SCHOOL OF COMPUTING, ENGINEERING AND MATHEMATICS

BIOFILM GROWTH AND CHLORINE STABILITY IN THE RECYCLED WATER DISTRIBUTION SYSTEM

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Declaration

To the best of my knowledge and belief this thesis contains no material previously published by any other person except where due acknowledgment has been made.

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ABSTRACT

Wastewater recycling is widely practiced to solve water crises created by increasing demand due to rapid population growth and scarcity of resources arising from climate change. Certain treatment is always provided to meet the appropriate health guidelines of the recycled water. When water is distributed over the pipelines and tanks, microbes can regrow and deteriorate water quality, and hence a disinfectant, usually chlorine, is added to the water. Chlorine can still decay while in transport and the problem can exist. Optimal chlorine levels are therefore to be maintained after the treatment to ensure the water quality does not deteriorate. For such fundamental understanding of factors impacting chlorine residuals are needed in the recycled water. While there are a lot of studies on drinking water systems, there is a scarcity of information on recycled water chlorine stability. This work is aimed to fill the knowledge gap by investigating the Sydney Olympic Park Authority (SOPA) recycled water pipelines and pilot-scale biofilm reactor system set up at the water treatment plant to elucidate the fundamentals.

An investigation was carried out to examine the water quality parameters that may degrade chlorine residuals in the recycled water distribution system of SOPA. Physicochemical parameters such as free chlorine, total chlorine, DOC, ammonia, nitrite, nitrate and pH were measured in the bulk water samples. Biofilm growth potential of two different pipe materials under the similar environment, especially chlorine residuals of the storage reservoir before supplying to the distribution system was investigated to determine the role of biofilm components in decaying chlorine. Three flow cell columns of bioreactors containing biofilm coupons of polyvinyl chloride (PVC) and high-density polyethylene (HDPE) pipes were continuously operated for 105 days.

Results were obtained from the examination of chlorine stability in the bulk water samples shows organic chloramine has reduced the effectiveness of chlorination. Chlorine decay in the water system occurred mainly due to inorganic and organic compounds such as metals deposition and bacterial activities, which were supported by the results of biofilm development data from the pilot-scale bioreactor system. Biofilm thickness, volume, mass and visualized images contribute an important role on understanding the decay of chlorine residuals. Over 15 weeks of biofilm development, the fluctuating chlorine trend of the

recycled water in Sydney Olympic Park system has an adverse impact on biofilm constituents, bacteria cells and extracellular polymeric substance (EPS) production. As compared to traditional measurements, confocal laser scanning microscope (CLSM) provided different recording of multiple biofilm parameters with their subsequent visualization and quantification. In addition, discoloured water factors such as metals such as Fe, Mn deposition within biofilms were observed and the results illustrate that the discoloured water event could be related to presence of Fe and Mn in the recycled chlorinated systems.

In all the samples, organic chloramine was found to be the dominant chlorine species in the recycled water distribution system. According to monitoring data, biofilms did not grow as fast as expected due to the presence of chlorine, organic chloramine, other unknown inhibitors and/or high flow rate. Free chlorine and slow-growing biofilms may oxidise Fe or Mn and influence the retention of these elements within the biofilm. Both PVC and HDPE had the same trend of increasing biofilm thickness as well as the biomass. HDPE pipe surfaces were more susceptible towards biofouling than PVC. EPS volume was usually higher than the bacterial cell volume in both pipe materials whereas EPS volume was higher in HDPE than PVC. The highest volume of EPS was approximately 4000 $\mu m^3/m^2$ compared to the highest volume of the bacterial cell about 2400 $\mu m^3/m^2$. The biofilm is not enough in the pipe materials to show the impact on decaying chlorine at concentrations range between 1 - 3 mg/L. Organic chloramine possibly plays a critical, but an unknown, role in determining the growth of biofilm and dirty water complaints through release of metals.

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TABLE OF CONTENTS

CHAPTER 1 1
INTRODUCTION 1
1.1. Background 1
1.2. Problem statement and significance of the research 2
1.3. Research Objectives 3
1.4. Research Approach 3
CHAPTER 2 5
LITERATURE REVIEW 5
2.1. Recycled water, uses and risks 5
2.2. Water disinfection: chlorination 7
2.3. Instability of chlorine residuals in water distribution system 12
2.3.1. Biofilm 13
2.3.2. Biofilm definition 15
2.3.3. Biofilm composition 15
2.3.4. Biofilm structure 16
2.3.5. Biofilm formation process 17
2.4. Biofilm control strategies 19
2.4.1. Nutrient limitation 19
2.4.2. Addition of disinfectants 19
2.4.3. Hydrodynamics 20
2.4.4. Pipe materials 21
2.5. Discoloured water events 22
2.6. Types of reactors used for biofilm study in water distribution system 23
2.7. The measurement of biofilm growth 25
CHAPTER 3 28
MATERIALS AND METHODS 28
3.1. Project framework 28
3.2. Water quality investigation 29
3.2.1. Sydney Olympic Park WRAMS 29
3.2.2. Investigation of water pipelines in Sydney Olympic Park 30
3.2.3. Physical and chemical analyses 31
3.3. Biofilm monitoring setup and experiment 33
3.3.1. Flow cell bioreactors 33
3.3.2. Biofilm sampling and reactor monitoring 34
3.3.3. Biofilm analysis 35
CHAPTER 4 39
RESULTS – PART I 39
4.1. Chemical parameters 39
4.2. BDOC 40
4.3. Chlorine stability in the bulk water samples: 41
4.4. Chlorine stability in the mixture of stormwater and recycled water: 43
4.5. Chlorine stability by re-chlorination: 44

CHAPTER 5 48

RESULTS – PART II 48

5.1. Monitoring bioreactors..48
5.2. Metal deposition on biofilm..52
5.3. Total carbohydrates on biofilm..53
5.4. Biofilm thickness and biomass by gravimetric measurements..54
5.5. Compared biofilm thickness between gravimetric measurements and CLSM quantification 55
5.6. Biofilm volume by CLSM quantification..56
5.7. Biofilm morphology..57
5.8. Chlorine decay with biofilms..59

CHAPTER 6 64

DISCUSSION..64

CHAPTER 7 66

CONCLUSION..66

REFERENCES 68

LIST OF TABLES

Table 2. 1 Classes and appropriate uses of RW (source: EPA Victoria 2003). 6
Table 2. 2 Different types of disinfectants and their advantages and disadvantages (White, 2010 and Hammer, 2008) 8
Table 2. 3 Quantification methods of biofilms in water distribution system........................ 27

Table 4. 1 The average chemical parameters in the bulk water samples collected over 10 days from 15/08/2016 to 25/08/2016. 39
Table 5. 1 Monitor flow rate, retention time, velocity and Reynold's number in each reactor 51

LIST OF FIGURES

Figure 2. 1: Relative risk associated with RW reuse (source: Department of Health and Ageing 2012). 7
Figure 2. 2: Graph of typical breakpoint chlorination curve (source: Hammer 2008). 10
Figure 2. 3: Flowchart displaying links between biofilms, RWDS problems and control strategies 14
Figure 2. 4: Images of biofilms and corrosion products in drinking water distribution systems. 16
Figure 2. 5: Diagram of main steps of biofilm formation. 18
Figure 2. 6 Figures of commonly used biofilm reactors, cross-sectional view, (a) annular reactor, (b) propeller reactor and (c) flow cell reactor (source: Gomes, Simoes & Simoes 2014). 24

Figure 3. 1: Flow diagram of water processing at WRAMS facility (source: SOPA 2017) 29
Figure 3. 2: WRAMS building – WTP (source: SOPA 2017) 30
Figure 3. 3: Photographs of water sample collection points and equipment. 31
Figure 3. 4: Photos and diagram of experimental setup flow cell system 33
Figure 3. 5: Gravimetric concept for quantifying biofilm 36

Figure 4. 1: BDOC and DOC for samples collected on 06/12/2016 41
Figure 4. 2: Total chlorine residuals in the recycled bulk water samples after dosing initial total chlorine residuals of 3.3 mg/L 42
Figure 4. 3: Total chlorine residuals in the recycled bulk water samples after dosing initial total chlorine residuals of 5.1 mg/L 42
Figure 4. 4: Total chlorine residuals in the mixed water samples after dosing initial total chlorine residuals of 3.3 mg/L 43
Figure 4. 5: Total chlorine residuals in the mixed water samples after dosing initial total chlorine residuals of 5.1 mg/L 43
Figure 4. 6: Initial chlorination and re-chlorination of water samples after dosing total chlorine residuals of 3 mg/L: (a) ICC, (b) OCC and (c) OSR 46

Figure 5. 1: Online chlorine concentration during biofilm sampling (source: SOPA 2017) 49
Figure 5. 2: Onsite monitoring of chlorine residuals concentration 50
Figure 5. 3: Monitoring DOC during sampling biofilms 52
Figure 5. 4: Fe and Mn deposition on biofilms 52
Figure 5. 5: Total carbohydrate content on biofilms 53
Figure 5. 6: Biofilm thickness and biomass by gravimetric measurements 54
Figure 5. 7: Comparison of biofilm thickness obtained by CLSM and calculated by gravimetric measurement 55
Figure 5. 8: EPS volume and cell volume of biofilms quantified by CLSM and IMARIS 56
Figure 5. 9 Images from CLSM (green channel: EPS; red channel: cells) of biofilms developed on PVC/HDPE 58
Figure 5. 10: Total chlorine decay profiles with biofilms on PVC and HDPE 60
Figure 5. 11: Free chlorine decay profiles with biofilms on PVC and HDPE 62

LIST OF ABBREVIATIONS

BAC: biologically activated carbon

BDOC: biodegradable dissolved organic carbon

CLSM: confocal laser scanning microscopy

DOC: dissolved organic carbon

DO: dissolved oxygen

DON: dissolved organic nitrogen

EPA: Environmental Protection Agency

EPS: extracellular polymeric substances

ICP-OES: Induced Coupled Plasma Optical Emission Spectrometer

NOM: natural organic matter

HDPE: high density polyethylene

PVC: polyvinyl chloride

RW: recycled water

SOPA: Sydney Olympic Park Authority

WRAMS: Sydney Olympic Park Reclamation Management System

WRP: Water Reclamation Plant

WTP: Water Treatment Plant

WHO: World Health Organization

CHAPTER 1

INTRODUCTION

1.1. Background

Climate change and urbanization are imposing a huge stress on fresh water supplies, which has now become a limited resource. In a worldwide context, the World Health Organization (WHO) has recognised that recycled water (RW) is set to become the principal driving force in building a sustainable water supply solution (World Health Organization, 2006). In Australia, the response to this has been an active push for water conservation and the development of alternative water sources. This has been embraced by many individuals, local, state, and federal governments, with the result that significant financial and scientific resources have been directed to encouraging and supporting water reclamation and the development of RW schemes (Power, 2010).

Most of the water sources are able to reclaim for a wide range of reuse purposes. For example, in Sydney Olympic Park Reclamation Management System (WRAMS), the RW is produced froma combination of stormwater and sewage. This RW is suitable for residential use, for instance, gardening, car washing, toilet flushing, and laundry. Furthermore, a number of applications for RW can be considered such as landscaping, agricultural irrigation, industrial use, groundwater recharge, dust suppression at construction sites and infrastructure such as road construction. However, there are potential health risks that are associated with human contact in the case of public areas under RW irrigation, and with exposure to aerosols generated by spray irrigation. RW is also unsuitable for human consumption because potable supplies could pose the highest risk to human health (Department of Health and Ageing, 2012). Therefore, none of the water reuse regulations recommends reclaiming wastewater directly for drinking purpose (Asano, 2009).

Due to the strong concern about waterborne diseases caused by pathogens, viruses, bacteria, protozoa, RW requires physical, chemical and biological treatments at multiple levels. Treatment techniques for removal of pathogens are called disinfection, such as chlorination, ozone, ultraviolet radiation, membrane system and media filters (Power, 2010). Disinfection

by chemical oxidation is an effective technique to inactivate microbial pathogens. Among disinfectants, chlorine, chlorine dioxide and chloramine can evoke oxidation, hydrolysis and deamination reactions with a variety of chemical substances in bacterial cells including structural regions of metabolic enzymes or membrane proteins (Schwartz et al., 2003). This explains the early adoption of chlorination in the water distribution systems as a means of disinfectant for its secondary treatment. It is still widely used because it is more economical than other alternative disinfectants (Hammer, 2008). Based on the benefits of chlorination, Sydney Olympic Park Authority (SOPA) has chosen chlorine as a key step to disinfect their RW.

1.2. Problem statement and significance of the research

Despite chlorination, bacterial regrowth is observed when chlorine concentrations decline along a distribution network. As chlorine concentration gradually decreases from the chlorination tank inlet through the system, the "chlorine residuals" provide a minimum level of disinfection effectiveness. The chlorine residuals are reduced due to their reaction with water constituents, biofilms and sediments. Previous studies show that bacteria are able to overcome disinfection barriers using various survival strategies and form biofilms in the distribution system. Biofilms in water distribution system cause chlorine decay, slime growths, biofouling, scaling, corrosion, foaming and support the regrowth of opportunistic and pathogenic bacteria (Jjemba et al., 2010, Jungfer et al., 2013). In fact, SOPA customer service has recently faced complaints over their RW related to aesthetic issues like odour and discoloration of the water. This situation calls for a greater understanding of the microbial activities in RW and their role in degrading water quality. There is a growing interest in the water industry in understanding microbial deterioration of the treated water during storage and distribution. Many studies have examined various problems of drinking water biofilms. However, there is little understanding of RW biofilm growth in different pipe materials and its impact on chlorine stability. Therefore, there is a need for an in-depth research to investigate biofilm formation and its impact on chlorine residuals under hydrodynamic flow and similar environmental conditions in the RW distribution system.

1.3. Research Objectives

The aim of this study is to understand biofilm growth in different pipe materials and their impact on chlorine decay in the SOPA RW distribution system. This involves the following objectives:

- Investigating the water quality parameters that degrade chlorine residuals in the SOPA RW distribution system.
- Investigating the biofilm growth potential of different pipe materials under various chlorine residuals.
- Determining the role of biofilm components in decaying chlorine.

1.4. Research Approach

A full-scale water distribution system is dynamic in terms of water quality and it is almost impossible to obtain consistent characteristics across water samples to conduct experiments as per the needs of the research aims. Thus, based on the research objectives to determine the role of biofilms a pilot-scale system was set up in the WRAMS building. The system contained three flow cell types of reactors (connected in series) which were operated under similar environmental conditions in SOPA RW distribution pipelines. The pilot-scale system was able to simulate a full-scale distribution system and appropriate conditions were provided to achieve engineered microbial activities in each reactor. The biofilms were developed on the surfaces of polyvinyl chloride (PVC) and high-density polyethylene (HDPE) pipes materials and their structures and role on decaying chlorine residuals were investigated. The system was able to provide sufficient samples for the study of the mechanisms behind chlorine decay in more details (i.e. biofilm structure and biofilm components).

This thesis consists of 6 chapters.

Chapter 1 presents the introduction including research objectives, their significance and the approach employed in this study.

Chapter 2 reviews the literatures, which include current research efforts to understand biofilm characteristics and chlorine decay that have been practiced for maintaining adequate chlorine residuals in the system.

Chapter 3 organizes the common materials and methods employed in this research, which include the sampling methods, pilot-scale system setup, operation and chemical analytical

procedures.

Chapter 4 reports the validation of results obtained from bulk water quality investigation, chlorine stability and re-chlorination.

Chapter 5 reports biofilm development in the pilot-scale systems presented in Chapters 3.

Chapter 6 discuss the results from Chapter 4 and Chapter 5

Chapter 7 provides the conclusions of this study and recommendations for future study.

CHAPTER 2

LITERATURE REVIEW

The objective of recycling water is to provide a non-potable supply. However, the recycled water (RW) should be chemically and microbiologically safe. For domestic uses, RW must be aesthetically acceptable, free from apparent turbidity, colour and odour. Distribution systems represent a large part of the infrastructure of the RW industry. The vast surface of these systems is in contact with bulk liquid and can support the growth of microorganisms, which further leads to the growth of biofilms. Biofilms can have negative impacts on RW quality during distribution, and is considered as an unknown factor in risk assessment of RW consumption. Chlorination is a common practice across the world in water and wastewater disinfection treatment to destroy pathogens and control nuisance microorganisms, and for oxidation of iron and manganese removal, destruction of taste and odour compounds. Thus, understanding how biofilms can survive under chlorination is important to improve chlorine stability and possibly predict the performance of RW distribution systems, as well as to better assess the risks associated with RW.

2.1. Recycled water, uses and risks

RW is defined as a general term for water reclamation and reuse. It has been reported that Sydney Olympic Park Authority (SOPA) saves over 850 million liters of drinking water each year. Water collection includes stormwater and sewage water. Water obtained from both sources is treated by Water Reclamation and Management Scheme (WRAMS) at Homebush Bay. The stormwater and treated sewage effluent are mixed together at a different ratio depending on the water demand and supply. The average mixing ratio of storm water and treated wastewater is 30 : 70. However, this ratio may fluctuate depending upon public demand (SOPA, 2017).

Moreover, RW can be sourced from wastewater treatment plants, agricultural industries, industrial wastewaters and stormwater (Environment Protection and Heritage Council, 2009). In some cases, on-site treated greywater from household also results in RW for non-potable purposes (Environment Protection and Heritage Council, 2006).

Australia has four RW classes (Table 2.1); A, B, C and D for non-drinking purposes which are distinguished by their relative E.coli count. E.coli is the bacteria that are found in the intestines of warm-blooded animals and the presence of which indicate faecal contamination. The four RW classes represent four minimum standards of biological treatment and pathogen reduction (EPA Victorria, 2003). Under the current management framework, treatment processes for Class B, C and D do not require validation. The Environmental Protection Agency (EPA) Victoria approves environment improvement plans for these schemes. Other states' guidelines also set log reduction targets required for Class A schemes and for schemes intended to augment drinking water supplies (Power, 2010). Table 2.1 provides a guide to the classes followed by appropriate uses for each class of RW.

Table 2. 1 Classes and appropriate uses of RW (source: EPA Victoria 2003).

Class	E.coli Measure	Appropriate Uses
A	<10 E.coli org/100ml	Residential garden watering, toilet flushing, clothes washing, food crop irrigation, parks, gardens & sports ground irrigation, industrial processes
B	<100 E.coli org/100ml	Urban uses with restricted public access, livestock drinking water, closed industrial systems, irrigation of dairy cattle grazing fodder
C	<1000 E.coli org/100ml	Urban uses with restricted public access, cooked / processed human food crops, some crops not exposed directly to water (e.g. apples)
D	<10000 E.coli org/100ml	Non-food crops, e.g. woodlots, turf, flowers

The suitability of RW for final consumption must be assessed to ensure it meets legislative health requirements and/or is suitable for desired construction applications. There are potential health risks that are associated with a human contact in the case of public places irrigated with RW, and with exposure to aerosols generated by spray irrigation. A risk assessment should be undertaken to ensure risks are minimised and managed using a combination of treatment requirements, site controls or sourcing water with a higher water quality rating (Department of Planning Transport and Infrastructure, 2013). Figure 2.1 provides an indication of the relevant risk associated with RW reuse based on the water

source and end use.

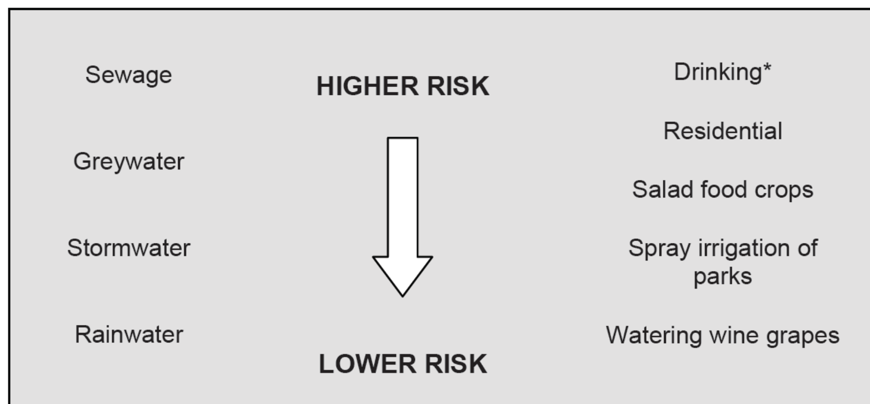


Figure 2. 1: Relative risk associated with RW reuse (source: Department of Health and Ageing 2012).

2.2. Water disinfection: chlorination

Inactivating pathogens and other harmful microbes to protect public health is the aim of disinfecting reclaimed water. In particular, to bring RW up to Class A standard, a rating that makes it suitable for a wide range of uses, yet still unsuitable for drinking purposes, the treatment standard must be close to those for drinking water, which is free of coliforms and all pathogenic microbes. Disinfection is normally carried out in two steps. The first one is primary disinfection, which is carried out to inactivate harmful microbes (mainly pathogens) in the source water, consequently preventing their introduction into the treatment plants. Secondary disinfection is carried out to maintain a sufficient disinfectant residual within the distribution systems, protecting water against microbial intrusion and regrowth (Harrington et al., 2003).

2.2.1. Different forms of disinfection

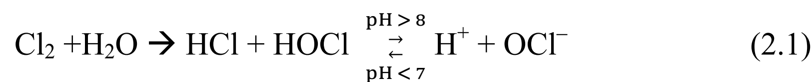
The common disinfection agents that have been used in the water distribution systems include chlorine, chloramine, chlorine dioxide, ultraviolet radiation, and ozone. Among them, chlorine is the most extensively used as a means of disinfection because of their advantages over other disinfecting agents. Table 2.2 shows different forms of disinfection and their advantages and disadvantages.

Table 2. 2 Different types of disinfectants and their advantages and disadvantages (White, 2010 and Hammer, 2008)

Disinfectants	Advantages	Disadvantages
Chlorine : forms of hypochlorous acid and/or hypochloride ion	- Chlorine in these forms are effective for pathogens and virus inactivation, used in primary disinfection; - Strong oxidation for iron and manganese removal; - Low cost.	- The power of chlorine decreases with increasing pH; - Toxic chlorinated by-products: such as trihalomethanes (THMs).
Chlorine dioxide	- Strong disinfection over a wide pH range; - No reaction with ammonia to form chloramines nor with humic acids to form THMs; - Formation of residual maintained in the treated water entering the distribution system.	- Limited use due to formation of toxic chlorate and chlorite residuals; - High cost.
Chloramines	- A protective residual can be maintained for a longer period of time without rechlorination to control bacterial growths in the distribution piping network - Low cost	- Less reactive than chlorine, used in secondary disinfection.
Ozone	- Effective disinfectant; - Strong oxidant to control taste and odour compounds; - Absence of THMs and other health-related by-products.	- Ozone does not produce a disinfecting residual, must applied combined with chlorine; - Should be generated on the treatment plant site because of rapid decay; - High cost.

