................................................................. 28
  3.2 Testbed Preparation ....................................................................................................................... 31
  3.3 Test Phases .................................................................................................................................... 32
    3.3.1 Phase I (March 16 – Apr 3, 2020) ....................................................................................... 32
    3.3.2 Phase II (July 6 – July 15, 2020) ......................................................................................... 33
    3.3.3 Phase III (July 15 - July 16, 2020) ..................................................................................... 34
    3.3.4 Phase IV (July 27 – August 27, 2020) ............................................................................... 36

CHAPTER 4: METHODS ......................................................................................................................... 38
  4.1 Overview ....................................................................................................................................... 38
  4.2 Environmental Parameters and Water Chemistry ......................................................................... 41
  4.3 Flow Cytometry ............................................................................................................................ 42
    4.3.1 Flow Cytometry Gating Procedure ....................................................................................... 43

CHAPTER 5: RESULTS ........................................................................................................................... 49
  5.1 Testbed Conditions ....................................................................................................................... 49
  5.2 Phase I: Slow Flow ....................................................................................................................... 52
  5.3 Phase II: Effect of Stagnation ....................................................................................................... 54
  5.4 Phase III: Pipe Flushing ............................................................................................................... 58
  5.5 Phase IV: Continuous versus Intermittent Operations ............................................................... 65
  5.6 Summary ..................................................................................................................................... 73

CHAPTER 6: SUMMARY, CONCLUSIONS, AND RECOMMENDATIONS ............................................. 74
List of Figures

Figure 2.1 Biofilm life cycle in DWDS .......................................................... 18
Figure 2.2 Cross-section of annular reactor for studying biofilm development in simulated DWDS hydraulic conditions .................................................. 19
Figure 2.3 Tubular biofilm reactor with recirculating flow system .................. 20
Figure 2.4 Time series measurements of turbidity and flow rate for periodic flushing of DWDS water pipes ........................................................................ 22
Figure 2.5 Impact of stagnation time on the increase in intact bacterial cell counts measured at household taps ............................................................... 23
Figure 2.6 Comparison of biofilm within shower pipes .................................. 24
Figure 3.1 Testbed location ........................................................................... 28
Figure 3.2 NTU testbed .................................................................................. 29
Figure 3.3 Water supply for testbed ............................................................... 30
Figure 3.4 Layout of testbed ......................................................................... 31
Figure 4.1 Basic principles of flow cytometry (FCM) analysis .......................... 41
Figure 4.2 Phase I negative control sample .................................................. 44
Figure 4.3 Phase I positive control ............................................................... 45
Figure 4.4 Phase I heat-treated positive control .......................................... 46
Figure 4.5 Phase I filtered sample ............................................................... 47
Figure 5.1 Concentration of monochloramine measured during Phase IV ........ 50
Figure 5.2 Phase I-IV water sample temperatures ....................................... 50
Figure 5.3 Phase I-IV sample conductivity .................................................. 51
Figure 5.4 Phase I characterization ............................................................... 53
Figure 5.5 Phase IIa characterization ........................................................... 55
Figure 5.6 Phase IIb and IIc characterization ............................................... 57
Figure 5.7 Phase III cell counts from July 15, 2020 ................................................................. 59
Figure 5.8 Phase III cell counts from July 16, 2020 ................................................................. 60
Figure 5.9 Phase III nitrogen parameters .................................................................................. 63
Figure 5.10 Phase III carbon parameters .................................................................................. 64
Figure 5.11 Phase IVa and IVb cell counts ............................................................................... 66
Figure 5.12 Phase IVc cell counts ......................................................................................... 67
Figure 5.13 Phase IVc flow rate ............................................................................................ 68
Figure 5.14 Phase IVa CWS flow .......................................................................................... 70
Figure 5.15 Phase IVb CWS flow .......................................................................................... 71
Figure 5.16 Phase IVc IWS flow .......................................................................................... 72
List of Tables

Table 2.1 Relative abundance of most abundant taxa ............................................................ 15
Table 3.1 Sampling in Phase I ................................................................................................. 33
Table 3.2 Summary of Phase III flushing conditions ............................................................... 35
Table A.1 Phase IVb Sampling Flow Rate ............................................................................... 77
Table A.2 Phase IVc Sampling Flow Rate ............................................................................... 77
Chapter 1: Introduction

Piped Drinking Water Distribution Systems (DWDS) represent an important exposure route for disease transmission (Anaissie et al., 2002, Brunkard et al., 2011). In most developed countries, chemical disinfectants (most commonly chlorine or monochloramine) are added to finished water that is supplied continuously through DWDS to consumers at pressures greater than 14-20 m (20-28 psi). These Continuous Water Supply (CWS) systems operate 24 hours a day, 7 days a week. Filtration and disinfection of drinking water distributed under CWS are intended to suppress opportunistic and obligate pathogens (Dai et al., 2020). However, there are nearly one billion people worldwide who currently obtain their water from distribution networks that operate intermittently (i.e. water supply is only available during limited time windows each day). These Intermittent Water Supply (IWS) systems constitute more than 40% of the distribution networks found in lower- and middle-income countries (Lapsidou & Spyropoulou, 2017), including 97% of utilities in South Asia (Van den Berg & Danilenko, 2017). Totsuka et al. (2004) summarize three possible reasons for the persistence of IWS systems: 1) absolute scarcity where there is insufficient quantities of source water; 2) economic scarcity where consumer demand exceeds the water available and the governing utility may not have the financial means to expand existing infrastructure; 3) technical scarcity where there is mismanagement of water supply, and the intermittent operation prevents excessive water losses associated with degraded infrastructure.

The most pervasive problem of IWS is the lack of equitable access to drinking water, as consumers downstream at the extremities of the pipe network have the least access, while wealthier consumers can install their own suction pumps to draw water from the available supply. It has long been accepted that IWS systems can also compromise the quality of water.
delivered to the consumers: pipes are only pressurized during supply periods and are either partially filled or empty at atmospheric pressure at all other times. In these situations, water is allowed to stagnate within the pipes and, due to the lack of pressure, there is potential for ingress of contaminants from the surrounding ground (Solgi et al., 2015; Kumpel & Nelson, 2013). While these processes are widely discussed, there have been no systematic studies to quantify how regular cycles of depressurization and re-pressurization, filling and emptying of pipes, associated with IWS, affect water quality (Kumpel & Nelson, 2014).

Bivins et al. (2018) used Quantitative Microbial Risk Assessment (QMRA) methods to estimate that IWS may account for more than 17.2M infections, 4.52M cases of diarrhea and 1560 deaths per year. This is likely to be a gross underestimate of the actual public health risk since it is based on limited data and conventional measurements using fecal indicator bacteria such as E. coli that are either readily suppressed at source, through high levels of disinfection, or locally present at points of ingress. Meyer et al. (2020) found broad regulatory compliance in levels of E. coli (at point of use) from a sampling campaign in New Delhi, India, while levels of free chlorine (the most common disinfectant) were often excessive (leading to potential for creation of carcinogenic disinfection by-products; Kalenkesh et al., 2019). To date, there have been no dedicated studies on the occurrence of opportunistic pathogens that can grow within biofilms that form inside the pipes as the water drains and stagnates between supply periods. There have also been no studies on the potential contamination in IWS systems from biofilm detachment due to the flushing of water. These represent an uncharted risk for IWS systems.

This thesis aims to investigate how the cycles of flushing and draining, associated with IWS, affect the amount of suspended biomass through a program of controlled field experiments as compared to CWS. Chapter 2 reviews the related literature on the relationship between health
risk, water quality, and IWS systems as well as microbial communities in DWDS. Chapter 3 describes the testbed constructed specifically for the current program of experiments, together with priming protocols and conditions. Chapter 4 summarizes the methods utilized for characterization and analysis of water samples with particular focus on the interpretation of flow cytometry data. Chapter 5 presents the results from a series of tests performed at the testbed. These include stagnation experiments (unplanned and made by the COVID-19 pandemic) and preliminary results comparing the production of biomass from IWS and CWS conditions. Chapter 6 summarizes the findings to date, offers some preliminary conclusions and makes recommendations for the next program of experiments.
Chapter 2: Background

2.1 Water-borne Diseases and IWS Systems

Water quality in distribution systems has long been a health concern. In the mid-19th to 20th centuries, outbreaks of two water-borne disease, typhoid and cholera, were conclusively linked to water-borne microbes by public health pioneers Jon Snow and Robert Koch during epidemics in London and Hamburg (Szewzyk et al., 2000). There are a number of opportunistic pathogens that have been found in water distribution systems and can cause infections including i) non-tuberculous mycobacteria (such *Mycobacterium avium*, *M. intracellulare*, *M. kansasii* and *M. fortuitum*) that have been linked to pulmonary and lymphatic disease; ii) *Legionella pneumophila*, the causative agent of Legionnaires’ disease (pneumonia) and the milder Pontiac fever, that result in thousands of hospitalizations annually (8,000-18,000 est. in 2008 by CDC); iii) *Pseudomonas aeruginosa* which commonly occurs in low nutrient environments such as water supplies and can infect the eyes, ears, and skin; and iv) protozoa such as *Acanthamoeba* (keratitis, encephalitis) and *Naegleria* (encephalitis) which are endemic to distribution systems.

It is well recognized that there are many opportunities for contamination and degradation of water quality in IWS systems principally associated with backflow (adverse pressure gradients) and intrusion mechanisms (seepage from the surrounding ground). Bivins et al (2018) have evaluated the global burden of diarrheal disease associated with IWS using Quantitative Microbial Risk Assessment (QMRA). Assuming 925 Million IWS consumers worldwide, they estimate 17.2M annual infections, 4.52M diarrheal cases, 1560 deaths and 109,000 disability adjusted life years that can be attributed annually to the water distribution system. Their methodology uses fecal indicator bacteria rather than direct pathogen measurements from
samples in the distribution networks\(^\text{1}\) and makes assumptions about the ratios of pathogens to indicator bacteria to estimate the burden. The dataset did not include measurements during the first flush of the pipes (i.e. the transient phase where pipes become repressurized during a supply period), where higher microbial concentrations are expected (Kumpel & Nelson, 2015).

Few studies have been published where disease outbreaks are linked directly to intermittent water supply: Jeandron et al. (2015) correlated the admission of patients to the Cholera Treatment Center in Uviva, Congo to periods where water supply was interrupted. Following a day of non-supply, the disease incidence rate increased by 155% over the next 12 days with 23.2% of total admissions attributable to the reduced water supply. In Hubli-Dharwad, India, Ercumen et al. (2015) collected health data on nearly four thousand households over the course of fifteen months and included neighborhoods that were served by continuous supply and intermittent supply. The health data included occurrences of diarrhea, highly credible gastrointestinal illness, bloody diarrhea, typhoid fever, cholera, hepatitis and the deaths of children under two years old. They mapped health incidences according to areas serviced by CWS vs IWS. The CWS was associated with lower incidence of dysentery and typhoid fever incidence compared to IWS. They concluded that IWS is a transmission source for water-borne pathogens.

