A study on biofilm formation in pipelines

by

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Abstract

Biofilms are organic microbiological matters that attach to the wall surface inside drinking water pipelines, forming a ‗mossy' or ‗slimy' layer. The biofilms affect the carrying capacity of pipes, increase head losses, lead to microbiologically induced corrosion in steel pipes, and can affect the health of downstream consumers of the water. Although the growth of biofilms cannot be completely stopped, it can be controlled by the choice of pipe material and/or the disinfectant system used.

The aim of this research was to compare initial biofilm growth in the presence of chlorine and monochloramine disinfectants, and between mortar and plastic substrates, and to investigate the nutrient dynamics associated with the formation of biofilms. This was achieved by conducting laboratory-based experiments using pipe coupons placed in beakers.

Although chloramine was less reactive and had a longer standing than chlorine time in the test water, a relationship between disinfectants and biofilm was not found as the disinfectants were allowed to decay. Due to a silt deposition on the mortar coupons, no analysis of biofilms on mortar coupons was made. Hence, no comparison could be made for biofilm growth on plastic coupons and biofilm growth on mortar coupons. The change in effective diameter due to biofilms is proportional to the diameter of the pipe. The change in roughness increases the head loss of a pipe and decreases its carrying capacity.

Total organic Carbon analysis did not produce useful results since the instrumentation used gave inexplicable null readings. It was found that the inorganic nitrogen has a key role in biofilm development. Ammonia present ultimately oxidizes into nitrates which act as a ‗food' source for nutrients. A depletion of nitrates leads to loss of biofilms present. Phosphorous concentrations, while much lower than the nitrogen concentrations, also showed clear variations associated with biofilm growth.

Declaration

I, Unais Kadwa, hereby declare that the whole of this dissertation is a presentation of my own work and has not been submitted in part, or in whole to any other institution. Wherever contributions of others are involved, every effort is made to indicate this clearly, with due reference to the literature, and acknowledgement of collaborative research and discussions.

This research was conducted under the supervision of Professor Derek Stretch and Doctor Muthukrishnavellaisamy Kumarasamy of the School of Civil Engineering, Surveying and Construction, University of KwaZulu-Natal, Durban.

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U.Kadwa Date

As the candidate‘s supervisor/s, I have approved this dissertation for submission

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Prof. D Stretch Date

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Dr M. Kumarasamy Date

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1: Introduction

This chapter introduces the reader to the concepts of biofilms in pipe networks and the motivation underlying research in finding the relationship between biofilm growth, pipe material and disinfectant. This chapter also provides an overview of the aims and objectives of this research and provides an overview of the structure of this dissertation.

1.1. Background and motivation

Biofilms are basically layers of micro-organisms that are attached to a substrate such as a pipe wall (Momba, et al., 2000) and are held together by a polymeric matrix (van Vuuren & van Dijk, 2012). Biofilms are not just simple life forms living on the pipe walls but are complex communities that survive in water distribution networks (van Vuuren & van Dijk, 2012). The growth of biofilms are influenced by many factors such as temperature (Donlan & Pipes, 1988), water quality and nutrient availability (LeChevallier, et al., 1988), disinfectant used (Lechevallier, et al., 1980), pipe material (van Vuuren & van Dijk, 2012) and shear stresses and flow conditions in the pipes (Molobela & Ho, 2011).

Biofilms have an effect on the pipe networks; they can reduce the carrying capacity of water (van Vuuren & van Dijk, 2012), cause microbially induced corrosion (MIC) on steel pipes, increase surface roughness leading to increased head losses, and affect the health of downstream water consumers, particularly babies and/or people who have compromised immune systems (Ringas, 2007; van Vuuren & van Dijk, 2012; World Health Organisation, 2011).

Although biofilms cannot be completely removed from the water distribution networks, there are ways of limiting the growth of biofilms. Biofilms can be controlled by changing the disinfectant or increasing the dosage of disinfectant (Kerr, et al., 2003), changing the pipe material (Hallam, et al., 2001), and/or reducing the carbon content in the water (van der Kooij, 1987)

Over the past few years, monochloramine is being used as a disinfectant as an alternative to chlorine. Monochloramine is more stable than chlorine and can maintain higher disinfectant residuals for longer periods of time (US Environmental Protection Agency, 1999). However,

a potential problem with monochloramine is nitrification which is caused by a reaction between ammonia-oxidising bacteria and excess ammonia due to incorrect dosing (Health Services Scotland, 2013).

1.2. Research question

What effect will a change in the disinfectant (chlorine or chloramine), pipe material (plastic or mortar) and/or nutrient availability have on biofilm formation on the substrate?

1.3. Aims

The aim of this study is to determine what effect the pipe material and the disinfectant will have on limiting biofilm growth and what role the nutrients present will play in inhibiting biofilm.

1.4. Objectives

The intended objectives to fulfil the aims are as follows:

- To review of relevant literature to broaden the understanding of biofilms, microbiological activity in drinking water systems and the hydrodynamic effects of biofilms
- To perform Lab-based experiments placing pipe coupons of different materials in disinfected water samples inoculated with primary colonizers.
- Monitor of disinfectant residuals to quantify the decay kinetics
- Use scanning electron microscopy (SEM) to view the biofilm attachment onto the coupon substrate
- Analyse and quantify of biofilm cover on substrate using ImageJ image analysis software
- Monitor total organic carbon (TOC), nitrate, nitrite and ammonia and relate these nutrients to biofilms present

1.5. Outline of dissertation

Chapter 2 is a review of existing research and literature on the form and structure of biofilms, the effects of biofilm on consumers and the water network at large and factors that control the growth of biofilm. Chapter 2 also contains a review of existing research in this field.

Chapter 3 describes the methodology developed to carry out the laboratory experiments using coupons cut off from a used water pipe. This chapter will also explain how the UV spectrophotometer and the TOC analyzer were used for disinfectant and nutrient monitoring

and how scanning electron microscopy (SEM) was performed to analyze images to determine biofilm cover on the substrate. The limitations of the methodology uses are discussed here as well.

Chapter 4 presents the results and discussion of this research. This chapter discusses the relationships between biofilm formation, substrate material, nutrient availability and disinfectant decay, along with the decay kinetics of chlorine and chloramines disinfectants.

Chapter 5 discusses the conclusions and summary of this research including recommendations for further research.

2: Literature review

This chapter of the dissertation contains a review of existing literature. The shape, form and structure of biofilms and the factors that affect the formation of biofilms are presented here. This chapter will also look at developments made in the relationship between biofilms and disinfectants and biofilms and nutrients. The biofilm effect on tuberculation will also be presented in this chapter.

2.1 Introduction

Microbiological growth that adheres to the inside pipe surfaces are biofilms. The growth of these biofilms has an effect on the water distribution network and the end consumers. Biofilms effect the hydrodynamics of pipe networks, lead to microbially induced corrosion (MIC), and may have an effect on downstream consumers of water. The formation of biofilms cannot be prevented but can be limited by changing disinfectant dosage or type of disinfectant and/or changing the pipe material.

2.2 Biofilms

A biofilm is defined as ~~a~~n assemblage of microscopic animals, plants and bacteria attached to a surface" (van Vuuren & van Dijk, 2012). A biofilm is a natural build-up of micro-organisms at an interface such as between a liquid and fixed boundary (van Vuuren & van Dijk, 2012). Momba et al. (2000) define biofilms as ~~a~~ layer of microorganisms in an aquatic environment held together in a polymeric matrix attached to a substratum such as pipes." Biofilm is also known as slime, microbial mat, or biological deposits (van Vuuren & van Dijk, 2012). Biofilms can form on solid and liquid surfaces where water and nutrients are present (Mains, 2008). About 95% of the biomass present in piped water will attach to the pipe wall (Simoes & Simoes, 2013). The presence of biofilm in a pipe is dependent on a combination of chemical, biological and physical processes. These processes either increase or decrease the amount of biofilm present on the substratum (van Vuuren & van Dijk, 2012). Figure 2.1 shows the presence of biofilms on a pipe wall from a water distribution network:

Figure 2.1: Biofilm growth and corrosion by-products on a ductile iron pipe (Simoes & Simoes, 2013).

2.2.1 Development and structure of biofilm

A biofilm is basically a layer of micro-organisms in an aquatic environment (Momba, et al., 2000) and consists mainly of water and is held together by extracellular polymer substances, commonly referred to as EPS (van Vuuren & van Dijk, 2012). The surface on which the biofilm forms (the pipe wall) is known as the substratum (van Vuuren & van Dijk, 2012).

Microorganisms can enter the pipelines either by surviving the treatment process or by re-contamination (Mains, 2008). The pioneer species (primary colonizers) attach to the pipe surface. Since it attaches to the pipe wall, the composition of the biofilm is influenced by the substratum and also by the inorganic molecules (Bos, et al., 1996).

As conditions in the pipelines change, secondary colonizers enter and the conditioning film can be used as a substrate for growth. Secondary colonization occurs when microbes adsorb to the conditioned surface (Kerr, et al., 2003). This adsorption is a 2-stage process. There is reversible adhesion, followed by irreversible adhesion (Marshall, et al., 1971). When local conditions are suitable for biofilm growth, irreversible adhesion takes place and if the local conditions are not suitable, the micro-organisms migrate until suitable conditions

are found (Kerr, et al., 2003). Adhesion between cells is one of the major influences for bacterial succession (Kerr, et al., 2003). There are two types of adhesion, co-aggregation and co-adhesion. Co-aggregation occurs between suspended cells while co-adhesion occurs on surfaces (Bos, et al., 1996).

After irreversible attachment, cells multiply and form micro-colonies and produce large amounts of extracellular polymeric substances (EPS), forming a matrix that surrounds and embeds the cells present (Kerr, et al., 2003). The EPS gives the biofilm its slimy nature (Mains, 2008) The EPS is supporting structure of the biofilm. It serves 2 main functions; firstly it maintains adhesion onto the substrate and secondly it holds cells together inside the colony (Kerr, et al., 2003). The EPS is a matrix that consists of organic polymers that are produced and excreted by the micro-organisms present in the biofilm (Momba, et al., 2000). It contributes 70-95% of the organic matter of biofilms and, by comparison, micro-organisms only represent a minor part (by volume and by weight) of the biofilm (van Vuuren & van Dijk, 2012).The chemical make-up of the EPS differs among the different types of organisms and is also dependent on the surrounding environment (Momba, et al., 2000).

Biofilm most commonly comprises of bacteria and usually, these bacteria form the major portion of the biofilm population (Kambam, 2006). The bacteria that require organic compounds as sources of energy and carbon are known as heterotrophic bacteria and this is the most common bacteria found in biofilms (Kambam, 2006).

Besides bacteria, there are other micro-organisms that make up the biofilm. Other micro-organisms include opportunistic pathogens, protozoa, algae, fungi, helminthes and other invertebrates (Kambam, 2006). After formation of the slime layer, the slime layer helps trap organic particles that bacteria can use as a source of energy and food (Mains, 2008).Figure 2.2 summarizes the life cycle of biofilms:

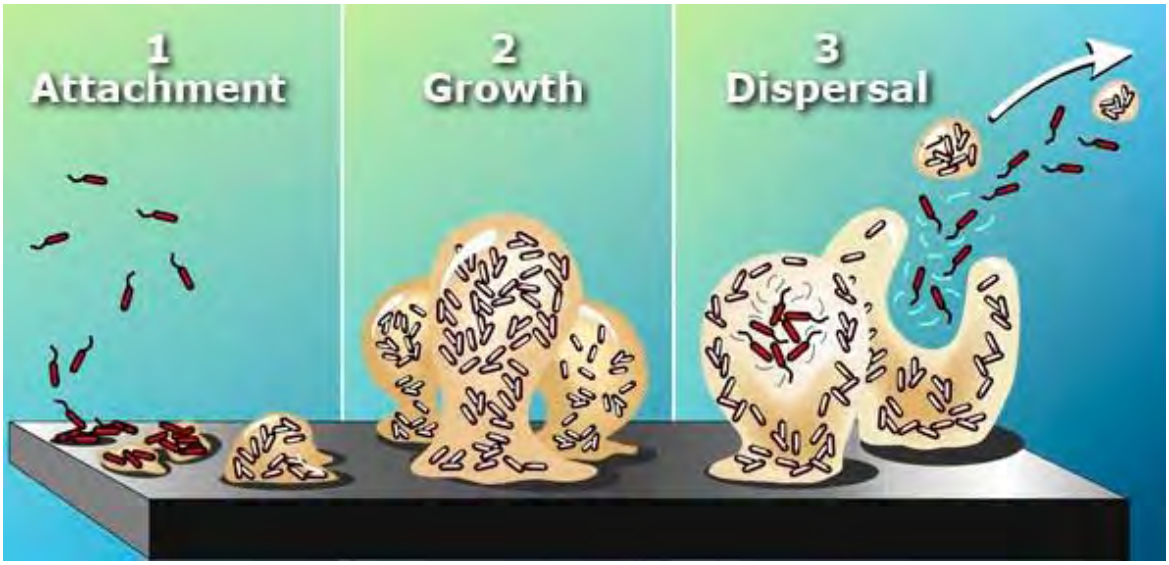


Figure 2.2: Life cycle of biofilms showing the attachment phase, growth phase and dispersal phase (Cunningham, et al., 2011).

2.2.2.1 Environmental factors

Temperature

Temperature is the most important influencing factor controlling biofilm growth (Kerr, et al., 2003). An increase in temperature leads to an increase in the number of bacteria present in water (Donlan & Pipes, 1988) but equivalent studies of the temperature-bacteria relationship in pipes is lacking(Kerr, et al., 2003). At lower temperatures, bacteria are more likely to be washed out from pipes before significant growth of biofilm has occurred (Kerr, et al., 2003). Although the temperature is the most important factor governing the growth of biofilms, water companies are unable to regulate the temperature of water (Kerr, et al., 2003).

Nutrient availability

The availability of nutrients also governs the growth rate of cells and metabolic activity, which influence the nature of biofilms (LeChevallier, et al., 1988). For heterotrophic bacteria (bacteria that uses carbon-containing compounds as a source of energy), the principal nutrients are carbon, nitrogen and phosphorous. The ratio of the principal nutrients for optimum bacterial growth is 100:10:1 (C:N:P). As can be seen from the ratio, carbon is usually the growth limiting nutrient. By reducing the carbon content, the growth of the biofilm can be limited and water treatment reduces the amount of carbon present in the water (Kerr, et al., 2003). Carbon is present in the water network in humic acids, carbohydrates, carboxylic acids and proteins (LeChevallier, et al., 1997).

Disinfectant residual

The disinfectant residual is the excess disinfectant added to the water at the water works, to react with bacteria that may enter or is present in the pipeline downstream of the water works (Kerr, et al., 2003). Experiments carried out by Lechevallier et al. (1980) found that dead-end distribution pipes where no free chlorine was detected had 23 times greater bacterial count as compared to pipes where free chlorine was detected. The chlorine decay kinetics is dependent on pipe material and hydraulic conditions (Lechevallier, et al., 1980). The disinfectant does not eradicate the biofilm completely, but decreases the rate of growth and adhesion (van Vuuren & van Dijk, 2006). A very high amount of disinfectant residual leaves a chlorinous taste in the water (Kerr, et al., 2003).

In South Africa, the most common disinfectants used are chlorine or chloramines (Meier, 2013). Although chloramine is less reactive than chlorine, chloramine is a more effective disinfectant since it is more persistent and maintains a higher residual throughout the network and also penetrates the biofilm more effectively (van Der Wende & Characklis, 1990).

2.2.2.2. Pipe Material

Pipe material plays an important role in the formation of biofilms and also influences the effectiveness of the disinfectant (Kerr, et al., 2003). Biofilms can be present on any pipe surface (van Vuuren & van Dijk, 2006) and there is no surface that is free from biofilm growth.

Plastic

Plastic pipe material, such as medium density polyethylene (MDPE) and unplasticised polyvinyl chloride (uPVC) are replacing older cast iron pipes (Momba, et al., 2000) and they support fewer bacteria than metal pipes. They have a smoother surface and hence provide less area for biofilm growth and these pipes are not subject to corrosion or bio-deterioration (Kerr, et al., 2003). The smoothness of these pipes also results in a decline in rate of adhesion and growth (van Vuuren & van Dijk, 2006). Rougher substrates provide a greater surface area and provide more shielding from shearing forces (Pederson, 1990). The biofilm forming potential for plastic pipes has not been fully being investigated (Momba, et al., 2000).

Metallic

Biofilm growth and development is encouraged on a pipe surface if the pipe material supplies the required nutrients that promote bacterial growth (Momba, et al., 2000). Poulton and Mixon (1992) found that there is uniform microbial attachment on mild steel, epoxy coated steel and mortar and concrete lined substrates. Lechevallier et al. (1990) found that 3-4 times more disinfectant is required to inactivate bacteria on iron pipes as opposed to copper pipes. This occurs since chlorine reacts preferentially with the iron surface (Lechevalliar, et al., 1990).

In copper pipes, scanning electron microscopy (SEM) has found that there are 2 distinct layers: a layer of EPS in direct copper with the copper substrate and a second layer of bacteria not embedded in the EPS (Momba, et al., 2000).

Cementious

Poulton and Mixon (1992) found that there is a uniform microbial attachment on mortar and concrete lined pipes. Momba et al. (1998) found that cement substrate has a much lower bacteria count than a stainless steel substrate, but with prolonged exposure times, the difference is diminished.

2.2.2.3. Hydraulic factors

The hydraulic behaviour of a water system changes daily and seasonally (Kerr, et al., 2003). An increase in the flow or velocity in the pipeline leads to a greater transport of disinfectants (Characklis, 1988) while at the same time leading to a greater shearing of biofilms from the substrate (Dumbleton, 1995). Shear stress has a significant impact on biofilm sloughing (Kerr, et al., 2003). Biofilms respond to high shear stresses by developing filamentous stacks (streamers) and in very high shear stresses, streamers may break off (Stoodley, et al., 1999).van Vuuren & van Dijk (2006) found that each substrate has its own detaching velocity, where adhesion is overcome and these velocities are in the range of 3-4 m/s. However, for ordinary water networks such a velocity is unlikely (van Vuuren & van Dijk, 2012).

On the other hand, stagnation may occur in the water distribution network. This leads to a loss in the transport of disinfectant residual (Kerr, et al., 2003). This may also lead to the sedimentation of particles, which causes biofilm to be shielded from the disinfectant and increase the surface area available for biofilm growth (Kerr, et al., 2003). As stated earlier, Lechevallier et al.(1980) found that in a dead-end distribution line, there was a 23 times higher bacterial count as compared to other points in the network.

2.3. Resistance of biofilms to disinfectants

Some bacteria develop resistance to disinfectants and can survive and multiply, even in the presence of the disinfectants (LeChevallier, et al., 1988). Bacteria present in biofilms are protected more than 600 times as compared to free living bacteria (Lechevallier, et al., 1980).In biofilms, the EPS reacts with the chlorine, neutralizing it, so less chlorine is available to inactivate the bacteria (Brown & Gilbert, 1993).

Ridgway & Olson (1982) suggested that chlorination gave rise to chlorine-resistant bacteria. The age of the biofilm and the previous growth conditions increases resistance from 2-fold to 10-fold (van Vuuren & van Dijk, 2012). Attachment is another major factor in resistance of disinfectants as attachment to substrate shields and protects the bacteria from the disinfectant residual (Camper, et al., 1998).

2.4. Effects of biofilm on water network

2.4.1. Hydraulics

The biofilm presence has an effect on the hydraulic capacity of the pipeline but it is difficult to quantify this effect since the biofilm growth is always fluctuating (van Vuuren & van Dijk, 2012) due to changes in the water quality, flow conditions, temperature and pH levels (Kerr, et al., 2003). In addition to the normal pipe wall roughness, there is additional change to pipe roughness due to presence of biofilms (van Vuuren & van Dijk, 2012).The severity of the biofilm impact was highlighted by van Vuuren et al. (2012) where tests carried out showed that increase in the friction of the pipeline was not due to pipeline degradation but due to biofilm growth in the pipeline. Their experiments on a 10 year old pipe found that the roughness after 10 years of the pipeline was 1.76mm while the designed roughness was 0.5mm. This leads to an increase in friction which leads to an increase in energy input costs since biofilms are visco-elastic in nature (van Vuuren & van Dijk, 2012).

The determination of the frictional head loss is important since it also influences the operating costs of the pipeline. For a given flow and pressure head, there is an increase in the friction per unit length due to biofilm growth due to the increase in hydraulic roughness, which means a higher energy input and increased costs.(van Vuuren & van Dijk, 2006).

2.4.2. Public health effects

Due to the ability of biofilms to harbour opportunistic pathogens, biofilms are seen as the prime cause of water quality deterioration (Kerr, et al., 2003). Opportunistic pathogens are those that cause diseases in people with compromised immune systems such as AIDS

patients, diabetic patients, cancer patients and other susceptible groups such as children and old people (Mains, 2008). If the pathogenic micro-organisms are not removed by disinfection, they may reach end users, and may cause outbreaks of disease within a community (Simoes & Simoes, 2013). Some opportunist pathogens show high resistance to chlorine disinfectant whilst many others show moderate resistance (Simoes & Simoes, 2013).

Bacterial contamination of the water network occurs in two ways: either micro-organisms are not eliminated at the disinfection plant, or micro-organisms detach from the biofilm present on the pipe walls (Mathieu, et al., 1993). Opportunistic bacteria that can survive free chlorine residuals of 0.5-1.0 mg/l include species of Mycobacterium, Pseudomonas aeruginosa, Klebsiella spp., Serratia spp., Legionella spp. and Flavobacterium spp. (Ridgway & Olson, 1982).

The most identified disease associated with waterborne outbreaks in developed countries is gastroenteritis (Simoes & Simoes, 2013). The health effects vary in severity and can range from mild gastroenteritis to severe (and sometimes fatal) diarrhoea, dysentery, hepatitis and typhoid fever (World Health Organisation, 2011).

2.4.3. Microbiologically induced corrosion

Corrosion, in general, refers to the degradation of a metal by chemical and electrochemical reactions with its environment or by the physical wearing away of the metal (DeBerry, et al., 1982) and also has an influence on the water quality (Bondonno, et al., 1999). Microbiologically induced corrosion (MIC) is defined as an electrochemical process in which the presence of micro-organisms is able to initiate, facilitate or accelerate the corrosion reaction without altering its electrochemical nature (Videla, 2001). This form of corrosion is found extensively in pipes carrying treated water, raw water and wastewater (Bondonno, et al., 1999).

MIC is caused by a variety of micro-organisms, usually bacteria, yeasts and algae (Ringas, 2007). Different species of micro-organisms affect the corrosion processes in different ways (Chintan, 2004). Sulphate responsible bacteria (SRB) are responsible for most of MIC. SRB locate themselves at the interface between the biofilm and metallic substrate and since SRBs are anaerobic, they can survive in that environment shielded from the bulk fluid. SRBs derive energy from converting sulphates and phosphates into sulphides which react to form either hydrogen sulphides or iron sulphides. Hydrogen sulphides are extremely aggressive and attacks metal surfaces (Ringas, 2007).