2.2.2. Chlorine chemistry

There are many benefits of chlorine which have been exploited by the RW industry. For instance, as free chlorine is a strong oxidizing agent and effective in inactivating pathogens and control nuisance microorganisms, it reacts quickly with organic and inorganic compounds, thus the free chlorine is not persistent in the system (Haas, 2000). As an oxidant, it is used for iron and manganese removal, the destruction of taste and odor compounds, and the elimination of hydrogen sulphide. In chemical terms, chlorine combines with water forming hypochlorous acid, which in turn can ionize to the hypochlorite ion. Below pH 7 the bulk of the HOCl remains un-ionised, while above pH 8 the majority is in the form of OCl^- (Eq. 2.1).



$$Cl_2 + H_2O \rightarrow HCl + HOCl \underset{pH<7}{\overset{pH>8}{\rightleftarrows}} H^+ + OCl^- \quad (2.1)$$

Chlorine existing in water as HOCl and OCl^- is defined as free chlorine. Chlorine existing in chemical combination with ammonia nitrogen or organic nitrogen compounds is defined as combined chlorine. When RW is chlorinated, chlorine reacts readily with various nitrogenous compounds naturally present in the water. Inorganic chloramines may be formed when ammonia-N reacts with free chlorine as follows (White, 2010).

$$HOCl + NH_3 \rightarrow H_2O + NH_2Cl \text{ (monochloramine)} \quad (2.2)$$

$$HOCl + NH_2Cl \rightarrow H_2O + NHCl_2 \text{ (dichloramine)} \quad (2.3)$$

$$HOCl + NHCl_2 \rightarrow H_2O + NCl_3 \text{ (trichloramine)} \quad (2.4)$$

These reactions involve successive chlorine substitutions. These reactions compete with each other and are heavily dependent on initial chlorine-to-nitrogen (Cl : N) ratio, pH, temperature and contact time. At low Cl : N ratio and typical water treatment conditions pH 7–9, reaction (2.2) will dominate (>95%) and virtually all free chlorine will be converted to monochloramine. Monochloramine can exist as a relatively stable form of inorganic combined chlorine in neutral or alkaline solutions, and is generally present as the dominant form in chloramination operations (Lee and Westerhoff, 2009). Upon further addition of free chlorine, dichloramine will proceed which is susceptible to degrade by oxidation of nitrogen to N_2 and NO_3^- and reduction of chlorine to chloride. This results in relatively rapid depletion of reactive chlorine residual. Inorganic mono-, di- and trichloramine contain +1-valent

chlorine and have been shown to demonstrate germicidal effects. Inorganic combined chlorine, predominantly in the form of monochloramine, is used in water treatment as a disinfectant. It is generally not as potent as free chlorine in terms of disinfection efficacy but provides a stable disinfecting residual that can be maintained over a long period of time during and post chlorination (Bryant et al., 1992).

When chlorine is added to water containing ammonia, the residuals that develop yield a curve similar to that shown in Figure 2.2. The straight line from the origin is the concentration of chlorine applied. The curved line represents chlorine residuals, corresponding to various dosages, remaining after a specified contact time, such as 20 minutes. Chlorine demand at a given dosage is measured by the vertical distance between the applied and residual lines. This represents the amount of chlorine reduced in chemical reactions, and therefore, the amount that is no longer available. Chloramine residuals decline to a minimum value referred to as the breakpoint. If the addition of free chlorine is continued beyond the breakpoint, free chlorine residual accumulates. The breakpoint curve is unique for each water test since chlorine demand depends on the concentration of ammonia, presence of other reducing agents, the contact time between chlorine application and residual testing and other factors (Hammer, 2008).

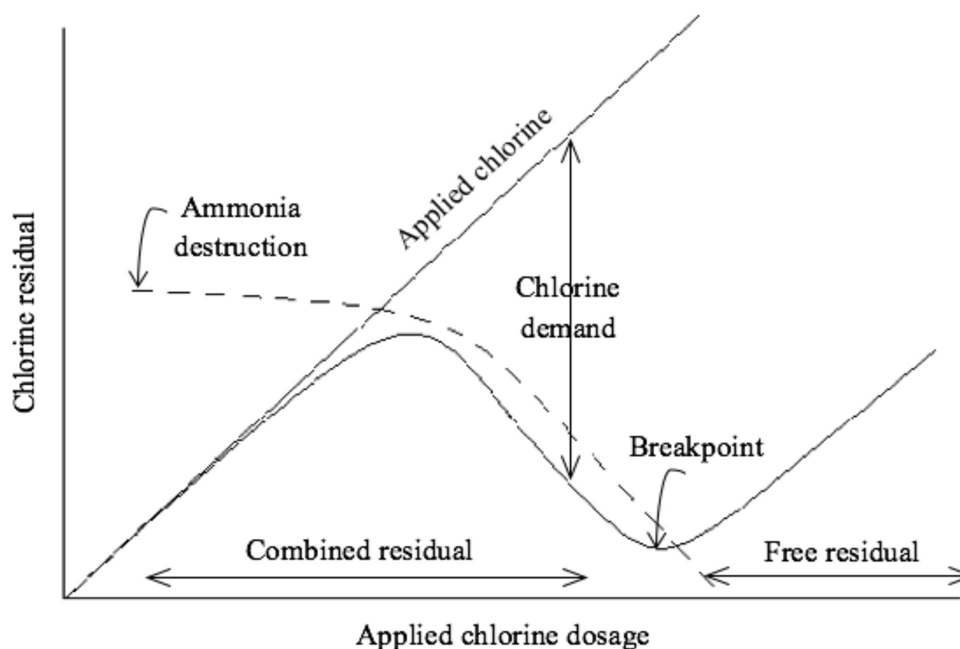
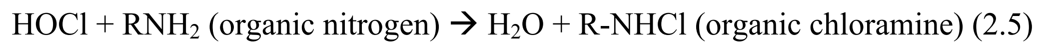


Figure 2. 2: Graph of typical breakpoint chlorination curve (Hammer 2008).

Organic chloramines may be formed when dissolved organic nitrogen (DON), represented by

functional groups such as amino acids, amides and amines reacts with free chlorine (Smith, 1967) or inorganic chloramines (Snyder and Margerum, 1982).



$$HOCl + RNH_2 \text{ (organic nitrogen)} \rightarrow H_2O + R\text{-}NHCl \text{ (organic chloramine)} \quad (2.5)$$

The reactions between amino acids and free chlorine are the main formations of organic chloramines. Reaction rates between them are 2-80 times faster than reaction rates between free chlorine and ammonia. Once formed, organic chloramines are relatively more stable than free chlorine and inorganic chloramines (Yoon and Jensen, 1993b). The distribution of chlorine between inorganic and organic chloramines depends mainly on the relative affinity of +1-valent chlorine for the inorganic and organic N-compounds, the concentration of the nitrogenous compounds and pH (Yoon and Jensen, 1993a). Active chlorine is transferred from inorganic chloramines to amino acids and peptides, and also from organic chloramines to ammonia (Ferriol et al., 1989, Isaac and Morris, 1983, Yoon and Jensen, 1995). The transfer of active chlorine between chloramines and nitrogenous compounds can either occur by hydrolysis of the chloramine to form free chlorine with subsequent N-chlorination or by direct chlorine transfer. If organic nitrogen is available in high amounts such as in RW, there is a very good chance to form organic chloramine. Organic nitrogen compounds may exert high chlorine demand and therefore reduce the concentration of free chlorine and inorganic chloramines, thereby diminishing their availability in disinfection. The formation of organic chloramines during the chlorination process also poses a problem with regard to analytical methods for chlorine residual determination.

Disinfection of water to kill or inactivate microorganisms that cause disease in humans is the most common application of chlorination. The disinfecting action of chlorine results from a chemical reaction between HOCl and the microbial cell structure, inactivating life processes. Disinfection rate depends on the concentration and form of available chlorine residual, contact time, pH, and temperature. The practice of satisfying the demand of high chlorine in RW to accomplish an effective disinfection could succeed in just the opposite, producing an abundance of organic chloramines of no disinfecting capacity (Feng, 1966). The disinfecting action of combined chlorine is significantly less than that of free chlorine residuals. HOCl is more effective than hypochlorite ion, and the power of free chlorine residual decreases with increasing pH (Hammer 2008). These organic chloramines are probably the measurable residual chlorine of low lethal activities. In the view of the possibility that the death of a

microbe in chlorination can be caused either by diffusion of chlorine into the organism followed by chemical reactions of the chlorine with its metabolism system (e.g., enzyme system) or by lesion of the cell wall by chlorine or both, and the difficulty of transferring combined chlorine from one organic substance to another, an organic chloramine should not have significant disinfecting power. Therefore, minimum chlorine residuals and contact times for virus inactivation and protozoal cyst destruction are considerably greater than for bacteria. Consequently, treatment of wastewaters includes coagulation, filtration to physically remove protozoal cysts and helminth eggs and reduce the density of viruses and bacteria (Harrington et al., 2003). Establishing a free chlorine residual for disinfection and maintaining a residual in the water entering the distribution system has proven to be satisfactory for protection. This requires breakpoint chlorination if the wastewater contains ammonia.

Sometimes combined chlorine residuals (monochloramine), rather than free chlorine residual, is established in the treated water entering the distribution system to maintain a protective residual and to control bacterial growths in the distribution piping network (Williams and Braun-Howland, 2003). Compared to chlorine, the advantages are that chloramines are less active and a residual can be maintained for a longer period of time without re-chlorination. For instance, a combined residual can be applied to treated water before it is pumped through a long pipeline to a municipal distribution system. If insufficient natural ammonia is present in the treated water, gaseous anhydrous ammonia is applied by feeding equipment similar to that used for chlorine.

2.3. Instability of chlorine residuals in water distribution system

RW contains a high level of organic matter, which potentially react with the disinfectants, and provides nutrients for microorganisms in the form of organic carbon. Owing to its higher nutrient content than drinking water, RW provides a highly conducive environment for microbes to grow within. (Jjemba et al., 2010). The depletion of chlorine or chlorine demand in the water phase is due to soluble oxidize inorganic compounds, soluble organic compounds, microbial cells, substratum and other particulates in the bulk water. These oxidize species compete with the biofilm for available chlorine and often reduce the effectiveness of chlorine for biofouling control. In other words, biofilms and inorganic particles (e.g. sediments, corrosion products, clays, sand, etc.) play a significant role in the

instability of chlorine residuals in water distribution systems (Chaves Simes and Simes, 2013). Additionally, there are a number of abiotic agents, such as pH, temperature that may physically cause chlorine instability to some extent. The rate of chlorine demand determines the amount of chlorine available for biofouling control. Typical methods for measuring chlorine demand is stoichiometric quantity.

2.3.1. Biofilm

Figure 2.3 summarises relevant information about biofilm formation in the RW distribution system, its impact on disinfectant stability and the main control strategies.

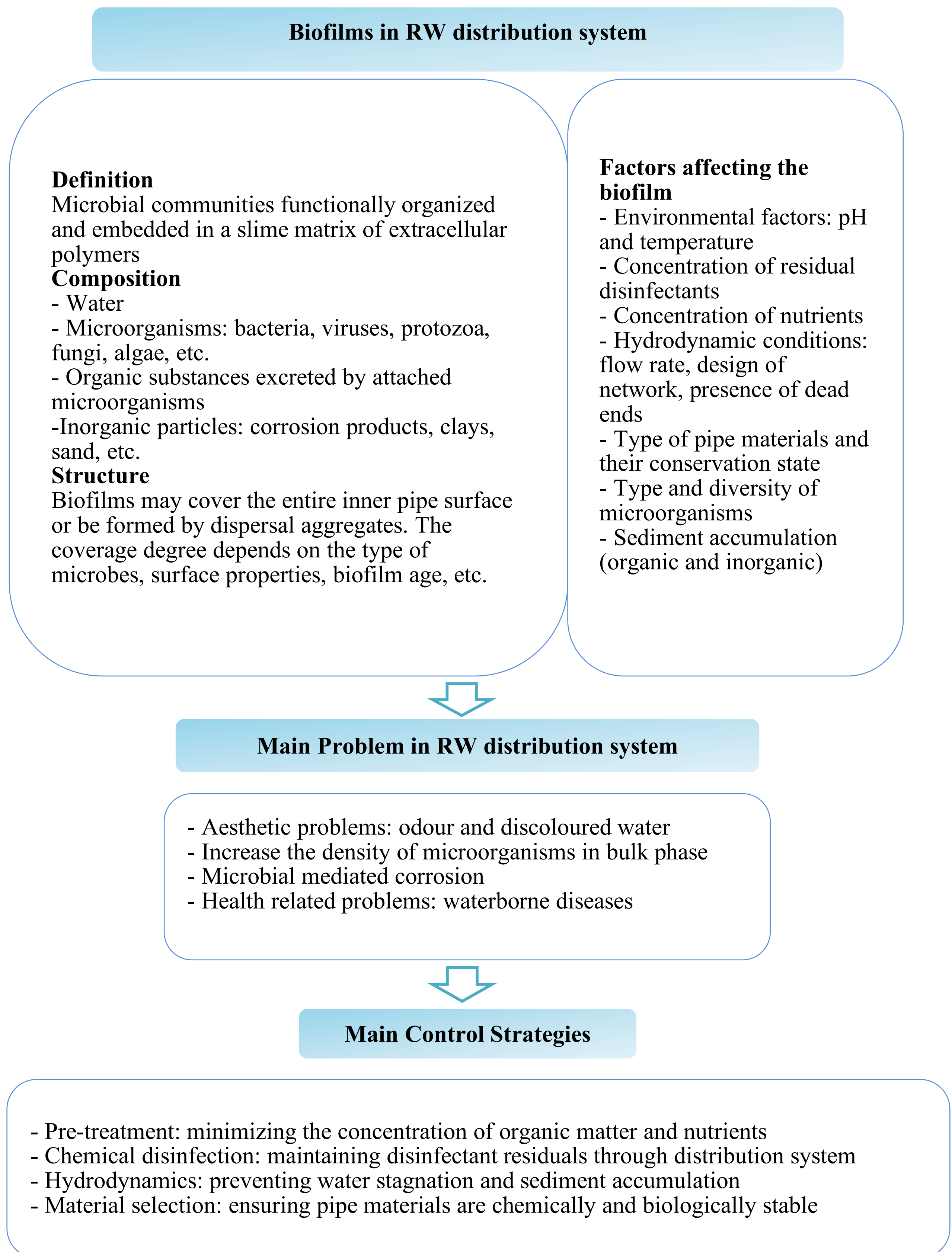


Figure 2. 3: Flowchart displaying links between biofilms, RWDS problems and control strategies

2.3.2. Biofilm definition

Biofilms in a water distribution network can be defined as multi-species microbial communities that colonize the inner pipe surfaces of the system. Biofilms are heterogeneous, in which microorganisms can change dramatically in response to changes in their environment and to adapt to live on the surface (O'Toole et al., 2000). The water distribution network offers a very large surface area in contact with a biological fluid for the adhesion of biofilms. These biofilms are made of discontinuous, non-uniform arrangement of a number of microbial communities, around 10^7cells/cm^2 (Abe et al., 2012). About 95% of total microbial cells reside inside biofilms, while only 5% are floating in the bulk phase (Wingender and Flemming, 2004). On the other hand, biofilms act as biological filters by mineralising biologically degradable material from the water and forming locally immobilized biomass. Moreover, biofilms may unpredictably emerge in the distribution system and may cause diverse problems of bacterial contamination with hygienically relevant bacteria or spontaneous increases in bacterial cell counts in bulk water (Schwartz et al., 2003).

2.3.3. Biofilm composition

Biofilms are composed of complex microbial structures functionally organised and characterised in a slime matrix of exopolymers, mainly proteins and polysaccharides, excreted by microorganisms. It is also known as extracellular polymeric substances (EPS) (Figure 2.4), an essential component in biofilms, which maintains the structural stability of the biofilm and provides protection from stressful conditions for the entrapped cells. It performs this with the irreversible adhesion of hydrogen bonding and dipole–dipole interactions between the microbes, attaching them to the surface (Abe et al., 2011, Gomes et al., 2014, Simões et al., 2010). In oligotrophic environments, such as drinking water, the synthesis of biofilm cells producing EPS is greatly affected by environmental conditions such as availability of nutrients, shear stress, pH and the existence of toxic substances. The formation of biofilm structures also depends on biotic factors, such as the type and diversity of microorganisms, as well as abiotic factors, namely temperature, pH, disinfectants (types and concentrations), nutrients concentrations, pipe materials, hydrodynamic conditions (flow rate and types of distribution networks) and sediment accumulation (Kim and Lee, 2010, Simoes et al., 2012).

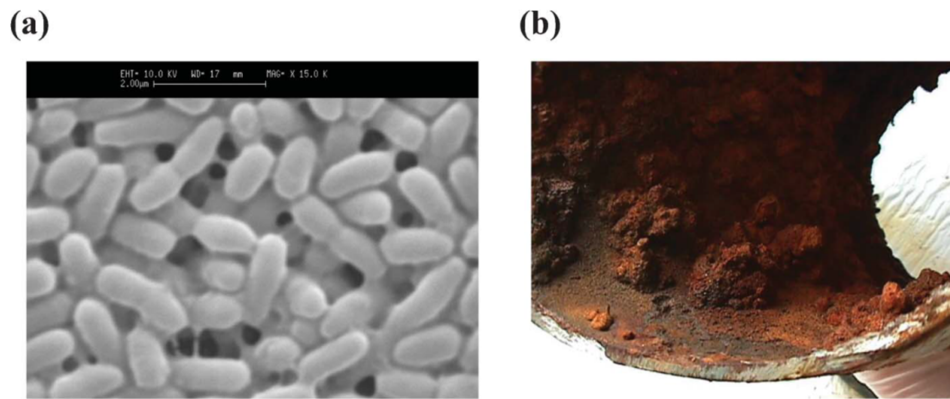


Figure 2. 4: Images of biofilms and corrosion products in drinking water distribution systems. (a) Scanning electron microscopy photomicrograph of 24 h old biofilms formed by opportunistic Gram-negative B. cepacia, evidencing the presence of an extracellular polymeric matrix (615 000 magnification; bar = 2 mm). The biofilm was developed in R2A broth as a growth medium on polystyrene surfaces of microtiter plates. (b) Ductile iron pipe section from a DWDS with a biofilm and high amounts of corrosion products. This section of DWDS pipe was obtained as result of a pipe break in the DWDS (source: Chaves Simes & Simes 2013).

2.3.4. Biofilm structure

Based on earlier studies, biofilm formation is known to be a well-regulated developmental process that results in the formation of a complex community of organisms. To form these communities, microorganisms must integrate external and internal signals, take stock of their neighbours by determining their density and type and coordinate a time series of multi-cellular behaviors that are associated with morphological changes. Multispecies biofilms demand the ability for interspecies communication suggesting the possibility of certain organisms performing specialized roles in the community (O'Toole et al., 2000). Biofilms often have a patchy appearance and non-uniform structure, from clumps ranging from a few cells to 100 μm in depth (Martiny et al., 2003, Abe et al., 2012). Biofilms have a filamentous and fluffy structure, thick and not very dense, and very susceptible to sloughing. Underneath this irregular easily detachable structure, hides the so-called basal biofilm layer, which is denser, more shear-resistant and with a higher content of biopolymers (Coufort et al., 2007). Slow growing biofilms, those formed by autotrophic bacteria, are more likely to be stable due to corrosion causing the biofilm to become denser and thinner (Pellicer-Nacher and Smets,

2014).

2.3.5. Biofilm formation process

The formation of biofilms in an aquatic environment takes place as a result of several consecutive steps including the formation of an initial conditioning layer (reversible), followed by irreversible adhesion of microbial cells to the conditioned surface, excretion of extracellular macromolecules to reinforce bacterial adhesion (steady state), and finally detachment which releases microorganisms into the bulk fluid *(2007)*. Biofilm formation could be viewed as a successional process in structure and composition. According to O'Toole et al. (2000), the biofilm formation process begins with the preconditioning of the pipe surface by organic and inorganic macromolecules that facilitate the bacterial adhesion process. Thereafter, cells can absorb to the surface reversibly or irreversibly. This stochastic primary attachment of a number of species recruited from the bulk water population forming a monolayer on the surface. After adhesion, a stage of active biofilm growth occurs by cell replication, EPS production, the release of quorum-sensing molecules and exchange of substances between the biofilm and the bulk. As subsequent steps, the occurrence of biofilm dispersion and formation/colonization in other clean areas can take place after biofilm detachment from pipe walls (Codony et al., 2005). The amount of a biofilm in a given system, after a certain time period, depends on a dynamic biofilm formation process, which has been defined as the balance between bacterial attachment from the planktonic phase, bacterial growth within the biofilm and dynamic detachment from the surface (Stoodley et al., 1998). When the balance is null, the biofilm is said to have reached a steady state. The final amount of biofilm in that state, which can be assessed by cell counts or biomass determination, is directly related to its formation potential in the system (van der Kooij, 1998).

Biofilms not only support the immobilisation of bacteria by reducing the shear force on cells and by increasing the number of adsorption sites, but also by developing other biofilm colonies. A significant advantage of the biofilm mode of growth is the potential for dispersion via detachment. Under the direction of fluid flow, detached microorganisms travel to other regions to attach and promote biofilm formation on clean areas (Stewart, 2012). Therefore, this advantage allows a persistent bacterial population, usually resistant to

antimicrobial agents (Gagnon et al., 2005), while at the same time enabling the continuous shedding to promote bacterial spread. Steps of biofilm formation can be observed in Figure 2.5.

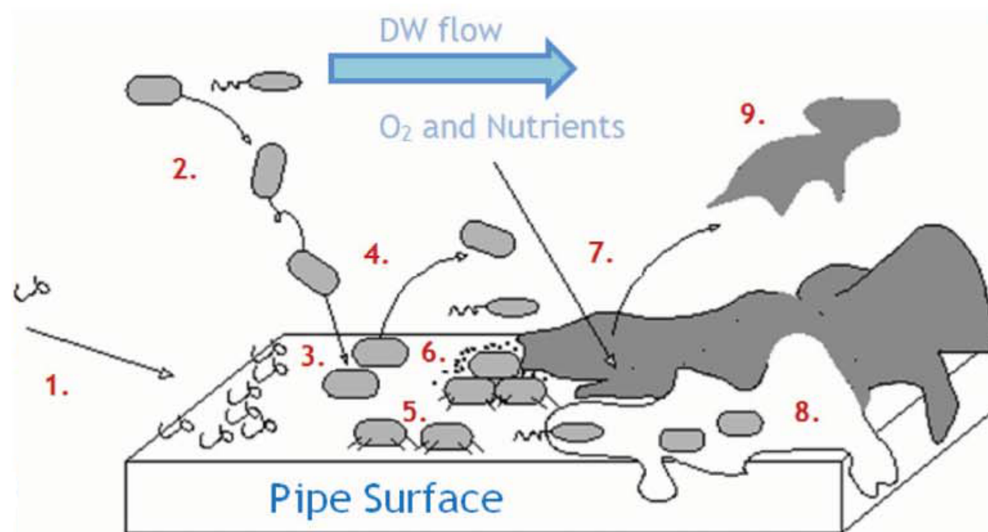


Figure 2. 5: Diagram of main steps of biofilm formation.