2.2 Microbial Communities within DWDS

The microbial communities within in DWDS comprise of biofilms which grow on pipe walls and bacteria in the bulk water (Kumpel & Nelson, 2015). Approximately 95% of the

\(^\text{1}\) The analyses do not include data from some key geographic areas such as South America and sub-Saharan Africa, where IWS systems are prevalent.
biomass within piped water systems exist in the biofilm and the remaining 5% is in the bulk water (Flemming et al., 2002). The composition of bulk water bacteria and biofilm bacteria differs greatly and is influenced by many different factors (e.g., Martiny et al., 2005). Biofilms can serve as a source of contamination for DWDS by harboring potentially pathogenic bacteria such as *Pseudomonas, Legionella, Aeromonas, Yersinia, Campylobacter, Mycobacterium, E. coli*, coliforms, amoebae and viruses (Flemming et al., 2002).

The microbial population of the bulk water is influenced by the source water, disinfection (treatment) methods, and climatic factors such as temperature and humidity, which explains why microbial communities within piped water systems vary across regions in the world. They are influenced by the local ecology present in the source water and contamination points from the environment through intrusion (Martiny et al., 2005; Kumpel & Nelson, 2015). Compared to biofilms, bulk water bacteria are more homogenous over time, and are influenced strongly by disinfectant conditions (Martiny et al., 2005)

Martiny et al. (2005) compared the microbial community in the biofilm and bulk water species from a model CWS system. Samples were taken from a pipe reactor system over the course of nine months. The biofilms ages varied from two weeks to three years. The bacterial species identified in bulk water were very similar between samples despite the large time gap. However, the bulk water bacteria differed greatly from the biofilm bacteria species, as shown in Table 2.1 (Martiny et al. 2005). Bacterial species exclusive to the bulk water included *Legionella, Methylomonas*, while some species were only present in the biofilm such as *Nitrospira and Verrucomicrobia*. The bacterial species abundance was more similar in younger biofilms and bulk water compared to older biofilms and bulk water. Specifically, the two-week
old biofilm shared *Nitrospira, Bacteriodetes,* and *Rhodobacter* similarities with the bulk water samples.

Chemical disinfectants are used to control the microbiome in DWDS. Chlorine (Cl₂) is still the commonly used disinfectant in drinking water systems worldwide and is known to be effective at reducing, but not preventing microbial growth (Liu et al. 2016). Certain bacterial species, such as *Mycobacterium avium,* are more resistant to chlorine than others which creates growth environments that favor specific bacterial species while suppressing others. Chlorine concentration levels decay over time, creating temporal differences in population dynamics.

### TABLE 1. Relative abundance of strains isolated on R2A for 10 days at 23°C and cloned 16S rRNA sequences from young biofilm, old biofilm, and bulk water

<table>
<thead>
<tr>
<th>Phylum and genus</th>
<th>% of sequences from:</th>
<th>R2A medium</th>
<th>Direct extraction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Biofilm</td>
<td>Bulk water</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(n = 97)</td>
<td>(n = 74)</td>
</tr>
<tr>
<td><strong>Proteobacteria</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brevundimonas</td>
<td></td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Devosia/Rhodobium</td>
<td>75.0</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Hydrogenophaga</td>
<td>–</td>
<td>18.9</td>
<td>–</td>
</tr>
<tr>
<td>Aquabacterium</td>
<td>–</td>
<td>10.8</td>
<td>1.8</td>
</tr>
<tr>
<td>Lateimonas</td>
<td>24.0</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Legionella</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Methylomonas</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Pseudomonas</td>
<td>–</td>
<td>1.4</td>
<td>3.6</td>
</tr>
<tr>
<td>Thiothrix</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><strong>Acidobacteria</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acidobacterium</td>
<td></td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><strong>Planctomycetes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Planctomycyes</td>
<td></td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><strong>Nitrospirae</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nitrospira</td>
<td>–</td>
<td>–</td>
<td>25.0</td>
</tr>
<tr>
<td>Unknown</td>
<td>–</td>
<td>–</td>
<td>39.3</td>
</tr>
<tr>
<td><strong>Verrucomicrobia</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Verrucomicrobiaceae</td>
<td></td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Not identified</td>
<td>1.0</td>
<td>47.3</td>
<td>0</td>
</tr>
<tr>
<td>Genera not listed</td>
<td>0</td>
<td>14.8</td>
<td>19.5</td>
</tr>
</tbody>
</table>

*Young biofilm, 1 to 256 days; old biofilm, 571 to 1,093 days; bulk water, inlet and outlet. n values in parentheses indicate total numbers of clones or isolates from each source. Percentages in the body of the table are out of these totals. a Not found.*

Table 2.1 Relative abundance of most abundant taxa (at Phylum and Genus level) in a model DWDS (Martiny et al., 2015) from 16S rRNA sequences. Biofilm samples were obtained from stainless steel coupons. There was no residual disinfectant in the bulk water.
Chloramines, particularly monochloramine (NH\textsubscript{2}Cl), are also widely used in DWDS and are more effective in penetrating biofilms. Chloramines also have a longer residual time than chlorines (Liu et al., 2016). However, chloramines have also been found to encourage growth of nitrifying bacteria (*Nitrosomonas* and *Nitrospira*), which in turn degrades water quality through the production of harmful nitrites (Martiny et al., 2005, Kitajima et al., 2020).

An important characteristic (and quality indicator) of bulk water is the residence time (water age), between finished water treatment and consumption. As chemical disinfectant concentrations decay exponentially over time, water with longer residence times tends to have increased microbial activity. A recent study of the DWDS system in Singapore reported population differences between samples with low residence times and high disinfectant residuals and samples with high residence times and decayed disinfectant residuals (Kitajima et al., 2020).

Other means of controlling bulk water quality include biofiltration, nutrient limitation, UV disinfection and ozonation (Liu et al., 2016). These are hard to achieve once the drinking water has been released from the water treatment plant into the DWDS (Kitajima et al., 2020).

The current testbed is located in Singapore, and uses monochloramine to treat its DWDS.

2.2.1 Biofilms

Biofilms, ubiquitous in all natural and engineered aquatic environments, are typically composed of heterogenous communities of bacteria that serve different ecological functions. The bacteria communicate with each other by releasing extracellular polymeric substances (EPS), which provide signals to indicate hospitable growing environments and provide structural integrity for the community (Douterelo et al., 2013). Some bacterial species cannot adhere directly to the pipe walls, but rather attach to other species and becoming incorporated into
biofilms through a bridging mechanism. For example, in high-density polyethylene (HDPE) pipes, *Pseudomonas, Janthinobacterium*, and *Methylophilus* genera bacteria often serve as the initial colonizers attaching to and forming biofilms on the pipes; while other species, such as *Acinetobacter calcoaceticus*, serve as a bridging species in biofilm formation (Simoes et al., 2008). Pathogens are then able to grow in the DWDS with the biofilms protecting them from disinfectants and hydraulic shear stress from the system (Kumpel & Nelson, 2015). Notably, pathogens such as *Pseudomonas aeruginosa, Legionella pneumophila*, and *Mycobacterium* have all been found in DWDS biofilms (Douterelo et al., 2013).

The biofilm growth cycle is cyclical (Figure 2.1). Species floating in the bulk water attach to the pipe substrate. Nutrients within the bulk water can create locally rich environments that allow these microbes to grow. They form a conditioning film of EPS that encourages other microcolonies to grow and mature within the protective layer. As the biofilm matures, the cells proliferate, and eventually spontaneous erosion or sloughing occurs. The protective layer is broken, and the biofilm bacteria are dispersed into the bulk water until the cycle repeats.

In order to understand how biofilms affect water quality, the factors affecting biofilm growth must be understood. Research concerning biofilm growth has largely revolved around modeling and laboratory experiments. Some research has been conducted on operational water distribution systems, but access to these networks is difficult (Douterelo et al., 2014). Barriers to access are namely the logistical challenges associated with permitting and accessing an active water supply pipe. The following sections describe some of the prior work to investigate biofilm growth in water pipes. In all of the laboratory experiments, the pipes are filled with continuously circulating water, while some in situ experiments have measured biofilms in stagnant conditions.
within pressurized pipes. None of the prior studies have considered IWS conditions where pipes are partially filled or empty between supply periods.

Figure 1. (a) Biofilm growth on different pipe materials. Reprinted with permission from Ren et al. Copyright 2015, Springer. (b) Biofilm life cycle in DWDS.

Figure 2.1 Biofilm life cycle in DWDS (after Ren et al., 2015)

2.2.2 Laboratory Experiments

Laboratory experiments modeling piped water systems typically focus on understanding the mechanisms affecting biofilm formation and behavior. Devices have been developed to control mechanical, physical and biological factors for biofilm growth with different devices being more suitable for different applications. The most common configuration is an annular reactor (Figure 2.2) with a rotating inner cylinder. Gomes et al. (2014) have reviewed bench-scale biofilm reactor designs and experimental results reported in the literature using a range of disinfectants and micro-organisms. Annular reactors are typically designed to simulate flow conditions found in CWS systems (with fluid velocity, v = 0.3 – 0.6 m/s and interface shear
stress, $\tau_s = 0.25 - 0.68$ N/m$^2$). The tests enable control of basic environmental variables in the circulating water, while biofilm samples are collected periodically by sampling coupons that are attached to the inner cylinder surface.

Annular reactor experiments have been used to investigate how changes in flow regimes can affect biofilm growth. For example, Tsagkari and Sloan (2018) compared the biofilm development under three different flow regimes. They primed the reactor by allowing initial biofilm colonization in a stagnant/no flow condition (batch mode) over a period of 4 weeks (adding a growth medium to the tap water), and then studied the accumulation and density of biofilms (using microscopy and gravimetric methods) for recirculating flow at three different velocities ($v = 0.03$ m/s, $0.07$ m/s, $0.25$ m/s, corresponding to laminar, transitional and turbulent regimes; with $\tau_s = 0.007 - 0.07$ N/m$^2$) flow over intervals of 24 hrs. They found that turbulence accelerated the development of biofilms on accessible surfaces, and that biofilms responded rapidly to changes in the flow regimes, indicating the importance of the interface shear stress in affecting sloughing processes (also reported by Douterelo et al., 2016).

Figure 2.2 Cross-section of annular reactor for studying biofilm development in simulated DWDS hydraulic conditions (Gomes et al., 2014)
Telgmann et al. (2004) used a tubular reactor (Figure 2.3) to investigate the stability of pipe biofilm when experiencing backwashing events. The reactor is inoculated/primed by flowing activated sludge through the tube; and then recirculating a deionized water with a growth medium (glucose and salt). Biofilm detachment was induced by incrementally increasing the flow rate from a steady condition (laminar flow at \( Q = 500 \text{ mL/min}, \ v = 0.016 \text{ m/s} \)) to between \( 1000 \text{ – } 5600 \text{ mL/min} \) (up to \( v = 0.18 \text{ m/s} \)) for a period of three to thirteen minutes. Biofilm growth was quantified by measuring changes in the weight of the reactor. Telgmann et al. (2004) showed that the growth history of biofilm influences future sloughing and erosion events. Sloughing is strongly affected by shear stress at the pipe wall. Biofilms growing in a laminar flow regime have a rougher surface structure compared to biofilms experiencing a turbulent flow regime. Furthermore, smoother biofilms are stronger and tend to either resist sloughing or detach completely.