2.5. Limiting the formation of biofilm

Although the formation of biofilms are influenced by many characteristics, (such as temperature, water quality etc), there are ways to limit the formation of biofilms in potable water pipelines. The methods used to limit biofilm formation are presented below:

2.5.1. Disinfectant

Increasing disinfectant residual

In South Africa, water travels, on average, 350 km before it is delivered to end users (van Vuuren & van Dijk, 2006). Although the water is treated at the treatment plant before it enters the pipe networks, there are still microorganisms present in the pipelines that may enter through open reservoirs or cracks, joints, valves, cross-connection and backflow in the pipeline (Mains, 2008; Momba, et al., 2000) or through incomplete disinfection at the treatment plant (Mains, 2008).The excess chlorine added to the water at the treatment work is the residual (Kerr, et al., 2003). The demand for the residual is immediate (Haas, et al., 2002) and, is therefore unable to maintain its residual throughout (LeChevallier, et al., 1996).

Nagy et al. (1982) performed tests on drinking water pipelines and found that maintaining a chlorine residual of 3-4 mg/l would reduce the bacterial biofilms by more than 99.9 %. They also found that a free chlorine residual of 1-2 mg/l did not prevent the growth of biofilms. In another study, there was no correlation found between the free chlorine residuals (0.15 to 0.94mg/l of chlorine) and the densities of HPC bacteria (Nagy, et al., 1982). Pastre et al. (2003) studied the Wiggins reservoir, part of the uMgeni Water distribution network and found that the chlorine concentration in the reservoir was kept between 0.9mg/l and 1.2 mg/l.

Although having a higher chorine residual in the water is beneficial, excess chlorine residual can also lead to problems in the water network. Excess chlorine affects the water quality delivered to the end user and the end user can experience a chlorinous taste in the water (Satterfield, 2006).

High chlorines residuals can also lead to the formation of trihalomethanes (THMs) (Nozaic, 2004). THMs are formed during the reaction that occurs between chlorine and natural organic material (Chowdhury & Champagne, 2008). Long term exposure to THMs can have devastating impacts on health, such as an increased risk of cancer, delivery problems at birth, and bladder problems (Water Quality Association, 2004). The US EPA has set a maximum THM level of 0.08 mg/l to protect the American public. High chlorine residual can also lead to formation of chlorine-resistant bacteria (Ridgway & Olson, 1982).

2.5.2. Changing the disinfectant

Any disinfectant chosen for water treatment must be capable of penetrating the biofilm and de-activating attached microorganisms and should be potable, stable and persistent in the water network (Kerr, et al., 2003). The more common disinfectants are discussed further below:

Chlorination

Chlorine is the most cost-effective disinfectant (Solomon, et al., 1998). It is the most widely used disinfectant in South Africa (Nozaic, 2004). Chlorine is a very effective disinfectant, is relatively easy to handle, simple to dose, control and measure and has a relatively good residual effect (Nozaic, 2004). Although there are disinfectants that are better than chlorine in some aspects, there is no disinfectant to date that offers as many advantages as chlorine in terms of convenience, reliability, ease of use, control and running and capital costs (Nozaic, 2004).

For large-scale disinfection plants, chlorine gas is the most common form of chlorine used. Chlorine gas (Cl_2) is delivered to the disinfection plant in gas cylinders and chlorinators are used to dose the water with the chlorine disinfectant (Water Research commision, 2002).

Chlorine gas is dissolved in water at a given concentration for a minimum contact time. Calcium hypochlorite, $Ca(OCL)_2$ is available in a solid form and is a convenient way to add chlorine to smaller disinfection plants (Water Research commision, 2002). Sodium hypochorite, NaOCL (commonly known as household bleach) exists as a solution and can also be used to add chlorine to water (Water Research commision, 2002).

Chloramination

Chloramination involves the addition of anhydrous or aqueous ammonia (NH_3) before or after the addition of chlorine (HOCl) to produce monochloramine (NH_2Cl).Before or after chlorine (HOCL) is added to the water, anhydrous or aqueous ammonia (NH3) is added to produce monochloramine (NH_2Cl) (Water Quality Association, 2004). The reaction is presented as follows:

NH_3+ HOCl = NH_2Cl + H_20

Monochloramine is 200 times less effective as a disinfectant than chlorine, but it is an alternative to chlorine since it does not react readily with organic materials to form THMs (Water Quality Association, 2004). It is a more stable and longer-lasting disinfectant than

chlorine and chlorine dioxide and is a more effective disinfectant in biofilm control because of its greater ability to penetrate biofilms (US Environmental Protection Agency, 1999). A potential problem when using chloramines is nitrification. Bacteria oxidises ammonia to produce nitrate, which is subsequently converted into organic carbon and nitrates (US Environmental Protection Agency, 1999). There are health concerns associated with excess nitrate in drinking water, and excessive levels of nitrate and nitrite may affect the capacity of the blood to carry oxygen to the heart (Health Services Scotland, 2013).

Chlorine dioxide

Chlorine dioxide (ClO_2) is a widely used disinfectant for treating potable water (Clark & Boutin, 2001). Conventionally, a chlorine-chorite solution method is used to generate chlorine dioxide. Chlorine gas reacts with water to form hypochloric acid and hydrochloric acid. These acids are then reacted with sodium chlorite ($NaClO_2$) to form chlorine dioxide (US Environmental Protection Agency, 1999).

One of the most important properties of chlorine dioxide is its high solubility in water. At greater than 10^0 C, it is approximately 10 times more soluble than chlorine (US Environmental Protection Agency, 1999). Chlorine dioxide is able to maintain measurable residuals through the distribution network (Gagnon, et al., 2005). Disinfection using chlorine dioxide has a much lower formation of THMs and haloacetic acids as compared to free chlorine (Hoff, 1986). Two water treatments work in Quebec, Canada replaced chlorine treatment with chlorine dioxide treatment and it was found (through experiments) that there was an 85% reduction in THMs and a 60% reduction in haloacetic acids (Volk, et al., 2002). Chlorine dioxide can inactivate chlorine resistant parasitic pathogens (Chauret, et al., 2001). Chlorine dioxide can also control taste and odours in drinking water networks (US Environmental Protection Agency, 1999) and is effective over a wide range of pH (Chauret, et al., 2001).

The disinfection by-products of chlorine dioxide disinfectant are chlorite and chlorate (Gagnon, et al., 2005). Chlorate and chlorite concentrations in a water network need to be monitored and the costs training, sampling and testing of chlorates and chlorites are high (US Environmental Protection Agency, 1999). Chlorine dioxide decomposes when in contact with sunlight and is explosive so it has to be generated on site (US Environmental Protection Agency, 1999).

Ozonation

The EPA has carried out much research in the field of potable water and disinfectants and has described ozone as the ~~"m~~most potent biocide" (Clark & Boutin, 2001). Ozone is formed when oxygen atoms combine with oxygen molecules as presented below:

$3O2 \rightarrow 2O3$

(US Environmental Protection Agency, 1999)

Although being the most potent disinfectant, it is very unstable and highly reactive. Maintaining stable residues in the water network is very difficult due to the unstableness and volatility of ozone (Hoff, 1986). The process required to produce ozone is a costly and complex process (US Environmental Protection Agency, 1999).

2.5.3. Changing the pipe material

As discussed in section 2.2.2.2, biofilm growth will occur on any pipe material, and there is no pipe surface in any water network that is completely free of biofilm attachment (van Vuuren & van Dijk, 2006). However, plastic pipes are better at limiting the formation of biofilms as compared to metallic and mortar pipes (Kerr, et al., 2003).

2.5.4. Reduce the carbon content of the water

As can be seen in section 2.2.2.1, carbon is the controlling nutrient for growth of microorganisms. The assimilable organic carbon is the carbon that is available to microorganisms for growth (Kerr, et al., 2003). Zacheus & Martikainen (1995) did experiments and found no relationship between the total organic compound and microorganism growth.

The water treatment works is designed to remove nutrients from the source water (Kerr, et al., 2003). Water treatment works have granular activated carbon filters (GACs) hold onto and adsorb assimable organic carbon (AOC's), and this also aids in removing taste and odour problems (van der Kooij, 1987).

Le Chavellier et al. (1987) found that AOC levels declined in drinking water as it flowed through the distribution system. AOC levels decreased with an increase in distance away from the water networks. Le Chavellier et al. (1987) also stated that AOC levels in distribution systems should be lesser than 100 µg/l to control growth of coliform bacteria in biofilms.

2.6. Current research methods in the field of microbiological aspects of drinking water networks

Biofilm research in drinking water is a new multi-disciplinary research area. This field has an overlap with civil engineering, biochemistry, biology, biochemistry, microbiology and material science. Since the 90s, there have been numerous articles published regarding biofilms in drinking water systems.

South African research in this area was carried out by Van Vuuren & van Dijk (2012). They determined the change in the hydraulic roughness in a pipeline by measuring the head loss over a known length of pipe. The Colebrook-White equation was then used to determine the hydraulic roughness from the head loss. The hydraulic roughnesses were then compared to hydraulic roughnesses for the same material from existing literature. However, they only compared the roughness from the field test to a reference roughness and did not develop trends for biofilm and roughness, age and roughness, biofilm and age as their data was not done over a large enough range of pipe ages. They used a biofilm thickness mesurement apparatus (BTMA) to measure the thickness of the biofilm.

Le Chavellier et al. (1980) performed testing by taking treated chlorinated water from a water distribution network in Oregon, USA and from the raw water intake at the same treatment plant. They then performed a standard plate count (SPC) to enumerate bacterial colonies, by the pour plate technique and membrane filtration (MF) to perform an analysis of the bacteria present. The pour plate technique involves placing the specimen in agar where the bacteria present will grow in the presence of agar and the membrane filtration involves passing the test water through a 0.45 µm sieve and enumerate the bacteria that collects on the sieve. They enumerated the bacteria present and classified the bacteria into their respective genus, species and group. This allowed them to identify opportunistic pathogens which could affect the health of downstream consumers. They looked at the bacteria from test water, but not at the microbial activity that occurred at the pipe-water interface, so their reseach was not indicative of the biofilm formation on the pipe walls.

Momba et. al. (2002) carried out lab-based studies to test the difference in biofilm growth between different materials and different disinfectant conditions. They used a lab-based model network at low velocities, using a Pederson device (Pederson, 1982) that allows pipe coupons to be taken off and analyzed at any time during the experimental run. When coupons were removed aseptically from the sample, the coupons were placed in a vortex to detach the biofilms from the substrate and then enumerated using the spread plate and

membrane filtration techniques. They also took SEM images of coupons to illustrate the absence/presence of biofilms. Their findings show that due to the instability of chlorine, it is not an effective disinfectant for bacterial inhibition. A combined chlorine-monochloramine disinfectant system (where chloramine is a secondary disinfectant) is more effective in bacterial inhibition due to it being more stable. Also, their research found that plastic coupons (PVC, uPVC and MDPE) pipes supported a greater density of biofilms than cement-based coupons, hence cement has a better biofilm-limiting ability than plastic. Based on their findings, they recommend cement-based pipes for distribution of chlorine-monochloramine disinfected water for effective bacterial inhibition.

Yu et al. (2010) cut pipes of different plastic, steel and copper materials into 3 cm x 2cm coupons. These coupons were then placed in sample test waters. Their test waters were tap water, drinking water inoculated with river water (90:10 ratio by volume), and drinking water inoculated with a known concentration of E.Coli cultures. They incubated the coupons in the respective test waters for 90 days. Thereafter, they analysed biofilm attachment by vortexing pipe coupons and conducting standard biological enumeration techniques. They also viewed coupons before testing and after testing under SEM to complete a qualitative analysis of the biofilms present. They found that the coupons with a smoother substrate supported less biofilm growth as it was more difficult for the biological cells to attach. Hence, plastic coupons support less microbiological organisms than copper or steel coupons.

2.7. Conclusion

From the literature reviewed, it is evident that biofilms are an encumbrance on a pipe network that connects the treatment works to end consumers. Even though they may appear to be only a slimy layer, biofilms are dynamic communities of microbiological cells and colonies with symbiotic relationships between organisms present. Biofilms increase the head losses in pipes by increasing the effective roughness, which requires an increased energy supply to compensate for the losses, which in turn drives up running costs. Biofilms may lead to microbiologically induced corrosion (MIC) in steel pipelines, which lead to pipe deterioration. The bacteria that attach to pipe walls may be pathogenic, which may affect the health of downstream consumers that have weakened immune systems.

The literature reviewed shows that there has been interest amongst researchers concerning microbial activity in drinking water systems over the years. Of late, there has been research done in the field of biofilms. Even for biofilm formation, many researchers have analyzed their results using standard biological and microbiological techniques such as standard plate

count (SPC) and membrane filtration (MF). The effect of change in roughness has been researched by in-situ tests (van vuuren & van Dijk, 2012) and observed roughnesses were compared to theoretical roughnesses. Morphology of biofilms has been looked at under SEM (Yu, et al., 2010). There is a gap that exists in the quantification of biofilms using visual means such as microscopy. Atomic force microscopy (AFM) can provide 3D images that allow for quantification of roughness while scanning electron microscopy (SEM) can provide a _plan view' of a given surface area that allows the for the enumeration of biofilm.

3: Methodology

This chapter provides a detailed description of the investigation and experimental procedure used to determine the rate at which attachment, growth and detachment of biofilms occur for different pipe materials (cement mortar and steel) and for different disinfectants. This chapter will look at how current literature in this field was reviewed, the lab-based experiments carried out, and how the results from the experiments were analyzed to present meaningful findings from the research.

3.1. Introduction

This research is concerned with the behaviour of biofilms on different pipe surfaces and in different disinfectant conditions. The aim of this chapter is to show how the experiments were carried out and what measurements were taken and how these measurements were interpreted into meaningful, useful conclusions. The lab experiments below are subdivided into 2 parts-Experiment A and Experiment B. Experiment A (section 3.4) examines the relationship between biofilm cover and disinfectants, and disinfectant decay while Experiment B (section 3.5) examines the relationship between biofilm cover and nutrients.

Furtheremore, the case study, data acquisition, analysis and limitations of the procedure used for this research will also be presented.

3.2. Literature review

The literature review is the first step towards conducting research. The literature review was conducted to gain a better understanding of the mechanisms and stages of biofilms, the factors that influence or are influenced by biofilms and the overlap between material science, water engineering, and microbiology. Reliable literature that is relevant to this research topic was acquired. These literatures are critically discussed and reviewed in the literature review.

One of the reasons why the literature review was conducted was to find out what research was conducted previously in this research area and where is the research niche or knowledge gap that exists. Previous research, experiments and real life applications in the field of biological activity on pipe surfaces were discussed in the literature review.

3.3. Case study: uMgeni Water pipe network

A case study is required to answer the research question.

3.3.1. In-situ testing

The initial plan was to prepare in-situ testing of large diameter pipelines in the uMgeni Water distribution network conveying potable, disinfected water. The uMgeni Water distribution network was chosen since this research was commissioned by uMgeni Water. The aim of the initial methodology was to determine the change in the friction factor (and roughness) with age and with biofilm present. Unfortunately, due to non-availability of pressure and flow recording devices and non-availability of reliable data (pipe ages, demand patterns etc.), the planned methodology was not executed.

It was planned, that from the GIS files supplied from uMgeni Water, pipes used for analysis would be selected. The pipes selected would cover a wide range of pipe ages, pipe diameters and pipe materials. Pressure tests and corresponding flow tests would have been carried out over each of the pipes identified. Pressure tests would have been carried out over 2 points along the pipeline using pressure transducers. The tests would have been carried out during off-peak and peak demand periods to get a range of velocities and flows as the velocity and flow in a pipeline influence the head loss or loss due to friction.

The total energy at any point along a length of pipeline, according to the steady flow energy equation (modified Bernoulli's equation) may be represented as the sum of the pressure, elevation and velocity head plus the head loss:

$$[p/\gamma+Z+V^2/2g]_{Point\ 1}= [p/\gamma + Z +V^2/2g]_{point\ 2}+h_f$$

Since that the velocities are the same for each point and cancel each other out, the equation can be rewritten as:

$$h_f=\Delta p/\gamma +\Delta Z.$$

Hence, the head loss between the 2 points is equal to the sum of the pressure drop and the elevation change. Knowing the head loss along a length of pipeline makes it simple to determine the pipe friction factor by means of the Darcy Weisbach equation:

$$h_f = \frac{fl}{D}\frac{v^2}{2g}$$

where:

hf = Friction head loss in conduit (m)

f = Pipe friction factor (dimensionless)

L = Length of conduit (m)

V = Flow velocity of fluid inside conduit (m/s)

g = Gravitational acceleration (m/s²)

D = Internal diameter of conduit (m)

Hazen and Williams developed a simpler empirical formula for head loss. The formula is presented as:

$$h_f = \frac{10.69LQ^{1.852}}{C^{1.852}D^{4.87}}$$

Where C is a coefficient that ranges between 70 and 150. The value for C is assumed to be constant but in reality, C should change with Reynolds number (Chadwick, et al., 2004).

The Darcy-Weisbach equation is the most theoretically correct equation for head loss and the formula is applicable under all flow regimes (Rossman, 2000).

The pipe roughness can be determined using the Colebrook-White equation which is presented below as:

$$\frac{1}{\sqrt{f}} = -2\log\left(\frac{K_s}{3.7D} + \frac{2.51}{Re\sqrt{f}}\right)$$

(Chadwick, et al., 2004)

Moody (1944) also developed f-Re plot based on commercial pipes. He also presented an explicit formula for the friction factor. The formula presented by Moody is shown below:

$$f = 0.0055\left[1 + \left(\frac{2000f}{D} + \frac{10^6}{Re}\right)\right]^{\frac{1}{3}}$$

Having the head losses, friction factors and pipe roughness‘s over a range of pipe ages; relationships could have been developed between these properties. Trends could have been developed between pipe roughness and pipe age and between friction factor and pipe age.

3.4. Experiment A

Since this research was commissioned by uMgeni Water, the pipes used for this research were obtained from old, decommissioned pipes from the uMgeni Water network and from a site upgrade at the uMgeni Water facilities in Verulam. 2 pipe materials were collected, as listed in Table 3. 1.

Table 3. 1: Pipe materials obtained for lab experiments

Pipe Material	Diameter (mm)	Source	Approximate age (years)
Mortar lined steel pipe	450	Construction site at Hazelmere WW	20
PVC Plastic	100	uMgeni Water pipe yard	6

Figures 3.1 and 3.2 show the uncut pipe material that was used for pipe coupons:

Figure 3.1: uncut PVC Plastic Pipe obtained from site before being cut into plastic coupons.

Figure 3.2: uncut mortar pipe obtained from site before being cut into mortar coupon.

3.4.1. Preparation of sample coupons:

An angle grinder was used to slice the pipes into coupons. The coupons had to be of such size that they could easily be placed in a beaker, easily handled, be heavy enough to stay at the base of the beaker and not float, and also fit comfortably into the microscope vacuum chamber. Since this research is concerned only with biofilm growth on the surface inside the pipe, a layer of about 1 cm thick of the inside surface was cut using an angle grinder. The pipes were then cut into 1mm x 1mm coupons. Coupons of this size allowed for many coupons to be placed into the beaker while also providing a suitable area for SEM microscopy. The mortar coupons were much thicker than the plastic coupons as the bottom layer of the mortar pipe could not be removed due to safety concerns.

Figure 3.3 and Figure 3.4 show the plastic and mortar coupons respectively:

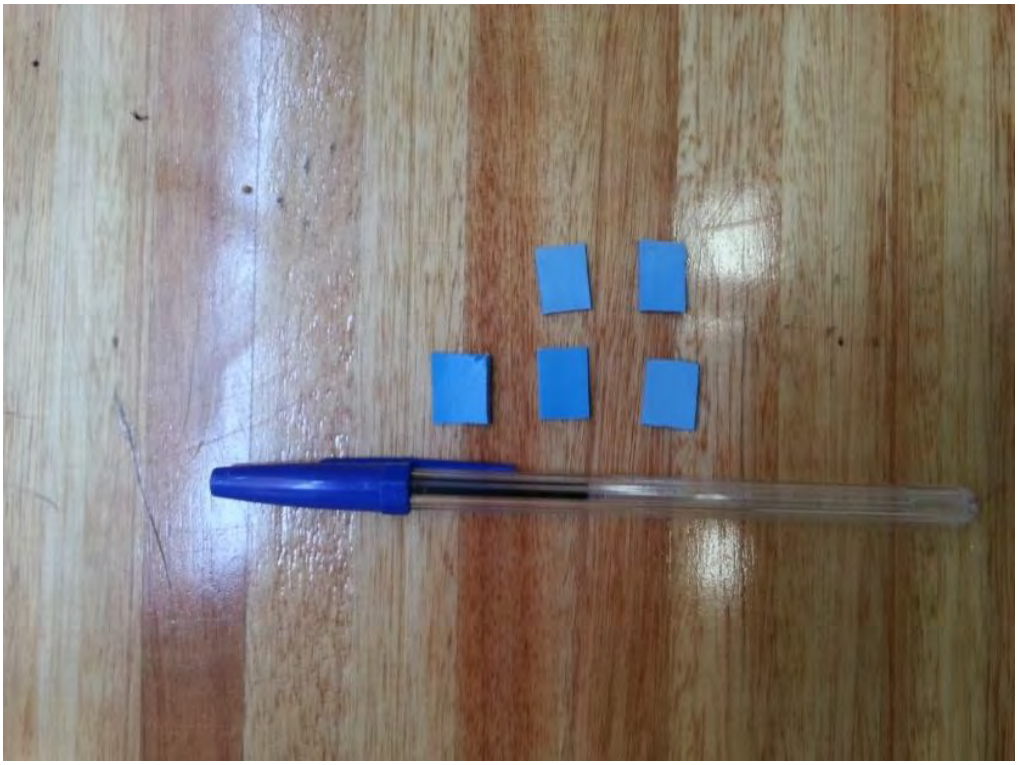


Figure 3.3: PVC plastic pipe coupons that were placed in beakers during testing for biofilm growth. A pen is used for scale.

Figure 3.4: Mortar coupons that were placed in beakers during testing for biofilm growth. A pen is used to for scale.

3.4.2. Preparation of disinfected waters

One of the aims of this experiment was to compare the effects of the 2 disinfectants, namely chlorine and monochloramine. Therefore, solutions of these 2 disinfectants were prepared. There were 2 disinfectants that were tested in this research and distilled water was used as a control. The disinfected waters were prepared as follows:

Chlorine:

A 2.78 mg/L of chlorine was made using standard procedure. After inoculation with pond water, the chlorine residual was 2.5 mg/l.

Chloramine:

A 2.78 mg/L of monochloramine was made using standard procedure. After inoculation with pond water, the monochloramine residual was 2.5 mg/l.

Distilled water:

Distilled water was used as a control.

Tap water:

Chlorinated potable water that is pumped through the uMgeni Water network was also used as test water. This was done to compare the results of water used in the laboratory to water that is flowing in a real-life distribution network. The only water available was directly from the tap (end user) and since it was at its furthest point from the disinfectant chlorination point, it had a very low chlorine residual (0.1 mg/L).