(1) Preconditioning the pipe surface by macromolecules (organic and inorganic) present in the water; (2) Transport of planktonic cells from water to pipe surfaces; (3) Adsorption of cells at the pipe surface; (4) Desorption of reversibly adsorbed cells; (5) Irreversible adsorption of cells; (6) Production of QS molecules; (7) Transport of substrates to and within the biofilm; (8) Substrate metabolism by the biofilm-bound cells and transport of products out of the biofilm, accompanied by cell growth, replication and production of EPS; (9) Biofilm removal by detachment or sloughing (source: Chaves Simes & Simes 2013).

Since the bacteria are initially highly dispersed within the water bulk phase and have a low selection pressure for attachment, a highly diverse community is initially formed. This attachment (facilitation) can be followed by a secondary colonization of bacteria that benefit from a protective environment in the biofilm and/or feed on the remnants of other bacteria. In this secondary community, better resource or space competitors may exclude less competitive organisms (Jackson et al., 2001). Further biofilm formation may cause a reduction in diversity, as a single or a few superior competitors start to dominate the community. As the bacterial biofilm matures, more niches are created due to the formation of gradients and the internal recycling of resources.

2.4. Biofilm control strategies

2.4.1. Nutrient limitation

Attached microbes in the biofilms are able to adopt a sessile lifestyle and gain a number of advantages over planktonic microbes. The first advantage of EPS is turning biofilms into a trap to capture and concentrate nutrients, such as carbon, nitrogen, and phosphate. Allison et al. (Allison et al., 1998) suggested that the amount of EPS bacteria synthesis within the biofilm depends greatly on the availability of carbon substrates (both inside and outside the cell) and on the balance between carbon and other limiting nutrients. Because the microbial growth may depend on whether the original water is phosphorus-limited or carbon-limited, a reduced level of phosphorus caused an increase in carbohydrate levels in EPS (Fang et al., 2010). The presence of excess available carbon substrate and limitations of other nutrients, such as nitrogen, phosphorus or potassium promotes the synthesis of EPS bacteria. On the contrary, as the carbon source becomes limiting, the cell may utilise more carbon to synthesise the cell components and produce energy instead of producing the EPS. As less EPS is produced, it may indicate the presence of a looser biofilm structure. As the addition of phosphorus to bacteria promotes higher metabolic potential but lower EPS production and homogeneity of biofilms, it also indicates that the biofilm growth will be promoted but spontaneously will be less resistant to the disinfectants (Bauman et al., 2009). This will promote the formation of biofilm spatial niches in response to environmental conditions and the activity of their neighbours to optimize nutritive resources. Under stress, the inherent resistance of biofilms to antimicrobials, can be mediated through very low metabolic levels and drastically down-regulated rates of cell division in the deeply embedded microorganisms (Simões et al., 2011).

2.4.2. Addition of disinfectants

Disinfectants have been widely used as means of controlling microbial regrowth. Yet, the EPS matrix also prevents the access of antibiotics to the bacterial cells (O'Toole et al., 2000). Chlorine can be neutralized by the organic constituents of the biofilm matrix and the neutralization reaction is faster when it diffuses into the biofilms (Simões et al., 2010). Bacteria in biofilms are often described to be more resistant against disinfection agents like

chlorine than planktonic microorganisms. Once trapped in biofilms, pathogens can be protected from disinfectants and antimicrobials through mass transfer resistance or the adoption of a persisting state. Several mechanisms explain that biofilms' resistance to biocides or antimicrobials, is due to biofilms acting as a diffusion barrier to disinfectants (Chaves Simes and Simes, 2013). As EPS components interact with chlorine or chloramine, this reduces the concentration of these disinfectants, limiting their effectiveness and slowing their penetration into biofilm bacteria. For large molecules, the exopolymer matrix of biofilms restricts their diffusion and binds to antimicrobials. The negatively charged exopolysaccharides are also efficient in protecting cells from positively charged biocides by restricting their permeation through cell binding (Schwartz et al., 2003). Additionally, enzymes destroying incoming antibiotics together with the effective synergy between the outer membrane and multi-drug resistance pumps complete bacterial resistance mechanisms in biofilms (Lehtola et al., 2004).

2.4.3. Hydrodynamics

Hydrodynamic conditions (flow rate, velocity, retention time, and shear stress) have a vital role in biofilm development and in determining biofilm stability (Stewart, 2012). The flow rate affects biofilm development by interfering with several phenomena, namely nutrient transport, bacterial adhesion, biofilm growth, and detachment. When the flow velocity is low (i.e. <0.5 m/s), there is a high resistance to mass transfer (such as nutrients, oxygen) from the bulk fluid to the microorganisms embedded in biofilms, impairing sessile cell growth (Bott, 1993). On the other hand, high flow velocity (i.e. > 0.5 m/s) causes high turbulence of the fluid bulk (Bott, 1993). This means that the mass transfer phenomena are enhanced, improving the biofilm growth. However, high velocity also causes high shear forces that can be responsible for higher biofilm erosion and detachment. Accordingly, it may cause a decrease of biofilm mass on surfaces (Lin et al., 2013, Gomes et al., 2014, Bott, 1993). A rule of thumb in the cooling water industry suggests that the velocity in pipes and heat exchangers should be greater than 1 m/s in order to reduce the extent of a biofilm growth. Velocity cannot be increased too much without penalty as velocity increases, the pumping energy increase and hence pumping cost increase (Bott, 1993). Moreover, as the bulk water flows through the water mains, like a transmission vehicle for nutrients, microbes, and particles to biofilms forming on the inner pipe surface of the distribution system. The particles are

transported throughout the network as colloids and suspended solids may accumulate/settle as loose deposits on the floor of the pipe. Any inorganic particle passing nearby (e.g. corrosion products, clays, sand, etc.) may also be incorporated to form the topography of biofilms (Liu et al., 2013). Biofilm control drinking water strategies in distribution systems are required including nutrient limitation, disinfectant addition and in some specific situations flushing out the pipes. This last action may be an effective remedy for soft deposits or sediment removal but relatively ineffective against biofilms due to their physical make-up and chemical properties (Carrière et al., 2005).

2.4.4. Pipe materials

Biofilms usually attach onto surfaces which are in contact with water. Therefore, biofilm formation commonly occurs in pipeline networks of water distribution systems. There have been many studies conducted on a wide range of commonly used materials and the effects of material on biofilm formation. It has been proven that the chemical composition of pipe materials strongly influence biofilm formation and adhesion. Some pipe materials, such as copper, iron and stainless steel are bactericidal, which reduces regrowth. Especially, copper exerts its toxicity by generating reactive oxygen species with copper-induced oxidative stress damaging the cell membrane through lipid peroxidation, leading to membrane permeability and cell death (Grass et al., 2011). On the other hand, some pipe materials may decay disinfectant residuals, leading to increased microbial regrowth. Several plastic pipes, such as polybutylene (PB) and polyethylene (PE) have recently been used as cost-effective replacements for traditional metal plumbing, but may release biodegradable organic compounds and phosphorus, which can promote biofilm formation (Kim and Lee, 2010). The tendency for elastomeric materials, such as ethylene propylene diene monomer (EPDM), and plastics, such as cross-linked polyethylene (PEX), to leach potential microbial nutrients such as phosphorus-based compounds into the water, is theorised to be a major contributing factor towards the formation of biofilms. Therefore, EPDM is an unsuitable material because of its ability to support biofilm formation at a consistently higher level than other materials (Waines et al., 2011).

Another important factor concerning materials is surface roughness which has a great influence on bacterial attachment and may vary not only between material types but also

between different grades of the same material. The roughness of a pipe is normally specified as *e* (mm or inches) and common values range from 0.0015 mm for PVC pipes to 3.0 mm for rough concrete pipes (Pipeflow, 2017). For instance, biofilm regrowth on pipes made with rough surfaces such as cast iron (*e* = 0.25 mm), concrete-lined cast iron (e = 0.30 mm), and galvanized steel (e = 0.15 mm) or stainless steel (SS) (e = 0.05 mm) was greater than that on smooth surface PVC pipe (Yu et al., 2010, Lin et al., 2013) supporting its suitability as plumbing material (Lin et al., 2013, Kim and Lee, 2010). In addition, SS is an alloy metal, depending on their corrosive- resistant layer, may be corroded under environmental conditions. SS corrosion certainly contributes to the bacterial regrowth in water distribution systems. Conversely, biofilms can also promote corrosion in metals (Teng et al., 2008). It is clear that pipe material properties and the disinfection treatment are two technical parameters which control biofilm quality.

2.5. Discoloured water events

Discoloured water events are a persistent cause for customer dissatisfaction in the Sydney Olympic Park RW industry. The build-up of Fe and Mn in distribution systems is considered to be a prime cause for discoloured water events (Slaats et al., 2003). Soluble Fe and Mn in natural waters often exist in their divalent ferrous and manganous forms (Deborde and Von Gunten, 2008). These forms of Fe and Mn tend to result in a metallic astringent or medicinal taste, discolouration, growth of biofilm, high chlorine and dissolved oxygen (DO) demand and hydraulic issues due to post deposition (Schock et al., 2008). Removal of soluble Fe and Mn from source waters is mainly achieved using oxidation, such as chlorine, ozone or permanganate and filtration processes (Wong, 1984). However, the corrosion of aging pipes in the SOPA distribution system will continue to introduce Fe into the bulk water and cause corrosion, thus challenging water utilities to continue to manage their discoloured water events.

The movement of oxidised forms of Fe and Mn through distribution systems is largely facilitated by the natural organic matter (NOM) content in water. Inorganic matter such as iron (Fe (III)) and manganese (Mn (IV)) ions complex with NOM and these metal–NOM complexes have increased solubility and mobility (Heitz and Mackenzie, 2006). NOM not only increases the solubility of metals, but also facilitates precipitation through its adsorptive

properties to solid surfaces (Amirbahman and Olson, 1993). Furthermore, metal–NOM complexes can be assimilated or sequestered into biofilms and coagulate to form precipitates that settle as sediments. NOM promotes biofilm growth and contributes towards formation of disinfection by-products and facilitates the accumulation of metals on pipe wall surfaces.

Although current pre-treatment processes remove a large proportion of NOM, distribution systems continue to have biofilm growth, even in the presence of chlorine residuals. These biofilms may enhance the accumulation of Fe, Mn, Al oxides, silica, calcium carbonate and other inorganic debris in areas beyond the bulk water phase. With spontaneous dislodgement of these accumulations into the bulk water, cloudy, brown, orange, red or black coloured water may reach the consumer. Besides biofilms oxidising Fe or Mn and expediting the retention of these elements within the distribution network, biofilms also contribute to water discolouration by enhancing the abiotic release of Fe from within corrosive scales or as particulates that discolour the water in suspension (Cerrato et al., 2010).

2.6. Types of reactors used for biofilm study in water distribution system

As the study of biofilms in real water distribution systems is difficult, several devices have been developed for the task. These devices allow for biofilm formation under controlled conditions of physical (flow velocity, shear stress, temperature, type of pipe material, etc.), chemical (type and amount of disinfectant and residuals, organic and inorganic particles, ions, etc.) and biological (composition of microbial community, type of microorganism and characteristics) parameters, ensuring that the operational conditions are as similar as possible to real water distribution system conditions in order to achieve results that can be applied to real scenarios (Gomes et al., 2014). There are two groups of reactors including bench top laboratorial devices and *in situ* reactors. The bench top laboratorial appliances were developed as water distribution system models to autonomously study biofilms in the laboratory, allowing different conditions to be tested with tap water or the appropriate medium or enriched water. The *in situ* devices were developed to study and monitor biofilms in pilot and real water distribution system. They are usually placed as a by-pass or directly connected to a water distribution system. Some bench top laboratorial appliances, namely Annular, Probella and flow cells reactors (Figure 2.6) have been used as *in situ* devices as well (Wilks and Keevil, 2003).

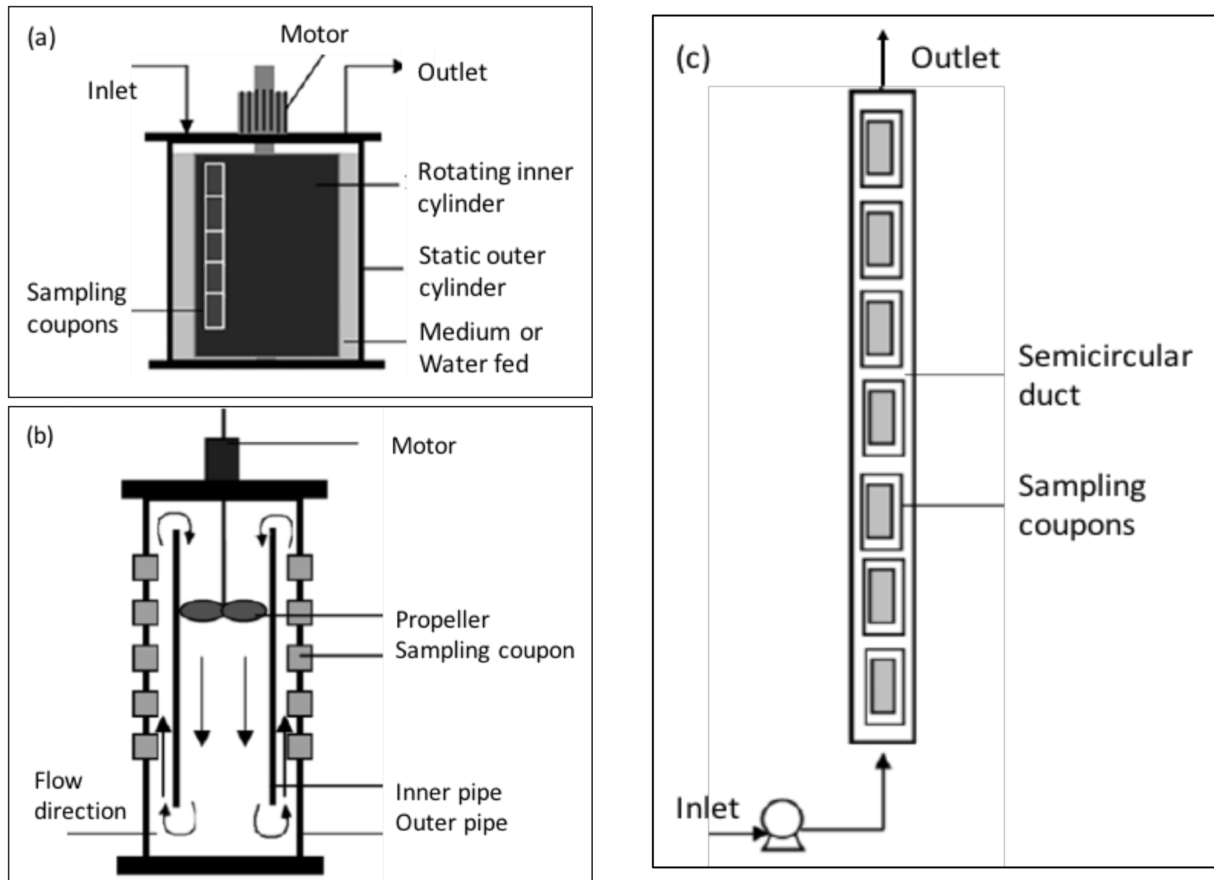


Figure 2. 6 Figures of commonly used biofilm reactors, cross-sectional view, (a) annular reactor, (b) propeller reactor and (c) flow cell reactor (source: Gomes, Simoes & Simoes 2014).

Every type of *in situ* reactors has its advantages and limitations based on design and the purpose of biofilm study. Generally, most of them are useful for the study of different materials simultaneously, easy to control environmental conditions, non-invasive biofilm formation and similar to operational conditions of the real water distribution system. Nevertheless, the surface coupons to grow biofilms must be flat, therefore, lack of sufficient sampling surface area is the common limitation of all in situ reactors. Sometimes periodical sampling coupons may change flow patterns around the boundary of the coupons which cause non-uniform biofilm formation and difficult to control sheer stress. The changes in water flow may be minimized by other advanced or modified devices (Gomes et al., 2014).

2.7. The measurement of biofilm growth

The biofilm studies require many appropriate methods to quantify biofilm formation and to provide information on its characteristics, particularly for the resident population. Biofilms can be quantified through the increase of biological activity or by the number of cells (Liu et al., 2013). Apart from the quantification of cell numbers, it is also important to obtain information on other biofilm constituents, particularly the EPS. Most of these methods require the biofilm detaching from the surfaces of pipe materials and dispersing to an adequate solution. It is necessary to use some physical approaches such as vortex and ultrasonication to achieve an efficient biofilm dispersion in the selected solution. Some microscopic methods, namely atomic force microscopy (AFM), scanning electron microscopy and confocal laser scanning microscopy (CLSM), which allow a direct and non-destructive analysis of biofilm. These methods can be advantageous since the possibility of biofilm loss in detachment process does not exist, but also allow the study of the entire biofilm structure (Abe et al., 2012, Fang et al., 2010, Jungfer et al., 2013, Lin et al., 2013, Mathieu et al., 2014). However, even if the direct microscopic analysis of coupon surfaces is important to provide information on the biofilm structure, these methods cannot determine all relevant aspects involving the biofilm formation process. Therefore, the combination of information from different methods will provide a more detailed picture on biofilm development and composition.

Cell enumeration, such as heterotrophic plate count (HPC) is the mostly used method to evaluate biofilm cell numbers through the enumeration of cultivable cells. The metabolic active, viable or total cells are able to accessed through microscopic analysis with fluorescent stain products including acridine orange, DAPI, SYTO 9 and propidium iodide (Gomes et al., 2014). EPS quantification methods are usually based on the analysis of carbohydrates and proteins. The reliability of the analysis is strongly dependent on the extraction methods used to separate the EPS from the biofilm cells. The EPS protein dye product and modified phenol-sulfuric acid method for total carbohydrates are the most common EPS quantification techniques (Fang et al., 2010). The study of EPS as a major component of biofilms has long been neglected in biofilm research. The reason may be the difficulty of analysing the large variety of EPS polymers, especially in environmental biofilms (Staudt et al., 2003). Polysaccharides may represent a major fraction of EPS, as a result, fluorescently labelled

lectins have been suggested as suitable probes for staining EPS in biofilms (Neu et al., 2001). Table 2.2 summarises the quantification methods of biofilms in water distribution system.

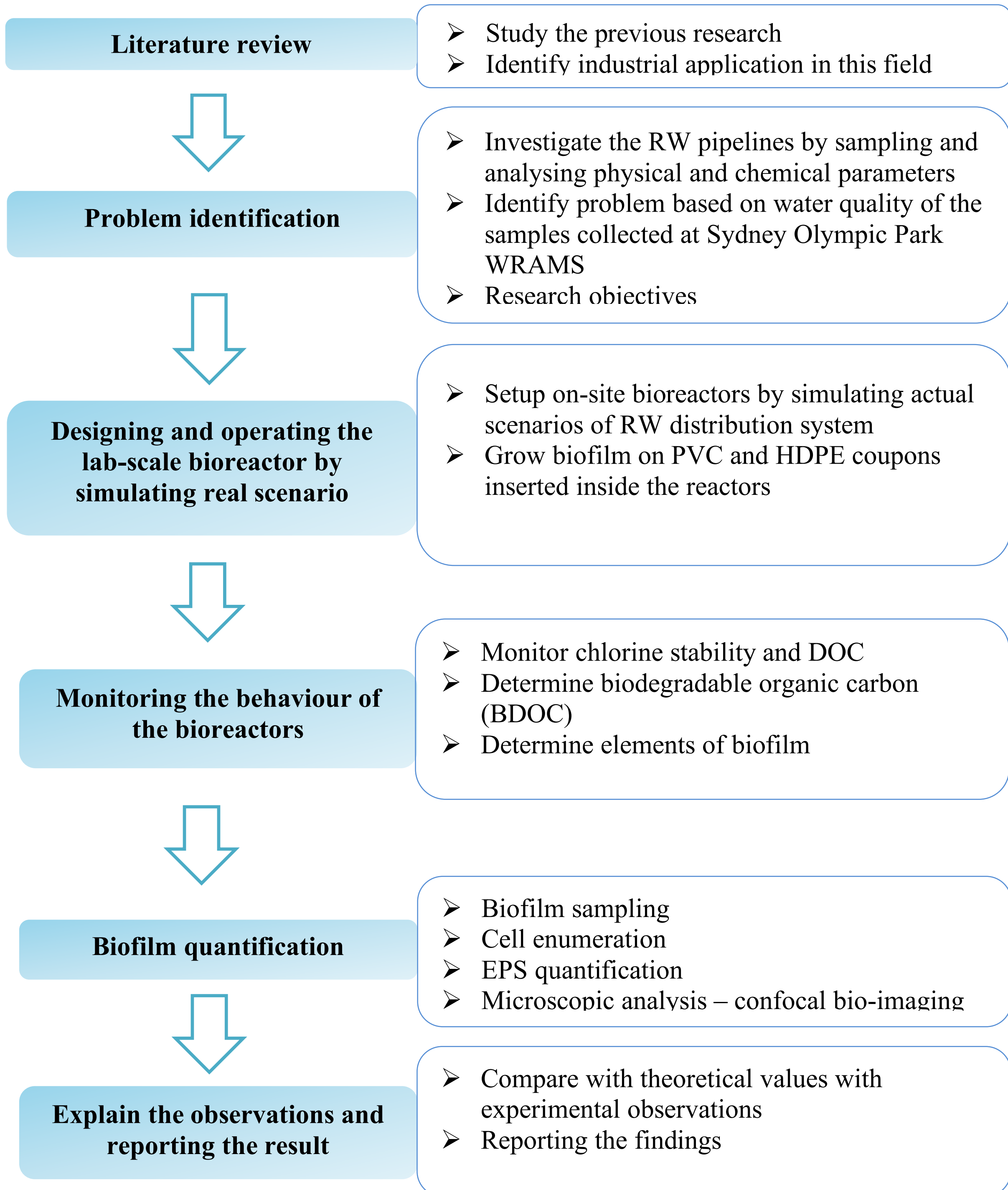
Table 2. 3 Quantification methods of biofilms in water distribution system

Biofilm quantification	Advantages and disadvantages	Analysis	Methods and Products	References
EPS quantification	Indirect measurement of biofilm quantity: specific biofilm constituent Damage biofilm structure	Total EPS	Lectin Triticum vulgaris TRITC	Neu et al., 2005
		Total carbohydrates	modified Phenol-Sulfuric acid method	Fang et al., 2009
		Proteins	Protein dye	Chandi and Angels, 2001
Cell enumeration	Indirect measurement of biofilm quantity: Microbial activity within biofilm	Heterotrophic plate count (HPC)	R2A agar	Manuel, Nunes and Melo, 2007
		Total cell count (TCC)	4',6-diamidino-2-phenylindole (DAPI); Acridine orange	Park, Choi and Hu, 2012
		Actively respiring cells	CTC 5-Cyano-2.3-ditolyl Tetrazolium Chloride	Jungfer et al., 2013
		Living and dead bacteria cells	BacLight: SYTO9-green and propidium iodide (PI)-red fluorescent nucleic acid stains	Gomes, Simoes and Simoes, 2013
		Viable cells	Adenosine triphosphate (ATP)	Liu et al., 2013
CLSM	Direct measurement of biofilm quantity No biofilm structure damage	Biofilm thickness, volume, 3D structure	Fluorescent nucleic acid stains: SYTO60, SYTO9 and labelled lectins	Fang, Hu and Ong, 2010
Gravimetric measurements	Direct measurement of biofilm quantity Damage biofilm structure	Biofilm thickness, biomass, mean biofilm density		Stautd et al., 2004

CHAPTER 3

MATERIALS AND METHODS

3.1.Project framework



3.2. Water quality investigation

3.2.1. Sydney Olympic Park WRAMS

SOPA is leading the way in urban water cycle management by integrating water supply, sewage, RW, and stormwater with a range of activities, services, functions and technologies. SOPA's integrated water cycle system plays a major role in protecting the local waterways and helping to maintain a supply of drinking water for Sydney through water conservation, waste minimisation, and pollution control. It is one of the most effective and comprehensive water recycling systems in the southern hemisphere and provides a model for future sustainability on one of the driest continents on Earth.