Figure 2.3 Tubular biofilm reactor with recirculating flow system (Telgmann et al., 2004). The biofilm tubular reactor comprises of 26 mm diameter glass pipe.
2.2.3 In situ Studies

The most effective approach to understand biofilm growth factors are studies done on operational in situ piped water systems. These have generally focused on the influence of real-world operations on microbial community compositions (e.g., Chan et al., 2019; Douterelo et al., 2016). In situ studies have been used to understand how flushing, temperature fluctuations, pipe material, bulk water nutrient availability, and pH affect the diversity, growth, structure, and attachment and detachment of biofilm.

Douterelo et al. (2016) investigated changes in microbial communities within an operational chlorinated DWDS system. They investigated how flushing affects the structure of microbial communities, how repeated flushing affects population dynamics and how biofilm is affected by bulk water conditions and pipe material. Sections of cast iron and polyethylene pipes were flushed at 4-month intervals at a volumetric flow rate varying from a rate of 1.5 L/s to 12 L/s (Figure 2.4).

Time series data show transient changes in turbidity of the flushed water, reflecting erosion and detachment of biofilm on the pipe walls. Water samples (five liter) were collected at inlet, before and during the flushing events for offline water quality and molecular analyses. The sample collection was timed to allow passage of one pipe volume of water after each step change in flow rate. After a year of sampling and observation on the in situ distribution network, the researchers concluded that microbial communities are strongly affected by the history of conditions, pipe material (cast iron vs plastic), seasonal temperature fluctuations, and water supply. Each flushing event changes the surface layer bacteria. The fraction of biofilm sloughed off from each flushing cycle depends on hydrodynamic factors and each instance exposes new
bacterial groups to the bulk water, thus changing the microbial composition and subsequent regrowth of biofilm. The pipe materials also influenced what kinds of bacteria were able to grow within the cast iron and plastic pipes. Microbial communities in plastic pipes showed greater diversity and were more heavily influenced by seasonal temperature fluctuations (this was attributed to differences in the surface textures of plastic versus metal pipe walls). Metal pipes provide a more favorable surface for biofilm detachment since corrosion of the metal creates pockets in the pipe surface. The seasonality created a 10 °C change in water temperature. For plastic pipes, higher temperatures favored bacterial groups such as *Actinobacteria*, while lower temperatures increased the growth of *Cyanobacteria.*
Lautenschlager et al. (2010) investigated the growth and compositional changes of biofilms in household taps. Their study measured changes in the cell concentration after periods of stagnation in household taps where the source water was produced from groundwater, lake water and spring water. The tap was gently flushed for five minutes and then closed for stagnation periods ranging from 8-20 hours. One liter samples were collected after stagnation and then after another five minutes of gentle flushing. Flow cytometry was utilized to analyze the total and intact cell counts within the bulk water. They found that after about 12 hours of stagnation, the intact cell concentration leveled off (Figure 2.5), likely due to insufficient nutrient concentration to continue growth. Cell counts increased by two- to three-fold after stagnation periods and dropped off after flushing, making it a simple mitigation strategy.

![Figure 2.5 Impact of stagnation time on the increase in intact bacterial cell counts measured at household taps (using flow cytometry) relative to conditions after flushing (log$_n$ = 2.303log$_{10}n$). Grey symbols used for linear regression. (Lautenschlager et al., 2010)](image)
Figure 2.6 Comparison of biofilm within shower pipes a) control pipe vs b) shower in regular use. Figures show 1.2cm long sections of pipe, biofilm thickness was measured using optical coherence microscopy, bacterial cell concentrations were measured using flow cytometry (Total Cell Count, TCC), and relative abundance using 16S rRNA gene sequencing. Each case shows the 3 most abundant taxa: a) Green: *Cytophagaceae*; blue: *TM6 [Dependentiae]*; red: *Bradyrhizobium spp.*; b) red: *Bradyrhizobium spp.*; yellow: *Altererythrobacter spp.*; purple: *Caulobacter spp.* (Neu et al., 2019)

Neu et al. (2019) demonstrated that biofilms experiencing real world growing conditions have a high amount of heterogeneity in composition relative to biofilms grown in controlled environments. Two PVC-P shower hoses were studied for a year where one served as the control and the other experienced actual shower usage from three residents. The control pipe was installed in a laboratory tap and was flushed at a flow rate of 0.3 L/min for fifteen minutes twice a day between eight- and sixteen-hour stagnation period, where the taps were closed and pipes sat full. The real world usage piped experienced random stagnation times, varied water temperature and flow velocities ranging from 8-12 L/min. Sections of the pipe were cut and imaged to measure biofilm thickness. Cell concentrations were measured with flow cytometry.
and 16s RNA sequencing was used to determine microbial taxonomy (Figure 2.6). The biofilm in the control hose grew to be considerably thicker with higher cell concentrations than that measured for the hose that was subject to variable usage and flow conditions. The usage patterns of the residents created a much more diverse microbial community relative to the control case, both at the small-scale local community and large-scale across the length of the hose.

2.2.4 Summary

There are many factors affecting biofilm growth, microbial biodiversity, structure, and attachment and detachment conditions. Parameters such as temperature (especially its fluctuations), pipe materials, bulk water chemistry and nutrient availability as well as pipe and water age have been reported to be the most relevant. Biofilms generally tend to grow more favorably in warmer weather (higher water temperatures), and plastic pipe biofilms exhibit greater microbial diversity in biofilms than cast iron pipe biofilms. Turbulence can increase nutrient supply to biofilms, create smoother biofilms and induce sloughing. (Tsagkari & Sloan 2018, Telgmann et al., 2004) as increased flow velocity supplies more nutrients to biofilms via the bulk water. Slow moving or stagnant water results in that biofilm growth plateaus once locally available resources are exhausted. Nitrogen, iron, and phosphorus have been shown to encourage biofilm growth (Liu et al., 2014) while increased flow rates of the bulk water results in increased occurrence of specific bacterial species that thrive in smooth surface biofilms (Telgmann et al., 2004). The changing nutrient concentrations of carbon, nitrogen and phosphorus specifically have been demonstrated to create compositional changes in communities. Specific species grew optimally or sub-optimally by altering the nutrient
composition in water. Altering both nutrient concentration and their availability is a potential method to control opportunistic pathogenic populations within continuous DWDS.

Biofilm detachment is strongly influenced by changes in interface shear stress but weakly affected by the absolute shear stress (Telgmann et al., 2004). This behavior may be significant for IWS systems where sudden changes in shear stress at the start of each water supply period potentially leads to greater detachment rates and subsequent increased consumer hazard when compared to CWS. The historic environment a biofilm experiences provides insight into the state of a biofilm community, but spontaneous sloughing and erosion from laminar flow also does occur. Sloughing changes the biofilm heterogeneity as it exposes previously protected bacterial species previously protected to the bulk water. The fraction of the biofilm left is dependent on the hydrodynamic factors from flushing (Douterelo et al., 2016). Given the changes to the composition of bacteria in the surface layer of bacteria, the recolonization of the pipe walls will not take place in the same way after a biofilm sloughing incident.

Different mechanisms are utilized to intentionally detach biofilms from pipe walls by water suppliers and agencies worldwide. Flushing by turbulent flow regimes, ice pigging, and introduction of air bubbles have been shown to effectively slough biofilms off of pipe walls (Liu et al., 2014). High fluid velocities increases shear stress between the biofilm and bulk water and detaches the biofilm. Ice pigging mixes crushed ice and freezing point depressants in water as a more effective mechanism to dislodge growth and buildup in pipes (Quarini, 2002). Mechanically inserting air bubbles is a more effective and efficient method to remove biofilm growth from pipe walls than flushing alone. (Tang et al., 2017) When air bubbles pass over the biofilm, the biofilm experiences a drop of shear stress and then a sudden increase when the air bubble passes and water flows over it again, thus sloughing biofilm off pipe walls.
All of the described laboratory and in situ studies have focused on either operational DWDS or premise plumbing networks, both CWS systems where the pipes are always filled and pressurized. To date, there have been no systematic studies characterizing bacterial growth in piped water systems where the pipes are periodically partially-filled (or empty), as found in IWS systems. The residual water left after the supply is disconnected can be prime breeding ground for opportunistic pathogens. While CWS growth conditions cannot be directly transferred to intermittent water supply scenarios since the growth environment is different, CWS systems do have similarities to IWS systems as operate in pressurized pipes and experience stagnation or low flow rates due to daily use patterns. This knowledge was used to inform the experimental design and anticipated results.
Chapter 3: Experimental Test Program

3.1 Overview of the Testbed

In February 2020, a testbed was constructed to investigate factors influencing the water quality in intermittent and continuous water distribution systems. The testbed is located on the campus of Nanyang Technological University (NTU) in Singapore is located at the top of Nanyang Hill adjacent to Nanyang House (1°20'58.5"N 103°41'12.9"E; Figure 3.1) Dr. Cheng Dan developed the design in cooperation with the research team at MIT and SCELSE as well as NTU’s Office for Development and Facility Management (ODFM) and supervised the construction in early 2020.

Figure 3.1 Testbed location a) Map of NTU campus with testbed location marked. b) Layout of testbed set up location. Both maps have approximate locations and show the general arrangement.

Figure 3.2 shows the general arrangement of the as-built testbed which comprises a parallel array of 7 PVC pipes (each ~30 m long, 100 mm diameter) that are gravity-fed through a common pipe (33 m long\(^2\)) from a surface water tank (nominal storage capacity of 8 m\(^3\)) that is elevated 0.4 m above the ground surface. The tank\(^3\) is made of fiberglass-reinforced plastic

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\(^2\) The entire testbed is approximately 250m in length and has a total volume 1.96m\(^3\).

\(^3\) The tank measures 2 m x 2 m x 2 m with an effective storage capacity of 4 m\(^3\).
(FRP) board. The outlet pipes are elevated 0.3 m above the level ground surface and are arranged with a downward gradient (0.5%). The flow paths through the pipes are controlled by a set of valves (Figure 3.2a), while the flow rate is controlled by adjusting the size of the exit orifice (Figure 3.2b shows the installation of 50mm discharge pipes) and the tank water level.

Figure 3.2 NTU testbed a) general arrangement showing water supply tank, parallel array of 100mm diameter PVC pipes and valves, common supply pipe and header; b) and c) discharge orifices; d) and e) removable coupon holder windows for collection of biofilm samples
Bulk water can be sampled at the point of discharge or through 25 mm side valves (Figure 3.2c), while biofilms can be collected from coupons attached to removable screw cap windows at the entrance and end of each replicate pipe (Figure 3.2d, 3.2e).

Water is supplied to the storage tank (Figure 3.3c) directly from the NTU campus water supply via a flexible hose from a valve chamber located at the top of Nanyang Hill (Figure 3.3b). The current set-up is able to supply approximately 7.8 L/min (flow meter in Figure 3.3d), and a float valve maintains a constant head (H = 0.9-1.1 m) at periods between the flushing experiments.