3.4.3. Pilot test

An initial test was run using tap water over a 20 day period to see if biofilms developed at a rate quick enough to produce enough biofilm coverage to provide meaningful results. It was found that having just tap water or just disinfected waters prepared from distilled water produced a very low percentage cover of biofilms. It was for this reason that all the samples were inoculated with pond water. Inoculation is discussed in section 3.5 of this chapter.

3.4.4. Inoculation of test waters

After observing very low biofilm growth in the pilot run, it was decided that pond water would be used to provide primary colonizers to initiate viable biofilm growth. The pond water provided a rich source of microbial activity that initiated the biofilm growth. The pond water used came from the pond at the Westville campus, UKZN. The pond is located on the North-Western side of the L-Block building and is shielded from direct sunlight for most of the day due to it being next to a large building. The pond was chosen since it is very rich in biological

and microbial activity. This can be seen by the ~~-~~mossy" layer seen growing on the pond walls, the varieties of fish present and abundance of hydrophilic flora. By volume, the pond water made up 10 % of each sample used in this experiment.

3.4.5. Sterilization of equipment and coupons

Coupons, beakers and tweezers used to set up the experiment were all rinsed 3 times with 70 % (by volume) ethanol to kill any microbiological activity that might have been present. 10 coupons were then placed in the beakers, and 1 L of the respective water was then poured into the beaker.

The beakers were then covered completely in aluminium foil and closed with a lid. All beakers were placed in a dark room inside a box. This was to prevent sunlight and UV rays from having any effect on the biofilms as UV light can destroy microbial life and sunlight encourages microbial growth.

3.4.6. Running the experiment

Coupons were placed under scanning electron microscope (SEM) to find the rate at which the biofilms formed and the UV spectrophotometer was used to measure the concentration of the chlorine and monochloramine residuals respectively. Figure 3.1 shows the test waters, closed to the atmosphere and to UV light, within which the pipe coupons are placed.

Figure 3.5: Experimental set-up of beakers containing coupons submerged in test waters and covered in aluminium foil to prevent UV light entering.

Samples were taken out of the water for microscope at time intervals:

1. 1 day (24 hours)
2. 4 days (96 hours)
3. 7 days (168 hours)
4. 10 days (240 hours)*
5. 14 days (336 hours)
6. 21 days (504 hours)
7. 28 days (672 hours)
8. 40 days (960 hours)
9. 50 days (1 200 hours)*
10. 60 days (1 440 hours)*

* Denotes phase 2 only. After phase 1 was analyzed, it was found that there is significant growth between day 7 and day 14 and an analysis needs to be taken between both those days. For phase 2, it was also decided that day 50 and day 60 samples should be taken as well to predict the future of biofilm growth in later stages.

These intervals were chosen as these are the times during which there is significant change in the biofilms (Momba, et al., 2000). The tests were run in 2 phases. For chlorine, chloramines, and distilled test waters, the tests were carried out twice (both phases), and for both experimental runs, the tests were run in duplicate. Tap water was only run during the first phase. Also, mortar coupons were only run during the first segment as there was crystallization that will be discussed further on in chapter 4. Table 3.2 shows the 2 phases of the tests.

Table 3.2: Number of trials that were run for each test water during each phase 1 and phase 2 testing

	Plastic	Mortar
Phase 1	2 x distilled water	2 x distilled water
	2 x chlorinated water	2 x chlorinated water
	2 x chloraminated water	2 x chloraminated water
	2 x tap water	2 x tap water
Phase 2	2 x distilled water	
	2 x chlorinated water	
	2 x chloraminated water	

For the second run, tap water (for plastic and mortar coupons) and all other mortar coupons were not tested. The advantages of staggering the tests into 2 phases instead of running all tests simultaneously are as follows:

- Fewer SEM preparation and viewing per day as the SEM preparation and viewing is a long and tedious process
- The ability to detect shortcomings and deficiencies ensures that the same shortcomings and deficiencies are not repeated in phase 2.
- More reliable, especially in the case of unexpected events that are not within the control of the researches (for example: failure of microscope), only half the tests are affected

3.4.7. Data collection and analysis:

3.4.7.1. Scanning electron microscopy:

At the specified times during the experiments, samples were removed and analyzed under the scanning electron microscope.

Before being placed into the SEM chamber, the samples have to be prepared. A standard SEM procedure was used (University of California, 2010). The sample was air dried for 25-30 minutes to remove all the water content. The sample is then attached to the microscope slide using double-sided carbon tape. The carbon tape makes the sample conductive. The mortar samples were coated with conductive glue on the side since, due to its thickness and material, it was not conductive. The samples were then placed in the sputter coater and gold coated. The sputter coating process takes about 15-20 minutes.

Once prepared, the samples were placed in the SEM vacuum chamber. The samples were then viewed under the microscope at magnifications between 300X to 500X. Contrast and brightness were adjusted as required. For each day where SEM testing was carried out, 3 coupons from each beaker were used.

3.4.7.2. UV spectrophotometer

A UV spectrophotometer was used to find the concentration of chlorine and monochloramine residuals. Ten ml samples of water were taken out daily from each test beaker and placed in a vial. A reagent test pillow is placed in the vial with the test water and is then swirled.

The test pillow used was either a chlorine or monochloramine pillow. The pillows contain a chemical, which when added to the test water react with the reactive disinfectant (chlorine or monochloramine), changing the colour of the liquid. The intensity of the colour depends on the amount of the disinfectant residual present in the test water. The UV spectrophotometer passes a UV beam through the sample in the vial, and then measures the amount of light that is absorbed by the sample. The machine then displays a reading of the concentration of disinfection based on the amount of absorbed light. Distilled water is used to calibrate the machine at the beginning of each batch test to get a zero reading.

Readings of the disinfectants were taken daily until the disinfectant residuals were below detectable limits. Figure 3.2 shows the difference in colour between a distilled water sample and a sample containing 2.4 mg/L chlorine residual after addition of chlorine test pillow.

Figure 3.6: Difference in colour between a distilled water (left) and 2.4 mg/L chlorinated water (right) sample after addition of test pillow.

3.4.7.3. Data Analysis

SEM data

The SEM data provided qualitative data (microscope images) that was transformed into quantitative data that indicated the rate at which biofilm formed on pipe surfaces and at what quantities. There were 2 analyses completed for the SEM viewing:

Percentage cover of biofilm on substrate

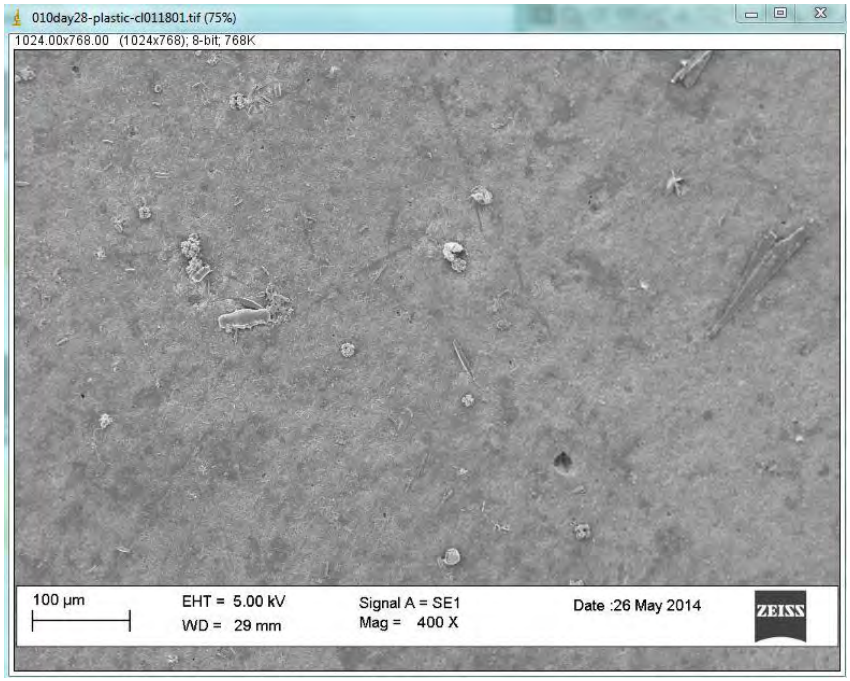
The images taken at the SEM on the chosen day were analyzed using ImageJ software. ImageJ is a small, yet powerful image processing and analysis tool. With ImageJ, the images were calibrated to scale, areas of biofilms were manually outlined and these areas were summed up and converted into a percentage of total substrate area. Figures 3.7(a) and 3.7(b) show how ImageJ software was used to trace out the biofilm cover.

The outlines of the biofilm cover on the substrate to get the percentage cover were all traced out manually. This was a time-consuming exercise as extra care had to be taken to ensure that the outline was as accurate as possible. The outlines were repeated 3-4 times for a few

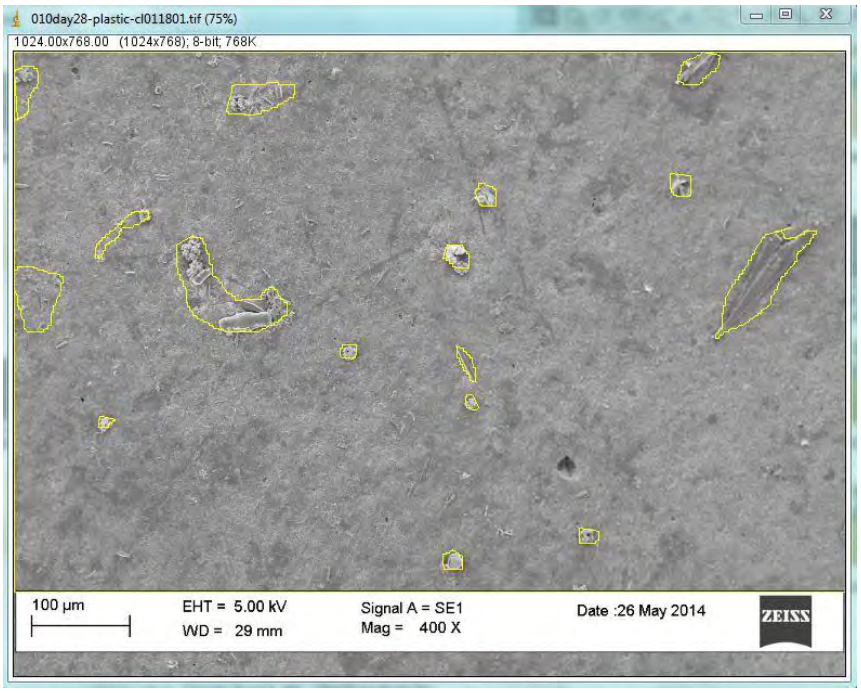
images and the results obtained for each test repeated were accurate to 3 significant figures (ie. 10 parts in 100 or 10 %), hence manual outlining is a reliable and consistent method of measuring biofilm growth on a substrate.

There is an automated method of analyzing the raw images that uses image thresholding to isolate the biofilm from the substrate. This is done by taking a greyscale image, then enhancing and segmenting the image into background (substrate) and foreground (biofilm). The images can then be analyzed using any area measurement widget on ImageJ. Although this method is faster than hand/manual outlining, it does not produce as accurate results as hand/manual outlining because:

- The threshold software isolates the non-uniformities such as ‗tears' or any damage on the substrate together with the biofilm. The software cannot differentiate between substrate non-uniformities and biofilm.
- The coupons were cut from a pipe. Due to the pipe radius, some coupons had a slight curvature, which created shadows under the microscope as the coupons were not ‗flush' on the SEM stage. These shadows are darker than the substrate and are therefore picked up as biofilm when image thresh holding is done.
- The contrast of the images varied as all the images were captured at different times. It is sometimes difficult to perform image thresholding when the substrate and the biofilm have a similar shade.



(a)



(b)

Figure 3.7: Illustration of image processing using ImageJ software (a) Raw SEM image before image processing (b) Processed image with yellow outlines demarcating assumed biofilm cover on substrate.

Average area of biofilm cover

Since all images used were of the same zoom level (300X), all images that were used had the same total area, the biofilm cover for each microscope image may be represented as:

Total average biofilm cover on coupons $=\frac{1}{n}\left(\frac{\Delta A_1}{A_1}+\frac{\Delta A_2}{A_2}+\frac{\Delta A_3}{A_3}+\cdots\frac{\Delta A_n}{A_n}\right)$

Where:

ΔA_n= biofilm cover on image n,

A_n= total surfce area of SEM image

And since all areas have the same total surface area, A_1=A_2=A_3=A_0

Total average biofilm cover on coupons=$\frac{1}{n}\left(\frac{\Delta A_1+\Delta A_2+\Delta A_3+\cdots\Delta A_n}{A_0}\right)$

So, total average biofilm cover on coupons=$\left(\frac{\Delta A1+\Delta A2+\Delta A1+\cdots\Delta An}{n.A_0}\right)$

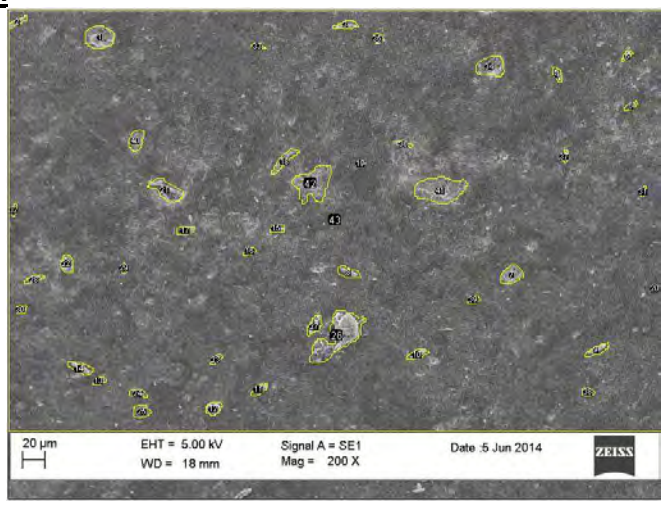
Hence, taking the sum of the average biofilm cover area of each image is the same as taking the sum of the biofilm cover across all images and dividing it by the sum of the total areas of all the images.

If areas of different zoom levels were used, the surface area for analysis across the images would have been different. In that case, weighted averages would have to be used where each zoom level would be assigned a different weight.‘

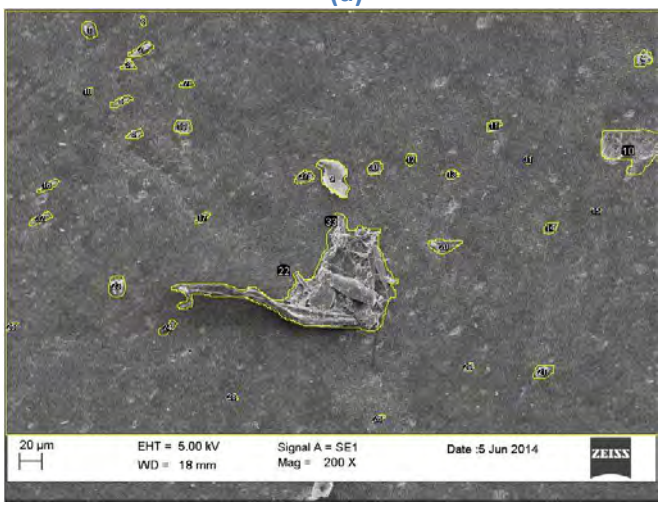
Sensitivity of results to zoom levels

A magnification of 300X was chosen since, at this zoom level, the difference between the biofilm and substrate can be clearly seen while providing a large enough area for analysis. Figures 3.8 to 3.10 provide a comparison of biofilm cover for zoom level 200X, 300X and 400X for day 7, monochloramine coupons. It can be seen that there is not much difference in the biofilm that can be visually observed between magnifications of 200X-400X. SEM images were taken at zoom levels of 1000X to 2000X to get a close-up of the biofilm cells and colonies, but these images were used not used in the quantitative analyses.

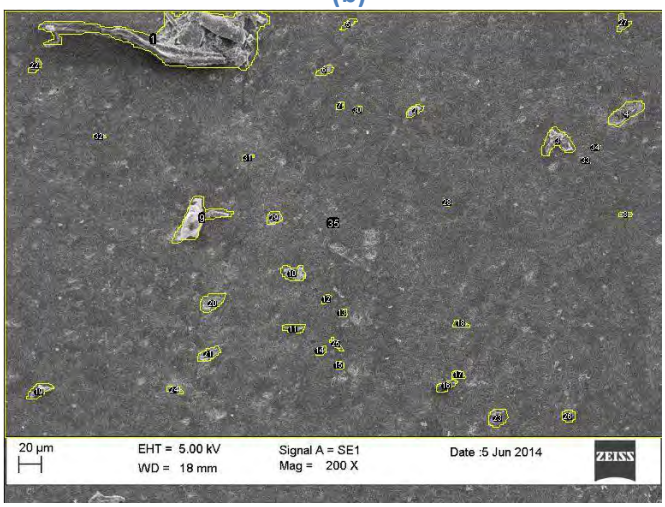
200X zoom level



(a)



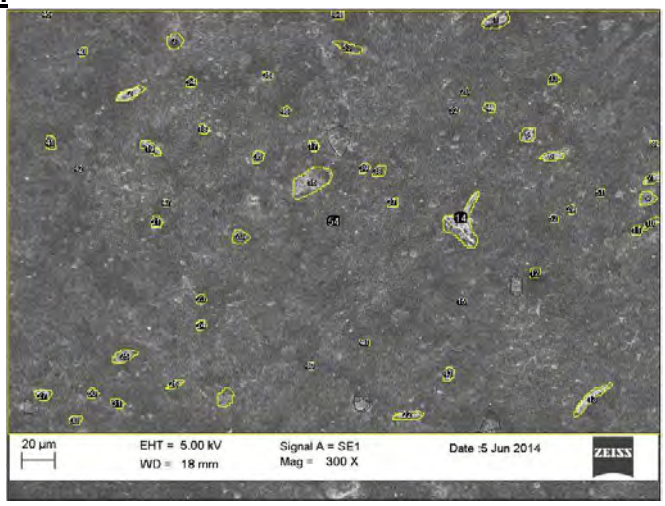
(b)



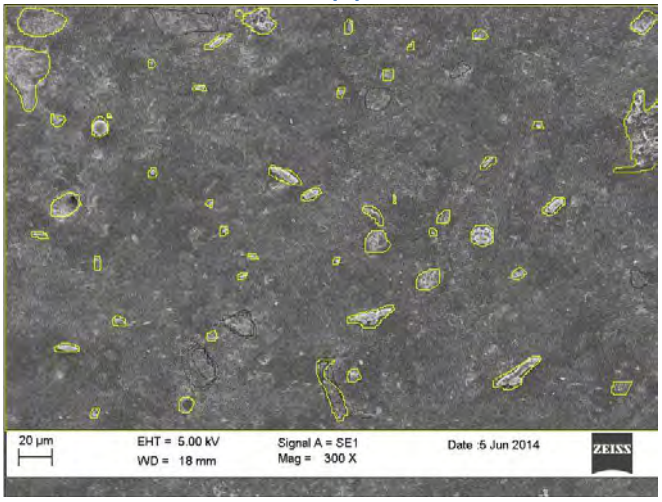
(c)

Figure 3.8: Sample SEM outlines, monochloramine plastic coupons at 200X magnification (a) 3.1 % biofilm cover (b) 5.7 % biofilm cover (c) 4.5 % biofilm cover.

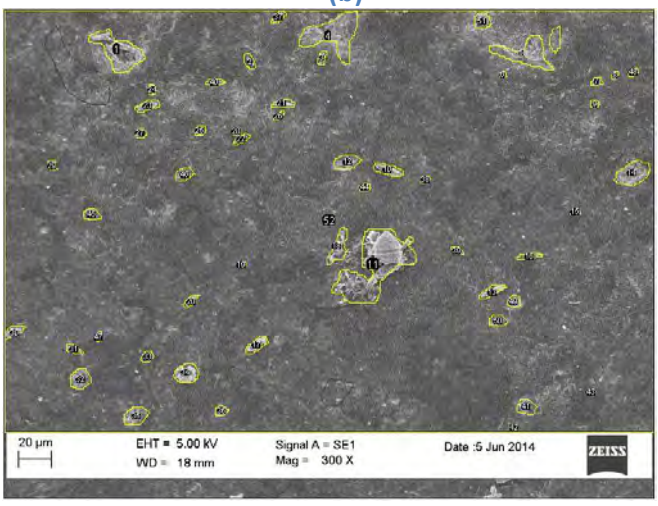
300X zoom level



(a)



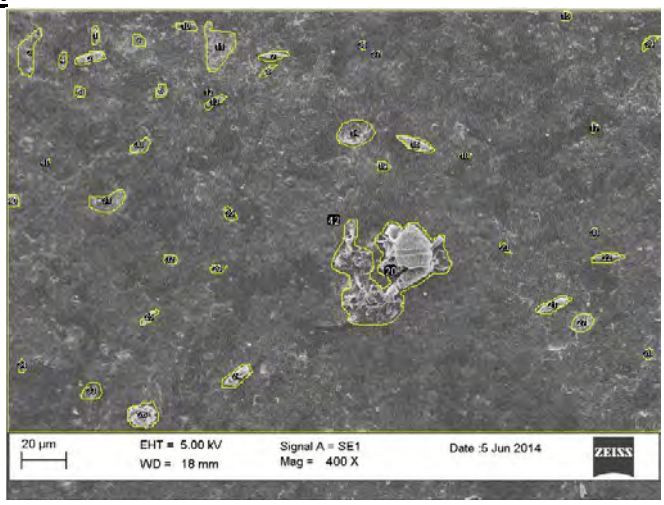
(b)



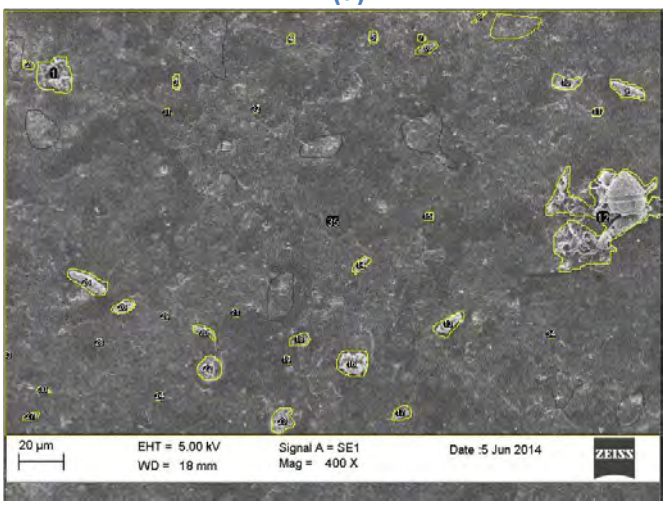
(c)

Figure 3.9: Sample SEM outlines, monochloramine plastic coupons at 300X magnification (a) 2.7 % biofilm cover (b) 4.7 % biofilm cover (c) 4.7 % biofilm cover.