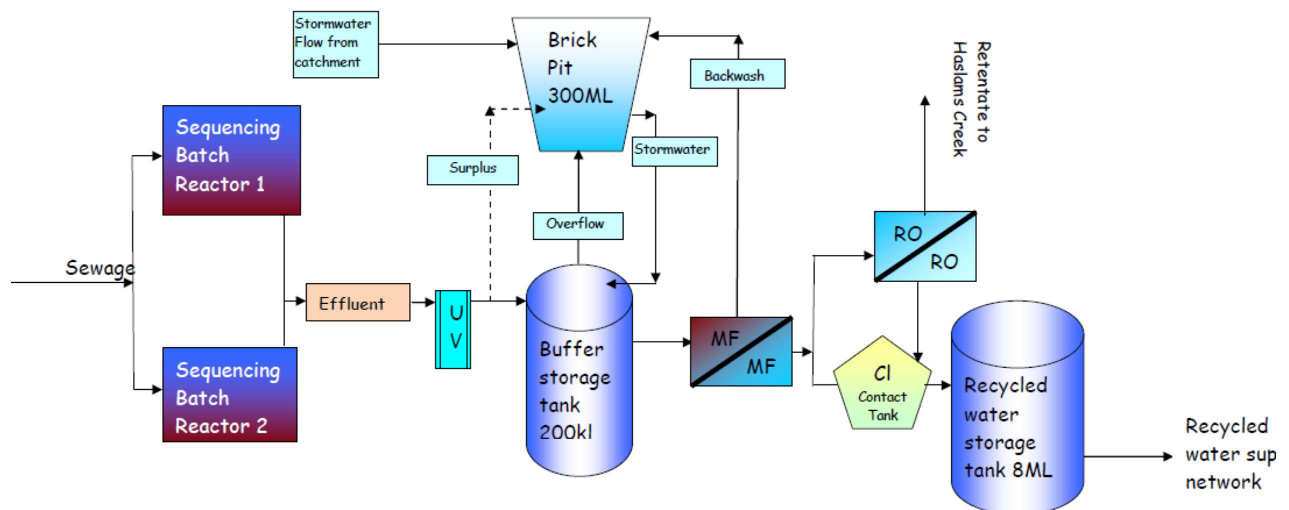


Figure 3. 1: Flow diagram of water processing at WRAMS facility (source: SOPA 2017).

Newington residential estate has a dual water (drinking and recycled) supply system. Drinking water is supplied by Sydney Water whilst RW is supplied by WRAMS. Figure 3.1 displays how all sewage types from Sydney Olympic Park and Newington residential estate is treated at the Water Reclamation Plant (WRP) using a biological process at a rate of 2.2 ML/day. The treated sewage water is then transferred to Water Treatment Plant (WTP) for further treatment. Alternatively, stormwater and runoff from roofs, roads, and pavements are collected to be recycled. Pollutant traps collect large debris before the water travels downstream to ponds and wetlands where it is stored. At the WTP, so-called WRAMS building (Figure 3.2), stormwater from the Brickpit reservoir and treated sewage water from WRP is combined in a 30 : 70 ratio before undergoing final processing using two further treatment methods that remove nutrients and pollutants. Firstly, water passes through a

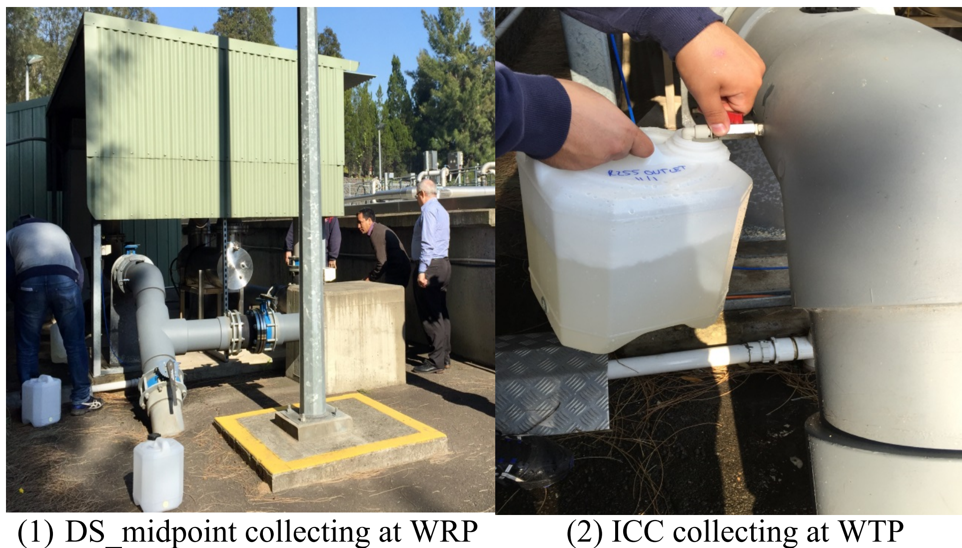
continuous microfiltration system to remove all particles larger than 0.2 μm (including viruses and bacteria), and secondly, through reverse osmosis to reduce salinity. The RW is then retained in a storage reservoir of 8 ML capacity for 2 to 3 days, before being distributed into local households for gardening, laundry and toilet flushing. More information about Sydney Olympic Park RW can be found at www.sydneyolympicpark.com.au.

Figure 3. 2: WRAMS building – WTP (source: SOPA 2017)

3.2.2. Investigation of water pipelines in Sydney Olympic Park

An investigation was carried out to examine the water quality parameters responsible for the degradation of chlorine residuals in the SOPA system. In this investigation, carbon-free glassware was used to avoid organic matter contamination as well as to minimise chlorine demand. They were prepared with thorough washing with detergent, rinsing with several times with MilliQ water, and then oven dried at 40 ^{o}C for 6 h. The plastic containers and caps were subjected to a similar procedure except that they were air-dried instead of being oven-

dried. Water samples were collected in container (15L) and then stored below 4 °C. Water samples were collected from the site with the cooperation and supervision of Sydney Olympic Park Authority from 6 locations: (1) stormwater, (2) inlet to chlorine contact tank (ICC), (3) outlet of chlorine contact tank (OCC), (4) outlet to storage reservoir (OSR), (5) Midpoint of distribution system (DS-midpoint), (6) End point of distribution system (DS-endpoint) (Figure 3.3). On 19/05/2016 and within 10 days from 15/08/2016 to 25/08/2016, these sources of water were collected daily and analysed to monitor their chlorine stability.



(1) DS_midpoint collecting at WRP (2) ICC collecting at WTP

(5) OSR collecting at WTP

Figure 3. 3: Photographs of water sample collection points and equipment.

3.2.3. Physical and chemical analyses

pH and free chlorine were measured via onsite instrumentation. Water samples were transported at room temperature to the environmental laboratory of Western Sydney University where all other physical and chemical parameters were analysed within 6 h from

the time of collection. All samples were filtered through 0.45 µm membrane filters (Millipore MF) and stored in carbon-free glass vials. Dissolved organic carbon (DOC) contents were analysed with a TOC-L instrument (Shimadzu, Japan). Ammonia nitrogen (NH_4-N), nitrite (NO_2) and nitrate (NO_3) were measured by Gallery (Thermo Scientific). Residual free chlorine and total chlorine were analysed using the DPD colorimetric method with a DPD Test and Tube kit (HACH 2800).

3.2.3.1. Chlorine decay profiles

To understand chlorine stability in the bulk water samples, it is very important to carry out chlorine decay profiles for all water sources. In particular, water samples were collected as raw water before chlorination, known as ICC and stormwater, which were then subjected to chlorination for chlorine decay profile. Because RW has been known for high demand of chlorine, and thus, two doses of total chlorine (3 and 5 mg/L) were added to the samples to determine chlorine demand which were lower than the actual dosage (≥ 6 mg/L) at the treatment plant. Similar chlorine dosages were also used in previous study (M Acharya et al., 2016) to understand bacterial community structure in the reclaimed water. The samples were stored in the incubator to maintain a constant temperature (25 ^oC). This test was carried out until total chlorine residuals dropped below the detection limit (< 0.1 mg/L). The same ICC samples were continued re-chlorination with only one repeated dosing of chlorine (3.0 mg/L). Other water sources were collected as disinfected water after chlorination, such as OCC, OSR, DS-midpoint and DS-endpoint, where initial chlorine decay was observed until total chlorine residuals dropped below the detection limit (<0.1 mg/L) and then subjected to re-chlorination with a single dose of chlorine (3 mg/L).

3.2.3.2. Biodegradable dissolved organic carbon (BDOC) test

The most common parameter for BDOC quantification is the measurement of DOC concentration in the bacteria containing water. The difference between the initial DOC and the final DOC during the incubation period is classified as the BDOC. The amount of biodegradable organic matter content can be used as a measure of bacterial growth. Biological activated carbon (BAC) was used as bacterial inoculums for 4 different water samples collected from ICC, OSR, DS-midpoint and DS-endpoint during the study period. The control of each sample had the BAC sterilised by heat-treatment at 70 ^oC for 2 hours. All samples and controls were individually stored in 500 ml glass bottles and were incubated

over 60 days. Samples were collected every week and filtered through 0.45 μm membrane filter prior to DOC analysis by TOC-L (Shimadzu, Japan).

3.3. Biofilm monitoring setup and experiment

3.3.1. Flow cell bioreactors

The selection of flow cell reactors among many different devices is evaluated based on its advantages in accordance with the aims of this study and to obtain reproducible results. The most important points worth noting are how flow conditions in flow cell systems mimic real distribution systems and are easy to control under different environmental conditions (Gomes et al., 2014). Flow cell reactors were used to monitor biofilm development and response to different disinfection control treatments as well as testing the influence pipe materials and hydrodynamic conditions on biofilm formation.

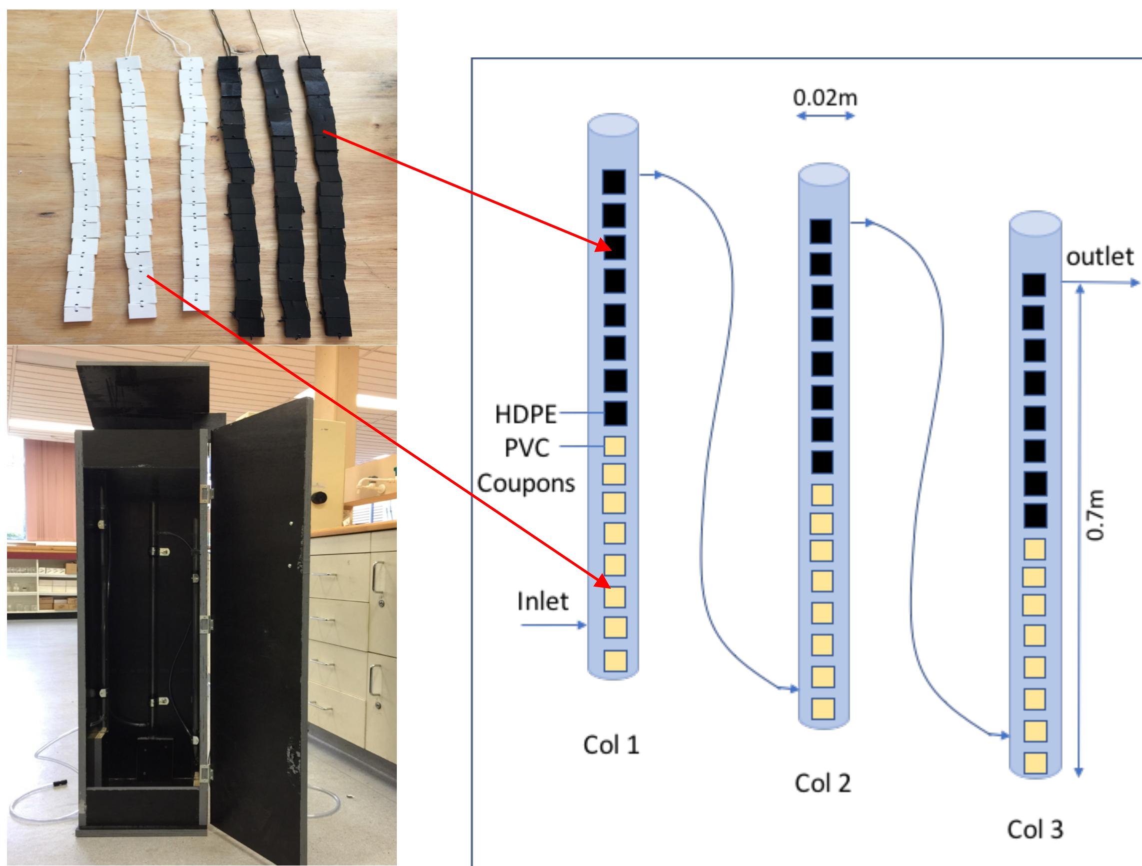


Figure 3. 4: Photos and diagram of experimental setup flow cell system

In this experiment, each flow cell reactor consists of a glass tube segment (800 mm length and 20 mm inner diameter), which has one inlet near the enclosed bottom and one outlet near the opened top. PVC and HDPE materials were cut to rectangular flat surfaces, average coupons size 14 mm x 10 mm, were washed with 70% ethanol and MilliQ water 3 times and oven dried for removal of bacteria. Both PVC and HDPE coupons were connected neatly together and each glass column was able to comprise a maximum of 72 coupons. Three flow cell columns were connected as shown in Figure 3.4 and were incubated in a dark chamber throughout the experiment. These flow cell bioreactors were installed in the glasshouse of WTP at Sydney Olympic Park, at the outlet of the storage reservoir. The reactors were fed directly by the main stream under various chlorine residuals over time. The flow cell reactors were used as a by-pass, therefore, temperature, pH, turbidity and conductivity in the reactors were similar as in the storage reservoir. Specifically, the water flow was set up at the minimum rate of 0.08 L min^{-1} and the maximum rate of 0.28 L min^{-1} by an adjustable valve or a peristaltic pump. The Reynold's number, which reflects flow characteristics was calculated using Eq. 3.1

$$Re = \frac{\rho * v_{ag} * D}{\mu} \qquad (3.1)$$

where, Re = Reynold's number

ρ = water density, kg/m^3

v_{ag} = average water velocity, m/s

D = pipe diameter, m

μ = dynamic viscosity, N.s/ m^2

3.3.2. Biofilm sampling and reactor monitoring

Biofilms were grown in the flow cell reactors for 105 days, from 14/11/2016 to 24/02/2017. Biofilms were sampled over 15 weeks at week 2, 4, 7, 10, 13 and 15 with sampling intervals of every 2 to 3 weeks. PVC coupons were taken from the bottom up, whilst HDPE coupons were taken from the top down. Water flow was unaffected by the sampling process as the coupons were removed without disturbing other biofilm coupons. Biofilm samples were handled with care at all times to avoid environmental contamination and to assure water retention within the inserted coupons in the reactors. Each PVC and HDPE coupon with attached biofilm was stored separately in MilliQ water containing centrifugal tube (15 ml) and preserved at 4 ^{o}C.

Bioreactor monitoring was a significant part of sampling and involved in many parameters that impacted on biofilm formation. From 5 different points of the reactors: inlet of the reactor, column 1, column 2, column 3 and outlet of the reactor, water samples were taken to monitor free chlorine, total chlorine, DOC at the times of biofilms sampling. Flow rate was also measured at each sampling. Regular reactor monitoring not only helped to understand the development on biofilm growth, but also compared the chlorine data between automatic records of the main system and the reactor monitoring records.

3.3.3. Biofilm analysis

Biofilm quantification includes:

- ✓ Fe and Mn analysis
- ✓ EPS production analysis as total carbohydrates content
- ✓ Biomass and biofilm thickness: using gravimetric measurement
- ✓ Biofilm thickness and biofilm volume: using CLSM
- ✓ EPS volume and bacterial cell volume: using CLSM and IMARIS
- ✓ Chlorine decay with biofilms

3.3.3.1. Metals (Fe and Mn) analysis

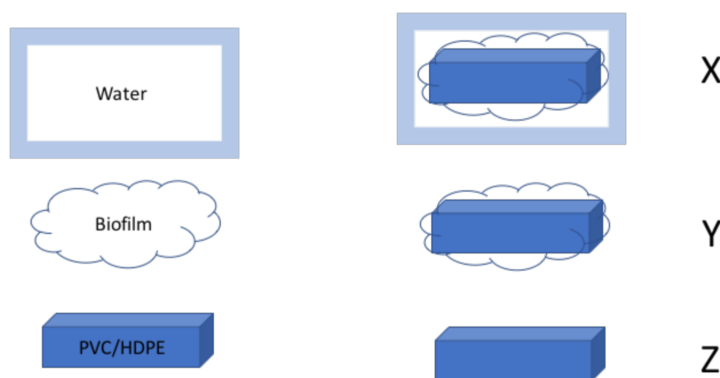
Besides biofilm-induced chlorine decay analysis, each coupon that was collected from the bioreactor was assigned a different method to quantify the biofilm composition. Biofilms were removed from the pipe material coupons for metal analysis. Ultrasonic (Unisonics 220, Australia) and vortex mixers (Ratek, VM1) were used to detach the biofilms from the PVC/HDPE coupons. Each biofilm coupon was individually pooled into a 10 ml 3% HNO_3 solution, which was sonicated for 2 hours and vortexed for 15 minutes. Subsequently 10ml of the suspended biofilm HNO_3 medium was decanted into new test tubes to analyse Fe and Mn concentration. Confocal laser scanning microscopy (CLSM) tests of the biofilm coupon showed that the longer time exposed to ultra-sonication, the more firmly attached biofilms could be eventually detached from the surfaces. Fe and Mn analysis were analysed by Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES) Agilent 700 Series (Agilent Technologies Inc. Malaysia), using wavelength calibration parameters of Fe 238.204 nm and Mn 257.610 nm.

3.3.3.2. Measurement of total carbohydrate quantity

Modified phenol-sulfuric method (Nielsen, 2010) was used to measure total carbohydrate concentrations in the biofilm sample with a glucose standard. Each detached biofilm sample was stored in 2ml Milliq water. A solution of 0.05 ml 80% phenol and 5ml concentrated H_2SO_4 was immediately added to the samples to improve mixing and vortex. Samples were allowed to stand for 10 minutes to react and were then cooled to room temperature in a water bath for 15 minutes. The concentrated H_2SO_4 breaks down polysaccharides to monosaccharides and then dehydrates it finally to furfural. These compounds then react with phenol to produce a yellow-gold colour, which can be colourimetrically detected using a Cary 60 UV-Vis recording spectroscope (Agilent Technologies, Malaysia). The absorbance at 490 nm was measured and total carbohydrate concentrations were obtained by comparing with a standard curve. The total carbohydrate quantity was expressed by the total carbohydrate concentrations divided by the surface area of PVC/HDPE coupon.

3.3.3.3. Gravimetric measurements for biofilm quantification

Gravimetric measurement is the traditional method to determine biofilm thickness and biomass. Firstly, biofilm coupons collected from the bioreactor, were drained for 5 minutes in a vertical position and weighed for X (g). Secondly, wet biofilm coupons were oven-dried for 24 h at 65°C, then weighed for Y (g). Thirdly, dried biofilms were pooled in MilliQ water and were detached by ultrasonication and vortexing, oven-dried for another 24 h at 65°C, then weighed for Z (g). Finally, biofilm wet mass (m_{WF}) was calculated from the weight difference between X and Z; and biofilm dry mass (m_{DF}) was calculated from weight difference between Y and Z as detailed somewhere else (Staudt et al., 2004).



X → oven-dried for 24hr → Y →removed biofilms, oven-dried for another 24hr → Z

Biofilm wet mass, m_{WF} = X – Z; biofilm dry mass, m_{DF} = Y – Z

Figure 3. 5: Gravimetric concept for quantifying biofilm

Biofilm thickness was calculated using Eq. 3.2

$$L_F = \frac{m_{WF}}{\rho_{WF}.A_F} \quad (3.2)$$

Where, L_F = biofilm thickness, cm converted to μm

m_{WF} = biofilm wet mass, g

ρ_{WF} = the wet density of the biofilm (ρ_{WF} = 1g/cm^3)

A_F = the surface area of the coupon, cm^2

The biomass was calculated by Eq. 3.3

$$M_F = \frac{m_{DF}}{A_F} \quad (3.3)$$

Where, M_F = biomass, g/cm^2

m_{WF} = biofilm wet mass, g

A_F = the surface area of the coupon, m^2

The mean biofilm density was estimated by Eq. 3.4

$$\rho_F = \frac{m_{DF}}{\frac{m_{WF}}{\rho_{WF}}} \quad (3.4)$$

Where, ρ_F = the mean biofilm density, g/cm^3

m_{DF} = biofilm dry mass, μg

m_{WF} = biofilm wet mas, μg

ρ_{WF} = the wet density of the biofilm (ρ_{WF} = 1g/cm^3)

3.3.3.4. Confocal laser scanning microscopy (CLSM) and digital image analysis IMARIS

The instrument used was an upright multi-channel confocal laser scanning microscope TCS-SP (Leica5, Heidelberg, Germany) equipped with:

- Software v2.00
- Lasers (Ar, Kr, He/Ne)
- Lens 50x dry
- Laser excitation: 488 nm (EPS) and 633 nm (bacteria cells)
- Laser emission signals: green channel 498-600 nm and red channel 640-750 nm

Staining products are:

- SYTO60 red fluorescent nucleic acid stain (Molecular Probes Inc., USA)
- Lectin from Triticum vulgaris EPS stain (Sigma Chemicals, USA)

First, biofilm sample was stained with a droplet of lectin at a concentration of 0.1 mg/ml as at 23°C for 15 minutes. Second, the sample was washed gently with MilliQ water three times, before it was counterstained with a droplet of SYTO60 as at 23°C for 5 minutes. The stock solution of SYTO60 was used at a dilution of 1:1000 in MilliQ water. Finally, the sample was washed gently with MilliQ water several times before bring visualized by the CLSM. By employing a range of CLSM-based imaging techniques in associated with IMARIS – digital image analysis v6.02 (Bitplane, Zurich, Switzerland), biofilm thickness, volume, biomass and three-dimensional structure were quantified.

3.3.3.5. Chlorine decay with biofilm profiles

Chlorine decay tests were carried out using the biofilm coupons collected from each column and the same source of water that was fed to the bioreactor. Control samples were made of clean PVC/HDPE coupons without biofilms and the same water source. From week 2 to week 10, a fixed dose of chlorine (3 mg/L) was added to all samples. However, the chlorine dose was reduced to 1.5 mg/ L and 1 mg/L on week 13 and week 15, respectively. A change in water source to MilliQ water was applied in order to limit chlorine demand from bulk water. All samples were stored in the incubator to maintain a constant temperature (25°C). Chlorine decay was carried out till total chlorine residuals of the samples dropped below the detection limit (<0.1 mg/L) using a colorimetric spectrophotometer (HACH DR 2800).