Figure 3.3 Water supply for testbed a) valve chamber connection to NTU DWDS; b) hose connection to storage tank; c) water tank with level gauge; d) flow rate measuring device at inlet

Figure 3.4 shows the layout and notation of the pipe testbed which includes 17 valves and 16 biofilm collection windows. While each of the 7 main pipes can be connected directly to the common header pipe, valves v16, v15, v14 connect between pairs of pipes enabling an extended flow path. For example, opening valves v1, v4, v10, v14, v9, and v2 while closing v17, v3, and v5 enables a flow path from the end of the common pipe to Outlet 1 through 90 m of pipe.

4 The original proposal had planned to re-purpose an active DWDS pipe section (100mm diameter, high density ductile iron, 270m long) from this same valve chamber and to study effects of IWS for conditions with a mature (30 years) pipe biofilm. The current testbed was developed when it became apparent that this pipe would remain in service for an extended time period. This pipe now serves as the source water for the tank.
Similarly, opening valves v1, v5, v11, v12, v7, and v13 while closing v17, v3, v10, v6, v8 and v16 will open a flow path to Outlet 6 through 90m of pipe. The current setup of experiments utilizes these two 90 m flow paths to simulate and compare conditions of CWS (Outlet 1) and IWS (Outlet 6) conditions. Outlets 0 and 7 are used for control measurements. Outlet 0 serves as a sampling point for inlet conditions, while Outlet 7 was used to investigate hydraulic conditions.

Figure 3.4 Layout of testbed showing configuration for comparing CWS conditions (Tank to Outlet 1 with v5 closed) vs IWS conditions (Tank to Outlet 6 with v4 closed) through 90m pipe section.

3.2 Testbed Preparation

After completion of the construction of the pipes in February 2020, the PVC pipes were cleaned with bleach three times according to ODFM’s standing operating protocol for the commissioning of drinking water tanks. This flushing was intended to remove any residual
particles originating from the construction process and the surrounding air. It also ensures that any microbial activity within the piped system originates from the source water and not residual colonies introduced during construction or storage of the pipes. The pipes were then filled with tap water without residual monochloramine and left stagnant for 4 weeks (February 17, 2020 to March 16, 2020). During this time, system integrity and water levels in the pipes and storage tank were checked daily. This priming period allowed for microbial communities to develop in the pipe under temperature and humidity conditions typical for Singapore’s tropical climate.

3.3 Test Phases

3.3.1 Phase I (March 16 – Apr 3, 2020)

Phase I of testing involved two different priming regimes. The storage tank was refilled\(^5\) intermittently at 3- to 5-day intervals.

1. CWS: For the 90 m flow path to Outlet 1, continuous flow conditions were simulated by opening the 25mm side valve at Outlet 0 and closing v5 (Figure 3.4). Water drained continuously with a volumetric flow rate between 400-600 mL/min (average velocity, \(v = 0.001 - 0.0008 \text{ m/s in the pipes}\)) depending on the gravitational pressure generated by the water head in the storage tank. Volumetric flow rate was measured by timing how long the flow took to fill a fixed volume. These flow conditions are slower than typically encountered in a CWS system and correspond to a laminar flow regime with \(R_e^6 = 85-120\). The water level in pipes 1, 2, and 3 were continuously full.

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\(^5\) Flow rate meter measuring inlet flow was not installed yet so true refill rate is unknown, but assumed to be similar.
\(^6\) Reynold’s Number: \(R_e = \frac{\rho v D}{\mu}\), where the pipe diameter, \(D = 100 \text{ mm}\); the mass density, \(\rho = 1000 \text{ kg/m}^3\); and the dynamic viscosity of water varies from \(\mu = 0.8 \times 10^{-3} \text{ Pa.s}\) for water at 30 °C (typical of conditions in Singapore).
2. IWS: The 90m flow path to Outlet 6 was flushed twice per day (around 9 am and 5pm). Outlet 6 was opened and valves v10, v6, v8 and v16 were closed. After draining, Outlet 6 were closed and then the pipes were refilled from the water tank. Since the main valves were closed during the resupply, pressure built in the pipes and the pipes only partially filled. Every 2-3 days, they were filled completely to simulate high pressure in the system. The pipes were then left stagnant for less than 24 hours.

Phase I priming continued throughout four different sampling events. When sampling from the CWS pipes, the samples were taken directly from the slow flow bulk water. For IWS samples, the system was drained and then samples were taken by running tank water through the pipes and collected at the outlet taps.

<table>
<thead>
<tr>
<th>Event #</th>
<th>Date</th>
<th>Outlets Sampled</th>
<th>Sampling Times (mins)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I-a</td>
<td>March 20</td>
<td>1</td>
<td>0, 5, 15</td>
</tr>
<tr>
<td>I-b</td>
<td>March 24</td>
<td>1; 6</td>
<td>0, 2, 4, 6, 8, 10, 12, 14</td>
</tr>
<tr>
<td>I-c</td>
<td>March 27</td>
<td>1, 3; 4, 6</td>
<td>0, 30, 60, 90</td>
</tr>
<tr>
<td>I-d</td>
<td>April 3</td>
<td>1, 3; 4, 6</td>
<td>0, 30, 60, 90, 120, 150, 180</td>
</tr>
</tbody>
</table>

3.3.2 Phase II (July 6 – July 15, 2020)

Research activities at NTU were suspended in early April 2020 due to the COVID-19 (SARS-cov-2) pandemic under Circuit Breaker (lockdown) regulations (Singapore Ministry of Health, 2020). The testbed was left in a dormant state with the pipes filled with all outlet valves closed (Outlets 0-7). The testbed could not be monitored during this period given severely restricted access to NTU. Water inside the pipes was effectively left to stagnate over a 10 week period (April 3 – July 6, 2020), coincidentally resembling the conditions in public buildings all over the world.

Phase IIa comprised of an initial sampling of the stagnant water taken from Outlets 0-6 on July 6, 2020, through the 25 mm side valves while the pipes were draining gently (Q = ~400
mL/min; laminar flow). In parallel, the tank was also drained and then refilled with fresh tap water. The pipes were subsequently refilled with the fresh tank water and left for a further period of 9 days, during which the rebooted analytical devices (e.g. flow cytometry) in SCELSE were validated.

Between Phase IIa and Phase IIb, a series of tests were conducted to measure the flowrates in the system to through the common pipe and Outlet 7. With Outlet 7 fully open (50 mm diameter orifice) and an initial tank elevation head \( H = 0.7 \) m, the maximum flow rate was calculated to be 213 L/min \( (v = 0.45 \text{ m/s}, R_e = 45,000, \text{turbulent regime}) \). Two tests were conducted on the small sampling valve (25 mm diameter was reduced to 12.5 mm). When the valve \( v1 \) was opened fully, the maximum flow rate was 2.6 L/min \( (v = 0.005 \text{ m/s}, R_e = 500, \text{laminar regime}) \). The flow rate stayed the same when valve \( v1 \) was opened partially, indicating that the small orifice was restricting the flow rate.

Samples obtained during Phase IIb and IIc served as replicates to those taken during Phase IIa. On July 15, 2020, a water sample was taken using the 25 mm valve for each outlet 1-6 while the pipes were first opened and allowed to drain gently \( (Q = \sim 400 \text{ mL/min}) \). After 60 minutes, a second sample (IIc) was taken from Outlet 0 before any flushing. All pipes were drained after 90 minutes and biofilm samples were obtained by swabbing from windows w10-w16. Particular care was taken to ensure that the swab only touched the biofilm and did not come into contact with residual water in the pipes.

3.3.3 Phase III (July 15 - July 16, 2020)

On July 15 and 16, a series of flushing experiments were conducted on each of the 6 main pipe outlets. The pipes were individually flushed \( (Q = 120 \text{ L/min}) \) from the tank through the
common pipe to each individual outlet in the following sequence Outlets 2, 3, 1 (July 15) and then Outlets 4, 5, 6 (July 16) to minimize cross contamination between the connecting pipes.

Prior to flushing each set of 3 pipes, the storage tank was refilled to ensure comparable hydraulic conditions prevail across each set of pipes (i.e., there should be identical hydraulic conditions for pairs of pipes 2 and 4, 3 and 5; and 1 and 6 due to the declining head in each set). However, this was partially achieved (Table 3.2) and the hydraulic conditions did vary among all six outlets. A series of timed water samples were collected at each outlet during the flushing events at 0, 10, 30, 120, 180, 240, and 300 s, (and at Outlet 0 at 0 and 300s) and additional data were obtained for Outlet 6 at 20, 40, 50, and 600 s. The time series measure transient changes in water quality due to flushing of pipe volumes from the fresh tank water.

<table>
<thead>
<tr>
<th>Date</th>
<th>July 15, 2020</th>
<th></th>
<th></th>
<th>July 16, 2020</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Sequence</td>
<td>(i)</td>
<td>(ii)</td>
<td>(iii)</td>
<td>(iv)</td>
<td>(v)</td>
<td>(vi)</td>
</tr>
<tr>
<td>Location</td>
<td>Outlet 2</td>
<td>Outlet 1</td>
<td>Outlet 3</td>
<td>Outlet 4</td>
<td>Outlet 5</td>
<td>Outlet 6</td>
</tr>
<tr>
<td>Q, average</td>
<td>240</td>
<td>72</td>
<td>48</td>
<td>80</td>
<td>56</td>
<td>36</td>
</tr>
<tr>
<td>volumetric</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>flow rate</td>
<td></td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>(L/min)</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>t, time to</td>
<td>58</td>
<td>196</td>
<td>294</td>
<td>176</td>
<td>252</td>
<td>392</td>
</tr>
<tr>
<td>displace</td>
<td></td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>30m pipe</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>volume (s)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Re,</td>
<td>55,600</td>
<td>16,700</td>
<td>11,000</td>
<td>18,900</td>
<td>13,300</td>
<td>7780</td>
</tr>
<tr>
<td>Reynolds</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>number</td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>Flow</td>
<td>Turbulent</td>
<td>Turbulent</td>
<td>Turbulent</td>
<td>Turbulent</td>
<td>Turbulent</td>
<td>Turbulent</td>
</tr>
<tr>
<td>Regime</td>
<td></td>
<td></td>
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<td></td>
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<td></td>
</tr>
</tbody>
</table>

Table 3.2 Summary of Phase III flushing conditions
3.3.4 Phase IV (July 27 – August 27, 2020)

One of the principal goals of this research is to compare qualitative and quantitative biofilm growth conditions that have a crucial influence on the water quality in DWDS under conditions of continuous vs intermittent water supply. Phase IV consisted of a priming during which two 90 m flow paths to Outlet 1 and Outlet 6 were exposed to flow regimes resembling continuous and intermittent flow over a four week period (July 27 to August 24, 2020). Discharge flow rates were controlled more precisely (and measured by flow rate meters) through reduced diameter outlets/orifices.

CWS conditions were emulated by continuous flow from the tank through pipes 3, 2, and 1 and discharged at Outlet 1 \((Q = 5.8 \text{ L/min}, v = .012 \text{ m/s - velocity in 100mm diameter pipe, } R_e = 1100, \text{ laminar regime})\) while maintaining a relatively constant head (steady state \(0.8 < H < 0.95\text{m}\) in the water tank (controlling by monitoring the inlet flow rate).