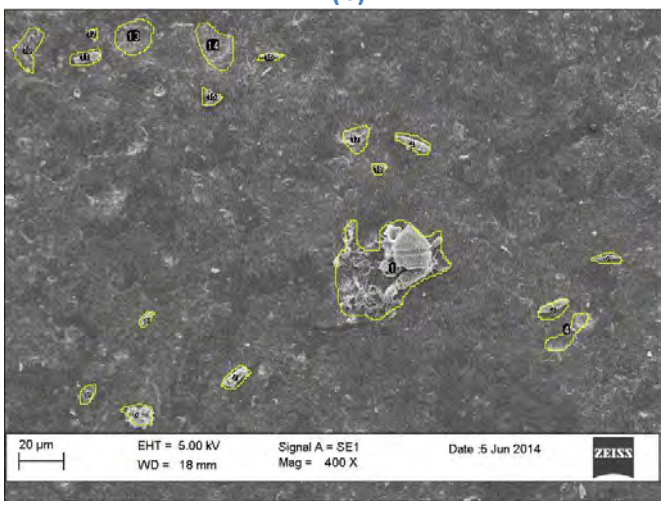
400X zoom level



(a)



(b)



(c)

Figure 3.10: Sample SEM outlines, monochloramine plastic coupons at 400X magnification (a) 5.7 % biofilm cover (b) 4. 7% biofilm cover (c) 2.0 % biofilm cover.

Table 3.3 provides a summary of biofilm cover for a sample set of data, in this case, monochloramine, day 7.

Table 3.3: percentage biofilm cover of coupons over a range of magnification levels

	200X magnification		**300X magnification**		**400X Magnification**	
	Image no	% biofilm cover	Image no	% biofilm cover	Image no	% biofilm cover
	9	2.61	15	2.68	19	5.7
	10	5.97	16	3.15	20	4.67
	11	3.14	17	4.69	21	1.96
	12	5.71	18	4.73	22	1.04
	13	4.46				
Average		4.38		3.81		3.34
Std Error		0.700		0.527		1.10

Viewing images over 400X zoom level would provide a very small area to work with, and won‘t be representative of the entire coupon. Table 3.2 provides a comparison between the biofilm cover analysed for monochloramine day 7 samples at 200X, 300X and 400X zoom levels. Figure 3.11 provides a schematic representation for SEM magnification levels, showing how the magnification affects the results obtained for percentage biofilm cover.

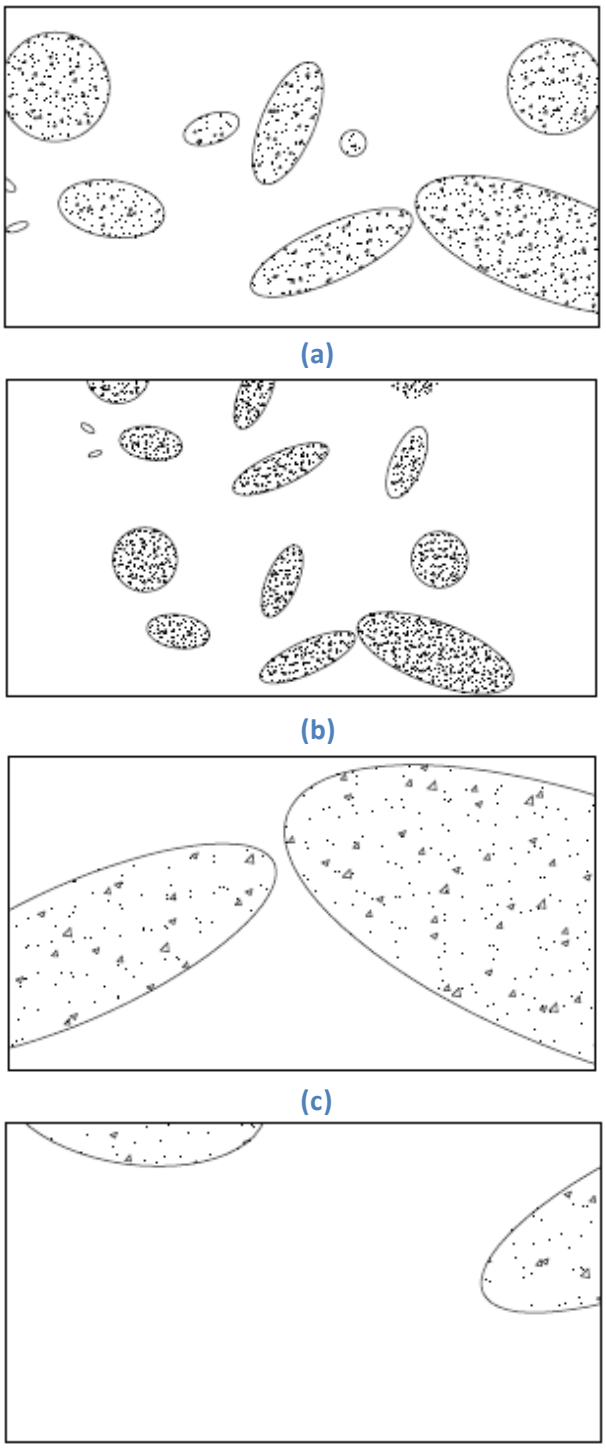


Figure 3.11: Schematic representation of SEM images at different zoom levels (a)'normal' zoom level (b) 'low zoom level' (c)'high' zoom level (d)'high zoom level'.

Figure 3.11(a) shows a reasonable magnification level, where there is adequate area provided and the biofilm can be clearly observed. Figure 3.11(b) shows a very low magnification. As can be seen in Figure 3.11(b), lowering the magnification provides a greater surface area for analysis, and biofilm over a greater surface area can be observed, but the smaller biofilm colonies/cells are either too small or cannot be seen altogether and will thus, in the quantitative analysis provide a lesser biofilm count than the actual biofilm present. Figure 3.11(c) and 3.11(d) show a high zoom level. As can be seen, at high zoom levels, a very small area of the image can be viewed under the microscope, and the very small area is not representative of the entire surface of the coupon. As seen on figure 3.11(c), a biofilm colony which might represent a very tiny portion of the area of the substrate may, under high magnification take up more than half of the SEM image returning a very high biofilm count. Figure 3.11(d) shows that a high magnification on an area with low biofilm count will return a very low biofilm cover, which again is not representative of the coupon as the focus area was too small and does not represent the entire coupon.

3.4.8. Quantitative analyses

Due to the resources available and the nature of this research, no identification of bacterial activity, DNA analysis etc. was carried out. The size, shape and form of the biofilms present will be analysed by means of visual analysis only.

The results will be able to provide the following relationships:

Biofilm growth and disinfectant

The growth over 60 day period was shown on the SEM images. These images were then analyzed using ImageJ software and the rate at which biofilms grow under different disinfectant conditions over the 40 days were recorded.

Biofilm growth and pipe material

Plastic and mortar samples were used in the test run. Unfortunately, the mortar samples were not used for the analysis. This will be discussed in Chapter 5.

Disinfectant decay

The decay of chlorine and monochloramine were compared. As expected, the decay was exponential. Decay constants were produced by performing linear regression analyses from the data to compare the decay over time and to compare the standing time of the disinfectants in the test waters. It was also determined if the disinfectant decay is constant throughout the period that the disinfectant is present in the water.

3.4.9. Qualitative analyses

The qualitative section of this report will examine and analyze the differences and/or similarities between the shape, structure and form of biofilms found between the different water qualities. No identification of microbial cells and/or bacteria was done and neither was any biological sampling methods (such as DNA replication or Fluorescence in situ hybridization) done.

3.4.10. Drinking water tests

Tests were carried out throughout the experiments on a regular basis to compare the quality of the water to ensure that the water being tested met the EPA drinking water guidelines and was safe to be considered as drinking water, even though it was inoculated with pond water rich in bacteria and other organisms. Although it was not feasible to test all the drinking water parameters, the essential parameters were tested. Table 3.4 shows which parameters were tested and the respective limits set by the EPA and DWAF. This allowed for comparison with local and international drinking water standards.

Table 3.4: Maximum observed results from drinking water tests carried out regularly during phase 1 and phase 2 of the experimental runs

Parameter (max)	Chlorine	Chloramine	Distilled water	Tap water	EPA limits*	DWAF limits*
Nitrates (mg/L)	1.5	1.7	1.1	1.4	10	11
Nitrites (mg/L)	0.1	0.1	0.1	0.2	1	0.9
pH (range)	6.7-7.0	6.8-7.2	6.9-7.1	7.5-7.8	6.5-8.5	5-9.7
Flouride (mg/L)	-	-	-	-	4	1.5
Temperature (^{o}C)	23-24	23-24	23-24	23-24		

Table 3.4 shows that the drinking water tests carried out for all test waters that were inoculated with pond water during both phases of the experimental run were within the water quality limits set by the EPA (2013) and DWAF(2011). The test waters can be deemed fit to be used for human consumption as the test results all fell within local (DWAF)and international (US EPA) drinking water regulations.

There should have been further testing done to monitor the biological/ physical and chemical parameters. The free nitrogen, carbon and phosphorous (and the corresponding C:N:P ratio) should have been recorded since these are the principal nutrients that control the growth rate of cells (Kerr, et al., 2003).

3.5. Experiment B

This test was used to find the relationship between the biofilm growth on coupons and the nutrients present. The nutrients tested were total organic carbon (TOC) and nitrogen (N). These are the nutrients that control biofilm growth (Kerr, et al., 2003). Organic carbon is the controlling nutrient for biofilm growth (Chu, et al., 2005; Lechevalliar, et al., 1990; van der Kooij, 1987). Although there are other nutrients present in water networks that influence biofilms such as phosphorous, nitrates, nitrites (Chu, et al., 2005), the greatest influence from nutrients comes from carbon and nitrogen (Kerr, et al., 2003).

3.5.1. Preparation of coupons

The plastic coupons were prepared according to section 3.3.2.1 and are the same as those shown in Figure 3.3. The same pipe length that was used for Experiment A was used for this experiment.

3.5.2. Preparation of test waters

Like Experiment A, this experiment used pond water from the pond at Westville campus to initiate growth and act as primary colonizers. As mentioned in section 3.4, on a trial run with tap water and distilled water, it was found that these waters had a very low microbiological growth. Therefore distilled water was inoculated with pond water. By volume, the pond water: distilled water ratio was 10:90. A single test was run in duplicate.

3.5.3. Running the experiment

The sterilization of all coupons and beakers was with 70 % (by volume) ethanol to kill any microbiological activity that was present.

The experimental setup was the same as Experiment A as shown in Figure 3.5, where coupons were placed in beakers. For this experiment, coupons were placed in beakers and recordings were taken as follows:

1. Day 0 (24 hours)
2. Day 3 (72 hours)
3. Day 6 (120 hours)
4. Day 9 (168 hours)
5. Day 12 (216 hours)

The tests were carrying out in duplicate, and beakers were named B1 and B2 for beaker 1 and beaker 2 respectively.

3.5.4. Data collection

3.5.4.1. SEM microscopy

On the day of the recordings, coupons were viewed under an SEM microscope. The process is similar to Experiment A and is shown in section 3.7.1.

3.5.4.2.TOC/TN analysis

The analytikJena multi N/C® 3100 was used for the determination of TOC. Standard methods for the total organic carbon (TOC) determination was used (Analytik Jena, 2014).The machine uses high temperature combustion at up to 950 ^{0}C to run the analyses (Analytik Jena, 2014)

3.5.4.3. UV spectrophotometer

Using reagents added to 10 ml of sample water from beakers, the spectrophotometer was used to determine the concentrations of:

- Nitrates
- Nitrites
- Ammonia
- Phosphates

Standard methods (Hach, 2010) were used for running the experiments on the UV spectrophotometer.

3.5.5. Data analysis

ImageJ software was used to find the amount of biofilm cover on the substrate. The whole image analysis process is presented in section 3.8. The biofilms present on the coupons were outlined and the area covered by the outlines was calculated.

The nutrient analysis results are provided directly by the machine and do not need further analysis.

The SEM data and the data from the nutrient analyses will be able to provide a relationship between:

- TOC and biofilm,
- Nitrates and biofilms,
- Nitrites and biofilms,
- Ammonia and biofilms,
- Phosphorous and biofilms

3.6. Limitation of methodology used

Although measures were taken to ensure that the lab experiments simulateded a real-life situation, there were many limitations on the experiments undertaken.

The biofilm was left to attach and grow on coupons left in beakers that contained stagnant water. In the pipe network, the velocities of the water cause shear stresses at pipe wall which leads to detachment of biofilms. In stagnant water in a beaker, there are no shear stresses to detach biofilms.

The laboratory wherein tests were being run was used for growth of bacteria and algae by other researchers. There is a possibility that there might have been microbial contamination from other sources in the lab.

In experiment A, the disinfectants were allowed to decay until they reached concentrations that were below detectable limits. The decay of disinfectants were not controlled and regulated. In a pipe network, there is always water being pumped from the water treatment works, so the disinfectant residual is, to an extent, constant.

The inoculation was carried out using water from the pond. There can be much variation from microbial quality of pond water as the biological activity present in the water varies according to temperature, UV light, depth at which water is taken, season amongst other factors.

Experiment A looked at disinfectant decay and biofilms whilst Experiment B looked at nutrients and biofilms. Hence, there was no relationship established between disinfectant concentration and nutrient availability.

SEM provided a plan view of the biofilms and cannot provide the actual roughness. The roughness influences the movement of water in the pipeline. Also, it is not possible to view biofilms beneath the top layer of biofilms if there is ‗stacking' of biofilms.

4: Results

This chapter presents the results of the lab experiments. The relationships between biofilm growth, pipe material, and disinfectant concentrations will be discussed. The results will be used to evaluate the most stable, biofilm-limiting disinfectant for different pipe materials. This chapter is made up of a quantitative analysis and a qualitative analysis. The relationships between biofilm growths, pipe roughness, head loss and flow rates are also examined.

4.1. Experiment A

4.1.1. Quantitative analysis

This section interprets and discusses the results obtained from the UV spectrophotometer and the SEM image analyses for Experiment A.

4.1.1.1. Disinfectant decay over time

A comparison was made between the disinfectant residuals to see which is more stable and has the ability to maintain a residual for the longest time. The disinfectant regimes tested were chlorine and monochloramine. Figure 4.1 shows the rate at which the disinfectants decayed for each test. It can be seen that both chlorine and chloramine decay, but chlorine decays quicker than chloramine. These results were obtained from the UV spectrophotometer. Tap water and distilled water are not shown since they have very low concentrations of disinfectant that were below the detectable limits of the UV spectrophotometer. It can be seen that an exponential decay trend is evident for disinfectant decay over time (i.e. approximately linear on a log scale), with the decay rate of chloramine being lower than that for chlorine.

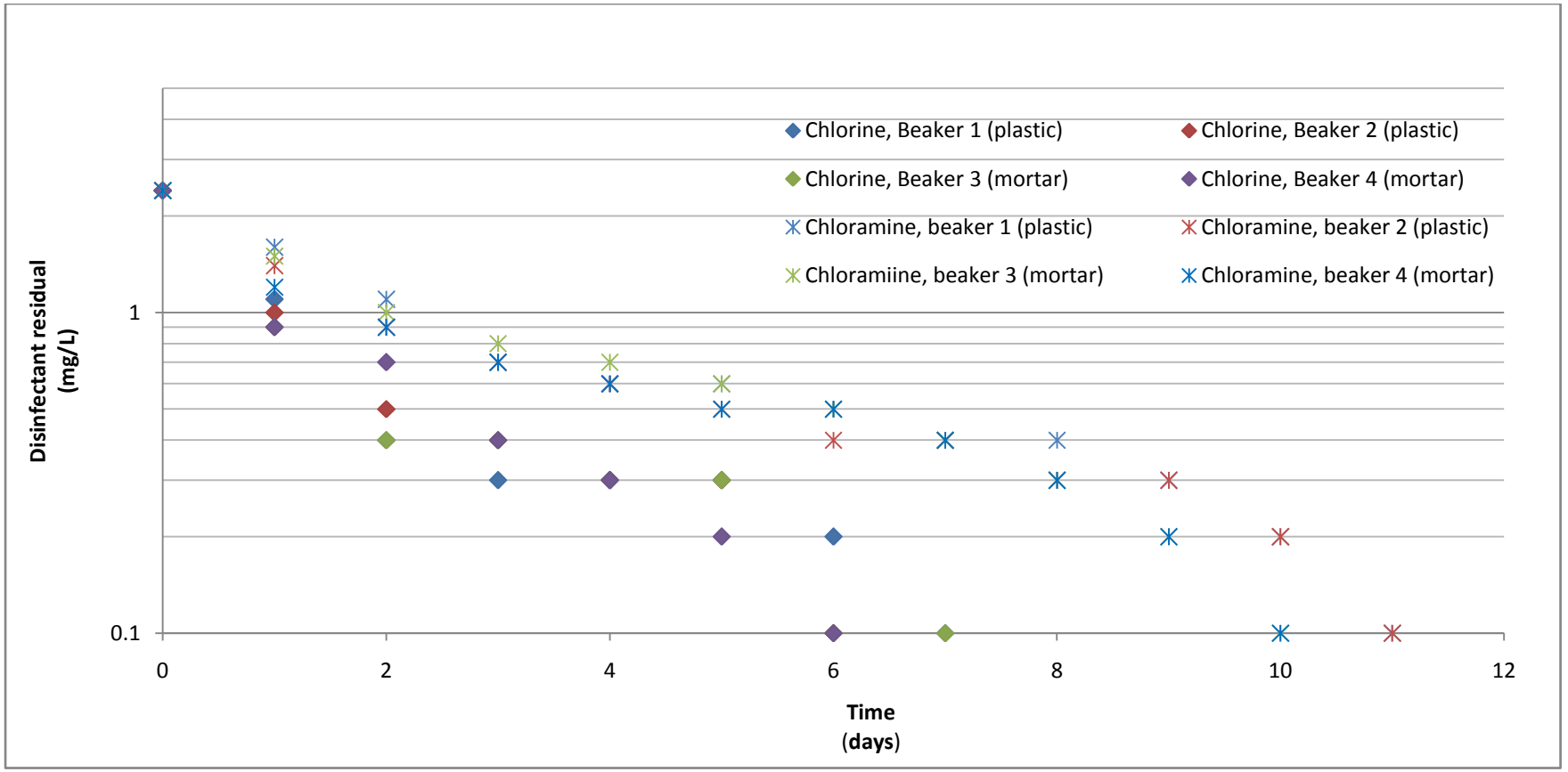


Figure 4.1: Decay of chlorine and chloramine disinfectant concentrations over 12 day period for each of 4 test beakers that contained coupons used for biofilm growth. The solid diamond symbols are for tests using Chlorine disinfectant, the open star symbols are for Chloramine disinfectant. Each beaker is represented by a different colour.

Although the tests ran for 40 days, only the first 12 days are shown since after that the disinfectant concentrations were below the detectable limits after 12 days.

4.1.1.2. Comparison between chlorine decay and chloramines decay

Figure 4.2 shows the decay for chlorine and chloramines. It can be seen that the decay kinetics follow first order decay.

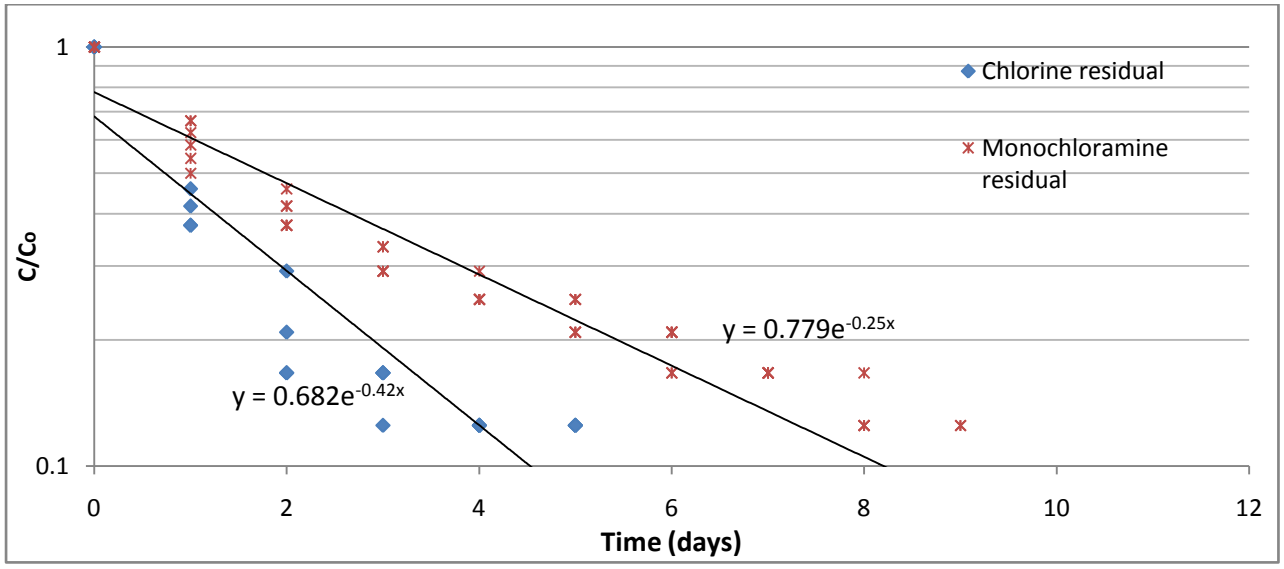


Figure 4.2: Dimensionless comparison between decay of chlorine and chloramine disinfectant decay during experimental run with linear regression analysis for the combined decay of chlorine and chloramine residuals. The solid diamond symbols are for beakers that had chlorine disinfectant, the open star symbols are for beakers that had chloramine disinfectant.

Both disinfectants had an initial concentration of 2.4 mg/L. A least square regression analysis for the disinfectant decay was carried out using the 4 water samples for chlorine and 4 samples for chloramines. The decay of disinfectant was assumed to be modelled as

$$C_{(t)}/C_0 = \exp[-k(t - t_0)]$$

where C(t) is the concentration at time t, and C_0 is the initial concentration at $t=t_0$, and k is a decay parameter (the inverse of a decay time scale).

Whence

$$k_{(t - t0)}=\ln(C_0/C_t)$$

A decay time scale T_d can be defined from the parameter k as $T_d = 1/k$. The regression analysis gave estimates of the decay time scale as 2.5 days and 4 days for Chorine and Chloramine respectively.

Both the disinfectants tested could be quantified using 1st order kinetics. The constant of decay, k for chlorine was 0.42 and for monochloramine was 0.25. The decay for chlorine was only 57 % the decay constant for chloramines. This shows that monochloramine is much more stable disinfectant than chlorine. Monochloramine lasted for about 11 days in the test waters while chlorine lasted for about 7 days.

The behaviour of the disinfectants is in agreement with findings of most researchers on this topic (Hart, 1991; Sharp, et al., 1991; Clark, et al., 1991; Chambers, et al., 1995). Most previous research (see section 2.2.2.1) has found that the decay of disinfectants follows first-order decay kinetics. However, the decay of disinfectant of chlorine and chloramines is dependent on many factors, is site-specific, and there are differences in the decay constants for different waters (Powell, et al., 2000). Hallam et al. (2001) found that the decay of disinfectant was dependant on temperature, presence of organic content, initial concentration of disinfectant, concentration of total organic carbon (TOC), and number of rechlorinations. Kiéné, et al.(1998) and Hua et al. (1999) also found that there was a first order reaction involved in the decay of disinfectants. Powell et al. (2000) concluded that chlorine decay behaves according to 2nd order kinetics but since this would be tedious, a 1st order kinetic equation factoring in organic content, UV, and temperature would be the most logical way of quantifying the decay.