CHAPTER 4

RESULTS – PART I

WATER QUALITY PARAMETERS AND CHORINE DECAY OF THE BULK WATER SAMPLES FROM SOPA

One of the aims of this study was to understand the stability of chlorine residuals in the bulk RW. Samples were collected continuously for 10 days from WTP and distribution system from six locations of the system which are stormwater, ICC, OCC, OSR and two different locations of the distribution pipelines. Chemical parameters in the bulk water samples including free chlorine, total chlorine, DOC, ammonia, nitrite, nitrate and pH were analysed and detailed in Table 4.1. To understand chlorine stability in the bulk water samples, two doses of chlorine (3.3 and 5.1 mg/L) were added to the samples and stored in the incubator to maintain a constant temperature (25°C). This test was carried out until total chlorine residuals of the samples have dropped close to detection limit (<0.1 mg/L).

4.1. Chemical parameters

The average chemical parameters and standard deviations measured in the bulk water samples are presented in Table 4.1.

Table 4. 1 The average chemical parameters in the bulk water samples collected over 10 days from 15/08/2016 to 25/08/2016.

Sampling locations	Free chlorine	Total chlorine	DOC	Ammonia	Nitrite	Nitrate	pH	DO
		mg/L		mg-N/L				mg/L
Storm water			9.2	0.074	0.40	0.54	7.40	7.45
ICC			8.33±1.22	0.096±0.04	0.02	1.60±0.29	7.50	7.42
OCC	2.35±1.06	4.20	5.28±0.84	0.054±0.015	0	2.00±0.24	7.48	7.68
OSR	0.38±0.22	0.54	5.15±0.43	0.066±0.011	0	1.93±0.07	7.83	8.08
DS-midpoint	0.26±0.23	0.12	5.12±1.28	0.031	0	0.66	7.55	8.36
DS-endpoint	0.12±0.06	0.03	5.11±1.44	0.032	0	0.64	7.52	8.45

A significant drop in free chlorine residuals (from 2.35 to 0.38 mg/L) was noted between OCC and OSR. Free chlorine residuals at OSR (inlet to the distribution system) were 0.38 mg/L which dropped to 0.26 mg/L at DS-midpoint and continuously dropped to 0.12 mg/L at DS-endpoint (Table 4.1). This chlorine profile was an average of the samples collected over 10 days. Similarly, DOC was observed to be decreased around 3 mg/L from ICC to OCC which is due to the dilution taking place in the chlorine contact tank (Table 4.1). In order to reduce the salinity and to meet distribution demand, the SOPA combines stormwater : ICC water at a ratio of 30 : 70. This may cause DOC to be reduced by the same ratio for samples after combination. In fact, Table 4.1 shows a reduction in DOC concentration between ICC and OCC, approximately 3 mg/L, equivalent to 30% of DOC in stormwater. Also, DOC decreased from ICC to DS-endpoint (Table 4.1) which could be due to the microbial activities and /or oxidation of DOC by chlorine. Nitrogen was found mostly in the oxidised (nitrate) form in the bulk water samples. The absence of nitrite (< 0.02 mg N/L), the presence of high levels of nitrate and low concentrations of ammonia-N prove that complete nitrification had occurred before the water was chlorinated (Table 4.1). The pH was stable at approximately range pH7.4 – pH7.8 (Table 4.1). Similarly, DO was steady in the bulk water samples which ranges between 7.42 and 8.45 mg/L.

4.2. BDOC

The quantity of BDOC which measures the potential growth of bacteria in the system was determined. This experiment was carried out with 4 water samples and 4 controls collected from ICC, OSR, DS-midpoint and DS-endpoint. All of them were in the condition of free of chlorine. Physical adsorption of DOC takes place in the control sample over time because they contained no bacteria. BDOC is the difference in the amount of DOC between the samples and the controls. BDOC did not witness a significant variation among the water samples. Approximately 1 mg/L BDOC in DS-midpoint and DS-endpoint samples shows that there is a great potential of regrowth of bacteria in the distribution system in the absence of/ or low disinfectant residuals (Figure 4.1).

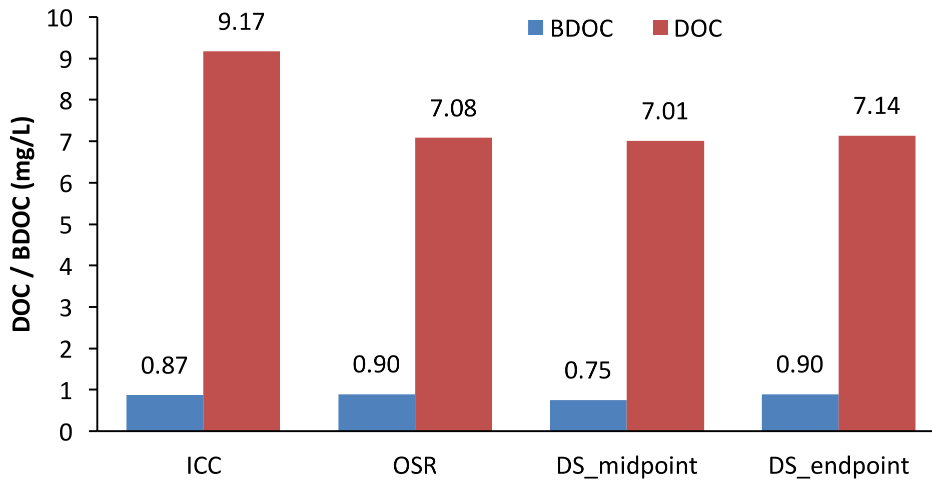


Figure 4. 1: BDOC and DOC for samples collected on 06/12/2016

4.3. Chlorine stability in the bulk water samples:

Chlorine decay in the bulk water samples during the course of experiment with two chlorine doses (3 and 5 mg/L) is depicted in Figures 4.2 and 4.3. A rapid total chlorine reduction within 3 hours and after that it slowed down, which was noted in the storm water and ICC for both doses of chlorine as they were collected prior to chlorination. The most stable total chlorine was noted in the water sample collected from the OSR (inlet to the distribution system) as it was collected after chlorination at the plant. Similar observations were noted in the samples collected from the distribution system which displayed greater chlorine stability in the DS-midpoint sample as opposed to DS-endpoint sample. At the time of collection, OCC had the highest measured chlorine residual (Table 4.1) which could have facilitated stable total chlorine residuals in the tested samples. However, less stable total chlorine was noted in the OCC water sample compared to other samples (DS-midpoint, DS-endpoint and OSR) (Figures 4.2 and 4.3). The varying chlorine decay rates might be proportional to the initial chlorine dose and cannot be explained by the simple first order decay. Such models have been developed to explain chlorine decay in drinking water and reclaimed water (Jabari Kohpaei and Sathasivan, 2011, Funamizu et al., 2004).

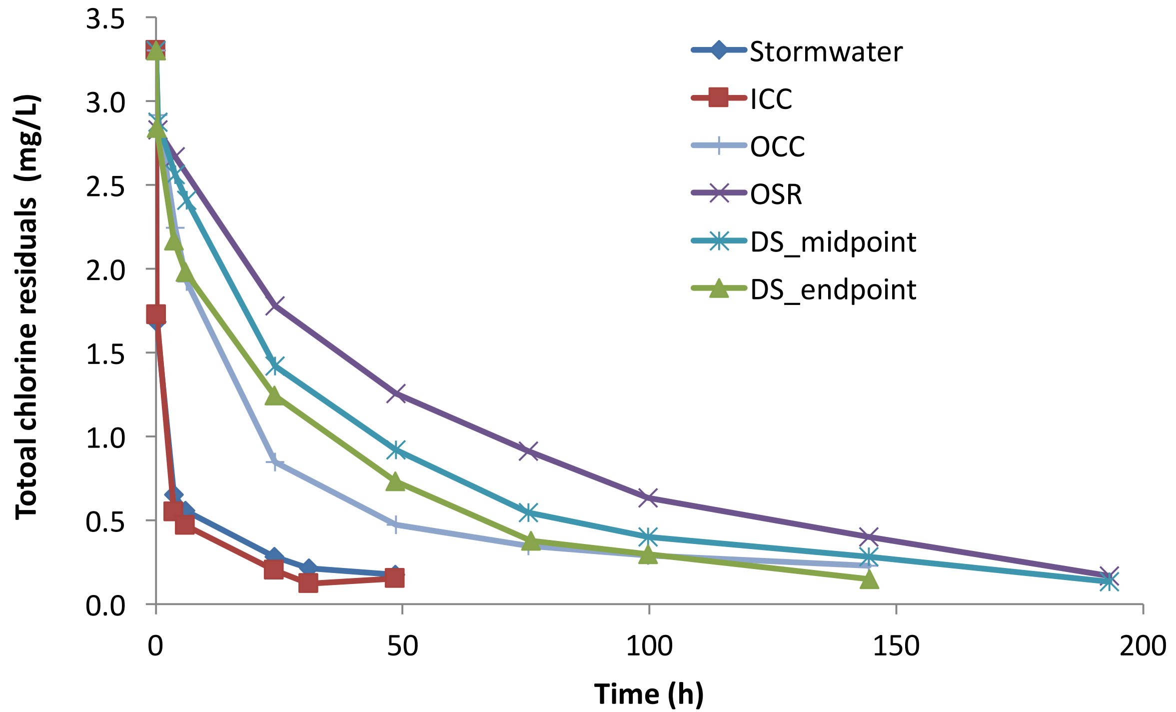


Figure 4. 2: Total chlorine residuals in the recycled bulk water samples after dosing initial total chlorine residuals of 3.3 mg/L

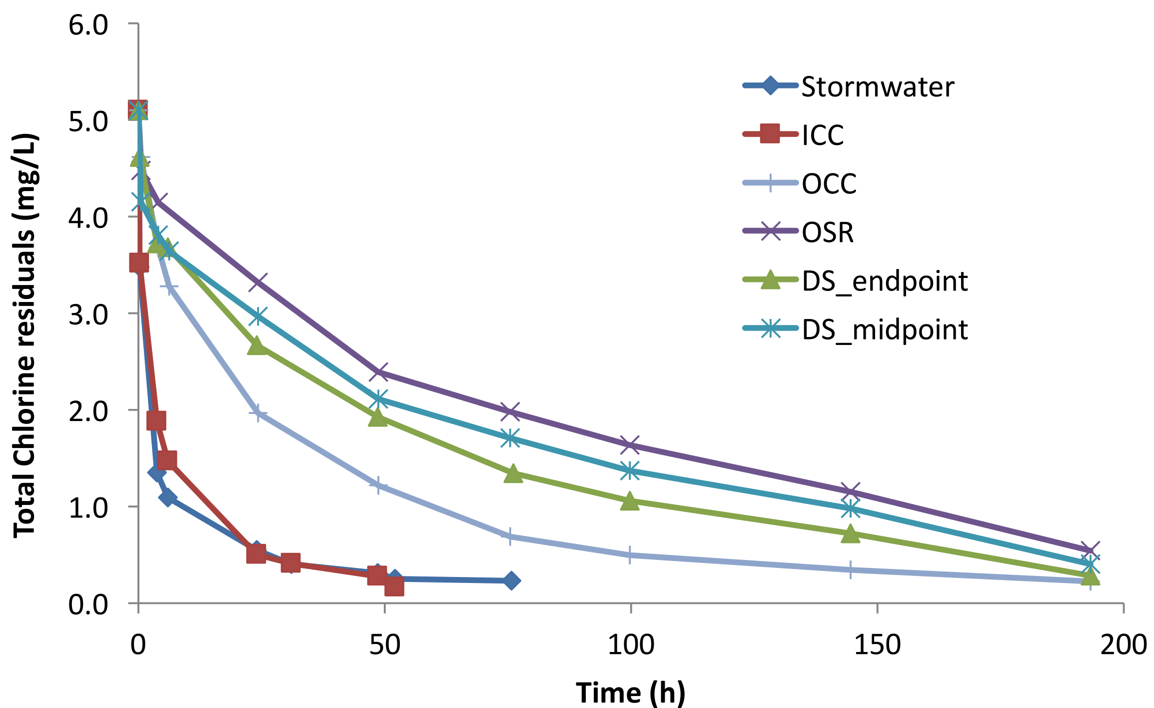


Figure 4. 3: Total chlorine residuals in the recycled bulk water samples after dosing initial total chlorine residuals of 5.1 mg/L

4.4. Chlorine stability in the mixture of stormwater and recycled water:

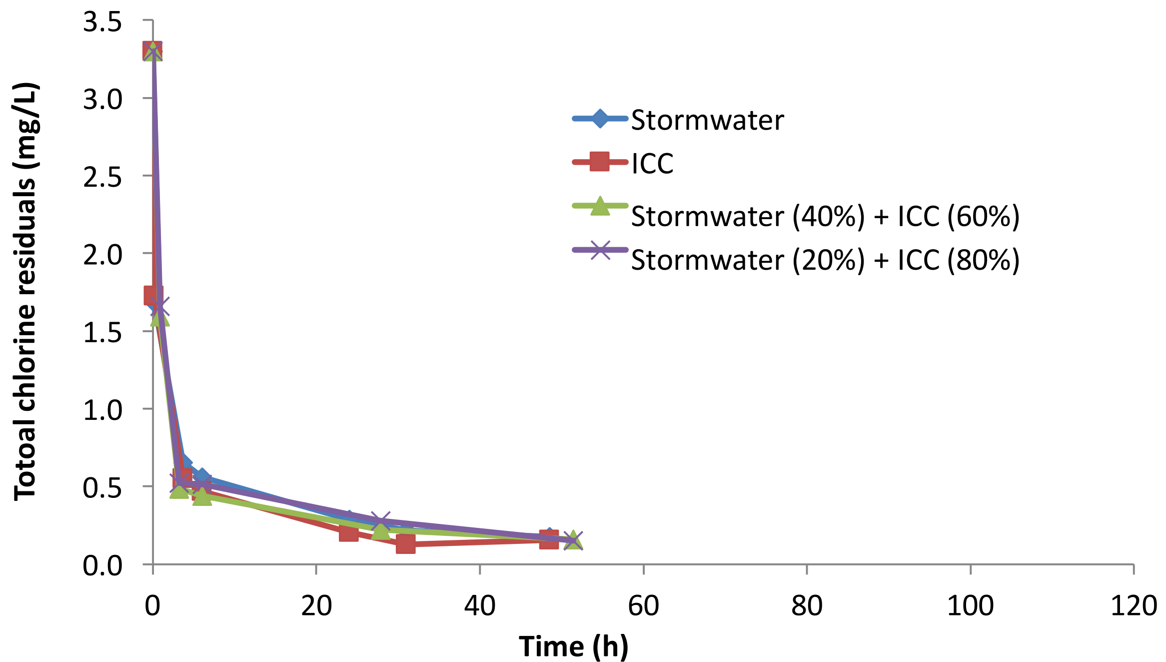


Figure 4. 4: Total chlorine residuals in the mixed water samples after dosing initial total chlorine residuals of 3.3 mg/L

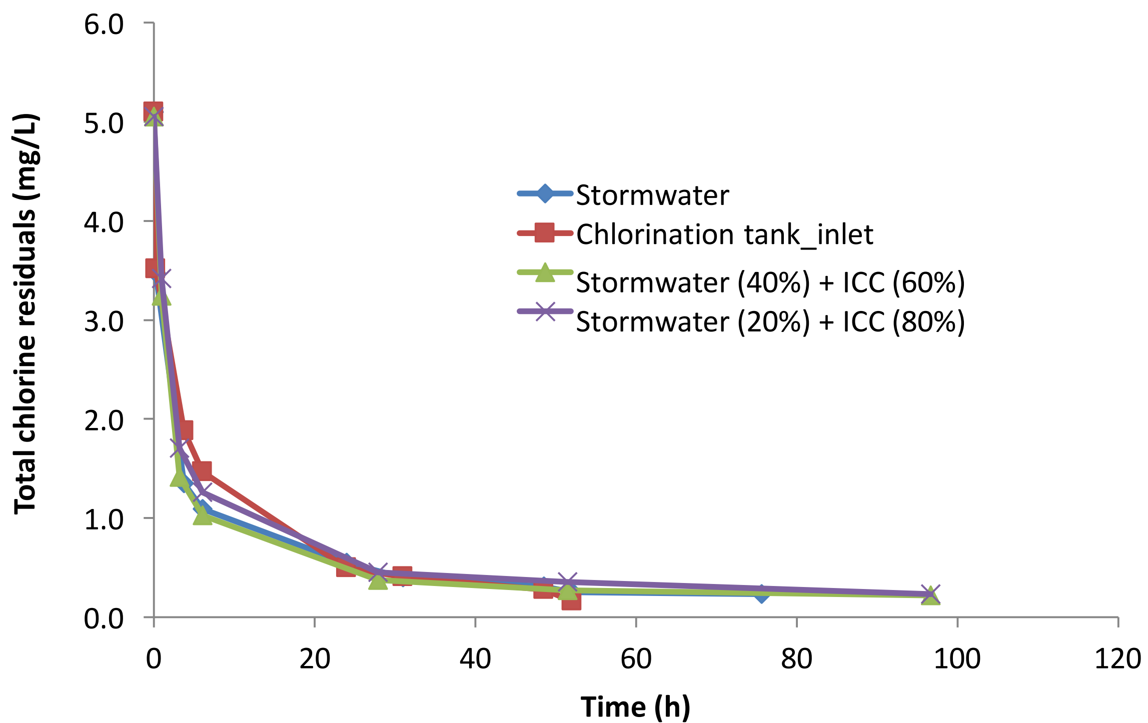


Figure 4. 5: Total chlorine residuals in the mixed water samples after dosing initial total chlorine residuals of 5.1 mg/L

Chlorine decay in the mixture of storm water and recycled water ICC during the course of experiment with two chlorine doses (3 and 5 mg/L) is depicted in Figures 4.4 and 4.5. The SOPA combines stormwater : ICC at a 30 : 70 which may fluctuate depending upon public demand. To replicate this supply, chlorine decay tests were carried out at two ratios. The stormwater: ICC mixing ratios used in this study were 40 : 60 and 20 : 80. Mixing of stormwater and ICC did not make any change to chlorine stability for both doses of the initial chlorine (Figures 4.4 and 4.5). However, this observation was based on a one-time sampling and the results could vary depending on stormwater and RW quality parameters such as pH, DOC, ammonia and nitrite. The varying chlorine decay rates might be proportional to the initial chlorine dose and cannot be explained by a simple first order decay model. Proper models have been developed to explain chlorine decay characteristics in drinking water and reclaimed water (Jabari Kohpaei and Sathasivan, 2011, Funamizu et al., 2004).

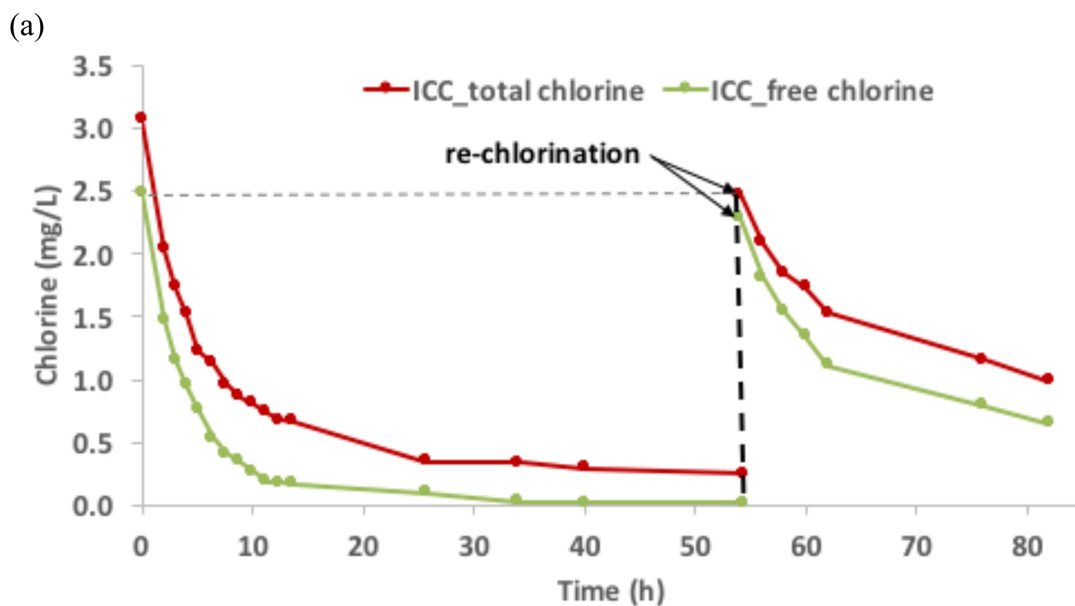
4.5. Chlorine stability by re-chlorination

In this experiment, the same total chlorine doses of 3 mg/L were used for all samples, whether initial- or re-chlorination. ICC water sample was unchlorinated at the time of collection. A rapid reduction in both total chlorine and free chlorine was noted at the initial chlorination stage of ICC water sample (Figure 4.6 a). Dissolved organic nitrogen (DON) compounds in untreated ICC sample might exert high chlorine demand and therefore reduced the concentration of free chlorine. The gap between free chlorine concentration and total chlorine concentration represents organic chloramine which is formed by reactions between DON and free chlorine. Although more stable chlorine was noted in re-chlorinated ICC, the organic chloramine still remained constant as the initial chlorination (Figure 4.6 a). The organic chloramine has poor germicidal properties, which makes their formation undesirable during chlorination and reduces the disinfection efficacy of chlorination process (Fisher et al., 2011).