IWS conditions were simulated by daily flushing the 90m flow path to Outlet 6 \((222 < Q < 300 \text{ L/min}, R_e = 411,000 –550,000, \text{ turbulent regime})\) for approximately 10 minutes. Outlet 6 was opened and valves v10, v6, v8, v16, Outlets 4 and 5 were closed. The water tank is the connected and the pipes flushed from the tank to Outlet 6. When the supply was disconnected (v11 closed), Outlets 4, 5, and 6 was left open to drain residual water for approximately 15 minutes until emptied. The pipes were left to stagnate in this emptied state prior to the next flush, corresponding to a daily stagnation of approximately 23.5 hours.

Following priming Phase IV comprised a series of sampling events for three experiments:

A. On August 25, 2020, a steady state time series was measured for the CWS flow rate of 5.8 L/min. The pipes experienced the same flow as during the priming phase. Water samples
were collected at Outlets 0, 3 and 1 over a period of 266 minutes at 38 minutes increments. (It takes approximately 38 minutes for one full pipe volume to flow through.)

B. On August 26, 2020, a transient response to a change in the flow regime by increasing the flow rate for the CWS pipes (Table A.2) Water samples were collected at 1, 40, 50, 90, 180, 240, 300, 360, 420, 480, 600, 720 and 780 seconds. The flow rate was calculated by monitoring water tank levels over time.

C. On August 27, 2020, the transient response from a flushing event was measured for the IWS pipes. Outlet 6 was opened and valves v10, v6, v8, v16, Outlets 4 and 5 were closed. The flushing flow rate averaged 1.25 L/s with a spike of 2.47 L/s (Figure A.2). Samples were taken at Outlet 6 at 0, 10, 20, 30, 40, 50, 60, 120, 150, 180, 210, 240, 270, 300, 520, 540, 570, 600, 630, 660 and 720 seconds. The flow rate was lower compared to the IWS priming to ensure that the transient response was captured.
Chapter 4: Methods

4.1 Overview

The principal goal of this research is to understand how intermittent water supply affects the water quality through measurements of biomass and microbial ecology. During this initial phase of the research the primary focus has been on the enumeration of bacterial cells in the bulk water.

Historically, this has been accomplished by incubating a filtered sample of water on plates of selective solid media like R2A agar\(^7\) and counting the number of resultant bacterial colonies that grow on each plate. The Heterotrophic Plate Count (HPC) is defined as the number of colony forming units (CFUs) on a plate divided by the volume of water filtered onto that plate (CFU/mL) over a specified incubation period (typically 2-7 days) and temperature (typically in the range 22°C to 37°C; Allen et al., 2004). While this method provides a general estimate of the bacteriological load in water samples it is limited to culturable bacteria and hence, can grossly underestimate the overall microbial community that also contains protozoa and bacteria in the viable but non-culturable state. Although plate-based culture methods are widely used worldwide, there is no standardized test method of approved health standard\(^8\) for HPC. In distribution systems where there is no detectable residual disinfectant, EPA (1989) requires utilities to maintain HPC levels at or below 500 CFU/mL in at least 95% of the samples each month.

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\(^7\) R2A agar is a [culture medium](#) developed to study [bacteria](#) which normally inhabit [potable water](#). These bacteria tend to be slow-growing species and would quickly be suppressed by faster-growing species on a richer culture medium.

\(^8\) WHO (2002) concluded that there is no direct relationship between HPC values in ingested water and human health effects in the population at large.
The development of molecular methods for environmental microbiology (extraction of nucleic acids, genome amplification using (quantitative) polymerase chain reaction and sequencing of market genes such as 16s rRNA) has shown that bacterial communities are much more abundant and complex than previously understood from culture-dependent methods\(^9\) (e.g., Berry & Raskin, 2006).

Another long-standing technique for quantifying the biomass of microorganisms is measuring the concentration of adenosine tri-phosphate (ATP), a metabolic compound associated with viability that is found in all living cells (Holm-Hanson & Booth, 1966). This method effectively assumes that there is a relatively uniform level of microbial cells, then the total microbial mass is reported assuming a fixed ratio of organic carbon to ATP, C: ATP (250:1 was first proposed by Holm-Hanson & Booth, 1966). However, it is difficult to interpret the total bacterial cell count as i) most assays do not distinguish between microbial ATP and extracellular ATP; and ii) concentrations of ATP are not uniform in bacterial cells and can vary significantly between species and physiological states (Hammes et al., 2010). The ATP concentration is proportional to the light emitted in a luciferin-luciferase assay and can be measured with high repeatability using a luminometer. ATP is considered a fast, low-cost tool for monitoring drinking water systems (van der Wielen & van der Kooij, 2010; Hammes et al., 2010) and is particularly useful as a routine indicator of microbial activity (and hence, of biological stability and biofilm growth) for DWDS.

The current research originally planned to estimate biomass from fluorescence measurements of bacterial hydrolase enzyme concentration, using the enzymatic Bactiquant™ (Mycometer, Denmark) assay. This method does not require chemical extraction of the enzyme.

\(^9\)The drinking water microbiome consists of as many as 9,000 distinct taxa, with total cell counts ranging from \(10^3\) – \(5\times10^5\) /mL (Bautista-de los Santos et al., 2016).
from within cells (as required for ATP) and has been found to correlate well with direct total cell count measurements (FCM; EPA, 2011; Cheng et al., 2020), but lacks the reliability and repeatability of direct cell counting methods. Among non-culture based methods, optical approaches such as fluorescence microscopy and flow cytometry have become established as the most reliable methods for quantifying microbial abundance (Douterelo et al., 2014; van Nevel et al., 2017). The current research makes extensive use of flow cytometry (FCM).

FCM characterizes and quantifies individual particles suspended in a fluid stream by passing them through a laser light source and measuring the scatter (forward and side) and emission of light from the fluorescent particles (Figure 4.1; Shapiro, 2003; Hammes & Egli, 2010). Specific fluorescence\(^\text{10}\) can be introduced into bacteria through staining using selected nucleic acid dyes. The application used for this project utilized a combination of SYTO 9\(^\text{TM}\) (green dye which can penetrate the membranes of both intact and damaged bacterial cells; and propidium iodide (PI; red dye) which is only able to penetrate damaged cell membranes. PI has a higher affinity for DNA than SYTO 9 and hence, replaces the latter when exposed to the same DNA. As a result, FCM measurements using the two stains are able to distinguish cells with intact membranes (live; green fluorescence) from those with damaged cell membrane (dead; red fluorescence). By staining only DNA, FCM also differentiates between microbial cells and inorganic particles. FCM results can typically be obtained within 15 minutes (van Nevel et al., 2017).

Figure 4.1 illustrates the basic principle of FCM. The device measures the number of particles, their scattered light intensity and fluorescence. Section 4.3 describes the specific flow

\(^{10}\) Some such as algae contain fluorescent pigments such as chlorophyll
cytometer used in this study and explains the gating methodology that has been used to identify total, live and dead cell counts for water samples.

Figure 4.1 Basic principles of flow cytometry (FCM) analysis. Device measure scattering and fluorescence of stained cells. Histogram of forward scattering (FSC) shows the relative proportions of bi-disperse beads (2 and 3mm) separated by particle size, while the two parameter plot (in this case green fluorescence, FL1, and FSC) can be used to identify clusters of similar particles through different types of gating (described in more detail in section 4.3) (Hammes & Egli, 2010)

4.2 Environmental Parameters and Water Chemistry

In addition to cell counts, environmental parameters and water chemistry measurements were performed. Temperature (T, °C), total dissolved solids (TDS, mg/L), conductivity (σ, µS/cm) were measured on site with the HACH HQ14D Portable Conductivity and TDS Meter (HACH Lange, Germany). During Phase I-III, the average volumetric flow rate (Q, L/min) was estimated from the measured time to fill a fixed volume from the 25 mm sampling tap. During Phase IV, two Gardena Water Computer FlexControl devices were installed to control and monitor volumetric flow rate (GARDENA, Germany). One was installed at the inlet from the
source tap to the water tank along with a float valve to control the refill rate. The second was installed at the exit hose of Outlet 1 to monitor the discharge rate.

Concentrations of various nutrients and ions were measured in triplicates. Ammonia (NH$_3$-N), nitrite (NO$_2$-N), nitrate (NO$_3$-N), orthophosphate (PO$_4$-P), and total phosphorus (TP) were determined with the HACH DR-3900 spectrophotometer (HACH Lange, Germany). The concentrations of total organic carbon (TOC), total carbon (TC), total inorganic carbon (TIC), and total nitrogen (TN) were obtained by Shimadzu TOC-L analyzer (SHIMADZU, Malaysia). Bound and free chloride (Cl$^-$), phosphorus (P) and sulphate (SO$_4^{2-}$) concentrations were determined by Shimadzu ion chromatography (Prominence HIC-SP, SHIMADZU, Malaysia). The analysis for these parameters were done in the lab.

During each Phase, two liters of water was collected for each sampling event in a sterile beaker before being transferred to two sterile one liter glass bottles (DURAN, Germany). Between sampling times, the collected samples were kept in a cooler until they could be transferred to the lab and stored at 4 °C for subsequent physio-chemical analysis.

The biofilm on pipe walls were also selectively swabbed by first draining the water from the pipes, opening the respective sampling window after wiping the outside with ethanol before four to five swabs on the same area were taken, transferred to 1 ml of a sterile 0.8% NaCl solution in 2 ml reaction tubes and then put on ice. This thesis does not address the subsequent analysis and results from these swabs.

4.3 Flow Cytometry

Flow cytometry measurements (FCM) have been obtained using the Molecular Probes LIVE/DEAD$^\text{TM}$ BacLight$^\text{TM}$ Bacterial Viability and Counting Kit (ThermoFisher, USA) was
used to quantify total and live bacterial cell counts using the Beckman Coulter CytoFlex flow cytometer (Beckman Coulter, USA). The test procedure uses a 1 ml sample stained with 1.5 µL of SYTO9 stain (3.34 mM) and 1.5 µL of propidium iodide (PI) (20 mM) in filtered dimethyl sulfoxide (DSMO) and incubated in the dark for 25 minutes at 35 °C. The FCM analysis was performed in triplicates for each sample at a flow rate of 60 µL/min and was calibrated (diluted; 1:4 with NaCl) to measure the bacterial count of 200 µL of the sample. Fluorescent dyes SYTO9 and PI were excited at a wavelength of 488 nm and emissions measured at 561 nm (green) and 640 nm (red), respectively. The samples were run through the flow cytometer within a few hours of sample collection.

After samples were run through the flow cytometer, FlowJo™ software (Beckman Coulter, USA) was used to analyze the result. A gating methodology was developed to identify areas of live, dead, and total cell counts using negative, positive, heat-killed, and filtered control samples consisting of regrown biofilm extracted from an operational DWDS (Section 4.3.1). This gating methodology was iteratively applied to recalibrate the gating areas between each sampling Phase I-IV to maintain consistency and ensure comparability between measurements.