The lab-based experiments found that monochloramine disinfectant is a more stable and less reactive disinfectant than chlorine. From the tests, chlorine took on average 7 days to decay to below detectable limits while monochloramine took on average 11 days to reach a value below detectable limits. Temperatures were monitored throughout the Experiment And were between 23 ^{0}C and 24 ^{0}C. Monochloramine is better able to maintain a residual and to last longer in the water. Monochloramine has a 60 % longer standing time in the test water than chlorine. The lab-based experiments confirmed what is already in contemporary research. All literature reviewed found that monochloramine is a more stable disinfectant than chlorine. Walt et al. (2004) monitored filtered water leaving Balkfontein and Virginia reservoirs in the Free State. They found that over a 3 day period, chlorine decayed from an initial concentration of 1.5 mg/L to 0.2 mg/L while over the same period, chloramines decayed from 1.5 mg/L to only 1.2 mg/L. Over 3 days, the C/C_0 value for chlorine and

chloramines from their testing was 0.13 and 0.8 respectively, showing that monochloramine is significantly more stable than chlorine, whilst the results from the UV spectrophotometer for the lab-based experiments conducted for this research project had a C/C_0 ratio of 0.15 and 0.35 for chlorine and chloramines respectively over a 3-day period. The environmental conditions (such as temperature, pH, shear stresses flow rates, nutrient conditions etc.) were different as this research was conducted in a lab environment and the tests carried out by Walt et al. (2004) were done in an actual pipeline. The US EPA (Clark & Sivaganesan, 1999) also carried out lab experiments in a simulated drinking water system, with an initial concentration of 1.4 mg/L. They found that monochloramine was more stable than chlorine with the C/C_0 value for chlorine and monochloramine being 0.08 and 0.5 respectively. Nguyen (2005), EPA (2011) and Chien (2012) have also found chloramines to be less reactive and more stable than chlorine.

4.1.1.3. Disinfectant decay and pipe material

Mortar samples and plastic samples were used for the experimental run. Table 4.1 shows the disinfectant decay in mortar and plastic coupons.

Table 4.1: Chlorine and monochloramine disinfectant residuals during experimental run obtained from the UV spectrophotometer for plastic and mortar coupons. Dashes (-) represent disinfectant concentrations below detectable limits. Shaded cells represent a difference in disinfectant concentration readings between plastic and mortar coupons

Day	Chlorine Residual (mg/L)		Monochloramine residual (mg/L)		Distilled water-chlorine residual (mg/L)		Tap water-chlorine residual (mg/L)	
	Plastic	Mortar	Plastic	Mortar	Plastic	Mortar	Plastic	Mortar
0	2.4	2.4	2.4	2.4	-	-	0.10	0.10
1	1.1	1.1	1.6	1.6	-	-	-	-
2	0.40	0.30	1.1	1.1	-	-	-	-
3	0.30	0.30	0.70	0.70	-	-	-	-
4	0.30	0.20	0.60	0.60	-	-	-	-
5	0.30	0.30	0.60	0.60	-	-	-	-
6	0.20	0.20	0.50	0.60	-	-	-	-
7	0.10	0.10	0.40	0.40	-	-	-	-
8	0.10	0.10	0.40	0.40	-	-	-	-
9	-	-	0.40	0.40	-	-	-	-
10	-	-	0.20	0.20	-	-	-	-
11	-	-	0.10	0.10	-	-	-	-
12	-	-	-	-	-	-	-	-

The shaded values on Table 4.1 indicate a difference in disinfectant residual readings between the water which had plastic coupons immersed in them and the water which had

mortar coupons immersed in them for a given day. This only occurred three times and the difference between them was minor. Hence, there is no difference between the disinfectant residuals due to the pipe material since the size of the coupons being approximately 1 mm x 1 mm in size, and having around 13 coupons per test water, the surface area of water exposed to the water was negligible; hence the pipe material had no effect on the disinfectant residuals over time. For chlorine and chloramine, the detection limit on the UV spectrophotometer was 0.1 mg/L.

The research on cement-lined pipes and disinfectant decay is limited. However, published research (Schwenk, 1990) shows that the substrates of plastic pipes are better at maintaining a disinfectant residual than concrete pipes. Schwenk (1990) compared mortar-lined pipes to plastic pipes in terms of both, rate of disinfectant decay and mechanism of decay. His findings showed that mortar-lined pipes had a greater reactivity with the disinfectant than the PVC pipes.

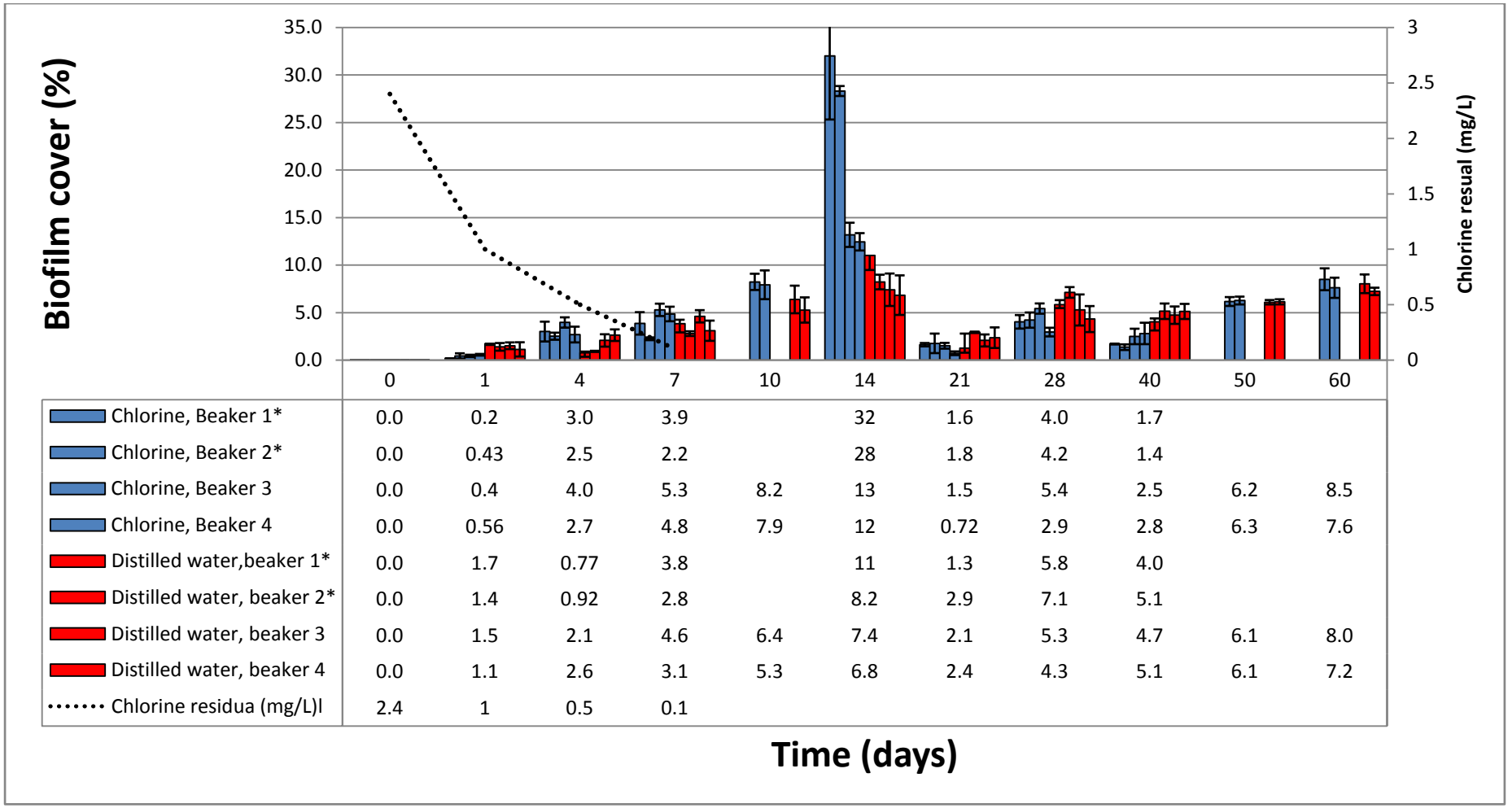
Due to the formation of a silty deposit on the mortar substrate, it was not possible to make a comparison between the biofilm growth on the plastic and mortar substrates, which will be discussed further on in this chapter.

4.1.1.4. Biofilm growth and disinfectant

From the results obtained, it can be seen that disinfectants do not totally kill off all biofilms that are present, but rather limit the formation of biofilms. In this section, a comparison will be made between the growth of biofilms and disinfectants. Distilled water was used as a control.

Chlorine

Figure 4.3 below shows the biofilm growth in chlorinated water over a 60 day period. On the same graph, the combined chlorine decay of all 4 test beakers is shown.



	0	1	4	7	10	14	21	28	40	50	60
Chlorine, Beaker 1*	0.0	0.2	3.0	3.9		32	1.6	4.0	1.7		
Chlorine, Beaker 2*	0.0	0.43	2.5	2.2		28	1.8	4.2	1.4		
Chlorine, Beaker 3	0.0	0.4	4.0	5.3	8.2	13	1.5	5.4	2.5	6.2	8.5
Chlorine, Beaker 4	0.0	0.56	2.7	4.8	7.9	12	0.72	2.9	2.8	6.3	7.6
Distilled water,beaker 1*	0.0	1.7	0.77	3.8		11	1.3	5.8	4.0		
Distilled water, beaker 2*	0.0	1.4	0.92	2.8		8.2	2.9	7.1	5.1		
Distilled water, beaker 3	0.0	1.5	2.1	4.6	6.4	7.4	2.1	5.3	4.7	6.1	8.0
Distilled water, beaker 4	0.0	1.1	2.6	3.1	5.3	6.8	2.4	4.3	5.1	6.1	7.2
Chlorine residua (mg/L)l	2.4	1	0.5	0.1							

Figure 4.3: Percentage biofilm cover on coupons placed in chlorinated water (blue) and distilled water (control) (red) over 60 day period for each of 4 test beakers. Error bars represent standard errors. The dashed line indicated combined average chlorine concentration of all 4 beakers. The blank spaces represent phase 1 wherein the tests were only carried out over a 40 day period and no day 10 reading was taken.

Beakers marked with * were carried out during the first phase of testing, where there were no readings taken on day 10 for chlorinated water and distilled water and the testing was done till day 40 only. Only after the analysis of phase 1 was compete, it was realized that there is significant growth between day 7 and day 14 and that another value for biofilm cover in-between would be required to quantify the behaviour of the increase in biofilm growth. The numeric values of the standard error used for the error bars are shown in Appendix A.

Figure 4.3 clearly shows that the biofilm growth present on the coupons placed in distilled water (control) and chlorinated water is very similar. This shows that for the nature of the biofilms present, the biofilms were chlorine resistant and the chlorine did not significantly limit the formation of biofilm on the pipe substrate. From day 1 to day 10, there was gradual growth of biofilms on the coupon for both, chlorine and distilled water.

At day 14, there was a significant difference between biofilm growth observed in distilled water and chlorinated water. On day 14, coupons from chlorine beakers 1 and 2 (i.e. phase 1 testing) experienced a biofilm cover of 32 % and 28 % respectively, whilst distilled water beakers 1 and 2 (i.e. Phase 1 testing) experienced a biofilm cover of 11 % and 8.2 % respectively. Although beakers 1 and 2 (i.e. phase 1) on day 14 for chlorinated water fit in with the observed trend, the values are unusually and uncharacteristically high. For coupons placed in chlorinated water, the day 14 coupons in beakers 3 and 4 had a biofilm cover of 13 % and 12 % respectively. The unusually high values for the biofilm cover observed on day 14 coupons for chlorinated water may due to bacterial contamination from within the lab or the presence of the EPS matrix which took up a large area on the pipe coupons (Figure 4.4). Looking at day 14 coupons placed in chlorinated water, the difference between phase 1 (beakers 1 and 2) and phase 2 (beakers 3 and 4) is significant, and the error bars do not overlap. Hence the results obtained from phase 1 on day 14 coupons placed in chlorinated water were not repeatable. The results for phase 2 on day 14 have a better ‗fit' with the rest of the data set for coupons placed in chlorinated water.

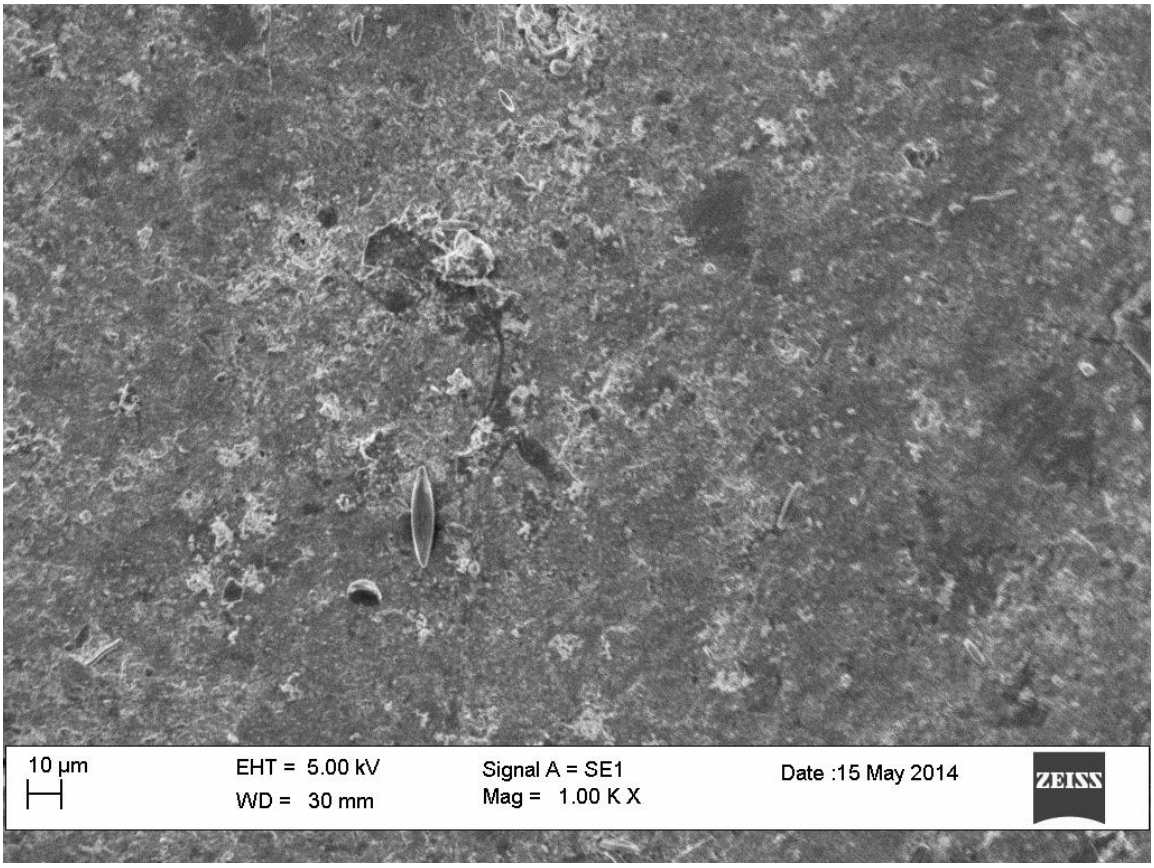


Figure 4.4: SEM image of chlorine test coupon on day 14 showing large area of substrate covered by EPS matrix that resulted in a large biofilm cover percentage reading.

For chlorine and distilled water, the biofilm cover present on coupons peaked on day 14. The next reading after day 14 was taken on day 21. There was a ‗crash' observed in the biofim cover observed from day 14 to day 21. This is attributed to the availability of nutrients, as a high biofilm concentration leads to increased consumption of nutrients (Winstanley, et al., 2010). The relationship between biofilm and nutrient availability is explained further in section 4.2. As the biofilm population increases, the nutrient availability decreases (i.e. the nutrients are used up, so the observed ‗crash' is due to nutrient limitation), which leads to the microbes that form the biofilm to die out and they lose their cohesion properties when they die (Winstanley, et al., 2010).

From day 14 until day 60, both chlorine and distilled water coupons experienced growth of biofilms on coupons. The growth observed for both chlorine and distilled water coupons from day 14 till day 60 was very similar.

Monochloramine

Figure 4.5 below shows the biofilm growth in water with monochloramine over a 14 day period. On the same graph, the combined monochloramine decay of all 4 test beakers is shown.

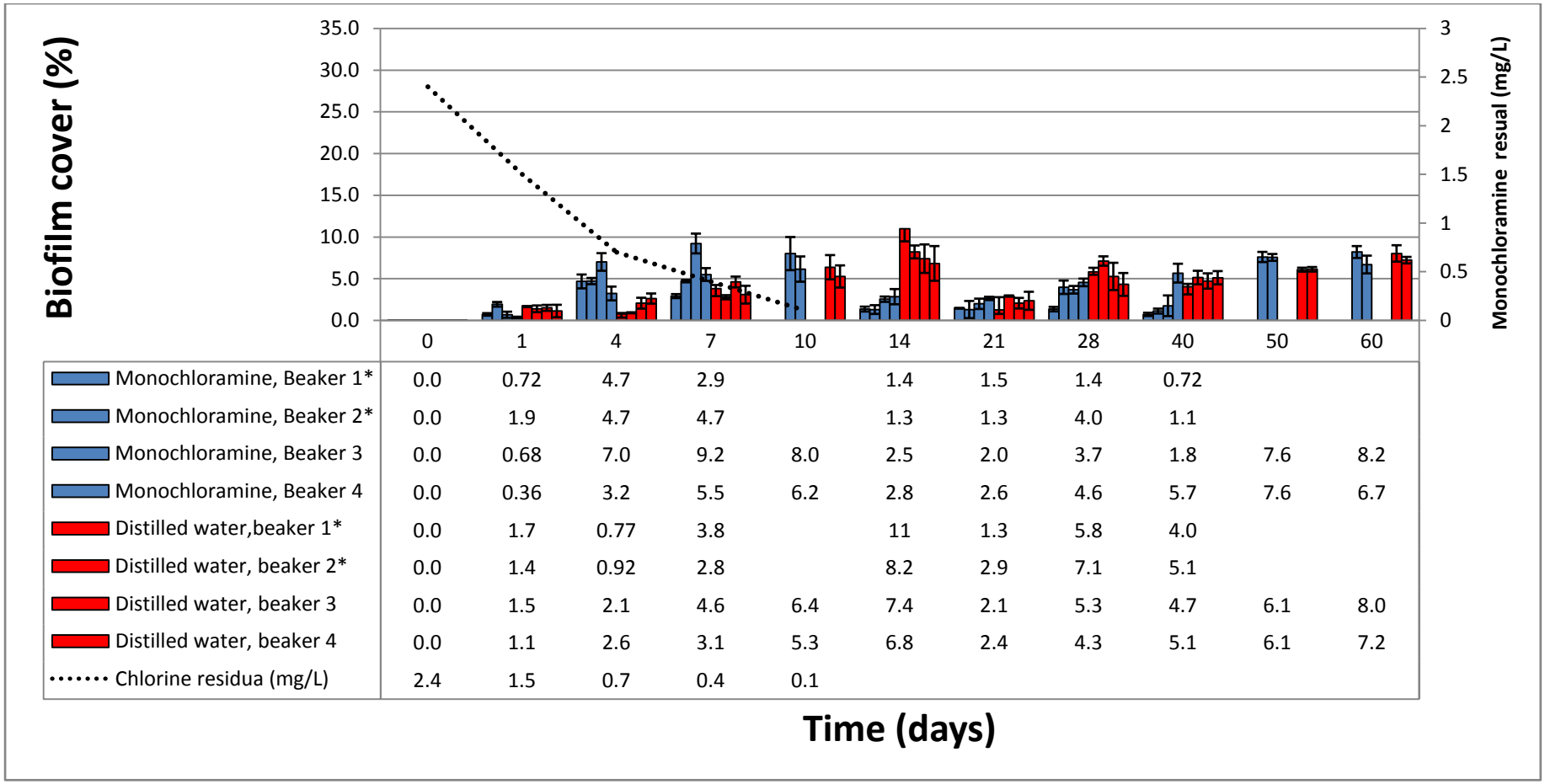


Figure 4.5: Percentage biofilm cover on coupons placed in monochloramine-disinfected water (blue) and distilled water (control) (red) over 60 day period for each of 4 test beakers. Error bars represent standard errors. The dashed line indicated combined average chlorine concentration of all 4 beakers. The blank spaces represent phase 1 wherein the tests were only carried out over a 40 day period and no day 10 reading was taken.

Beakers marked with * were carried out during the first phase of testing, where there were no readings taken on day 10 for chloramines-disinfected water and distilled water and the testing was done till day 40 only. Only after the analysis of phase 1 was compete, it was realized that there is significant growth between day 7 and day 14 and that another value for biofilm cover in-between would be required. The numeric values of the standard error used for the error bars are shown in Appendix A.

Figure 4.5 clearly shows that the biofilm growth present on the coupons placed in distilled water (control) and the chloramines test waters are very similar. Similar to chlorine, this shows that for the nature of the biofilms present, the biofilms were chloramine resistant and the chloramines disinfectant did not significantly limit the formation of biofilm on the pipe. From day 1 to day 10, there was gradual growth of biofilms on coupons in chloramines test water, while the distilled water coupons experienced gradual growth from day 1 to day 14. Biofilm cover on coupons in monochloramine peaked on day 10, and then ‗crashed', while the biofilm cover for distilled water coupons peaked on day 14, and then ‗crashed'. The peak of the biofilm growth with coupons placed in chloramine-disinfected waters occurred earlier than the peak for coupons placed in distilled water although the peak value for chloramines and distilled water were similar.

The crash is attributed to nutrient availability, as a high biofilm concentration leads to increased consumption of nutrients (Winstanley, et al., 2010), which is investigated further in section 4.2. As the biofilm population increases, the nutrient availability decreases, which leads to the microbes that form the biofilm to die out and they lose their cohesion properties when they die (Winstanley, et al., 2010).

From day 21 until day 60, both monochloramine and distilled water coupons experienced growth of biofilms on coupons. The growth observed for both chlorine and distilled water coupons from day 21 till day 60 was very similar due to their both having disinfectant residuals below detectable limits.

Comparison of biofilm cover between chlorine disinfectant and chloramines disinfectant

From Figure 4.3 and Figure 4.5, it can be noted that chlorine and monochloramine both experienced biofilm growth on coupons similar to distilled water coupons. Hence, both disinfectants have a minimal effect on limiting biofilm formation. Also, the primary colonizers present could have been both chlorine-resistant and chloramine-resistant.