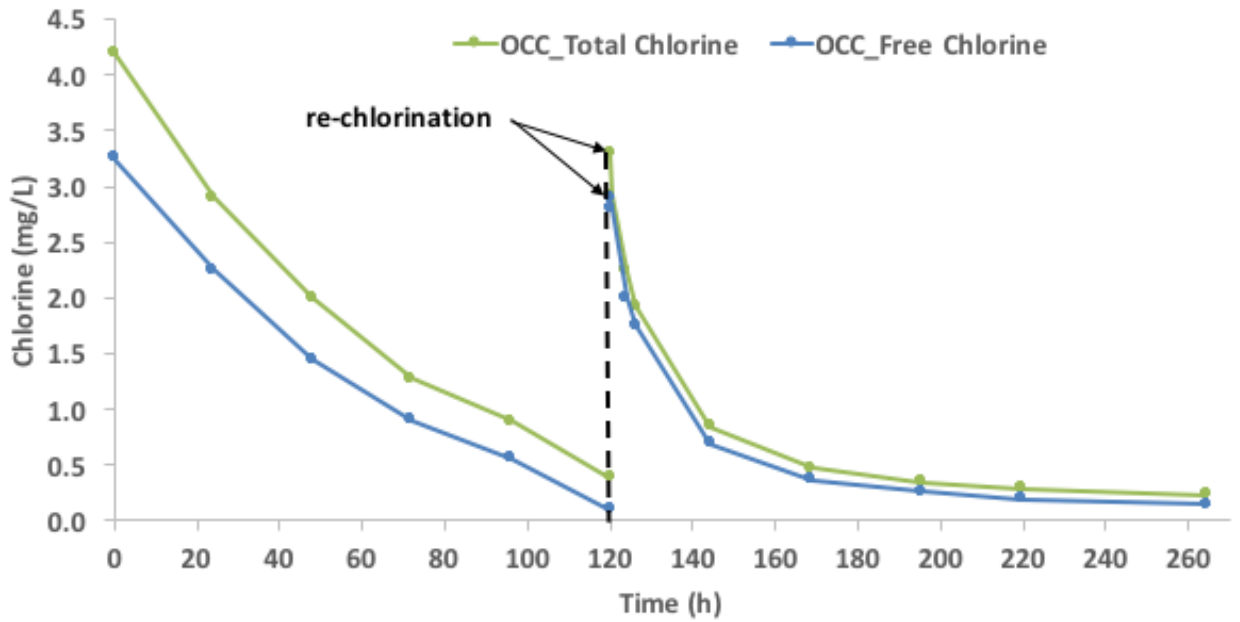
Water samples OCC and OSR, which were chlorinated by SOPA at the time of collection, were observed until total chlorine reached below the detection limit (< 0.1 mg/L). These were considered as initial chlorination profiles of OCC and OSR (Figures 4.6 b and c). The difference between total chlorine and free chlorine concentrations for both sampling locations, OCC (Figure 4.6 b) and ICC (Figure 4.6 a) were nearly the same which

demonstrates the formation of equal concentrations of organic chloramines in both locations. However, less stable chlorine and reduction in organic chloramines were noted for both OCC and OSR water samples in re-chlorination stage in comparison with their initial chlorination ones (Figures 4.6 b and c). Moreover, re-chlorinated OCC and OSR demonstrate greater chlorine stability than re-chlorinated ICC. This varying rate of chlorine could not be explained by simple first-order decay. Besides decay rate being proportional to initial chlorine dose, the rate could also be dependent upon the presence of 2 or more groups of chlorine-demanding components, which react with chlorine differently in their reactivity (Jegatheesan et al., 2004). Water retention time in the OSR tank is between 2 to 3 days, the re-chlorination profiles have shown that both free chlorine and total chlorine become stable in the OSR tank (Figure 4.6 c), thereby enabling the distribution system to maintain chlorine residuals after the re-chlorination.

(a)



(b)



(c)

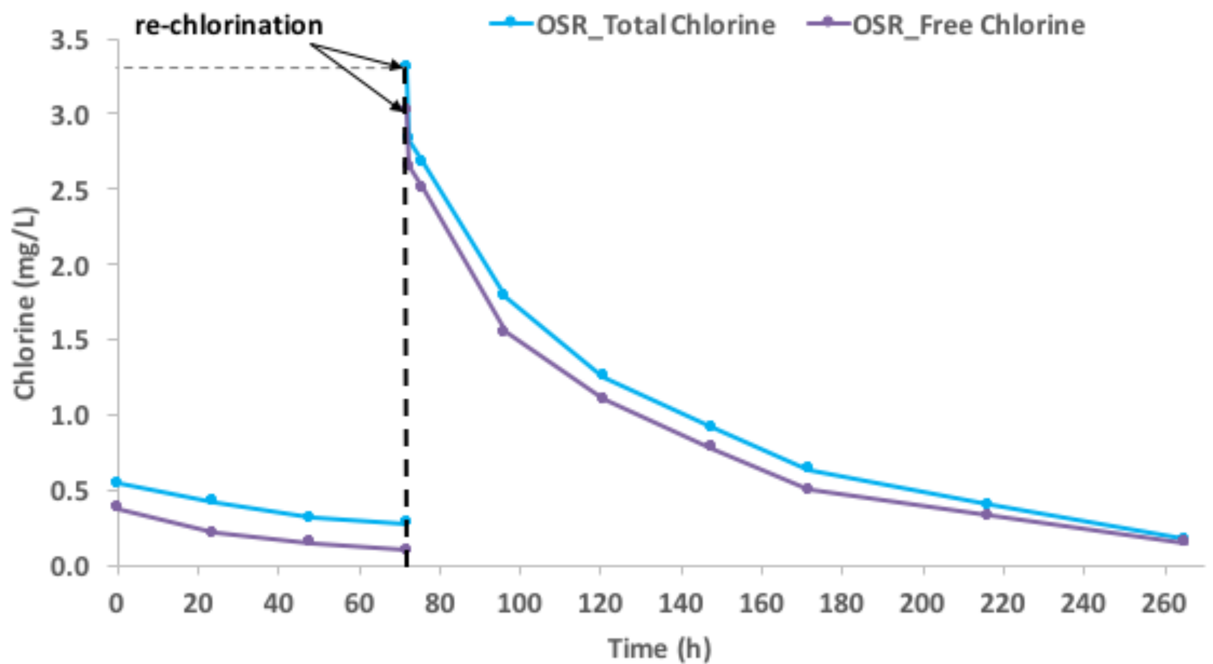


Figure 4. 6: Initial chlorination and re-chlorination of water samples after dosing total chlorine residuals of 3 mg/L: (a) ICC, (b) OCC and (c) OSR

In short, the bulk water samples collected over 10 days show a significant drop in free chlorine residuals in the system of SOPA. A rapid total chlorine reduction was noted in the stormwater and ICC as they were collected prior to chlorination. More stable total chlorine was noted in the water sample collected after chlorination, with an order of chlorine stability

as follows, OSR, DS-midpoint, DS-endpoint and OCC. Mixing of stormwater and ICC did not make any change to the chlorine stability. Similarly, DOC was decreased around 3 mg/L from the inlet to the outlet of the system which is due to the dilution taking place in the chlorine contact tank in order to reduce the salinity and to meet distribution demand. BDOC did not display any observable variation between the water samples. Approximately 1 mg/L BDOC in DS-midpoint and DS-endpoint samples shows that there is a great potential of regrowth of bacteria in the distribution system in the absence of/ or low disinfectant residuals. Inorganic nitrogen was found mostly in nitrate form in the bulk water samples. The absence of nitrite and the presence of high levels of nitrate and low concentrations of ammonia-N prove that complete nitrification had occurred before the sample was chlorinated. The pH was stable around pH 7.5±0.1.

DON compounds in untreated ICC sample might exert high chlorine demand and therefore reduce the concentration of free chlorine and inorganic combined chlorine. Although more stable chlorine was noted in re-chlorinated ICC, the organic chloramine still remained at the same level. The organic chloramines were also found in water samples OCC and OSR which were chlorinated by SOPA at the time of collection. However, reductions in organic chloramines were noted for both re-chlorinated OCC and OSR. The organic chloramines have poor germicidal properties, which makes their formation undesirable during chlorination and reduces the disinfection efficacy of the chlorination process. Finally, re-chlorinated OCC and OSR demonstrate greater chlorine stability than re-chlorinated ICC due to low organic chloramines.

CHAPTER 5

RESULTS – PART II

BIOFILM DEVELOPMENT IN THE PILOT-SCALE FLOW CELL REACTORS

The aim of this study was to understand biofilm growth on selective pipe materials and the impact of biofilm on chlorine decay in the RW distribution system. In order to investigate biofilm development, the flow cell reactors system containing PVC and HDPE coupons were installed and operated on the field at the outlet of the storage reservoir at WTP over 15 weeks exposed to similar chlorinated environmental conditions of the storage reservoir. The role of biofilm components in decaying chlorine were determined by periodically monitoring (water flow rates, chlorine residuals, DOC), sampling (biofilm coupons) and analysing a number of biofilm parameters (total carbohydrates, EPS, bacterial cells, metals, thickness and volume of biofilm). There were five different locations of the reactor system that were considered in this study, namely inlet, column 1, column 2, column 3 and outlet. Both online and onsite data of free chlorine residuals were compared. Biofilms coupons were analysed using gravimetric measurements for biofilm thickness and biomass. The assessment of biofilm structure and volume was carried out using CLSM. The results of biofilm development in the pilot-scale reactors are presented as follows.

5.1. Monitoring bioreactors

5.1.1. Details of chlorine residuals

Biofilms were grown from 15/11/2016 to 28/02/2017 (105 days or 15 weeks). There were 6 samplings at week 2, 4, 7, 10. 13 and 15. Free chlorine concentration monitoring using the online probe at the OSR of WTP over the operation time of the biofilm reactor setup is detailed in Figure 5.1. Both free chlorine and total chlorine were monitored on the site at each sampling. The level of free chlorine from both online and onsite monitoring appears correlated to each other (<0.5 mg/L) (Figures 5.1 and 5.2). Monitoring data shows total chlorine concentrations at all times were greater than free chlorine due to a part of free chlorine may react with organic matter to form organic chloramines. At the time of biofilm sampling, the different chlorine concentration between the inlet and outlet was the amount of chlorine consumed by the biofilms resident in each column bioreactor (Figure 5.2).

Performing linear regressions on the data for free chlorine concentration levels and the inlet/ outlet chlorine concentration differences with week of sampling as a random effect shows no significant relationship between the two variables (F=0.6741, df = 1.5, p=0.449).

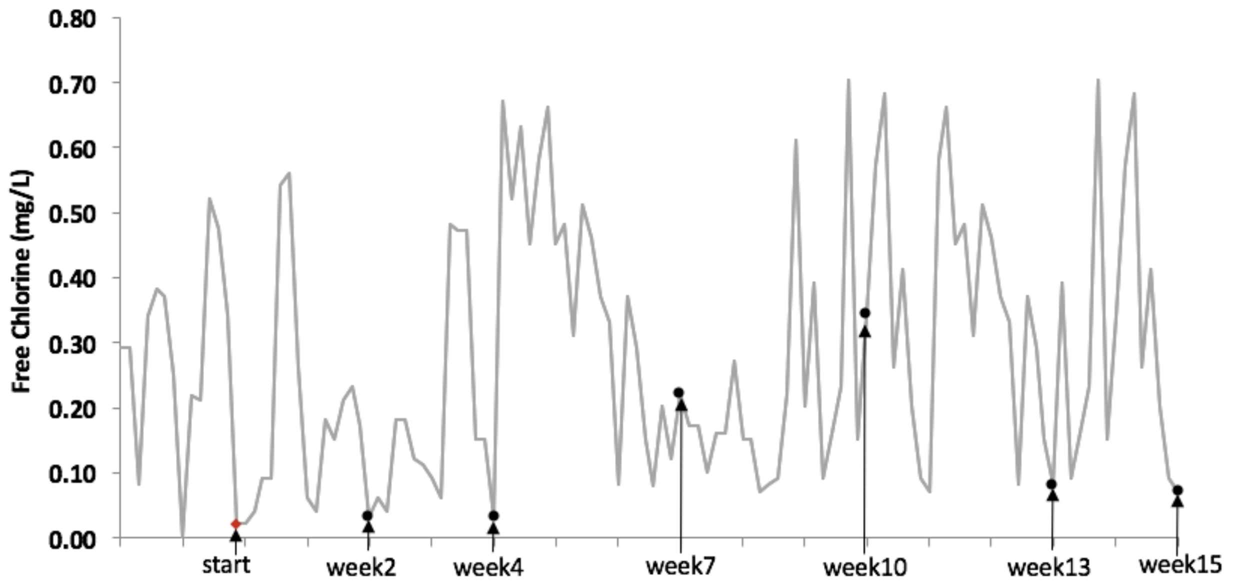


Figure 5. 1: Online chlorine concentration during biofilm sampling (source: SOPA 2017)

*start: the day when the reactor was installed and operated

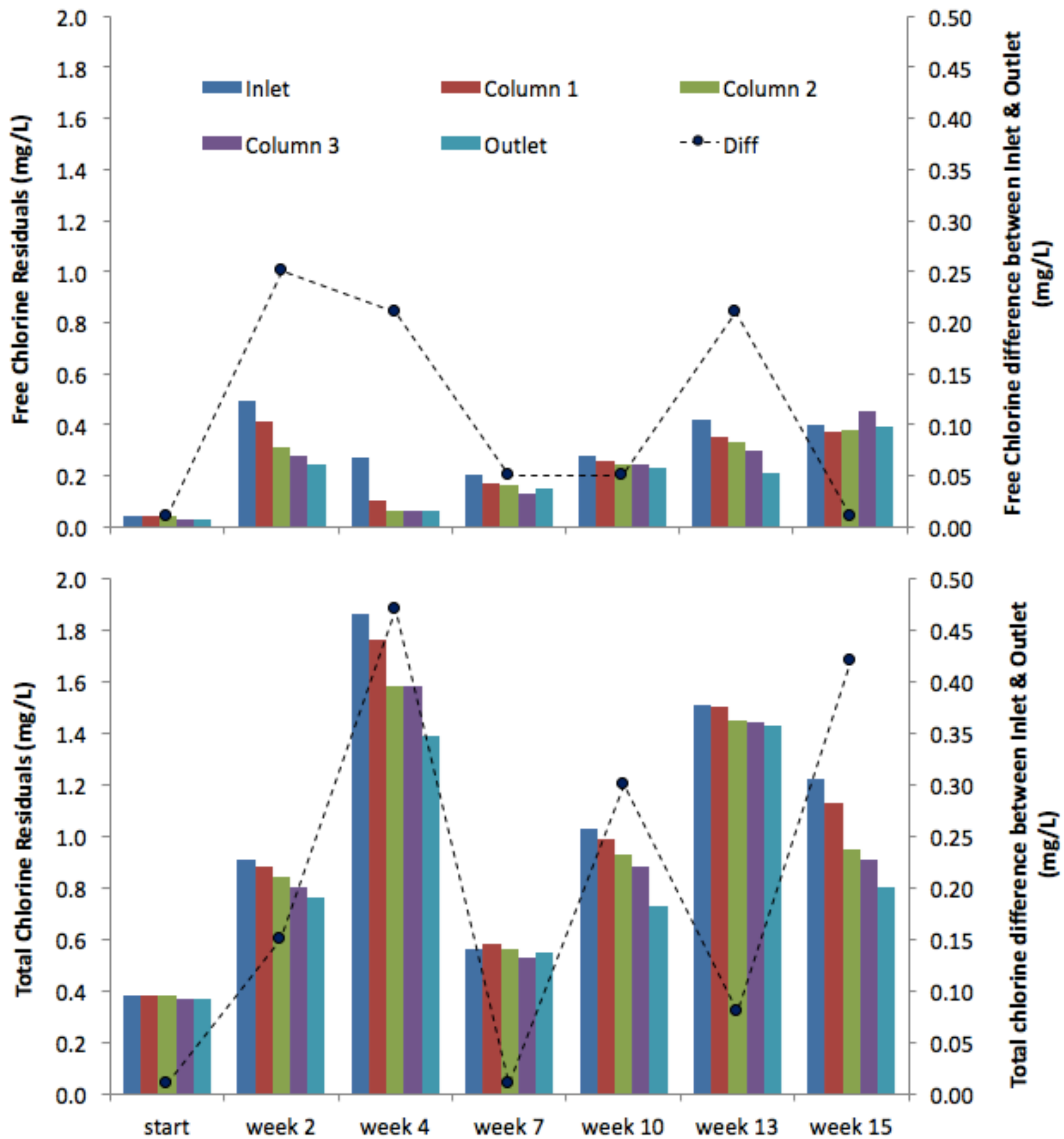


Figure 5. 2: Onsite monitoring of chlorine residuals concentration

5.1.2. Operational details of the biofilm reactors

From the beginning of reactor setup, flow rate monitoring at the outlet of the reactors shows that the flow rate was likely to increase and cause an overflow in the reactors at week 4. A significant decrease in flow rate was observed due to valve installation for adjusting the flow at the inlet of the reactors. However, the flow rate tended to rise in spite of adjusting the valve each sampling time. However, the laminar flow (Re < 2300) were maintained in biofilm reactor (Table 5.1) during the study period. Flow rate and the characteristics of the flow in

each column reactor were calculated using the following formulae of retention time, velocity and Reynold's number.

Water retention time (s), $t = \frac{V}{Q}$

Water velocity (m/s), $v = \frac{Q}{A}$

Reynold's number , $Re = \frac{\rho * v * D}{\mu}$

Dynamic viscosity, $\mu = 1.002 \times 10^{-3}$ N.s/ m^2

Where Q is flow rate (m^3/s), ρ is water density (~1000 kg/m^3), V is glass column volume (m^3) and A is glass column area (m^2).

Table 5. 1 Monitor flow rate, retention time, velocity and Reynold's number in each reactor

Sampling date	Reactor setup	Q	Q	t	v	Re
		L/min	m^3/s	s	m/s	
15/11/2016	start	0.15	2.50×10^{-6}	88	0.008	16
28/11/2016	week 2	0.20	3.33×10^{-6}	66	0.011	21
13/12/2016	week 4	0.30	5.00×10^{-6}	44	0.016	32
03/1/2017	week 7	0.21	3.50×10^{-6}	63	0.011	22
24/1/2017	week 10	0.08	1.33×10^{-6}	165	0.004	8
14/2/2017	week 13	0.15	2.50×10^{-6}	88	0.008	16
28/2/2017	week 15	0.15	2.50×10^{-6}	88	0.008	16

5.1.3. Details of the DOC levels

DOC level was also monitored in each column reactor, as well as at the inlet and outlet of the reactor. There was no significant difference in DOC (p>0.1) among reactor columns throughout the time of reactor operation, changes in DOC concentrations were due to the change in the inlet water quality over the period of 15 weeks. At the time of sampling biofilms, the difference in DOC concentrations between the inlet and outlet of the reactor system could arise from the instrumental error while monitoring the DOC or such difference

was potentially equivalent to the amount of DOC consumed by the biofilms resided within each column (Figure 5.3).

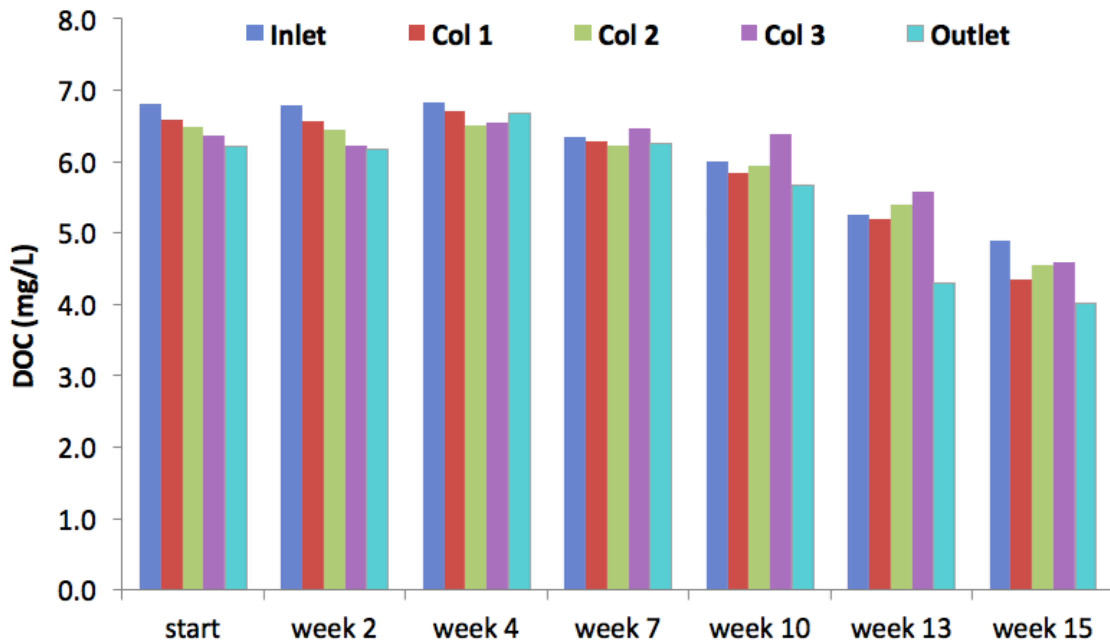


Figure 5. 3: Monitoring DOC during sampling biofilms

5.2.Metal deposition on biofilm

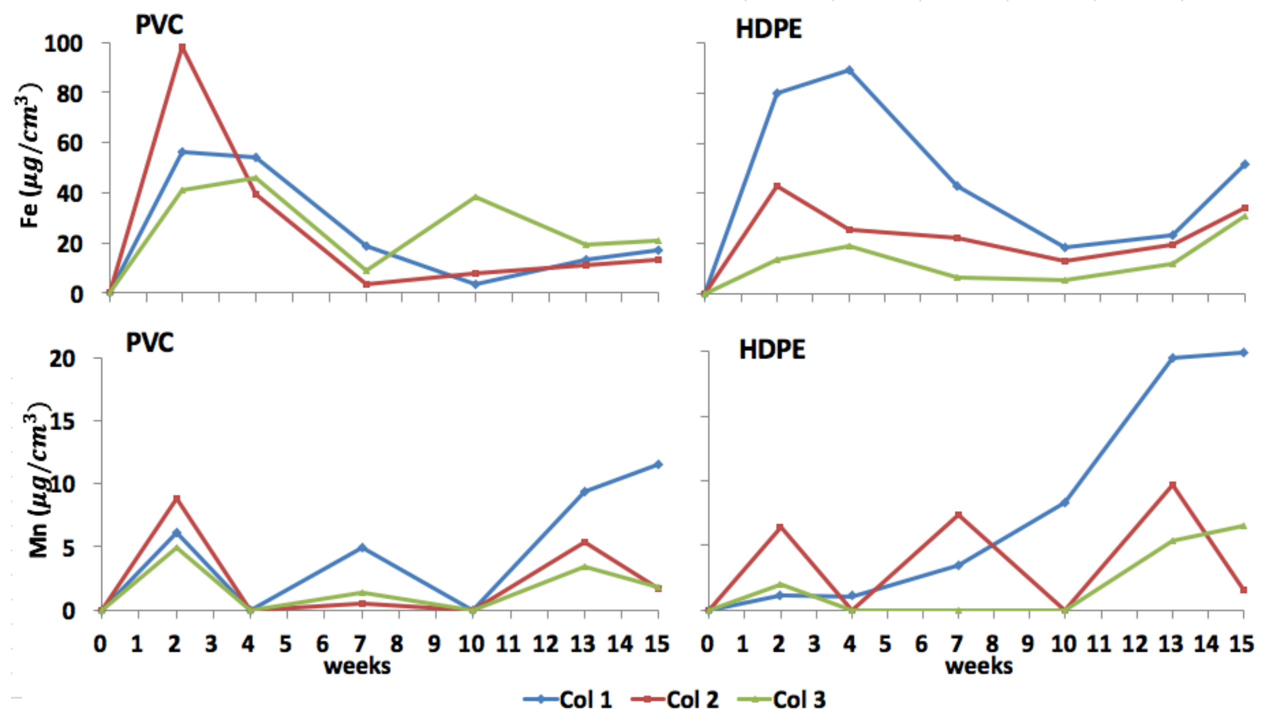


Figure 5. 4: Fe and Mn deposition on biofilms

Fe and Mn deposition on biofilms grown in RW bioreactors reached up to 100 μg/cm^3 for Fe and 20 μg/cm^3 for Mn deposits (Figure 5.4). Mn deposits appeared to have lower concentrations than Fe deposits on the same volume of biofilm. Fe deposits on PVC biofilms varied more dramatically than those on HDPE biofilms. As an oxidant, free chlorine oxidise Fe and Mn from bulk water thus lower the metal concentration in bulk water. Moreover, bacteria also possibly oxidise Fe and Mn, therefore, Fe and Mn deposit on PVC/HDPE biofilms did not increase (Ginige et al., 2011, Ginige et al., 2017). Since week 10 biofilm structures were well-built and Mn deposition on biofilms started to increase, however, the complex biofilm structures in the bioreactor were likely to facilitate the bacterial oxidation and reduction of Mn (Figure 5.4). Generally, biofilms may oxidise Fe or Mn and improve the retention of these elements within the bioreactors. On a larger scale, biofilms may also contribute to water discolouration by enhancing the abiotic release of Fe from within corrosion products into the distribution system (Ginige et al., 2017, Ginige et al., 2011).