4.3.1 Flow Cytometry Gating Procedure

FCM data was gated by graphing the Forward Scatter Height (FSC-H) against the SYBR Green (Comp-FL1-H: SYBR Green FITCH-H), which graphs cell size against fluorescence (McKinnon 2019). This initial graph identified the first gate to identify total cell counts by using a negative and positive control. The negative control was run using a 1 ml 0.8% NaCl sample (Figure 4.3). The reading identified the machine noise present in the equipment. For the positive control, a young sensor biofilm originating from an operational DWDS (obtained during a
previous experiment conducted by Dr. Cheng Dan) was used (Figure 4.4a). The positive control was incubated at 37 °C for 2-3 hours to reach log stage in which highest ratio of viable bacteria can be expected before being dissolved in 0.8% NaCl, diluted (1:5000\(^{11}\)) and split into two. One half remained at room temperature and was used for the positive control. By comparing the fluorescence to the negative control, the area for the first gate is identified. There is a clear lobe of fluorescence in the positive sample indicating the presence of bacteria.

![Graph showing fluorescence data](image)

**Figure 4.2** Phase I negative control sample (0.8% NaCl solution) stained with SYTO9 and PI. Data shown is instrumentation noise. Gated area shows expected area for bacterial cells.

\(^{11}\) Cell concentration needs to be below $10^6 – 10^7$ cell/mL for FCM.
Figure 4.3 Phase I positive control showing young sensor biofilm. a) Cluster of points defines the region for total cell count b) Clustering of green and red fluorescence intensity differentiates between dead (more intense red fluorescence), double-stained (red and green fluorescence) and live cells (more intense green fluorescence). Double-stained cells are considered dead because they are not viable.

Once the scatter identifying bacteria cells was determined, SYBR Green (intact fluorescence, Comp-FL1-H: SYBR Green FITC-H), was graphed against PI (dead fluorescence, COMP-FL3-H: PI PC5.5-H). The second half of the young sensor biofilm was heat treated at 95 °C for 10 minutes, killing all the bacteria in the sample (Figure 4.4). Then, the sample was run through the flow cytometer and the resulting scatter only identified dead bacteria. The positive control young sensor biofilm was compared against heat-treated young sensor biofilm to differentiate between live and dead cells. (Figure 4.3b, 4.4b)
Figure 4.4 Phase I heat-treated positive control of young sensor biofilm. a) cluster identifies the total cell counts b) differentiates between dead, double-stained and live cells. Double-stained cells are considered dead because they are not viable. Compared to Figure 4.4b, the data has shifted to the upper left, identifying the scatter expected from dead cells.

Filtered samples were used to further validate background noise from the flow cytometer and reagents (Figure 4.5). A sample was filtered with a 0.22 µm syringe filter to remove particles as well as bacteria and registered as a blank sample. Bacteria typically measure in the 0.2–2.0 µm in diameter and 2-10 µm in length. There is a clear similarity between Figure 4.3 and 4.6 where no cells were counted, as expected given the treatments both samples received.

Blanks and reference values in FCM are crucial in identifying bacterial load against background noise of the particularly sensitive CytoFlex FCM. Residue from other samples, machine noise, and inorganic particles can all affect the number of events/mL detected. The gating
Figure 4.5 Phase I filtered sample a) cluster gates where total cells should be, but only instrumentation noise is measured b) Total cell counts clustered by dead, double-stained and live. A negligible quantity of cells was counted and affirm the region of events that are instrumentation noise.

methodology was applied across all Phases with changes to the gating location depending on the scatter from control samples for each Phase. Once gating was complete, cell counts could be extracted from FlowJo. Subsequent analysis was completed using Excel (Microsoft, USA) RStudio (RStudio PBC, USA), and GraphPad Prism 8 (GraphPad Software, USA).

Flow cytometry has been commonly used for measuring cell counts with less than a 3% relative standard deviation on measurements from a single user and less than 7% variability across laboratories and instruments (van Nevel et al., 2017). The raw flow cytometry data generated from CytoFlex was analyzed independently by operator A (Dr. Mats Leifel, NTU) and operator B (Sophia Wu, MIT). Operator A used CytExpert software while Operator B used
FlowJo. Both applied the gating methodology described independently. Table 4.1 enumerates the ratio of cell counts between the two operators.

<table>
<thead>
<tr>
<th>Phase</th>
<th>Live Cell Counts</th>
<th>Dead Cell Counts</th>
<th>Total Cell Counts</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>1.27</td>
<td>0.62</td>
<td>0.76</td>
</tr>
<tr>
<td>IIa</td>
<td>0.78</td>
<td>0.97</td>
<td>1.02</td>
</tr>
<tr>
<td>IIb</td>
<td>1.05</td>
<td>1.08</td>
<td>1.04</td>
</tr>
<tr>
<td>IIc</td>
<td>1.03</td>
<td>1.06</td>
<td>1.01</td>
</tr>
<tr>
<td>III</td>
<td>1.08</td>
<td>1.09</td>
<td>1.06</td>
</tr>
<tr>
<td>IVa</td>
<td>0.96</td>
<td>1.07</td>
<td>1.06</td>
</tr>
<tr>
<td>IVb</td>
<td>1.01</td>
<td>1.17</td>
<td>1.15</td>
</tr>
<tr>
<td>IVc</td>
<td>0.95</td>
<td>1.05</td>
<td>1.02</td>
</tr>
</tbody>
</table>

Phase I had the greatest discrepancy between flow cytometry results (30% average – which may reflect inexperience of operator B). Phases II-IV had an average 3% deviation between Operators A and B for total, live, and dead cell counts using gating analyses.
Chapter 5: Results

5.1 Testbed Conditions

Phases I through IV took place from April to August 2020. The source water was obtained directly from the water distribution system on the NTU campus. NTU and SCELSE employ remote and real-time monitoring of the levels of monochloramine (1.08-3.23 mg/L), TOC (3.97±2.05 mg/L), TN (0.46±0.03 mg/L), ammonia (0.22±0.04 mg/L), nitrite (0.02±0.02 mg/L), and nitrate (0 mg/L), conductivity (250-430 µS/cm) in its DWDS. Monochloramine levels were also monitored manually directly from the tank and pipes during Phase IV (Figure 5.1). Given the proximity of the source to Nanyang House, a student activity center on NTU campus, as well as several canteens and student dormitories, variations in source water were expected depending on usage (local demand). This was not explicitly monitored during the initial experiments, but carefully monitored during Phase IV (first round of tests that compare IWS and CWS conditions).

The water tank was refilled through a hose inserted at the top of the tank. Tank water parameters were measured using samples from taps at the bottom of the tank. The temperature of water samples from the tank and pipes averaged between 26 and 32 °C across the Phases (Figure 5.2).

Conductivity, an indicator for source water changes, in the tank ranged from 250 to 280 µS/cm (Figure 5.3) across the Phases.
Figure 5.1 Concentration of monochloramine measured during Phase IV

Figure 5.2 Phase I-IV water sample temperatures from a) tank and pipes b) tank
Figure 5.3 Phase I-IV sample conductivity a) All samples including tank and pipe outlets b) tank samples
5.2 Phase I: Slow Flow

The Phase I test program used FCM to measure cell counts for slow flow. The testbed was primed during the preceding weeks (March 16 to April 3, 2020) by i) CWS flow to Outlet 1 (Q ≈ 0.5 L/min, v ≈ 0.001 m/s, Re ≈ 100); and ii) IWS flow to Outlet 6 with drainage twice per day and stagnant intermediate conditions where pipes are filled with water. Samples were taken as a time series every 30 minutes. Assuming a fixed velocity, these can be related to distances from the outlet along the pipes (Figure 5.4).

In the pipes primed for CWS, laminar flow conditions were maintained and (Figure 5.4a) show steady total cell counts with average, \( N_T = 35,000 \text{ cell/mL} \). The live cell count decreased with distance from Outlet 1 (\( N_L = 12,000 \) to 5500 cell/mL) such that the ratio of live to total cells (R) decreased 11% over the pipe distance measured (d = 43.8m). The pipes primed under IWS conditions (Figure 5.3b) show much more variability across Outlets 4 and 6, potentially reflecting the effect of the priming conditions over the pipe length. The live (\( N_L \)) and total (\( N_T \)) cell counts decreased from 143,000 to 5800 cell/mL and 165,000 to 48,000 cell/mL, respectively.

A Kruskal-Wallis was performed comparing the mean ranks of Outlets 1, 3, 4, and 6’s cell counts. No statistically significant difference was observed across outlets 1, 3, 4, and 6. This meant that the pre-treatment between the CWS and IWS pipes did not generate statistically significant differences between across the pipes. A Spearman correlation matrix was run comparing the live, dead, total cell counts and ratio of live to total cell counts against the TDS, temperature, conductivity, ammonia (NH\(_3\)-N), nitrite (NO\(_2\)-N), and nitrate (NO\(_3\)-N). None of the parameters were correlated the cell counts to the 1%, 5% or 10% significance level. Phase I results act as a baseline to compare the effects of the extended stagnation phase from COVID-19.
Figure 5.4 Phase I characterization a) Cell counts of CWS conditions \((Q \approx 0.5\ \text{L/min}, v \approx 0.001\ \text{m/s}, R_e \approx 100)\) b) Cell counts of IWS conditions (pipes stagnant drained gently twice per day) Ratio is number of live over number of total cells. c) Total dissolved solids (TDS, mg/L) and Conductivity (µS/cm)
5.3 Phase II: Effect of Stagnation

Phase II measured conditions in the testbed after a 10 week stagnation period caused by the Circuit Breaker regulations (Singapore Ministry of Health, 2020) resulting in a total lockdown of NTU campus. The pipes were full and stagnant throughout this period. Phase IIa (July 6, 2020) obtained samples from each of the seven pipe outlets separately as the stagnant water was slowly drained. The pipes were then refilled with a fresh water tank (water age less than 24 hours). Phase IIb samples (July 15, 2020) were obtained as the water was slowly drained. Phase IIc samples (July 15, 2020) were sampled 60 minutes after IIb as the water was slowly drained \((Q = 400 \text{ mL/min})\). Phase IIa samples characterize the water and microbial context after ten weeks of stagnation while IIb and IIc compare conditions for a shorter, week-long stagnation.

Given that pipe runs experienced the same stagnation event, it was expected that the cell counts should be similar across Outlets 1-6. The TDS and conductivity measurements (Figure 5.4b) would suggest similar cell counts. However, across Outlets 1-6, the live, dead, and total cell counts had a wide range. Dead cell counts varied the least and total cell counts varied the most (Figure 5.5a, \(30,800 < N_L < 120,600 \text{ cell/mL}; 67,200 < N_d < 110,800 \text{ cell/mL}; 111,000 < N_T < 215,000 \text{ cell/mL}\)). This indicates heterogeneous microbial activity across the pipes (that are exposed to slightly varying shading by surrounding vegetation that could not be maintained during Circuit Breaker). The tank water had decreased TDS and conductivity measurements (254 mg/L, 162 \(\mu\)S/cm) compared to the outlet pipes (317 mg/L, 204 \(\mu\)S/cm). This difference can be attributed to UV light on a larger mass of water. Outlet 0 also had a much lower TDS and conductivity measurement (176 mg/L, 277 \(\mu\)S/cm), which may be due to its spatial proximity to the tank.
Figure 5.5 Phase IIa characterization a) Cell counts b) TDS and Conductivity. Ratio is number of live over number of total cells.
Between Phase I and Phase IIa, the total, live, and dead cell counts increased. Average cell count increases over the 10 week stagnation are as follows: \( N_L = 9000 - 85,800 \text{ cell/mL} \), \( N_D = 56,000 - 87,200 \text{ cell/mL} \), \( N_T = 65,400 - 173,000 \text{ cell/mL} \). Due to the low flow rate in both sampling regimes, the difference is characteristic of bulk water changes. Average conductivity increased from 290 to 317 \( \mu \text{S/cm} \) and average TDS from 186 mg/L to 204 mg/L. This indicated growth of microbial communities in the bulk water over the stagnation period with total cell count growing by a factor of 2.6 and live cell counts by 9.4.