The main difference observed during testing between biofilms cover on coupons placed in chlorine and monochloramine test waters were the peak biofilm covers on coupons. The biofilm cover peaked on coupons placed in chlorinated water on day 14 with a biofilm cover of between 28 % and 32 %. However, these very high values of biofilm cover were not repeatable, as shown on Figure 4.3, when compared to the day 14 tests of phase 2 (beakers 3 and 4). The second phase peak at day 14 for chlorine is between 12 % and 13 %, which ‗fits' in with the observed trends. The biofilm cover peaked on coupons placed in monochloramine disinfected water on day 10 with a biofilm cover of between 6 % and 8 %. Monochloramine is a better disinfectant in the sense that its peak biofilm cover observed on coupons placed in chloramines-disinfected water is about half of the peak biofilm cover observed for chlorine-disinfected water.

The trends observed for both biofilm cover in chlorine and monochloramine test water are very similar. The biofilm cover increased from day 1 until the day that it peaks, and then ‗crashes'. After the ‗crash', until day 60, there is a steady increase in the biofilm cover on coupons. The biofilm cover did not slough off due to shear stresses as all beakers were kept motionless and there was no shear forces generated. The sudden decrease was due to the increasing biofilm populations and the limited nutrient availability. Bacteria reliant on the same nutrients become progressively starved, and then die, losing their adhesion to the substrate (Winstanley, et al., 2010).

Although the experiments carried out did not find a significant difference between chlorine and chloramines with respect to biofilm growth, the Water Quality Association (2004) reports that monochloramine is 200 times less effective as a disinfectant than chlorine, but it is an alternative to chlorine since it does not react readily with organic materials to form THMs. It is a more stable and longer-lasting disinfectant than chlorine and chlorine dioxide and is a more effective disinfectant in biofilm control because of its greater ability to penetrate the biofilms (US Environmental Protection Agency, 1999). Momba et al. (2000) reported that monochloramine is a more stable and persistent disinfectant and has a better ability to limit

biofilm formation. They also reported that the disinfectant does not have a major impact on biofilm formation, but rather the substrate has a larger influence on the biofilm formation and growth. Turetgen et al. (2007) carried out experiments by placing coupons in cooling towers that had chlorinated water and water with monochloramine disinfectant, and after a period of 30 days, performed biofilm analyses and found that monochloramine was a better disinfectant in limiting biofilm growth. On the other hand, Park (2011) compared HPC counts on chlorine-disinfected and chloramines-disinfected waters having organic matter and concluded that having a chlorine residual was more effective than having a monochloramine residual. The different results obtained by these research is possibly due to the fact that they used HPC counts and used other microbiological methods to quantify the growth of cells on the substrate, unlike this research which used a purely visual method to quantify the microbial activity. Also, the disinfectants residuals were not maintained through the experimental run, so this could have been the reason that the trends observed for biofilm growth in chlorine-disinfected water and chloramines-disinfected water were similar.

The limitation to this study was that by looking at biofilms on substrate in plan view, although it is representative of the microbial activity, it may not be a completely accurate indication of the microbial activity as the plan view might are not able show biofilms that are ~~-s~~tacked" on top of one another.

4.1.1.5. Biofilm cover and pipe material

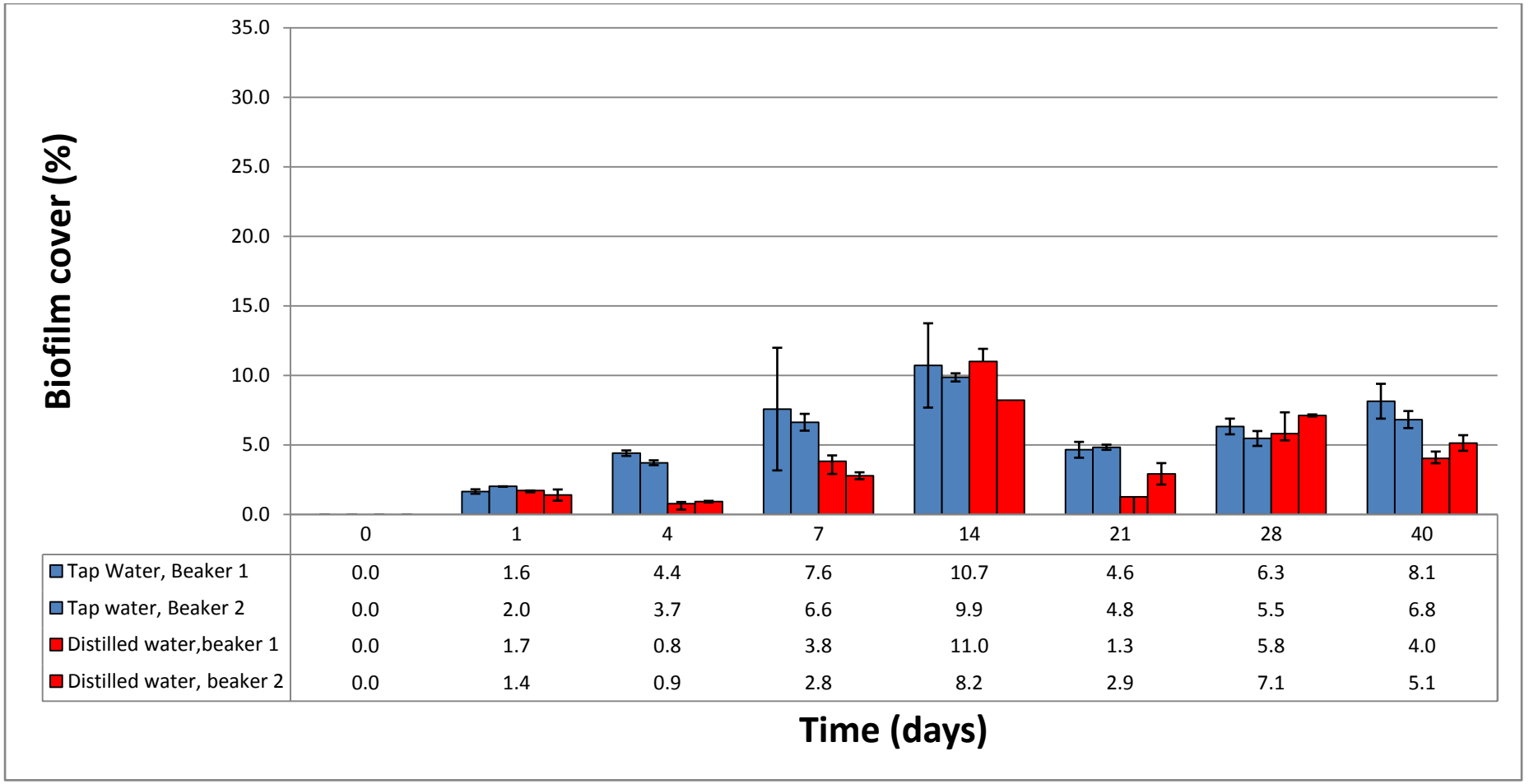
Due to the size of the coupons being approximately 1 mm x 1 mm in size, and having around 13 coupons per test water, the surface area exposed to the water was only 13 mm^2; hence there was no difference in the decay of disinfectants for plastic coupons and mortar coupons. However, published research shows that the substrates of plastic pipes are better at maintaining a disinfectant residual than concrete pipes. Due to the formation of a silty deposit on the mortar substrate, it was not possible to make a comparison between the biofilm growth on the plastic and mortar substrates. All papers and journals reviewed found that plastic was the preferred pipe material over mortar/concrete with respect to biofilm inhibition due to plastic having a smoother surface which makes it more difficult for biofilms to attach (Kerr, et al., 2003; Hallam, et al., 2001; Niquette, et al., 2000). Concrete pipes have a lesser attachment potential for biofilm growth as compared to steel but a greater potential as compared to plastic pipes (Hallam, et al., 2001). Niquette et al. (2000) carried out experiments by exposing different pipe materials to different source waters. The results of their experiments showed that PVC pipes had a lower biofilm forming potential than mortar-lined steel pipelines. Camper et al. (2003) carried out experiments by placing coupons of different materials in a lab reactor with test water. They found that mortar-based pipe

coupons experienced greater biofilm formation than PVC pipes coupons. Momba and Makala (2004) investigated the effect of different pipe materials and biofilm formation. They carried out HPC counts of coupons in the test waters and found that plastic-based substrates had a lesser biofilm-formation than cement-based substrates.

There has been very limited research in the field of biofilm limitation and cement/mortar-lined pipelines. The silt crystalline deposition seen under the SEM microscope is due to the chemical reactions that take place between the water and the compounds that make up the cement (Slaats, et al., 2004). The principal constituents of cement are aluminates ($Ca_3Al_2O_6$) and silicates (Ca_3SiO_5, Ca_2SiO_4). These compounds react with water and hydrate which forms calcium hydroxide ($Ca(OH)_2$). Calcium hydroxide further dissolves into Ca^{2+} and OH (Slaats, et al., 2004). The Ca^{2+} leads to calcium precipitation which is the silt deposition seen on the SEM images (Figure 4.14).

4.1.1.6. Biofilm growth in tap water

Tap water was tested as well to compare the lab results to real life situations. Tap water was used mainly to compare qualitatively the types, structure and forms of biofilms to lab results to get an indication of the differences in the biofilms observed between biofilms in a pipe network and the biofilms from a semi-controlled lab experiment. Since tap water is at the furthest point away from the treatment works in the drinking water network, and has experienced decay along the length of its conveyance to the end user, it had a very low chlorine residual (0.1 mg/L) which dropped to below detectable limits within the 1st 24 hours. Figure 4.6 shows the biofilm cover for tap water. As stated in Chapter 3, the tap water experiments were only carried out during phase 1. Hence, there are only results for 2 test beakers over a 40 day period. The numeric values of the standard error used for the error bars shown in figure 4.6 are shown in Appendix A.



	0	1	4	7	14	21	28	40
Tap Water, Beaker 1	0.0	1.6	4.4	7.6	10.7	4.6	6.3	8.1
Tap water, Beaker 2	0.0	2.0	3.7	6.6	9.9	4.8	5.5	6.8
Distilled water,beaker 1	0.0	1.7	0.8	3.8	11.0	1.3	5.8	4.0
Distilled water, beaker 2	0.0	1.4	0.9	2.8	8.2	2.9	7.1	5.1

Figure 4.6: Percentage biofilm cover on coupons placed in tap water (blue) and distilled water (control) (red) over 40 day period for each of 2 test beakers. Error bars represent standard errors.

As shown on Figure 4.6, the biofilm cover on coupons placed in tap water peaked on day 10. From day 1 to day 10, with the exception of day 4, tap water had a slightly higher biofilm cover on coupons than coupons placed in chlorine, monochloramine and distilled water. The slightly higher percentage of biofilm cover on coupons placed in tap water from day 1 to day 7 indicate that there are microbial organisms present in the water, and these organisms form primary colonizers once they enter the system. The primary colonizers from the tap water are in addition to the primary colonizers that entered due to inoculation with pond water. A qualitative analysis-where the differences between the tap water biofilms and the chlorine, chloramine and distilled water biofilms- are provided later on in this chapter.

It can be seen that the biofilm cover for chlorine, monochloramine, and distilled water are within a reasonable range of biofilm cover for tap water; hence the results obtained do reflect realistic field data.

4.1.2. Qualitative analysis

This research did not attempt to identify the type and/or strain of biofilm or bacteria present. However, the shape, size and structure of biofilms were analyzed. This section provides a qualitative analysis of the images in terms of shape and structure of biofilms present. The timeframe and availability of equipment did not allow for DNA analysis, and/or any other microbiological identification techniques.

4.1.2.1. Structure of biofilms present

Chlorine, monochloramine and distilled water

The shape, structure, size and type of biofilms found in coupons submerged in chlorine, monochloramine and distilled water coupons were similar. This shows that the pond water used for inoculation for all 3 of these coupons were consistent and provided the water samples with a good source of primary colonizers. The 3 most common structures for the biofilm found on the substrates were the ~~"J~~elly-baby", the ~~"l~~eaf" and the EPS gel-like biofilm. These shapes and forms were found through the 60 day period for all water qualities. The structures mentioned are shown in Figures 4.7 to 4.9:

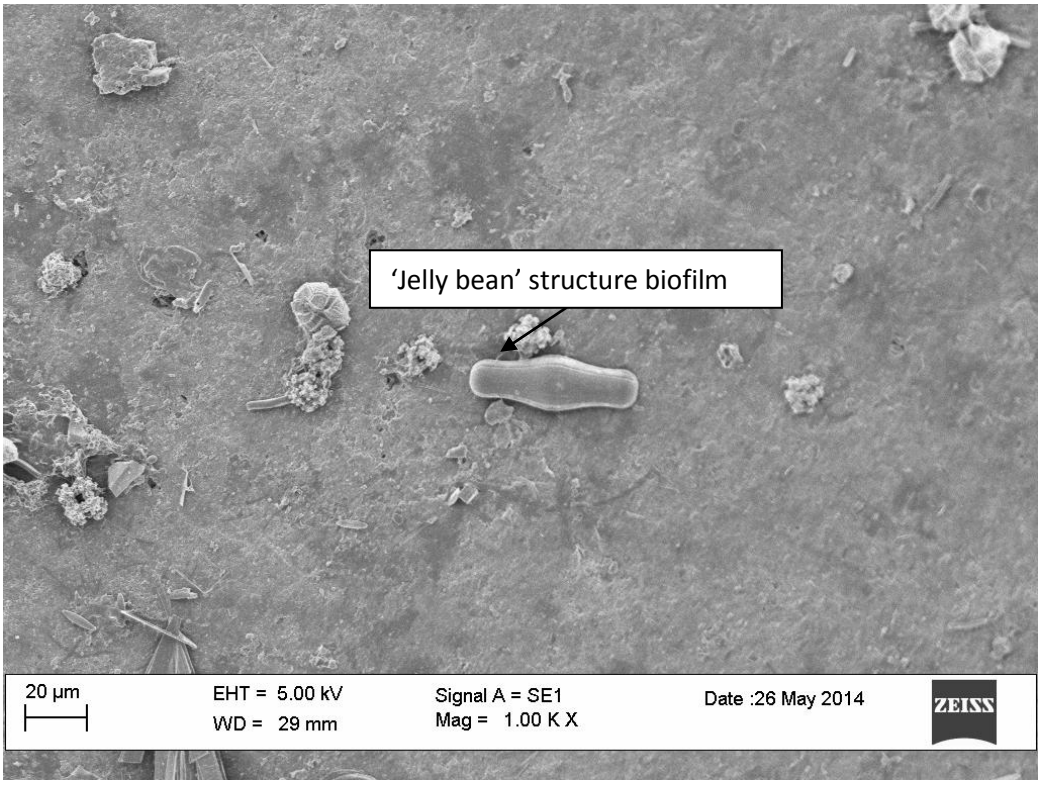


Figure 4.7: Jelly bean shaped biofilm cellstructureon plastic coupon substrate (day 28 chlorine water).

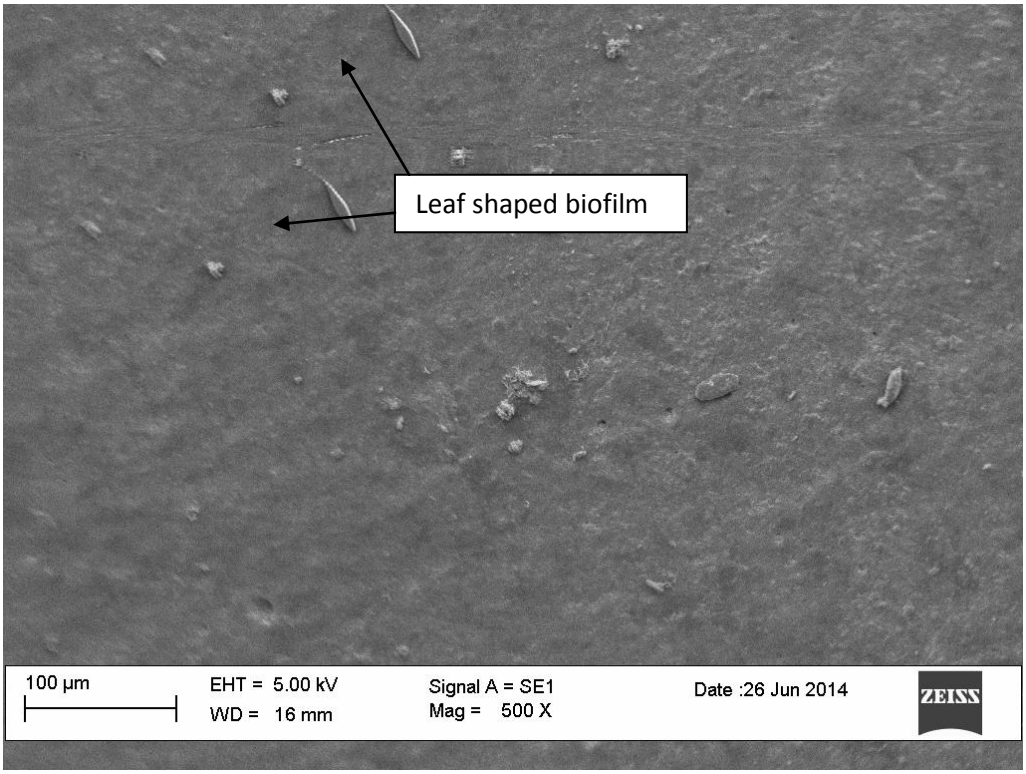


Figure 4.8: Leaf shaped biofilm cell structure on plastic coupon substrate (day 28 monochloramine).

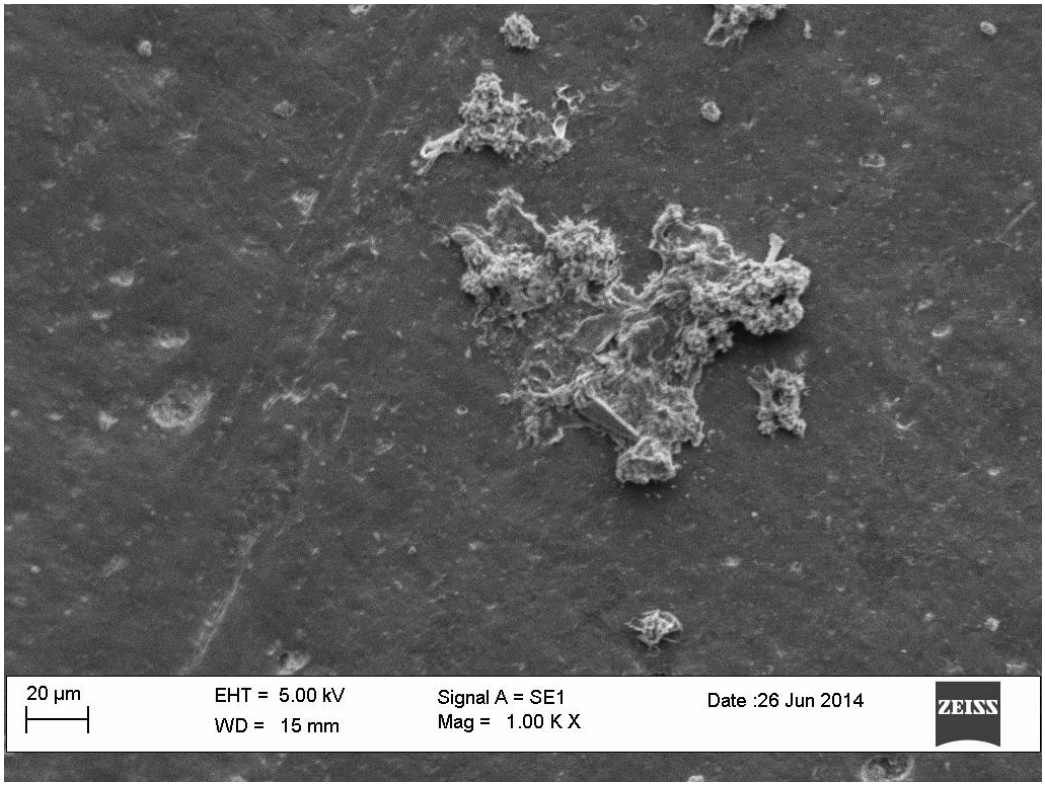


Figure 4.9: EPS structure present of biofilm on plastic coupon substrate (Day 7 distilled water).

On day 28, it was observed that there was plant matter that had settled on the surface. The plant matter resembles a blade of grass. By the definition of biofilms, as stated in Chapter 2 (that says that all biological matter dead or alive makes up the biofilm), that plant matter was considered as part of the biofilm when doing the image analysis. It can also be noted here that there is a greater biofilm cover present around the plant-like matter. Hence, plant matter present provides a better environment for biofilm growth as it makes it easier to adhere to the surface (Characklis, 1984). The plant-like material can be seen in Figure 4.10:

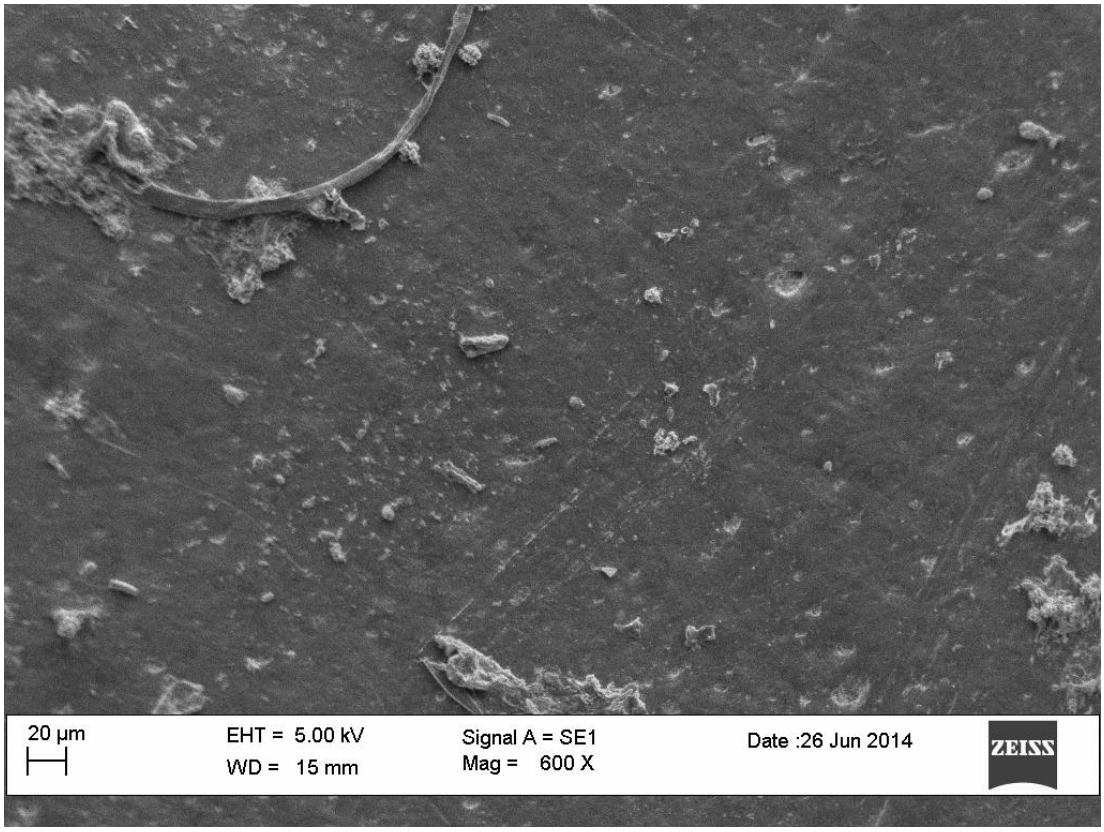


Figure 4.10: Plant-like matter present on plastic coupon substrate (Day 28 monochloramine).