5.3. Total carbohydrates on biofilm

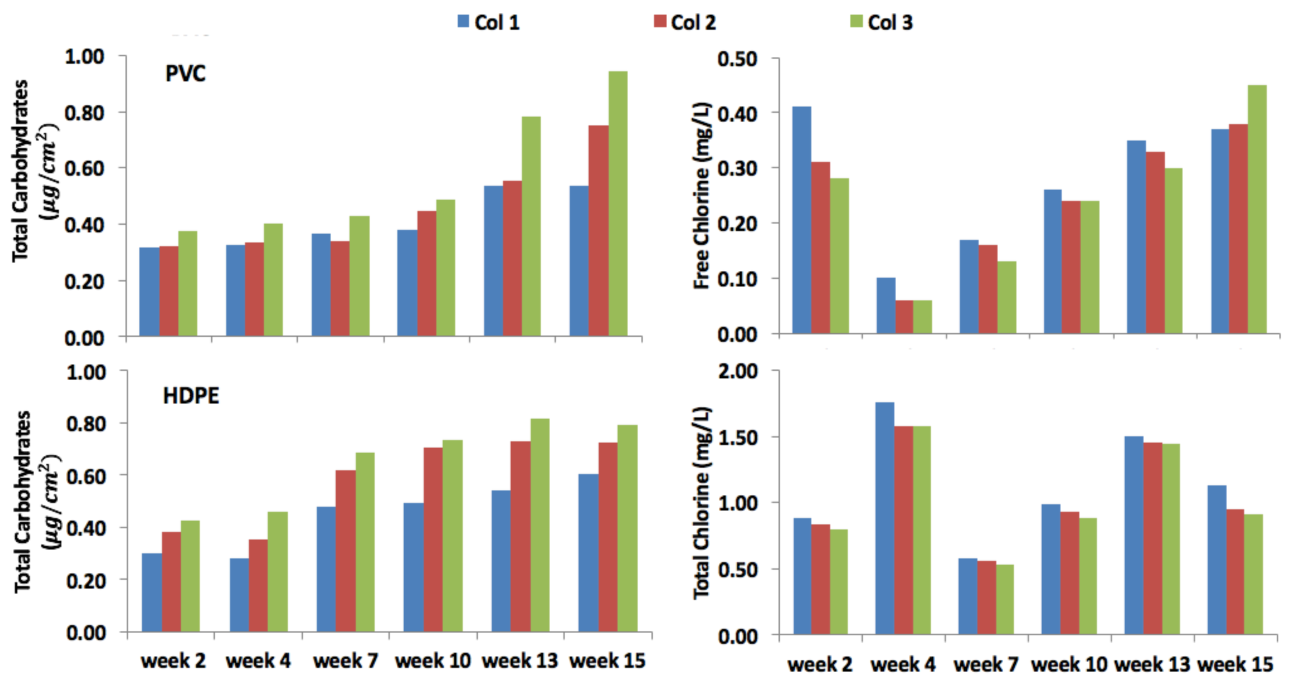


Figure 5. 5: Total carbohydrate content on biofilms

Quantification of biofilm growth was carried out based on total carbohydrate content on biofilm coupon area. Total carbohydrate contents on both PVC and HDPE biofilms were likely to increase over 15 weeks when the biofilm coupons were exposed to chlorine treatment (free chlorine <0.5 mg/L). Under chlorinated environments, young biofilms from week 2 to 4 did not show differences in total carbohydrate concentration in each column reactor. Biofilms in reactor column 3 always produced higher amounts of total carbohydrates than other columns, regardless of the application of chlorine or pipe material (Figure 5.5). Pipe materials of column 3 had exposed to low chlorine residuals than other two columns which could be the result in the increase of total carbohydrate. In general, total carbohydrate content has proven that biofilms in reactors were ongoing growth process.

5.4. Biofilm thickness and biomass by gravimetric measurements

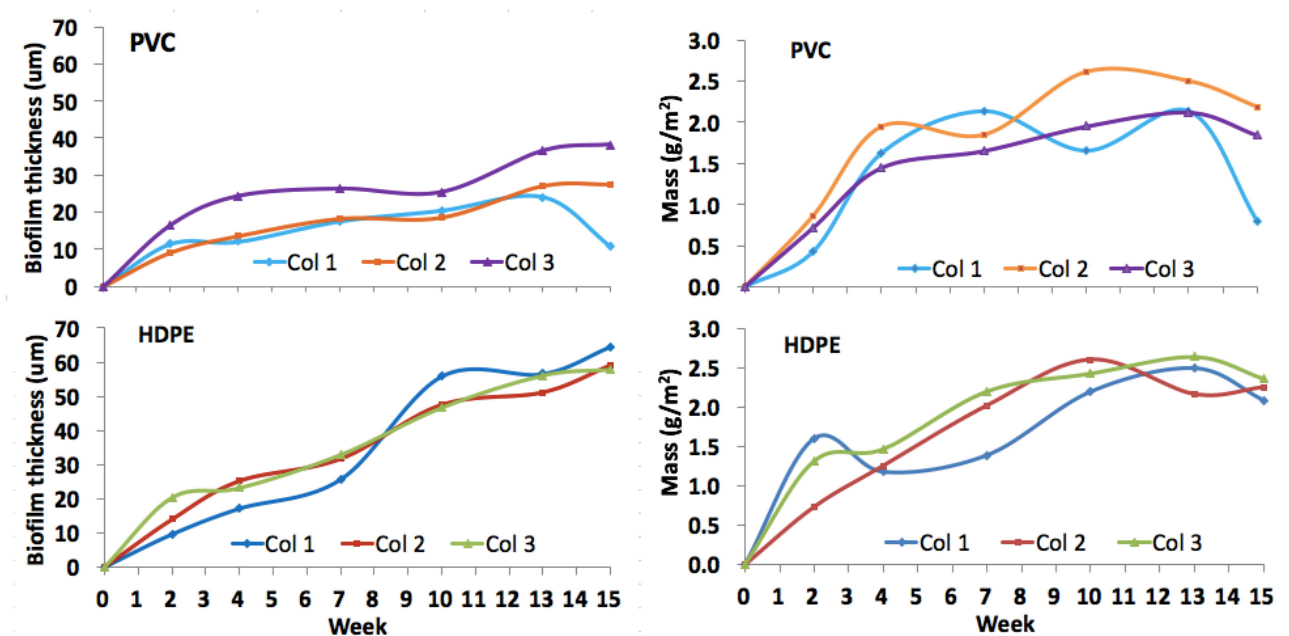


Figure 5. 6: Biofilm thickness and biomass by gravimetric measurements

Biofilms in each reactor column were analysed by gravimetry method, a traditional measurement method to quantify biofilm thickness and biomass. The results showed that biofilm formation potential of pipes differs depending on the materials used. The susceptibility of the tested materials to colonise bacteria and biofilm formation was HDPE > PVC. This research finding also corroborates with many other previous studies

on HDPE and PVC material-biofilm (Rożej et al., 2015, Kim and Lee, 2010). At the first glance, both PVC and HDPE had the same trend of biofilm thickness as well as biomass, but differed slightly between the columns. HDPE biofilm thickness increased considerably over 15 weeks in all three columns, while PVC biofilm thickness was expected to increase similarly with HDPE but its actual trend shows only a slight increase (Figure 5.6). There was an unexpected incident of overflow on week 15 which might have led to biofilm detachment and a combined loss of biomass. Either PVC or HDPE biofilm showed a fluctuation in biomass (Figure 5.6), which was calculated via biofilm dry mass measurements. The biomass data might not be measured accurately to some extent, which is greatly influenced by the success of dry biofilm detachment from PVC and HDPE surfaces.

5.5. Compared biofilm thickness between gravimetric measurements and CLSM quantification

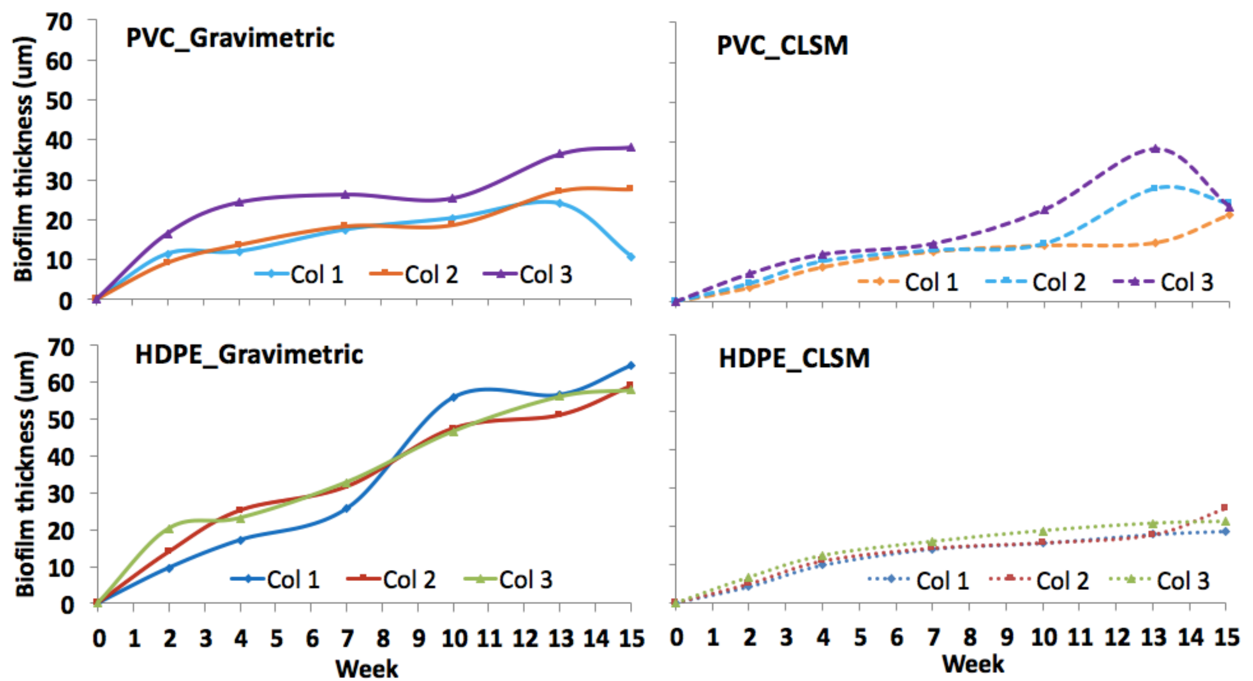


Figure 5. 7: Comparison of biofilm thickness obtained by CLSM and calculated by gravimetric measurement

The structure of microbial biofilms is closely linked to their function. In the present study, biofilm development was determined by both gravimetry and CLSM. Comparison of the two techniques confirms their validity. Biofilm thickness was calculated from the wet

biomass of the biofilm. Gravimetric data showed that 3-month-old biofilm thickness reached up to 25μm on PVC and 50μm on HDPE. By employing CLSM, biofilm thickness was averaged from 3D images of three locations on each coupon sample. 3-month-old biofilm depth ranged from 20 μm on PVC to 25 μm on HDPE surfaces. In each bio-reactor column, both methods showed the same trend in biofilm thickness, but did not exactly fit. The data from CLSM and gravimetric measurements of PVC biofilms showed far more correlation than that of HDPE biofilms. The samples from different reactors show very similar biofilm thickness because slow-growing biofilms were not subject to frequent sloughing (Figure 5.7).

5.6. Biofilm volume by CLSM quantification

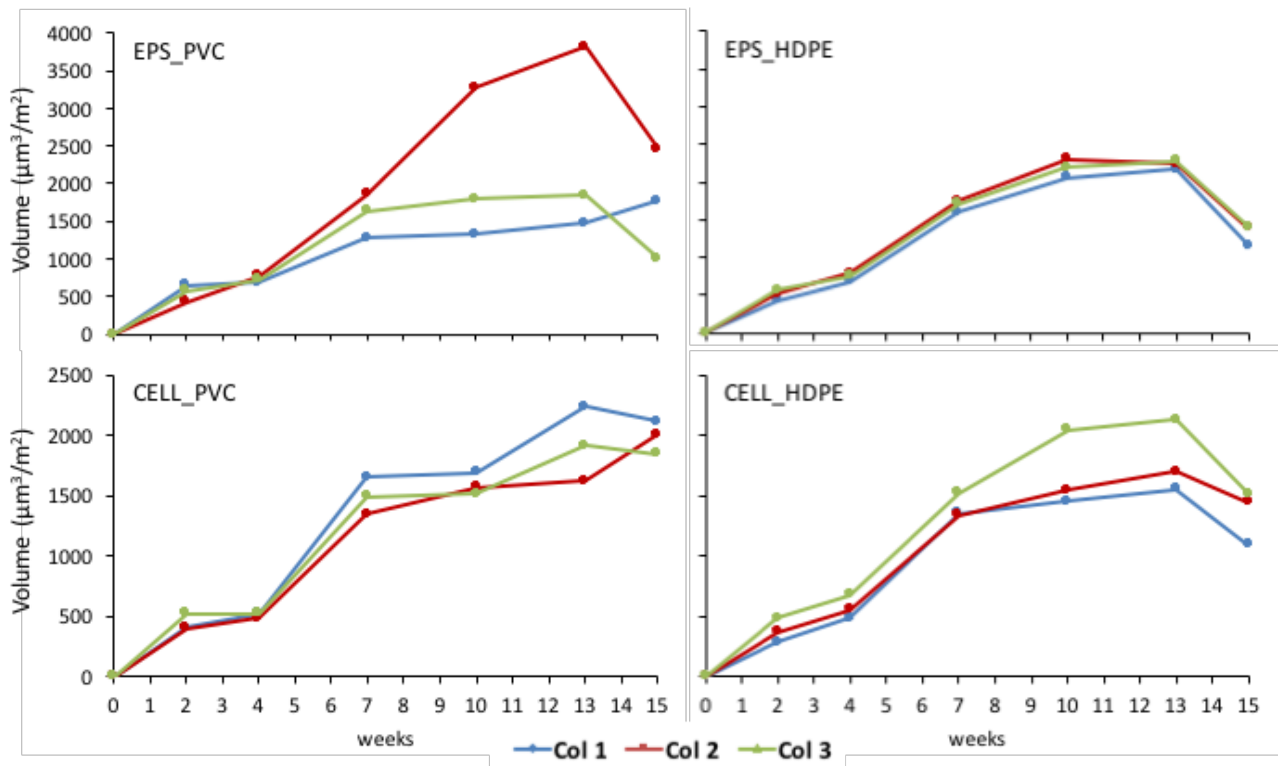


Figure 5. 8: EPS volume and cell volume of biofilms quantified by CLSM and IMARIS

CLSM allows for distinction between different biofilm constituents, such as cells and polymers. Quantification of CLSM data showed that the EPS volume was usually higher than the bacterial cell volume in most biofilms. This may be due to the intrinsic properties of cells and polymers and the growth rate of the bacteria. Under proper conditions, the

bacteria reproduce at a fast rate without producing much EPS. Slow-growing bacteria or bacteria growing under hostile environmental conditions may produce more EPS due to unbalanced growth conditions and as a protection against negative environmental factor. In this research, bacteria exposed to fluctuated chlorine and therefore biofilms were developed at a slow-growing pace, EPS volume was larger than cell volume, and HDPE EPS more developed in volume than PVC EPS (Figure 5.8).

5.7.Biofilm morphology

Figure 5.9 shows how biofilm morphology develops between weeks 2, 4, 7, 10, 13 and 15. There was only EPS in both materials in week 2 with no visible bacterial cells. Biofilm growth was found to increase from week 4, with isolated pockets of bacteria within the EPS on PVC, but HDPE surfaces still only contained EPS. The bacterial colonies can be seen to have successfully established themselves by week 7 with the number of cell growth and division forming a continuous structure that can be described as the establishment of the biofilm. To form these communities, microorganisms must integrate external and internal signals, take stock of their neighbours by determining their density and type and coordinate a time series of multi-cellular behaviors that are associated with morphological changes. The green fluorescence visualises non-bacterial polysaccharides of biofilm EPS and the red fluorescence visualises bacterial cells that make up the biofilm. As the biofilm develops a lower magnification is more suited to display this spread of the film with large patches of bacteria being interspersed by EPS and other polysaccharides on the surface. Both materials displayed a similar rate of biofilm development following week 7 with both materials developing a similar complexity by the final imaging time point. Whilst the biofilms were not completely uniform, images were taken of areas that were most representative of the whole biofilm.

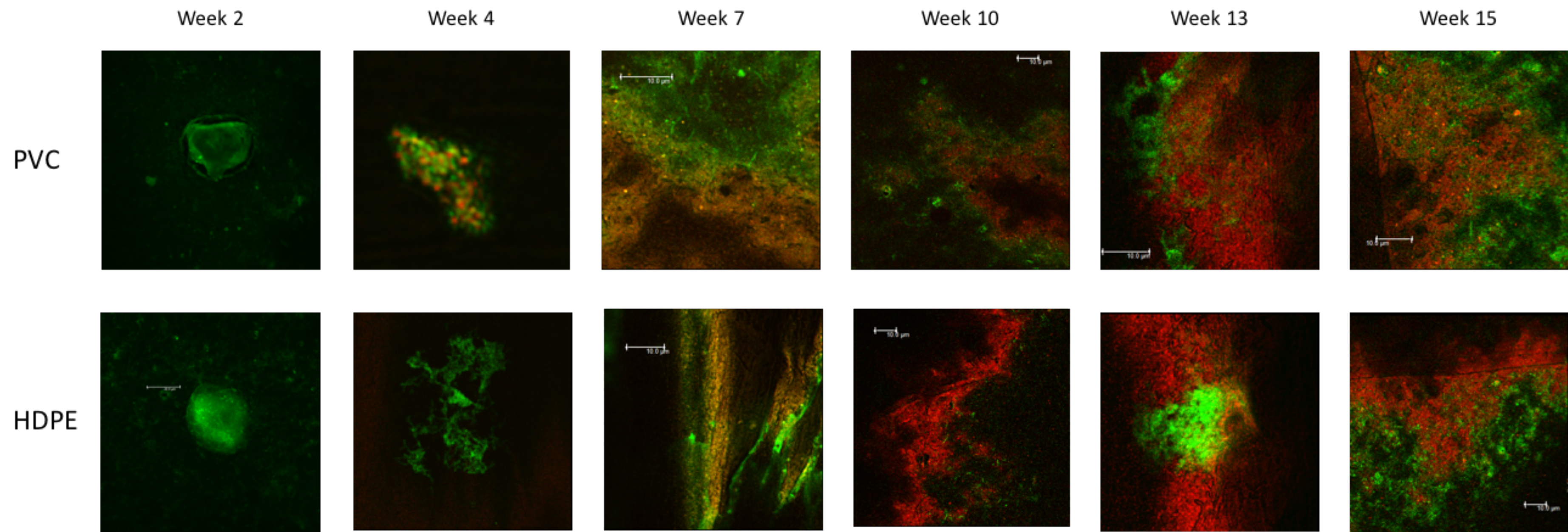
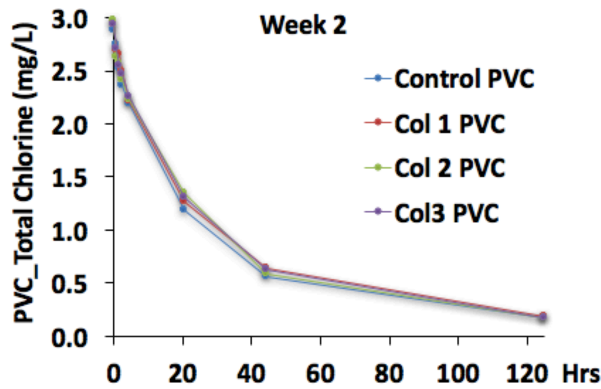
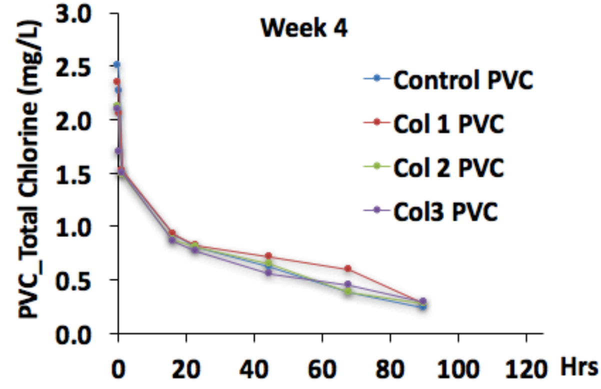
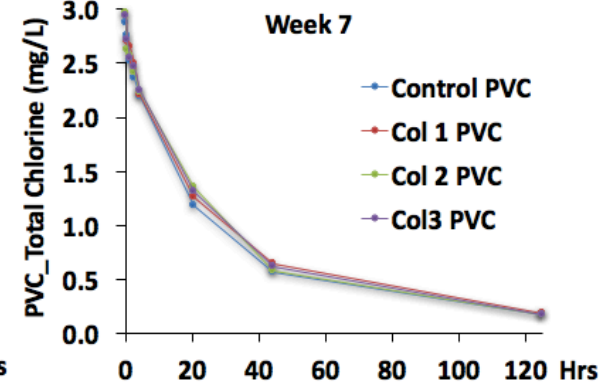


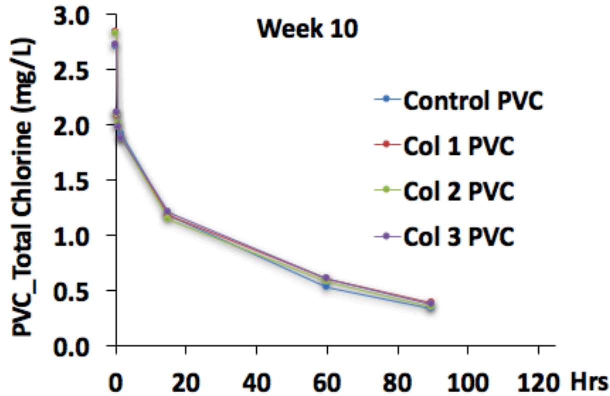
Figure 5. 9 Images from CLSM (green channel: EPS; red channel: cells) of biofilms developed on PVC/HDPE