A two-tailed Wilcoxon test was performed comparing the Phase I and Phase IIa data to determine if these groups are different in a statistically significant manner to the 5% significance level. For live cell counts, a p-value of less than 0.0001 was observed, concluding that the Phase I live cell counts and Phase IIa live cell counts are different at a significance level of 5%.

Both Phase IIb and IIc (Figure 5.6a) show clear increases in cell counts in the tank \( (N_T = 480,000 \text{ cell/mL}) \) and Outlets 0-6 \( (N_T = 177,800 \text{ cell/mL} \) and \( 183,000 \text{ cell/mL} \), for Phases IIb and IIc, respectively) compared to the source water \( (\text{tap } N_T = 54,000 \text{ cell/mL}) \). Since the tap water has a lower water age and monochloramine present from the NTU DWDS, low cell counts were to be expected. The tank and testbed pipes were not in contact with chemical disinfectant during the stagnation period and therefore, microbial growth was anticipated. The Phase IIb and IIc counts show no significant difference from each other as expected, given that IIc is a replicate sample taken 60 minutes later with the same flow conditions. The Phase IIb and IIc cell counts, TDS and conductivity remained steady across the one hour time difference between samples. The Phase IIb and IIc live cell counts (average, \( N_L = 121,700 \) and \( 110,000 \text{ cell/mL} \), for IIb and IIc, respectively) increased compared to Phase IIa \( (N_L = 85,800 \text{ cell/mL}) \) due to the additional week between samples where the microbes had the opportunity to grow. After Phase IIa
sampling, the pipes were refilled with newly supplied water from the tank. This introduced nutrients into the bulk water and created the opportunity for increased microbial activity. This is reflected in the increase of the ratio of live to total cell counts between the two sampling dates (45% to 70%).

Figure 5.6 Phase IIb and IIc characterization a) Cell counts b) TDS and Conductivity. Ratio is number of live over number of total cells.
5.4 Phase III: Pipe Flushing

Phase III involved collection of a time series of water flushing for each 30m pipe section (July 15 and July 16, 2020). Table 3.2 summarizes the average hydraulic conditions for the experiments over the course of the five minute time series. There is considerable reduction in flow rate in subsequent flushing experiments due to drops in the tank head.

Figure 5.7 and 5.8 summarize the results of the Phase III flushing tests from July 15 and 16, respectively. Each of the figures compares the time series measured at Outlets 1-6 with measurements from Outlet 0 at the start of flushing (t = 0 seconds) and the end (t = 300 seconds). In each experiment, the outlet cell count is elevated at the start of flushing and decreases with time. The initial conditions are expected to be similar across all 6 outlets. Differences in measured response should reflect differences in hydraulic conditions (Table 3.2) and other environment factors between the two days of flushing (temperature, drainage state, etc.).

Across all the outlets, there is an increase in cell count at each outlet’s first reading (10 seconds) compared to the inlet sample (Outlet 0, at t = 0 s), indicating the removal of cells from pipe walls from flushing. The total cell counts decrease with flushing time. Over time, the cell counts stabilize and are at similar levels to each inlet sample (Outlet 0). Outlets 1, 3, 4, 5, and 6 show downward trends in total cell counts, indicative of a decrease in the amount of biofilm sloughed off of pipe walls during the transient phase, before reaching a steady state. In general, there are higher cell counts produced after 300 seconds of flushing for Outlets 4, 5, and 6 (Figure 5.8; $N_T = 23,000$ cell/mL) compared to those for Outlets 2, 3, and 1 (Figure 5.7; $N_T = 38,000$ cell/mL).
Figure 5.7 Phase III cell counts from July 15, 2020 a) Outlet 2 b) Outlet 3 c) Outlet 1. Outlet 0 measured the inlet water during flushing.
Figure 5.8 Phase III cell counts from July 16, 2020 a) Outlet 4 b) Outlet 5 c) Outlet 6. Outlet 0 measures the inlet water during flushing.
During sampling on July 15, the hydraulics were controlled less precisely than during sampling on July 16. This was part of the learning curve when executing the flushing protocol. Phase III Outlet 2 shows a increase between the inlet ($N_T = 80,000$ cell/mL) and first sampling ($N_T = 267,000$ cell/mL). At 30 seconds, the total cell count drops to 46,000 cell/mL, which is very close to the steady state average ($N_T = 38,000$ cell/mL). This is likely due to high flushing rate ($Q = 240$ L/min) experienced relative to the other outlets (Table 3.2). The transient phase occurred during the first 30 seconds and the time intervals monitored the steady state. Outlet 3 had an anomalous inlet cell count of 237,000 cell/mL (Outlet 0 at $t = 0$ s) that was very similar to the initial reading of 305,000 cell/mL (Outlet 3 at $t = 10$ s). Then, cell counts decreased to 26,300 cell/mL over the next 300 seconds. Outlet 1, 4, 5, and 6 showed similar trends of low inlet cell counts (Outlet 0 at $t = 0$ s ) before increasing at the first sampling and then decreasing steadily ($N_T = 250,000 - 20,000$ cell/mL; $N_T = 210,000 - 40,000$ cell/mL; $N_T = 242,600 - 26,600$ cell/mL; $N_T = 160,600 - 50,400$ cell/mL for outlets 1, 4, 5, and 6, respectively).

Given the hydraulic conditions experienced by each outlet, it is expected that Outlets 1 and 4 ($Q_1 = 72$ L/min, $Q_4 = 80$ L/min), and Outlets 3 and 5 ($Q_3 = 48$ L/min, $Q_5 = 56$ L/min) look the most similar despite the order that the outlets were flushed. Both Outlets 1 and 4 reach the baseline cell counts at approximately 120 seconds of flushing, which is equivalent to approximately 0.66 and 0.68 flushed pipe volumes, respectively. Given the anomalous inlet (i.e. Outlet 0) measurements for Outlet 3, the results are inconclusive for the volume required to restore baseline cell counts. Outlet 5 required approximately 240 seconds (1.19 flushed pipe volumes) to reach baseline conditions. Outlet 5 had a lower volumetric flow rate, thus presumably sloughing off less biofilm per second. Outlet 6 has the slowest volumetric flow rate and did not reach steady state before the sampling window ended (inlet $N_T = 27,000$ cell/mL.
versus outlet $N_{T6} = 50,000 \text{ cell/mL after 300 seconds}$). Nevertheless, the data shows a clear trend of decreasing outlet cell count before reaching steady state conditions, where there is little further removal of cells from pipe walls (i.e. inlet cell counts are equal/similar to outlet cell counts).

Across all outlets, there are significantly higher levels of nitrate (average NO$_3$-N is 0.375 mg/L) compared to nitrite (average NO$_2$-N is 0.071 mg/L) and ammonia (average NH$_3$-N is 0.055 mg/L) across all outlets (Figure 5.9). This indicates the presence of ammonia-oxidizing bacteria (AOB), which oxidize ammonia to nitrite and nitrite-oxidizing bacteria (NOB) that produce nitrate. Total nitrogen stayed relatively steady over time across all of the outlets, averaging approximately 0.453 mg/L. Similarly, nitrate showed little variation over time with an average across all outlets of 0.375 mg/L. Outlet 2 shows steady values of ammonia, nitrite, and nitrate across the time series (0.055, 0.071, 0.373 mg/L, respectively). Outlets 1, 3, 4, and 5 show a noticeable drop in ammonia levels in the first sample taken at 10 seconds (0.01~ mg/L). Values for ammonia return to concentrations measured at the inlet after 30 (Outlet 3: 0.06 mg/L) or 60 seconds (Outlet 1, 4, 5: 0.07, 0.07, 0.06 mg/L respectively). Nitrite concentrations followed a similar trend at ammonia with an initial decrease before a return to inlet conditions after 30 (Outlet 3: 0.072 mg/L) or 60 seconds (Outlet 1, 4, 5 : 0.083 mg/L, .103 mg/L, 0.084 mg/L).

Total carbon (TC), inorganic carbon (IC), and total organic carbon (TOC) measurements for individual outlets over time are shown. TC, IC, and TOC concentrations averaged 14.25, 13.5 and 0.66 mg/L across all samples in Phase III. Water parameters relating to carbon remained steady over the time series with little variation (TC: 13.6 – 15.2 mg/L, TIC: 13-14.4 mg/L, TOC: 0.25 – 1.2 mg/L).
Figure 5.9 Phase III nitrogen parameters for a) Outlet 2 b) Outlet 3 c) Outlet 1 d) Outlet 4 e) Outlet 5 f) Outlet 6. Outlets are ordered by sampling sequence and graphed against time.
Figure 5.10 Phase III carbon parameters for a) Outlet 2 b) Outlet 3 c) Outlet 1 d) Outlet 4 e) Outlet 5 f) Outlet 6. Outlets are ordered by sampling sequence and graphed against time.
5.5 Phase IV: Continuous versus Intermittent Operations

The Phase IV experiments compare cell counts and water quality for two 90m pipe sections that were primed for a four week period with i) CWS (Q_{\text{CWS}} = 5.9 L/min, laminar flow discharging through Outlet 1) and ii) IWS with a daily flushing event (Q_{\text{IWS}} = 222 - 300 L/min L/min, turbulent flow discharging through Outlet 6) and left drained in between. Phases IVa and b compare a time series for CWS pipes (Outlet 1) at i) the same steady continuous slow flow rate (Q_{\text{IVa}} = 5.9 L/min) used during the priming phase and ii) steady flow (Q = 55 L/min) equivalent to turbulent conditions. Phase IVc obtains a time series flushing for the IWS pipe from Outlet 6 with an average flow rate, Q = 72 L/min.

Phase IVa samples (Figure 5.11a) to Outlet 1 (via pipes 3, 2 and 1) provide direct evidence for the steady state conditions in CWS pipes from the inlet (Outlet 0). Samples were collected at 38 minute increments corresponding to a 30 m pipe volume (i.e. at this flow rate Q = 5.8 L/min, Re = 1250, the 90 m pipe volume was replaced every 114 minutes). Figure 5.11a shows that all cell counts remain stable (N_T = 28,000 – 31,900 cell/mL; N_L = 4000-6000 cell/mL, and N_D = 21,500 – 27,000 cell/mL) over a period of 266 minutes (two full pipe volumes from the tank). The ratio of live to total cell counts was low (N_L/N_T = 15-24%). This is significantly lower than the ratio of live to total cell counts from Phase II and Phase III samples (N_L/N_T = 40% and 70% in Phases II and III; Figures 5.5, 5.6, 5.7).