Tap water

Because tap water is not as pure as distilled water, and tap water contains impurities (some of which are microbiological life forms, which later become primary colonizers on the substrate placed in tap water), the biofilm structures observed on coupons placed in tap water is different to the other 3 water qualities tested. Tap water coupons, besides having similar biofilm structures to that of chorine, chloramines and distilled water coupons, also had its own unique biofilms that had entered through the pipe network. There was a ~~"~~flower-shaped" organic material found on the tap water coupons that was unique to the tap water, as shown in Figure 4.11.

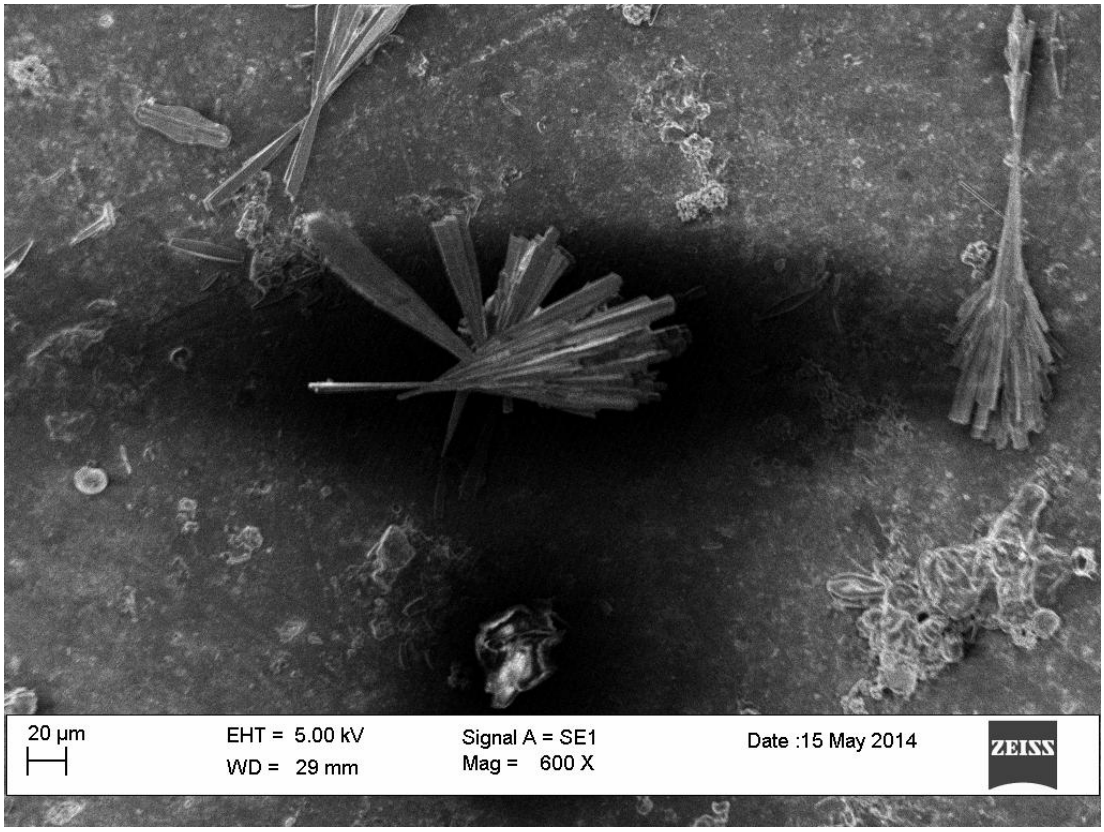


Figure 4.11: Biofilm 'flower' structure on cell present on tap water coupon (day 14).

There was also an elongated rod-like structure unique to tap water as shown in Figure 4.12:

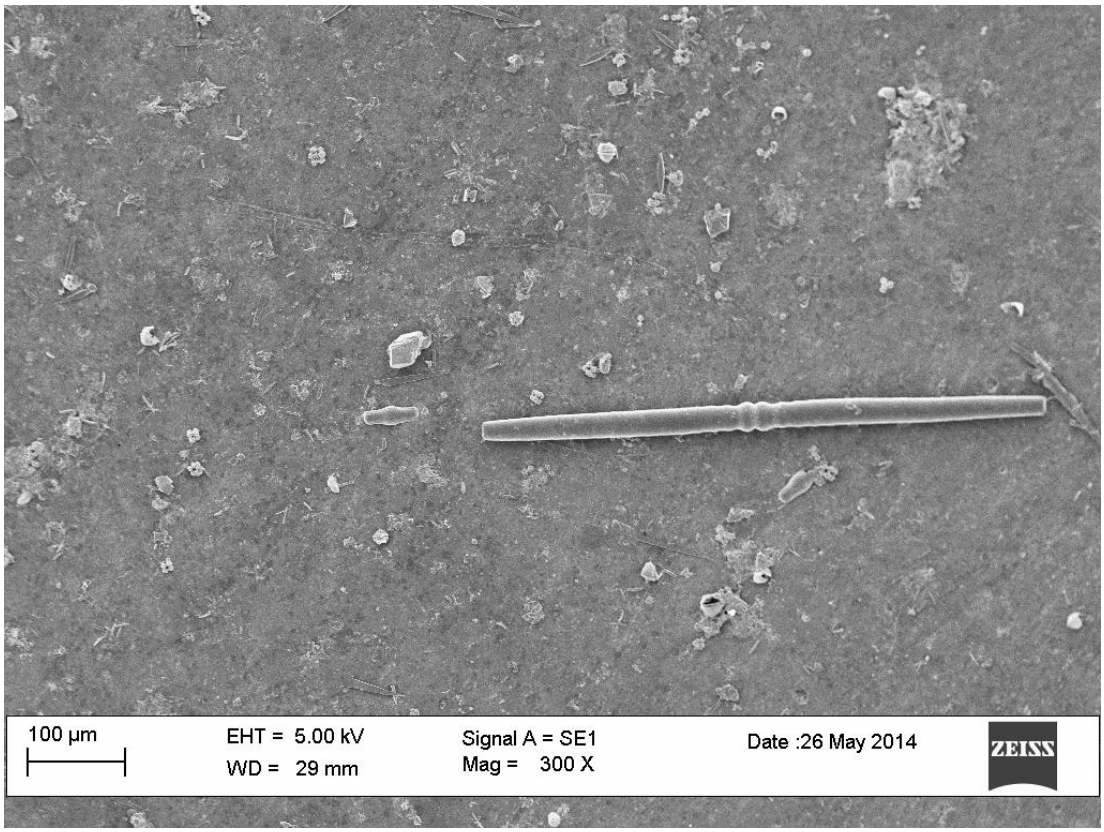


Figure 4.12: Elongated rod-like biofilm cell structure on tap water coupon (day 28).

The fact that there is additional biofilm growth for the tap water coupons means that there are microbial life forms (that are primary colonizers) present in the drinking water network and although the water is disinfected, the end user consumes water which is not 100 % pure from biological activity. Although water is treated, primary colonizers may enter the drinking water system from:

- incomplete disinfection at the water treatment works (Mains, 2008)
- open reservoirs and storage tanks (Mains, 2008)
- cracks, joints, valves, and backflows (Mains, 2008; Momba, et al., 2000)
- the pipes themselves (Farkas, et al., 2012)

4.1.2.2. Dimensions of microorganism structures

There was variation in the size of each of the different biofilm structures and/or shapes. This was due to the cell beginning its life at a much smaller size, and then progressively growing as it ages. The largest ‗jelly bean' cells observed, had a length of ±60 µm, a width of 25 µm at its widest point and a width of 14 µm at its narrowest. The ‗leaf cell' had a maximum length of 39 µm, and a width of 5 µm at its widest point. Although the cells had differences in their lengths and widths, the ratios between the length and width were almost always the same. Even visually, the proportions between the different dimensions were clearly similar.

4.1.2.3. Microbiological insect form

There was a single instance of a coupon (chlorine day 7, mortar) that had, what appeared to be a microbial insect. This insect measured roughly 264 µm in length and 132 µm in width (Figure 4.13). It is assumed that this life form entered the test water from the beaker or the tweezers used to extract coupons for analysis by surviving sterilization. Since this occurred just once in all of the coupons, there was insufficient information to make a solid judgement between insect life present and disinfectant and/or insect life present and pipe material. This was merely an interesting observation made. Figure 4.13 shows the insect that attached to the concrete pipe coupon, at a 600X magnification. No identification and classification was carried out as this was not one of the objectives of this research project.

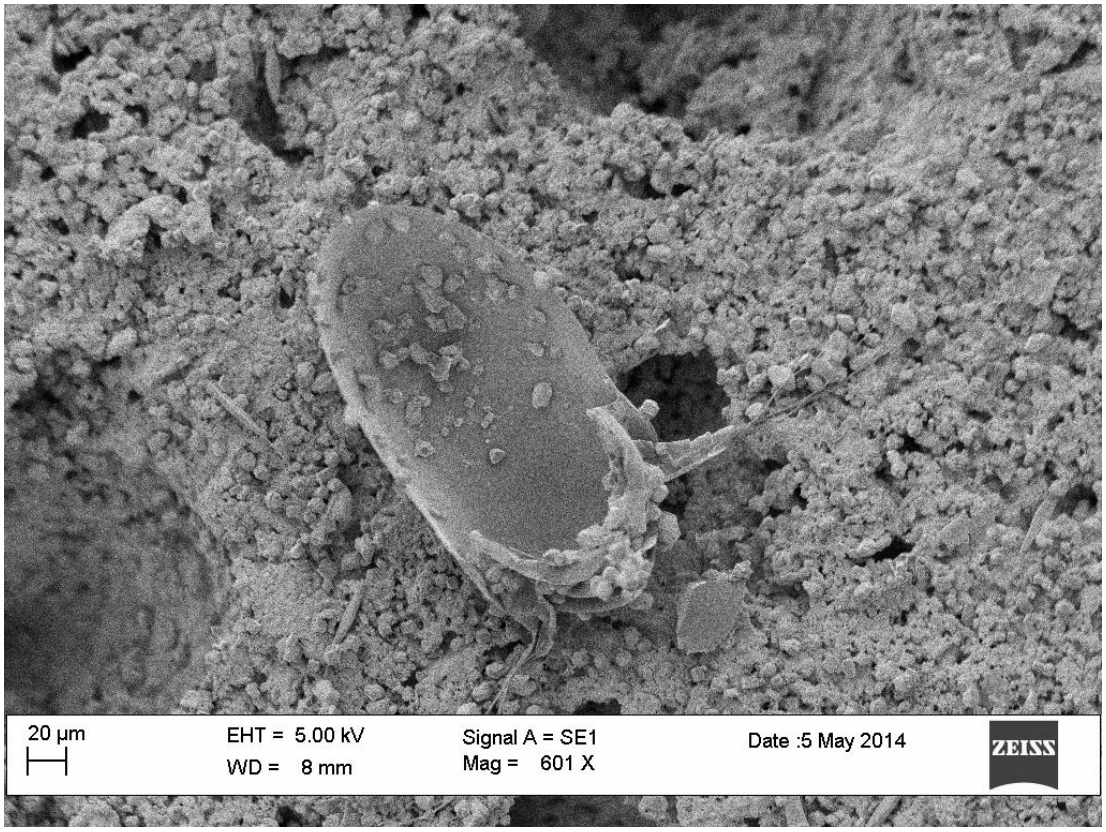


Figure 4.13: Microbial insect life form on mortar coupon observed on day 7, chlorine disinfectant substrate (600X zoom).

Figure 4.13 shows that the silt deposition that was discussed earlier on in this chapter, took place on the insect itself as well. Silt would most probably accumulate on a dead motionless object, proving that this organism was most probably lifeless for a while before the SEM imaging was done on this surface. Also, the fact that silt deposition occurred on the insect means that there might have been instances where the entire surface of an insect was covered in silt deposits and was therefore, unable to be detected by the SEM imaging.

4.1.2.4. Biofilm and pipe material

It was initially planned that biofilms would be grown on plastic and mortar coupons, and then a comparison would be made to determine which pipe material is better at limiting biofilm formation. However, it was found that with the mortar samples, after 4 days, had a crystal structure that had formed over the entire substrate and it was not possible to perform any biofilm analyses. Therefore no comparison could be performed. There is literature that shows that biofilms form less on plastic pipes than mortar pipes as there is a smoother surface on plastic pipes which makes it more difficult for adhesion (Kerr, et al., 2003; Hallam, et al., 2001). Figure 4.14 shows the mortar substrate at day 0 and the crystal structure that formed on the mortar coupons at day 7:

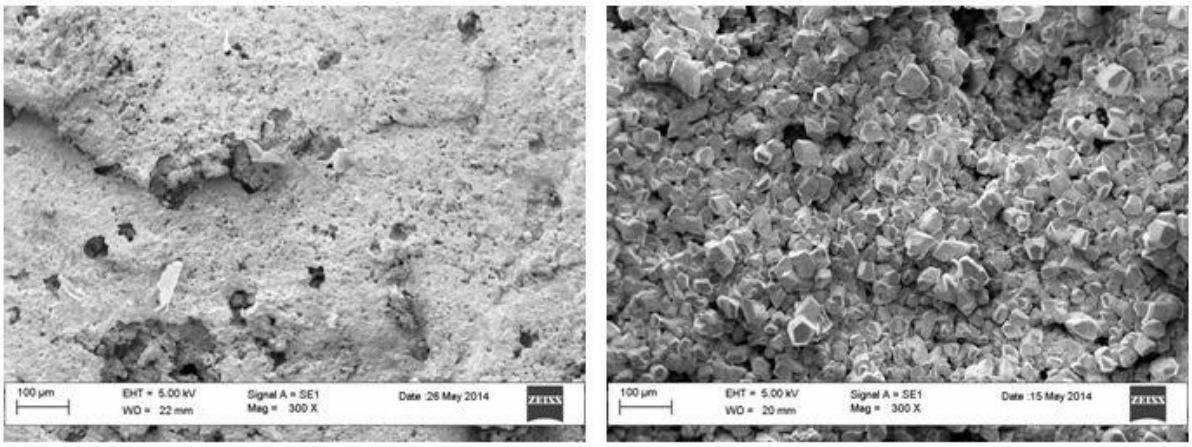


Figure 4.14: SEM image of day 0 concrete coupon with substrate fully visible (left) and SEM image of day 7 coupon placed in chlorinated water, with substrate fully covered in silt deposit (right).

4.1.3. Biofilm contribution to hydraulic roughness

SEM only provides a ‗plan view' of pipe coupons, unlike atomic force microscopy (AFM) which provides a 3D image of a surface by running a probe on the test surface and generating an image based on the deflections of the probe. Hence a detailed roughness profile and roughness analysis was not done due to limitations in the available laboratory equipment.

The jelly-bean structured biofilm cells shown in Figure 4.7 are ±20µm in width and this dimension was used to estimate the increase in pipe roughness due to biofilm formation.
There are many ways in which biofilm formation can attach to the pipe substrate: some examples are shown in schematically in Figure 4.15.

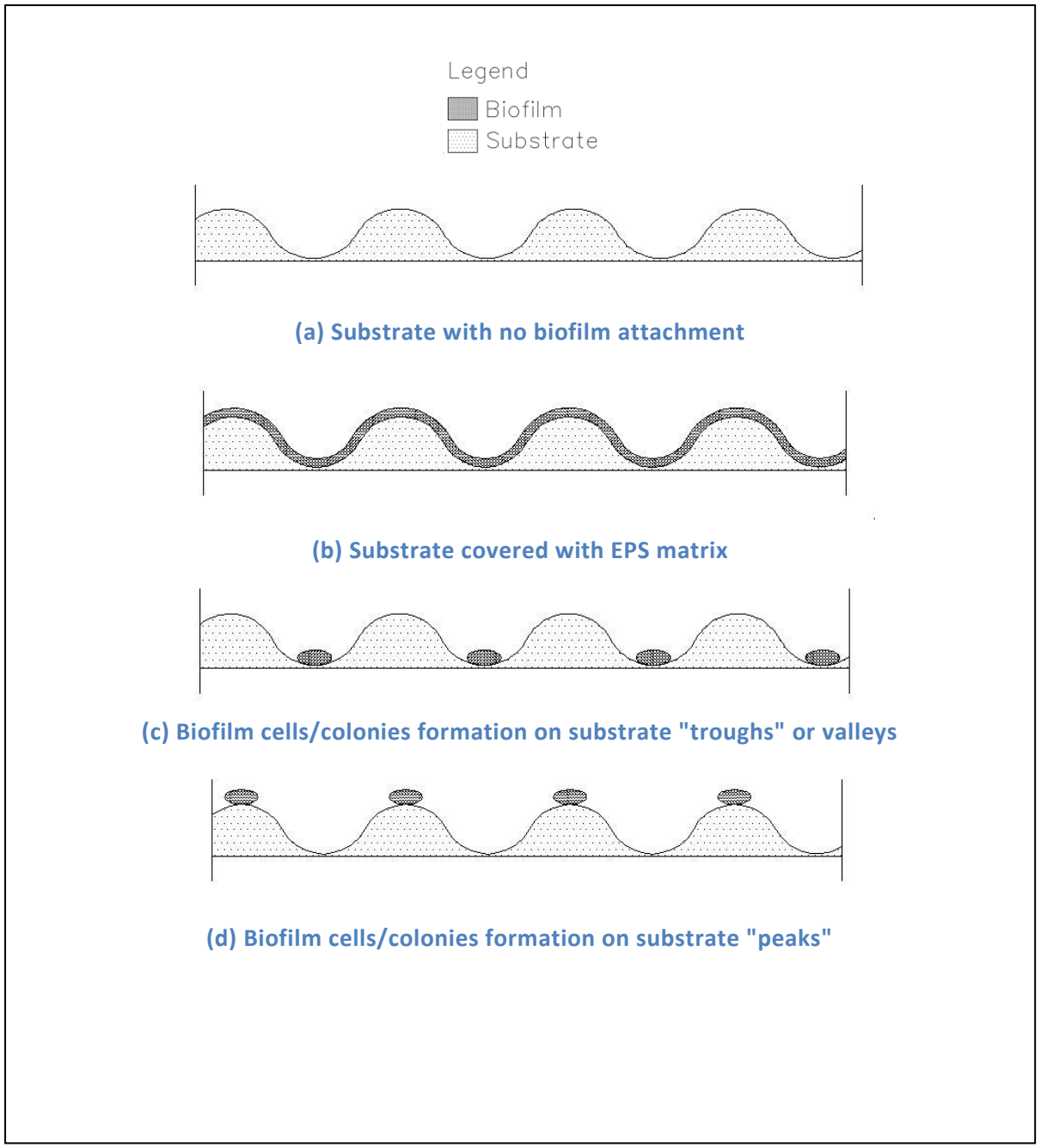


Figure 4.15: Schematic representation of biofilm influence on effective pipe roughness for different attachment conditions (exaggerated scale): (a) No biofilm attachment (b) substrate covered with uniform EPS matrix (c) biofilm cell/colonies attachment on substrate valleys (d) biofilm cell/colonies attachment on substrate peaks.

As can be seen from Figure 4.16, the contribution of biofilm to hydraulic roughness is dependent on the location of biofilm cells/colonies attachment on the substrate. A uniform cover by an EPS matrix (Fig. 4.16(b)) or attachment of cells/colonies in the ~~t~~roughs" or valleys (Fig. 4.16(c)) of the pipe substrate would cause a minimal change to pipe roughness characteristics. Biofilm cells/colonies that will have maximum contribution to hydraulic roughness are those that attach to the ~~p~~eaks" of the substrate (Fig. 4.16(d)), assuming that

this occurs over substantial areas of the substrate. The roughness depth changes from ε_0 to $\varepsilon_0+\Delta\varepsilon$, where ε_0 is the original pipe roughness and $\Delta\varepsilon$ is the change in roughness length due to biofilm growth.

4.1.3.1. Change in relative roughness due to biofilm growth

For localized increases in roughness as depicted in Fig. 4.16(d), the change in relative roughness can be estimated as:

$$\Delta\left(\frac{\varepsilon}{D}\right)=\frac{\varepsilon_0+\Delta\varepsilon}{D}$$

$$=\frac{\varepsilon_0}{D}\left(1+\frac{\Delta\varepsilon}{\varepsilon_0}\right)$$

Plastic pipes, generally have $\varepsilon_0\approx$ 30 μm (Chadwick, et al., 2004), whilst the average width of individual biofilm cells observed was typically about 20 microns. Hence the change in relative roughness for one cell thick biofilms observed in this research can be estimated as:

$$\text{Change in roughness}=\frac{\varepsilon_0}{D}\left(1+\frac{20}{30}\right)\approx 1.67\frac{\varepsilon_0}{D}.$$

Therefore for localised attachments to the peaks of the substrate material, there could be a 67 % increase in relative roughness, assuming that the whole surface area is similarly affected, and ignoring multi-layered attachments. From the Moody chart it can be seen that at high Reynold's numbers, the change in the relative roughness by a factor of a 1000 gives rise to a factor of 10 for the change in the friction factor$\left(\text{ie: } f\sim\left(\frac{\varepsilon_0}{D}\right)^{1/3}\right)$. Hence a 67 % increase in relative roughness could contribute to approximately18% increase in the friction factor).

However, the roughness values calculated may not be representative of the actual effective hydraulic roughnesses in pipes for the following reasons:

1. In a pipe network, the water velocities result in shear stresses that can lead to detachment of biofilms (Kerr, et al., 2003)
2. The roughness of the biofilm is not the actual effective roughness that influences the flow properties. The biofilm is not a solid interface and there is flow between the cells and cell matrices (Stoodley, et al., 1999). Even though there might be uniform layering of biofilm elements present, there is liquid flow between the biofilm channels (Stoodley, et al., 1994).

3. The biofilm cells may only influence a proportion of the surface area, which would decrease the overall roughness change proportionally and may therefore have a negligible effect on the overall mean roughness of the pipe.

4.1.3.2. Effect of reduction in effective pipe diameter and increase in relative roughness due to biofilm formation

A 20 µm constant thickness of biofilm relates to a 40 micron change in effective diameter of pipeline. For trunk mains, where all pipes are over 1 000 mm in diameter, this translates to a very small change in the effective diameter (less than 0.01 %). van Vuuren & van Dijk (2012) measured biofilm thicknesses of 2mm to 4mm in a pipeline with inside diameter of 1 462 mm. This also translates to a 0.27 % to 0.55 % change in the effective diameter, which is a very small change (<1 %).