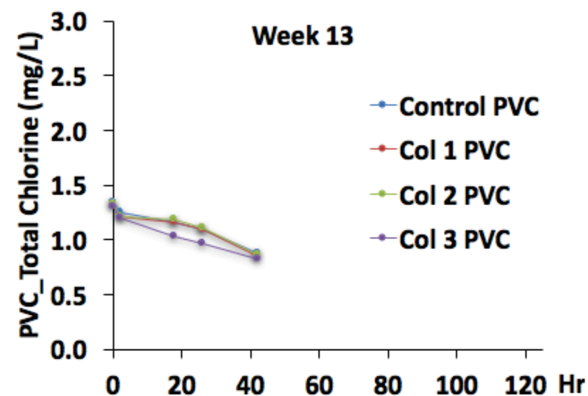
5.8. Chlorine decay with biofilms

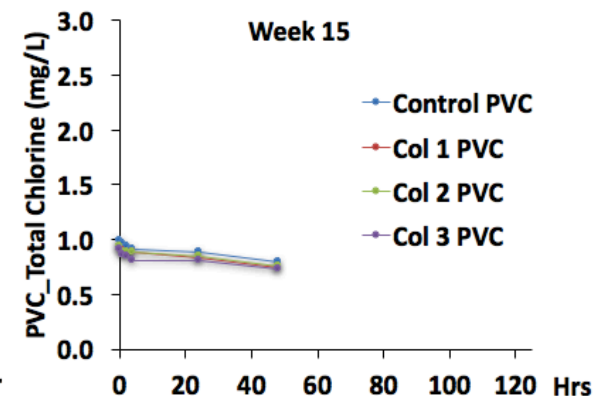












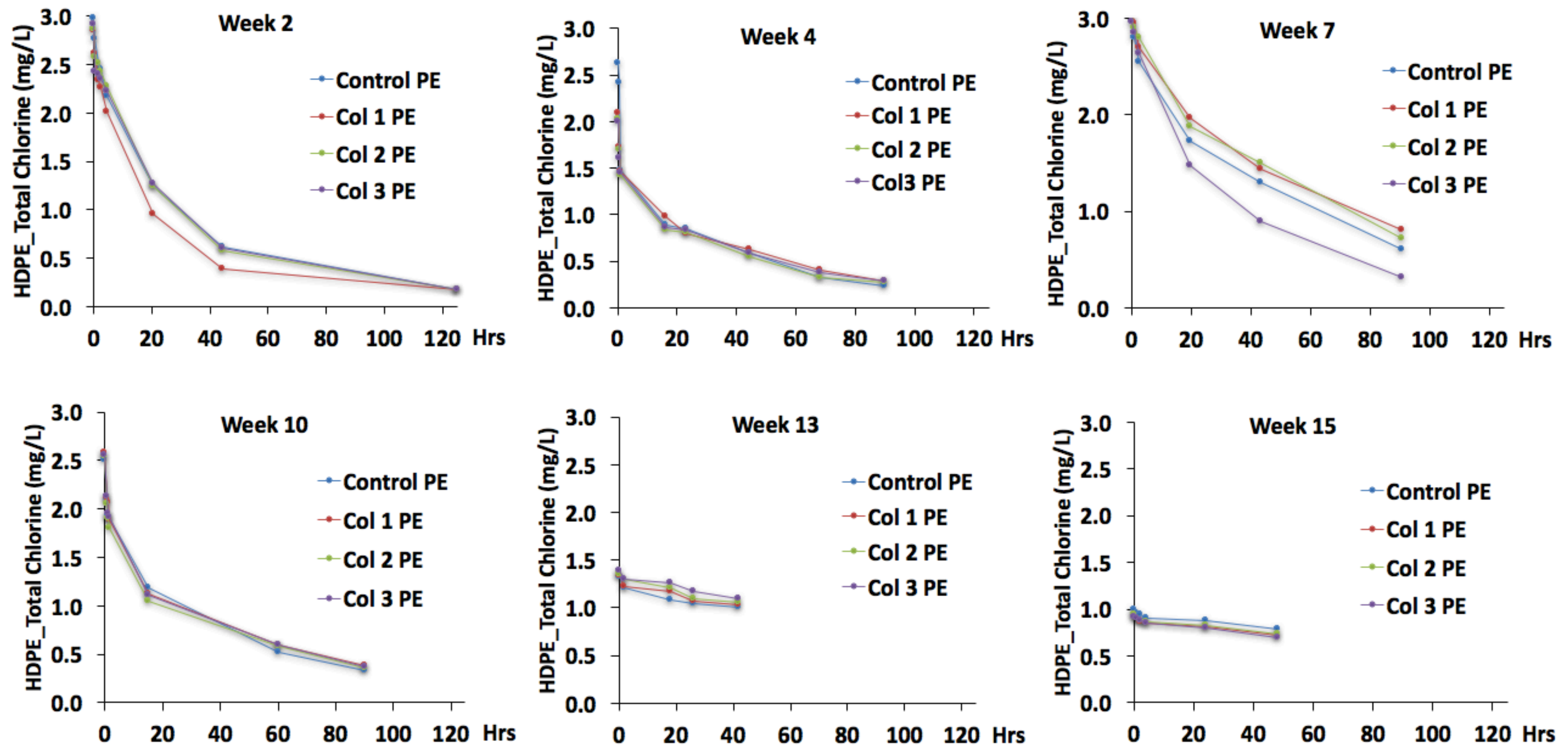
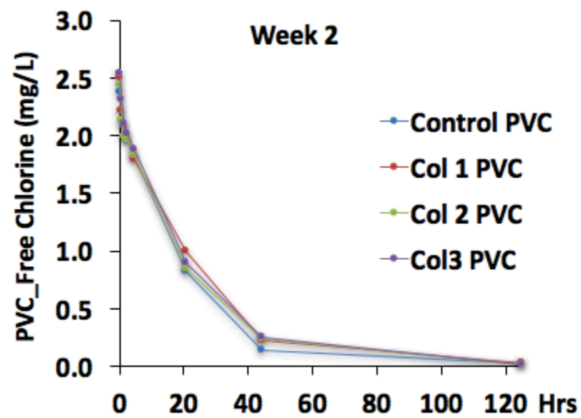
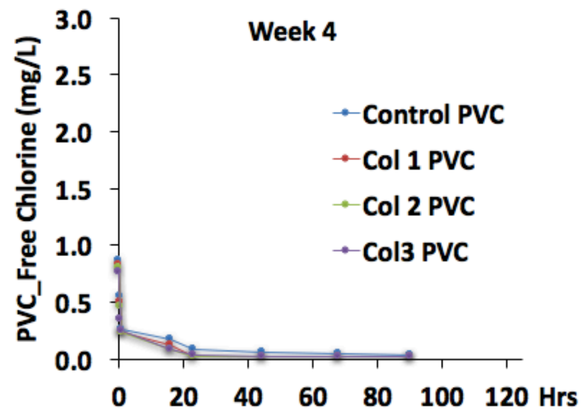
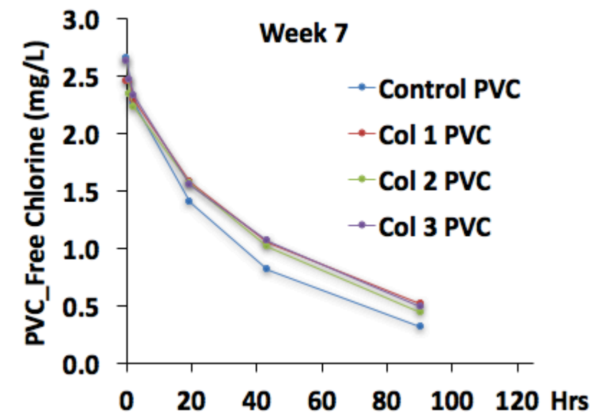
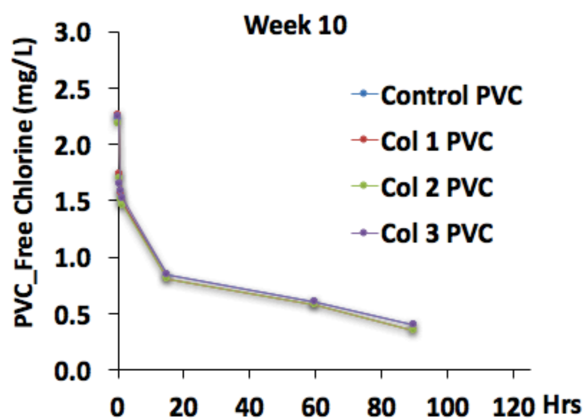


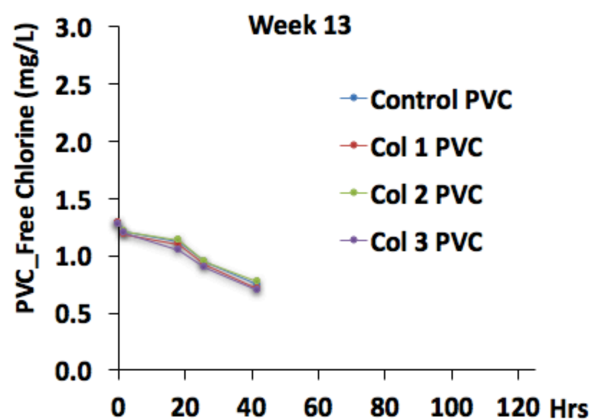
Figure 5. 10: Total chlorine decay profiles with biofilms on PVC and HDPE

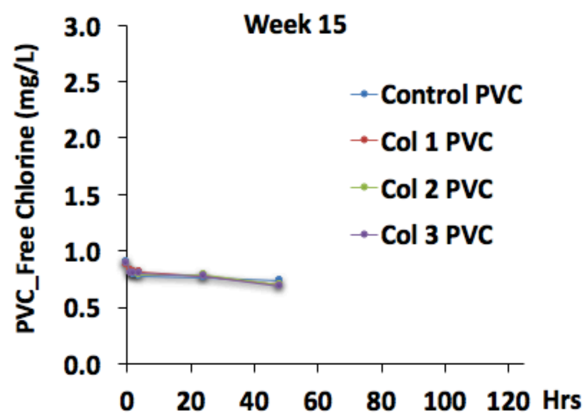

Week 2
PVC_Free Chlorine (mg/L)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
20
40
60
80
100
120
Hrs
Control PVC
Col 1 PVC
Col 2 PVC
Col3 PVC


Week 4
PVC_Free Chlorine (mg/L)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
20
40
60
80
100
120
Hrs
Control PVC
Col 1 PVC
Col 2 PVC
Col3 PVC


Week 7
PVC_Free Chlorine (mg/L)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
20
40
60
80
100
120
Hrs
Control PVC
Col 1 PVC
Col 2 PVC
Col 3 PVC


Week 10
PVC_Free Chlorine (mg/L)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
20
40
60
80
100
120
Hrs
Control PVC
Col 1 PVC
Col 2 PVC
Col 3 PVC


Week 13
PVC_Free Chlorine (mg/L)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
20
40
60
80
100
120
Hrs
Control PVC
Col 1 PVC
Col 2 PVC
Col 3 PVC


Week 15
PVC_Free Chlorine (mg/L)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
20
40
60
80
100
120
Hrs
Control PVC
Col 1 PVC
Col 2 PVC
Col 3 PVC

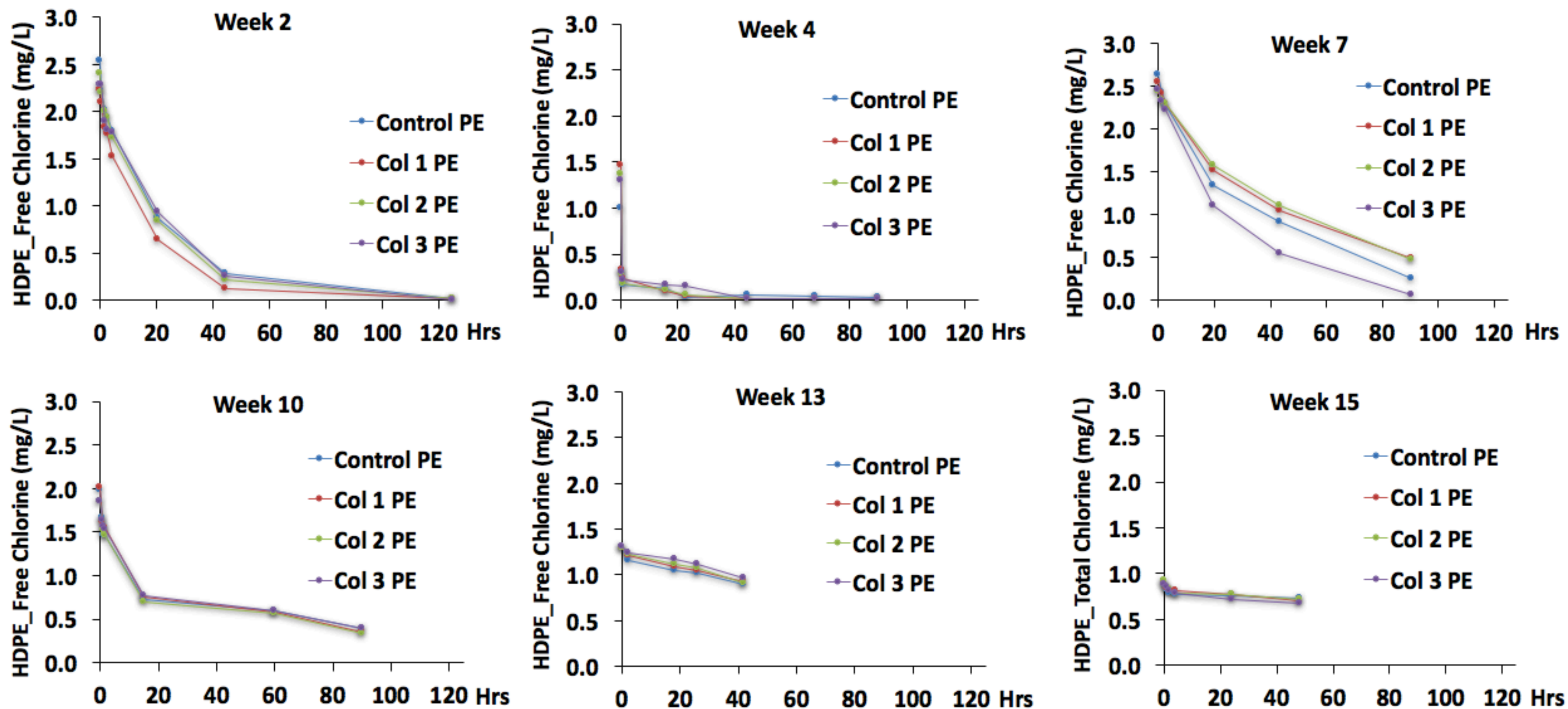


Figure 5. 11: Free chlorine decay profiles with biofilms on PVC and HDPE

Chlorine decay profiles showed no major differences between each column reactor until the 15-week period of biofilm development (Figures 5.10 and 5.11). Since the sample coupons contained very low amounts of biofilm which may not be enough to impact on decaying chlorine at concentrations up to 3 mg/L. Although a control strategy was taken into account by changing the water source from RW to MilliQ water, the chlorine decay profiles of week 13 and week 15 were not significant different, which proves that the biofilm is not enough in the pipe materials to show the impact on chlorine decay.

In summary, during 15 weeks operation time of the biofilm reactor, the levels of free chlorine from both online and onsite monitoring were low (<0.5 mg/L), which may be due to free chlorine reacting with organic matter to form organic chloramines. At the time of biofilm sampling, the difference in chlorine concentration between the inlet and outlet was the amount of chlorine consumed by the biofilms resident in each column bioreactor. Unstable flow rates posed an adverse effect on free chlorine residuals in the reactors. There was not much significant difference in DOC among reactor columns. Throughout the time of reactor operation changes in DOC concentrations were due to the change in the inlet water quality.

Free chlorine and biofilms may oxidise Fe or Mn and influence the retention of these elements within the biofilm. Total carbohydrate content has proven that biofilms in reactors were ongoing processes. Both PVC and HDPE had the same trend of increasing biofilm thickness as well as biomass, but differed slightly between the columns. Gravimetric measurement and CLSM showed the same trend in biofilm thickness, but did not exactly fit. The data from CLSM and gravimetric measurements of PVC biofilms showed far more correlation than that of HDPE biofilms. The samples from different reactors show very similar biofilm thickness because slow-growing biofilms were not subject to frequent sloughing. Biofilm depth ranged from 20 μm on PVC to 25 μm on HDPE surfaces. EPS volume was usually higher than the bacterial cell volume and HDPE EPS more developed in volume than PVC EPS, which was confirmed by the developed biofilm morphology over 15 weeks. Since the sample coupons contained very low amounts of biofilm which may not be enough to impact on decaying chlorine at concentrations up to 3 mg/L. The chlorine decay profiles did not show any significant different, which proves that the biofilm is not enough in the pipe materials to show the impact on chlorine decay.

CHAPTER 6

DISCUSSION

RW contains a high level of organic matter, which potentially reacts with chlorine, and provides nutrients for microorganisms in the form of organic carbon. RW provides a highly conducive environment for microbes to grow within the system. Free chlorine is a strong oxidizing agent and effective in inactivating pathogens and control nuisance microorganisms, it reacts quickly with organic and inorganic compounds, thus the free chlorine is not persistent in the system. The organic chloramines have poor germicidal properties, which makes their formation undesirable during chlorination and reduces the disinfection efficacy of chlorination process. Presence of higher chlorine demand in RW caused the chlorine levels to drop quickly, paving way for the regrowth of bacteria. Once free chlorine residuals have been declined below the detection limit (< 0.1 mg/L), there is a great potential for the bacterial regrowth as indicated in several literatures. This varying rate of chlorine could not be explained by simple first-order decay. Besides decay rate being proportional to initial chlorine dose, the rate could also be dependent upon the presence of two or more groups of chlorine-demanding components, which react with chlorine differently in their reactivity.

One of the chlorine-demanding groups is inorganic particles like metal deposition (Fe and Mn) in biofilms. However, the Fe and Mn could have been oxidised on the first chlorination in the chlorine contact tank leaving oxidised form to travel to biofilms, until chlorine is reduced to lower level. Once the chlorine is reduced to lower level, it is possible the Fe and Mn can be released back to the water, which may cause dirty water incidents. In the RW supply system, it frequently experiences the lower free chlorine concentration paving the way for continuous release of metals from the biofilms/sediments. When the system is operated on a diurnal cycle, the chlorine concentration remains high in the water during day time (high demand period) and remains low in the night. This can cause build up/release of metals. Most probably, only consistent higher level chlorine can help reduce this from occurring. To test theory samples have to be drawn early in the morning before water demand increases and during daytime to check the Fe/Mn content in the water.

EPS in biofilms is another component which plays a significant role in decaying chlorine residuals. EPS volume was usually higher than the bacterial cell volume in this study. Total carbohydrate content measured until week 15 has shown that biofilms growth in reactors was an ongoing process especially in recycled wastewater distribution system. Both PVC and HDPE had the same increasing trend of biofilm thickness as well as biomass, and HDPE EPS was higher in volume than PVC EPS. Slow-growing biofilms were not subjected to frequent sloughing and biofilm depth ranged from 20 µm on PVC to 25 µm on HDPE surfaces. Over 15-week development, biofilms was still not enough to impact on decaying chlorine at concentrations when different range of chlorine concentrations (1 to 3 mg/L) were used to test.

It is suspected that organic chloramine formation in the treated water entering into the distribution system which may reduce the effectiveness of the chlorination to control bacterial growth in the distribution piping network. Organic chloramines are less effective than inorganic chloramine (especially monochloramine). Cell volume, as measured by CLSM has consistently increased with retention time within the reactor and with the age. Although cell enumeration such as total cell count could be measured through microscopic analysis with fluorescent stains, this has not been carried out. Other EPS quantification methods are usually based on the analysis of total carbohydrates and proteins. This study focused on EPS carbohydrate quantification but excluded EPS proteins. It has been suggested that protein content which may impact chlorine decay, should be included in EPS quantification in further studies.

In drinking water supply system, biofilm is usually grown to full size within three weeks with free chlorine concentrations below 0.7 mg/L. However, in the RW supply system, the biofilm has not reached the highest level even after 15 weeks indicating the possible role of some inhibitors for the growth of biofilm. One possible reason can be pointed out to organic chloramine. Given a poor understanding of the role of organic chloramine on biofilm growth it is essential that a study is undertaken.

CHAPTER 7

CONCLUSION

To understand the stability of chlorine residuals in the bulk RW, physiochemical parameters (free chlorine, total chlorine, DOC, ammonia, nitrite, nitrate and pH) were analysed and chlorine decay profiles were carried out. The bulk water samples collected over 10 days show a significant drop in chlorine residuals along the system of SOPA. Mixing of stormwater and ICC did not make any change to the chlorine stability. DOC was decreased in the system which is due to the dilution taking place in order to reduce the salinity and to meet distribution demand. Approximately 1 mg/L BDOC shows that there is a great potential of regrowth of bacteria in the distribution system in the absence of/ or low disinfectant residuals. Inorganic nitrogen was found mostly in nitrate form, while DON compounds in the bulk water might exert high chlorine demand and therefore reduce the concentration of free chlorine and inorganic combined chlorine. Although more stable chlorine was noted in re-chlorinated ICC, the organic chloramine still remained at the same level. The organic chloramines were also found in OCC and OSR water which led to less chlorine stability in re-chlorinated OCC and OSR. The organic chloramines have poor germicidal properties, which makes their formation undesirable during chlorination and reduces the disinfection efficacy of the chlorination process. However, their role in inhibiting biofilm is not known. Nevertheless, re-chlorinated OCC and OSR demonstrate greater chlorine stability than re-chlorinated ICC due to low organic chloramines.

The purpose of this study was to address the contribution of pipe wall biofilms on selective pipe materials to chlorine decay in the RW distribution system. This study addressed the contribution of pipe wall biofilms to chlorine decay. The flow cell reactors were installed in the WRAMS building to investigate biofilm growth on PVC and HDPE and the role of biofilms on chlorine decay in SOPA distribution system. Biofilms were examined by a range of quantitative methods such as iron and manganese deposition, total carbohydrates, biofilm thickness, biomass and biofilm volume. The findings of the biofilms can be summarised as follows.

- Organic chloramine was the dominant chlorine species within the RW distribution system.
- According to monitoring data biofilms did not grow as fast as expected due to the presence of chlorine, organic chloramine, other unknown inhibitors and/or high flow rate.
- Free chlorine and slow-growing biofilms may oxidise Fe or Mn and influence the retention of these elements within the biofilm.
- Both PVC and HDPE had the same trend of increasing biofilm thickness as well as biomass.
- HDPE pipe surfaces were more susceptible towards biofouling than PVC because of some drawbacks to the use of HDPE pipe, for example, HDPE are susceptible to oxidative degradation when exposed to water disinfectants and are also susceptible to permeation in contaminated soils, even when contaminant concentrations are low.
- EPS volume was usually higher than the bacterial cell volume and HDPE EPS more developed in volume than PVC EPS, which was confirmed by the developed biofilm morphology over 15 weeks.
- The biofilm is not enough in the pipe materials to show the impact on decaying chlorine at concentrations range between 1 to 3 mg/L.
- Organic chloramine possibly plays a critical, but an unknown, role in determining the growth of biofilm and dirty water complaints through release of metals.

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