Phase IVb samples measure the transient effects of increasing the flow rate into a turbulent regime (Q = 55 L/min, Re = 11,600). There is an initially significant increase in the total cell count compared to inlet conditions at Outlet 0 (approximately a factor of 5). Live cell counts increase by a factor of nine compared to inlet conditions (Outlet 0). After 20 seconds
Figure 5.11 Phase IVa and IVb cell counts a) Phase IVa CWS (Q = 5.9 L/min) b) Phase IVb CWS (Q = 55~ L/min). Ratio is live to total cell count (N_L/N_T).

(equivalent to a 2.3 m length of pipe), the total cell count steadies out to approximately ~41,000 cell/mL. Live cell counts initially account for 50% of the biomass measured when the flow rate is increased, but only 25% after 30 seconds. This steady state cell count (Q_T = ~41,000 cell/mL) is higher than the steady state from laminar flow (Phase IVa, Q_T = 28,000 cell/mL).
Phase IVc IWS Outlet 6

![Graph showing cell counts over time](image)

Figure 5.12 Phase IVc cell counts for flushing from Outlet 6 after IWS priming

Phase IVc measured a time series of cell counts during flushing of the IWS pipe section that had experienced daily wet and dry cycles during the priming period. The pipes were flushed with water at approximately 220 L/min once per day, drained, and then left in an empty (partially filled or nearly dry state). During sampling, the flushing rate initially varied from ~50 to 155 L/min and settled out to approximately ~75 L/min. Flushing rate was calculated by monitoring the water level in the tank (Appendix Table A.3). The slower sampling rate (compared to priming rate) was to ensure that the transient data was captured. Samples taken from Outlet 0 corresponded to inlet conditions. Outlet 0 samples were consistent over time (Figure 5.12). Therefore, any difference measured at can be attributed to sloughing/erosion of biofilm from the pipe walls, which is then transported in the bulk water. Cell counts spike during initial flushing, but then decrease and revert toward inlet levels.
Figure 5.13 Phase IVc flow rate. Hydraulic conditions calculated by decrease in water tank levels over time (Table A.3)

Flow rates for the IWS primed section of pipe (Figure 5.13, Q = ~75 L/min) are similar to Phase IVb (Q = ~55 L/min). The principal difference in these two data sets is the initial filling of the pipe section (IVb starts full and IVc fills at the start of the flushing process). Phase IVc total cell counts are high for the first 60 seconds of flushing (peaking at 7 times the inlet count; Outlet 6 $N_T = 45,500$ cell/mL at 1 s versus $N_T = 7,000$ cell/mL at the inlet). After approximately seven minutes (420 s) of flushing, the Outlet 6 total cell count is similar to inlet total cell count. For live cell counts, Outlet 6 sees an initial increase of 18 times ($N_T = 23,400$ cell/mL at $t = 0$ s vs inlet $N_T = 1300$ cell/mL) before decreasing to approximately six times ($N_T = 8000$ cell/mL over 60 seconds). Similar to total cell count, it takes approximately 7 minutes of flushing for the live cell count at Outlet 6 to equal inlet cell conditions.

Figure 5.14, 5.15 and 5.16 summarize the measurements of physiochemical parameters during Phases IVa, IVb, and IVc, respectively, including total dissolved solids (TDS), conductivity, nitrogen (total nitrogen, ammonia, nitrite, nitrate) and carbon (total carbon, total inorganic carbon, total organic carbon) concentrations.
TDS and conductivity remained very stable in Phases IVa and IVb, as expected for CWS priming and flow (Figure 5.14a, 5.15a). However, TDS and conductivity in Phase IVc (Figure 5.16a) followed the same trend as cell counts for the IWS pipe. There was an initial increase for both TDS (166 to 172 mg/L) and conductivity (259 to 266 µS/cm) before decreasing back to inlet conditions after about 300 seconds.

Similar to the TDS and conductivity, all nitrogen parameters (total nitrogen, ammonia, nitrite, and nitrate) in Phase IVa and IVb remained steady over time (Figure 5.14b, 5.15b). For the IWS case, the flushing event affected the concentration of all parameters (Figure 5.16b). Ammonia and nitrite levels drop from 0.118 and 0.040 mg/L, respectively, to 0.001 and 0.007 mg/L at 10 seconds. Then, ammonia levels return to inlet levels (0.118 mg/L) at approximately 180 seconds of flushing (0.116 mg/L). After 30 seconds, nitrite levels exceed inlet levels (reaching 0.067 mg/L) before returning to inlet levels at 300 seconds (.040 mg/L). Inlet concentrations of total nitrogen (0.48 mg/L) and nitrate concentrations (0.38 mg/L) initially increased to (0.75 and 0.65 mg/L, respectively) before decreasing and reaching inlet levels at 240 and 180 seconds, respectively.

Across all of Phase IV, the hydraulic conditions did not affect TC, TIC, and TOC levels, evidenced by its steady state over time (Figure 5.14c, 5.15c and 5.16c), however, the priming conditions did slightly affect the concentrations of TC, TIC and TOC. Slow moving CWS conditions resulted in TC, TIC and TOC levels of 12.4, 10.7, and 1.7 mg/L, respectively (Phase IVa). Increasing the flow rate in the CWS pipes (Phase IVb) decreased TC, TIC and TOC levels to 11.6, 10.2, and 1.44 mg/L, respectively. The IWS pipes had TC, IC, TOC levels of 12.1, 10.85, and 1.25 mg/L, respectively. IWS conditions had lower levels of TOC compared to CWS.
Figure 5.14 Phase IVa CWS flow from Outlet 1 at 5.8~ L/min a) TDS and Conductivity b) Nitrogen parameters c) Carbon parameters
Figure 5.15 Phase IVb CWS flow from Outlet 1 (Q = 55~ L/min) a) TDS and Conductivity b) Nitrogen parameters c) Carbon parameters
Figure 5.16 Phase IVc IWS flow from Outlet 6  
a) TDS and Conductivity  
b) Nitrogen parameters  
c) Carbon parameters
5.6 Summary

The results show clear evidence of significant increases in biomass associated with higher flow rates and priming conditions. These results quantify the effects of transport of biomass from pipe walls in the testbed. Phase I quantified variations in biomass along sections of the testbed experiencing a near stagnant flow regime. Pipes that had constant slow flow had more stable cell counts ($N_T = 65,000$ cell/mL) compared to pipes that were gently drained twice per day ($48,000 < N_T < 165,000$ cell/mL). Phase II, sampled in a slow/laminar flow regime, measured biomass changes in stagnant and full pipes from the 10 week stagnation period (total and live cell counts by 2.6 and 9.4 times). Phase III obtained results for a time series flushing pipes over turbulent flow regimes from stagnant and full pipes. Cell counts initially increased due to initial sloughing of loose deposits and biofilm before decreasing back to inlet conditions. Phase IV quantified differences in biomass associated with two flow regimes (laminar and turbulent) in CWS conditions and how CWS compares to IWS conditions. In CWS conditions, increasing flow rate by a factor of 10 ($Q = 5.9$ L/min and 55 L/min for Phases IVa and IVb) increased the steady state cell count by approximately 13,000 cell/mL (46% increase). During the transient phase of IWS conditions, total cell counts initially increase by a factor of 7 and live cell counts by a factor of 18 times the inlet counts.
Chapter 6: Summary, Conclusions, and Recommendations

The overall goal of this research is to investigate how cycles of flushing and draining associated with intermittent water supply systems affect the amount of suspended biomass through a program of controlled field experiments. Through collaborations with researchers at SCELSE and NTU, a testbed comprising an array of 100mm diameter PVC pipes was constructed on the campus of Nanyang Technological University in Singapore. The pipe array is supplied with water through a common header pipe from a tank and flow paths are controlled through a set of valves, with samples obtained at up to 7 pipe outlets. To date, 4 Phases of tests have been performed. Total and live/intact cell counts have been measured using Flow Cytometry, together with a set of physiochemical water parameters.

The testbed was completed in February 2020 and initial biofilm formation was seeded by stagnating water in the pipes over a 4 week period. Two 90 m long flow paths were then primed under conditions of 1) steady laminar flow (CWS) and 2) a daily cycle of drainage and partially filling (initial emulation of IWS). The Phase I test program measured biomass and physiochemical water parameters in slow laminar flow. The test program was then interrupted by the COVID-19 pandemic and the testbed was shutdown for a period of 10 weeks (April – July 2020) due to lockdown of NTU. After resuming operations, Phase II investigated changes in biomass in the bulk water associated with the extended period of stagnation (pre- and post-shutdown). Phase III comprised a set of experiments to collect time series data for flushing of fresh tank water through six independent, 30 m long pipe sections. Phase IV involved careful priming of two 90 m long flow paths for a period of 4 weeks: 1) representing laminar flow in CWS; and 2) simulating a daily supply cycle for IWS (flushing over a short time window) and
then allowing the pipe to drain and remain in a partially-filled state until the next supply period. For the CWS system, we measured and compared inlet and outlet cell counts and physiochemical parameters for steady laminar flow ($Q = 5.8$ L/min over 266 minutes) and turbulent flow regimes ($Q = 55$ L/min over 12 minutes), Phases IVa and IVb. Similar measurements were obtained for the IWS section where flow rate varies between the initial filling and full-bore flow conditions (with average $Q = 75$ L/min; turbulent flow regime). The data from Phase IV provide the first definitive evidence quantifying how erosion of biofilm along the pipe walls contributes to the biomass transported in the bulk water. A step change in flow rate in the CWS section cause the initial cell count to increase by a factor of 10, but differences diminished over a period of 3-4 minutes. Initial filling of the IWS was associated with large increase in total and live cell counts (factors of 7 and 18, respectively).

At the time of this writing, the testbed is undergoing another 4 week priming phase under the same controlled CWS and IWS conditions as Phase IV. This process will be replicated twice to generate triplicate datasets to confirm repeatability of results. Future phases of work for this testbed could include priming six 30 m sections of pipe in parallel instead of two 90m sections to provide replicate datasets that have experienced identical priming conditions. Another set of experiments involving monitoring biomass changes while varying monochloramine concentrations

These preliminary results only quantify total biomass and analysis to understand the relative abundance of microbial taxa should be performed. Phenotyping analyses (PhenoFlow\textsuperscript{12}, 16s rRNA amplicon sequencing etc.) on collected samples will provide insight in how the

\textsuperscript{12} PhenoFlow translates flow cytometry data into diversity estimates of microbial communities.
bacterial diversity of microbial communities are affected by the different growth conditions experienced.

More experiments should then be conducted to better understand microbial community changes before any water policy recommendations regarding IWS are made. After microbial diversity and pathogen concentrations are analyzed, an applicable future question is to seek how operational strategies can be implemented to improve water quality and decrease pathogen concentration in intermittent water supply.
## Appendix

**Table A.1 Phase IVb Sampling Flow Rate**

<table>
<thead>
<tr>
<th>Time (sec)</th>
<th>Tank Level (m)</th>
<th>Flow Rate (L/sec)</th>
<th>Flow Rate (L/min)</th>
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<tr>
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<td>0.990</td>
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**Table A.2 Phase IVc Sampling Flow Rate**

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Bibliography