Change in velocity due to diameter changes

For a constant flow rate, using the law of conservation of mass:

$$Q = \frac{V\pi D^2}{4}$$

Whence $V = \frac{4Q}{\pi D^2}$

and $V + \Delta V = \frac{4Q}{\pi (D - \Delta D)^2}$

Therefore, using Taylor series expansions to 1st order in $\frac{\Delta D}{D}$

$$\frac{v + \Delta V}{v} = \frac{d^2}{(D - \Delta D)^2} \approx \frac{1}{\left(1 - 2\frac{\Delta D}{D}\right)} \approx 1 + \frac{2\Delta D}{D}$$

$$\frac{\Delta V}{V} \approx \frac{2\Delta D}{D} \qquad \text{Equation 4.1}$$

For the pipeline tested by van Vuuren & van Dijk (2012), with biofilm thicknesses of 2 mm to 4 mm in a 1462 mm diameter pipeline, the relative change in velocity is estimated to negligible using Equation 4.1, so that the biofilm causes no significant velocity change.

Change in the head loss, for a fixed flow rate

Using the Darcy-Weisbach equation:

$$\frac{h_f}{l} = \frac{8f}{g\pi^2}\frac{Q^2}{D^5}$$

Whence

$$\frac{h_f + \Delta h_f}{l} = \frac{8(f+\Delta f)}{g\pi^2(\mathrm{D}-\Delta\mathrm{D})^5}\frac{Q^2}{}$$

Hence changes in the hydraulic gradient can be estimated from:

$$\frac{(h_f + \Delta h_f)}{(h_f)} = \frac{(f+\Delta f)D^5}{f(\mathrm{D}-\Delta\mathrm{D})^5} = \frac{\left(1+\frac{\Delta f}{f}\right)}{\left(1-\frac{\Delta D}{D}\right)^5}$$

Therefore, using Taylor series expansions to 1st order in $\frac{\Delta D}{D}$ and $\frac{\Delta f}{f}$

$$\frac{\Delta h_f}{h_f} \approx \frac{\Delta f}{f} + \frac{5\Delta D}{D} \qquad \text{Equation 4.2}$$

Using the pipeline tested by van Vuuren & van Dijk (2012) where the roughness ε changes from 0.05 mm design roughness to a roughness of 1.78 mm after 12 years, the relative pipe roughness ε/D changes from 0.0003 to 0.02. Using the Moody chart, the corresponding change in friction factor is from 0.014 to 0.02. (i.e. a 44 % increase). Using Equation 4.2, the relative change in head loss is 44 % for a 2mm thick biofilm growth and 46% for a 4mm thick biofilm growth. The biofilm growth of 2mm to 4mm has a relatively large effect (44 %-46 %) on the head loss of a pipe. The relative change in the pipe diameter is very small $\left(\frac{5\Delta D}{D} \sim 1\ \%\right)$ and therefore the relative change in head loss is mainly a function of the relative change in the friction factor.

Change in the flow rate, for a fixed head loss

Using a modified Darcy-Weisbach equation:

$$Q = \left[\frac{h_f 2g\pi^2 D^5}{8f}\right]^{\frac{1}{2}}$$

Whence $Q - \Delta Q = \left[\frac{h_f 2g\pi^2 (D-\Delta D)^5}{8(f+\Delta f)}\right]^{\frac{1}{2}}$

$$\frac{Q-\Delta Q}{Q} = \left[\frac{(D-\Delta D)^5 f}{D^5(f+\Delta f)}\right]^{\frac{1}{2}} = \left[\frac{\left(1-\frac{\Delta D}{D}\right)^5}{1+\frac{\Delta f}{f}}\right]^{\frac{1}{2}}$$

Therefore, using Taylor series expansions to 1st order in $\frac{\Delta D}{D}$ and $\frac{\Delta f}{f}$

$$-\frac{\Delta Q}{Q} \approx \left[\frac{1-\frac{5\Delta D}{D}}{1+\frac{\Delta f}{f}}\right]^{\frac{1}{2}} \text{-1} \approx \frac{-\frac{\Delta f}{2f}-\frac{5\Delta D}{2D}}{1+\frac{\Delta f}{2f}}$$

So $\frac{\Delta Q}{Q} \approx \frac{\Delta f}{2f} + \frac{5\Delta D}{2D}$ Equation 4.3

Using the 1462 mm pipeline tested by van Vuuren & van Dijk (2012), and using the corresponding values for $\frac{\Delta f}{f}$ from the Moody chart (as shown in section 4.3.3.2), $\frac{\Delta Q}{Q}$ is approximately 0.18 for a 2mm thick biofilm growth and 0.19 for a 4mm thick biofilm pipeline. Hence, for a given head loss, a 2-4 mm thick biofilm will reduce the carrying capacity of a pipeline by approximately 18 % to 19 %. Again, as in section 4.3.3.2, the effective diameter change is too small to make a significant contribution to the change in flow.

It is most likely for a pipeline, that there is a given head loss, and that the flow changes according to the pipe conditions. In 12 years' time, the pipe tested by van Vuuren & van Dijk (2012) is likely to experience approximately 18 % to 19 % loss in its carrying capacity. The change in effective diameter causes a very small change in the hydraulics of the pipe due to the pipe being so large in diameter ($\frac{\Delta D}{D} \sim 0\ \%$). The large loss in the flow rate and the large increase in the friction factor are mainly due to the contribution of roughness from the

biofilms. However for the smaller diameter pipelines, such as the distribution lines, the change in the effective diameter may have a greater influence on the hydraulic conditions.

For biofilm thicknesses of 20 µm that were observed during the laboratory experiments, the change in effective diameter and the increase in roughness is too small to cause a noticeable change to flow conditions. The 60 day period was inadequate to grow biofilms large enough to make changes to the hydraulic changes.

4.1.4. Forecast of biofilm growth after 60 days

There was a sudden decrease in biofilms after day 10 peak for coupons placed in chloramines disinfected water and the day 14 peak for coupons placed in chlorinated and distilled water. After the ‗crash', all test waters (chlorine, chloramines and distilled water) biofilms observed a gradual increase in biofilm cover on coupons which was almost linear. There was no further decrease over time or ‗crash' in biofilms after the initial crash up till day 60. Hence, forecasting of future biofilm cover percentages were computed assuming that the increase in biofilms is steady and linear and that there are no further ‗crashes'. It was also assumed that the entire substrate would be colonized before a new layer of biofilm began to form on top of the present layer. However, from Figure 4.7, it can be noted that before the entire substrate is colonized, EPS starts accumulating in clusters.

The forecasting was computed based on biofilm cover data for the 1st 60 days of each respective test water. The forecasting was done using a linear regression model. The linear regression model was used to predict future values of biofilm cover on each respective substrate.

To quantify and comment on the accuracy of this prediction, prediction intervals were used. The complete linear regression model and the prediction intervals are shown in Appendix B. A Microsoft Excel macro developed by John McClain (2007) was used for predictions and prediction intervals. The output data from the spreadsheet provided by McClain (2007) was in agreement with sample hand calculations performed.

The prediction uncertainty limits are based on the assumption that the errors are randomly sampled from a Gaussian distribution. The prediction uncertainty limits are an indication of the expected scatter of the data. At a 95 % prediction interval, a randomly selected value will lie within 2 standard deviations of the mean. In some cases where the Gaussianity assumption is not strictly correct, the confidence limits for biofilm cover can become negative

or can return biofilm cover of more than 100 %, which is obviously unrealistic. Such cases have been truncated to zero for negative biofilm cover percentages and 100 % for biofilm cover percentages over 100 %. Table 4.2 provides a summary of the percentage cover of biofilm on coupons placed in each respective test water.

Table 4.2: Summary of results for percentage biofilm forecast with upper prediction limits

Disinfect. used in beaker	R^2	Time to reach 100% biofilm cover (days)	Time to reach 100% biofilm cover using upper prediction limit (days)	Time to reach 100% biofilm cover (yrs)	Time to reach 100% biofilm cover using upper prediction limit (yrs)	*a* (mm/yr)	*a* using upper limit (mm/yr)
Chlorine	0.732	680	340	1.88	0.94	0.01	0.01
Chloramine	0.756	770	440	2.13	1.22	0.009	0.009
Distilled water	0.611	900	430	2.5	1.19	0.008	0.008

The *R*-squared values are between 0.611 and 0.732, which means that there is a fairly strong linear correlation between the linear regression analysis and the actual data.

Using the linear regression model, it would take chlorine coupons 1.88 years (23 months) to reach a 100 % biofilm cover, while it would take monochloramine coupons 2.13 years (26 months) and it would take distilled water coupons 2.5 years (30 months) to reach a 100 % biofilm cover. This was done using a 30-day period for a month. The time taken to reach a 100 % biofilm cover on pipe coupons for all test coupons are very similar due to the water quality in all the beakers from day 14 were very similar (i.e. disinfectant concentrations below detectable limits during days 21 to 60 for distilled water and chlorinated water and during days 14 to 60 for chloramine-disinfected water).

In Table 4.2, a represents the coefficient or the slope at which roughness changes over time, from the equation developed by Echaves (1997):

$K_{(t)} = K_{(0)} + at$ Equation 4.4

where
$K_{(0)}$ – roughness of the new pipe (mm),
$K_{(t)}$ – roughness of pipe, at time t (mm),
a – coefficient (mm/year).

It is important to note that this equation is for pipe ageing as a whole and not specifically due to biofilm growth alone.

Assuming that the 100 % biofilm growth provides a roughness of 0.05 mm, and is, for the sake of this research, treated as a solid substrate boundary, and the roughness of the plastic pipe substrate is 0.03 mm, and using Equation 4.4 developed by Echaves, making a the subject of the formula:

$$a = \frac{K\,(t)\; - K\,(0)}{t}$$

As stated in section 4.3.1, the *K* values for pipe roughnesses are not the actual hydraulic roughnesses but the average roughnesses of the pipe coupons.

Again, it should be noted that the values for *a* are similar due to the water conditions in each beaker being similar (i.e. all have disinfectant residuals that are not detectable).

Van Vuuren & van Dijk (2012) took measurements of biofilm thicknesses in a 12 year old pipeline and found thicknesses of 2mm-4 mm. Using pressure and flow tests, and using the hydraulic gradient, they found that the roughness had increased from a maximum design roughness of 0.5 mm to 1.76 mm. This corresponds with a=0.128 mm/year (where a is defined by Equation 4.4 in section 4.4). The nature of the conditions in the pipeline tested by van Vuuren & van Dijk (2012) and to the lab experiments carried out by this research varies greatly due to:

- The test waters were placed in beakers on a table, unlike the pipeline where there was shear stresses due to the water velocity in the pipeline.
- The water conditions were different-water used for the lab experiment in this research was prepared from distilled water inoculated with pond water and addition of disinfectant. The pipeline tested by van vuuren & van Dijk (2012) conveyed raw water
- The lab experiments were carried out over a 60 day period whereas the roughness analysis carried out by van Vuuren & van Dijk (2012) was on a 12 year old pipeline.
- van Vuuren & van Dijk (2012) calculated the pipe roughness from the hydraulic gradient from pressure and flow tests, whilst this research made an assumption for pipe roughness from the dimensions of the biofilms present.
- van Vuuren & van Dijk (2012) found the increase in roughness due to biofilm growth for a steel pipeline whilst for the lab experiments for this research, plastic coupons were used. The attachment and cohesion of biofilms depend on the substrate material (Kerr, et al., 2003).

4.1.5. Error and accuracy

During each phase of the experiments, there were 2 beakers each of chlorine, monochloramine and distilled water. Tap water was only run during the first phase. Hence, for coupons placed in chlorine, chloramines and distilled water, there were biofilm cover and disinfectant decay results obtained from 4 beakers, whilst coupons placed in tap water had results obtained from 2 test beakers. The trends observed for biofilm growth were similar for all 4 test beakers, hence the experiments carried out for this research showed consistency, reliability, repeatability and dependability. The error bars on all the graphs in this chapter indicate that there was not much variability for all results obtained. The standard error and standard deviation are tabulated in Appendix A.

Around 6 microscopic images over 2 coupons were analyzed to find the average biofilm cover per coupon. A series of images were re- analyzed a few times to determine the margin of error on the biofilm cover. It was found that the ImageJ results provided from the analysis was, in most instances accurate to 2 decimal points.

For each batch that the average value of biofilm was determined, the standard error of the mean was calculated. The standard error is represented mathematically as:

$$SEM = \frac{s}{\sqrt{n}}$$

where s=standard deviation and n=sample size.

Six microscope images were taken per time period, per water quality. However, there were instances where for a given time and for a given water quality, there were only 5 images. This was due to the fact that there a single image was captured twice from the SEM microscope.

4.2. Experiment B

4.2.1. Quantitative Analysis

This section interprets and discusses the results obtained from the UV spectrophotometer and the SEM image analyses for Experiment B.

4.2.1.1. Nutrient concentrations at start of experiment

At day 0, there was a relatively high presence of ammonia. Also present were nitrites and really low levels of nitrate. The main source of ammonia in ponds is waste from dead and living organisms (Šraj, et al., 2014). The presence of nitrates and nitrates was due to bacteria already present in the inoculated pond water that converted ammonia into nitrates and nitrites. These will be discussed further on in the nitrogen cycle. In water distribution networks, bacteria enter the pipe network through tanks and reservoirs, incomplete disinfection, cracks, and backflows (Mains, 2008; Momba, et al., 2000).There was no total organic carbon present in the test water at day 0. Although the test water was innoculated with pond water.

4.2.1.2. Total Organic Carbon (TOC) present in test water

For both the samples, the TOC levels were below detection limits during the entire experimental run. The detection limit of the machine is 4 µg/L (Analytic Jena, 2013). There would also have been TOC present from the organic matter that entered the test water during inoculation with pond water. Although TOC is the controlling nutrient for biofilm growth (Kerr, et al., 2003), this experiment was unable to find the relationship between carbon and biofilm growth due to the TOC concentrations being below detection limits.

It is speculated that the null results returned by the laboratory technicians for the TOC analysis is due to inappropriate calibration. TOC analysis using tap water also returned null results when data showed that treated water leaving 11 of the uMgeni Water treatment Works had TOC concentrations ranging from 3.24 mg/L to 5.65 mg/L (uMgeni Water, 2013). The total bound nitrogen (TN_b) results from this machine also confirm that this machine returned incorrect results. The sum of ammonia, nitrates and nitrates when measured on the spectrophotometer was significantly higher than The TN_b results obtained from the Analytic Jena Analyzer. Hence for nitrogen analysis, the results from the Analytic Jena were not used. Alternative instrumentation was not available.

A survey carried out by the US EPA for drinking water over 80 locations found that TOC concentrations ranged from 0.05 mg/L to 12.2 mg/L, with median concentration of 1.5 mg/L (Symons, 1977). The TOC limit for drinking water set by DWAF is 10 mg/L (Department of Water Affairs and Forestry, 2011). LeChevallier, et al (1991) found that the most coliform bacteria present were associated with TOC concentrations greater than 2.4 mg/L. Zacheus and Martikainen (1995) found no correlation between the numbers of microorganisms and the amount of TOC present. However, according to Camper (2010) , biofilms can grow at very low concentrations of organics, so preventing such growth may not be feasible. According to Camper (2004) , TOC may not be an effective indicator of biological activity as portions of the TOC may be obstinate to microbial use.

4.2.1.3. The nitrogen cycle

The results from the monitoring of the inorganic species of nitrogen (nitrites, nitrates and ammonia) showed that there was a nitrogen cycle and that the inoculation of pond water brought in species ammonia oxidizing bacteria (AOB) and denitrifying bacteria. The nitrogen cycle and biofilm growth is shown on Figure 4.16:

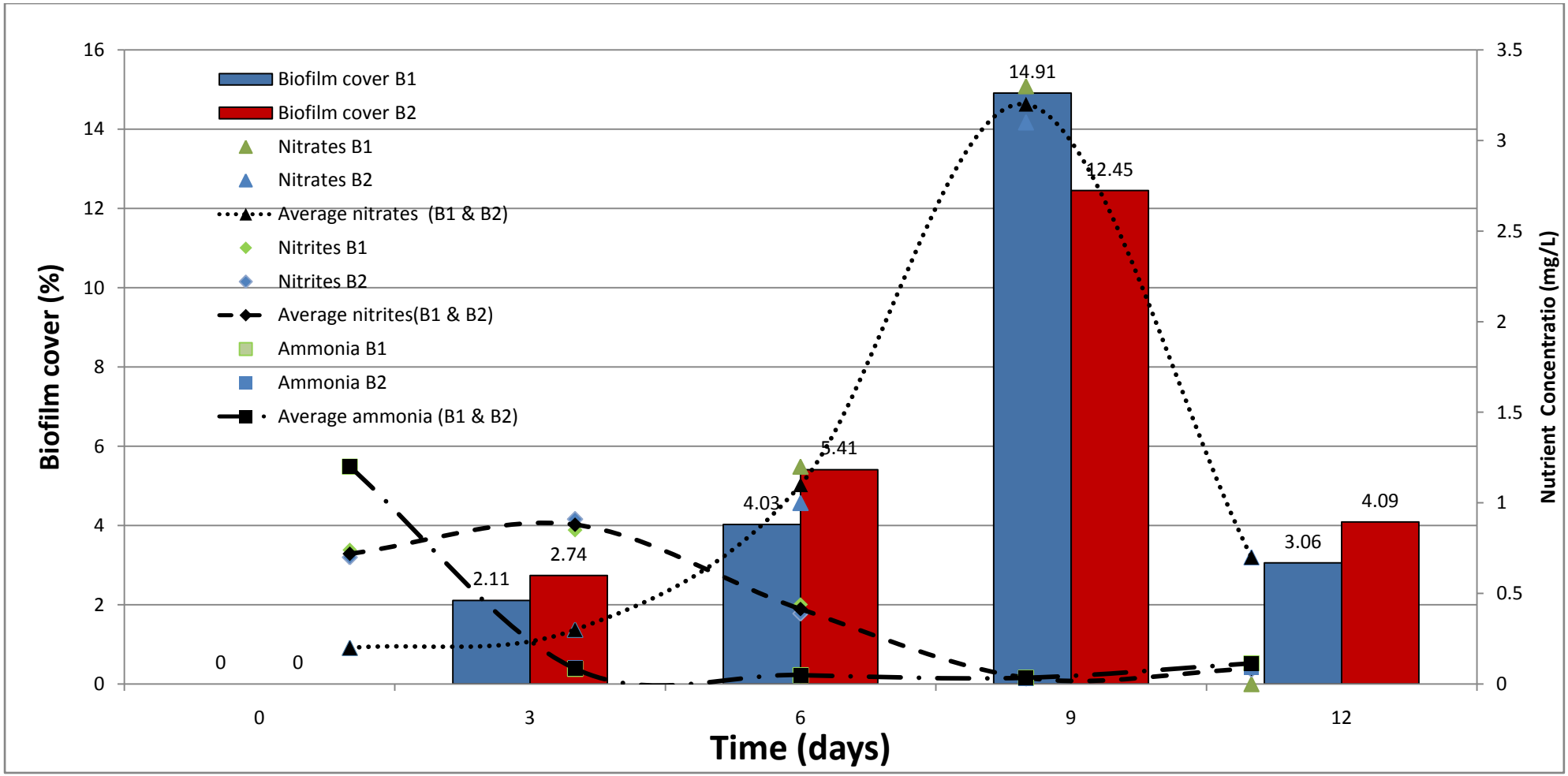


Figure 4.16: Biofilm growth and presence of inorganic nitrogen in Beaker 1 (B2) and Beaker 2 (B2) for the 12 day lab tests. The green markers indicate Beaker 1 (B1) and the blue markers indicate Beaker 2 (B2). The dashed black lines are the average of beaker 1 and beaker 2.

The numerical values for Figure 4.16 are shown in Appendix C.

In the pond water, nitrifying bacteria were present. The nitrifying bacteria convert ammonium nitrogen into nitrate nitrogen. This conversion takes place via a 2 stage process:

Ammonia to nitrite conversion

As shown in Figure 4.16, as the ammonia concentration decreases, the nitrite concentration increases. This is due to the *Nitrosomonas* bacteria oxidizing the ammonia into nitrite according to equation 4.5:

$2\ NH_4^+ + 3\ O_2 \rightarrow 2\ NO_2^- + 2\ H_2O + 4\ H^+$ Equation 4.5

(Grguric, et al., 1999)

Nitrate to nitrate conversion

The *Nitrobacter* bacteria further oxidize the nitrites into nitrates (Grguric, et al., 1999).

$NO_2^- + H_2O \rightarrow NO_3^- + 2H+ + 2e^-$ Equation 4.6

(Grguric, et al., 1999)

Besides the Nitrobacter bacteria, Nitrospina, Nitrococcus, and Nitrospira genus are common bacteria that oxidize nitrites into nitrate (Wolfe, et al., 1988).

Although the total nitrogen gas was not measured, it is known that the decrease of nitrates after day 9 takes place due to denitrifying bacteria reducing nitrates into nitrogen gas (Jördening & Winter, 2005; Shrimali & Singh, 2001). Equation 4.7 shows this reaction.

$NO_3^- + NO_2^- \rightarrow NO + N_2O + 2e^- + N_2(g)$ Equation 4.7

(Jördening & Winter, 2005)

Denitrifying bacteria reduce nitrates (and nitrites) into nitrogen-containing gasses (Shrimali & Singh, 2001). Denitrifying bacteria include the
Thiobacillus denitrificans, Micrococcus denitrificans, Paracoccusdenitrificans and Pseudomo nas species amongst others (Csuros & Csuros, 1999).

The nitrogen cycle in a closed system, with the conversion of ammonia into nitrites and ultimately nitrates is consistent with past published research. Fazio& Jannelli (2006) carried out 5 tests in an aquarium model in order to numerically model the nitrogen cycle. Their

results found a similar trend to the one presented here. Many other researchers have found the increase of nitrates over time (Adey & Loveland, 1998; Grguric & Coston, 1998; Spotte, 1979). Strottman and Windecker (1997) also found that ammonia present leads to formation of nitrate.

4.2.1.4. Nitrogen and biofilm growth

Ammonia and biofilm growth

Most of the ammonia was oxidized into nitrites. Hence it was not possible to compare the effect of ammonia with biofilms. However, research shows that biofilm ammonia can promote the growth of biofilms in drinking water systems (US Environmental Protection Agency, 1994). Rittmann and Soeyink (1984) found that ammonia concentrations present in ground water supplies were usually high enough to allow for growth and survival of bacteria. Batch tests carried out by Chu et al. (2005) from a drinking water system in an urban area showed that bacterial growth was stimulated by addition of nitrate, while the addition of nitrates did not stimulate bacterial growth in suburban areas.

Experiment A showed that monochloramine is a more stable disinfectant than chlorine. However, when chloramine decomposes, ammonia is a by-product (Dvorak & Skipton, 2013). As chloromine acts as a source of ammonia, the ammonia will ultimately be converted to nitrates that will promote biofilm growth, thus limiting its effectiveness as a disinfectant (Pressman, et al., 2012).

Nitrates, nitrites and biofilm growth

There is a correlation between nitrates and biofilm growth. Figure 4.16 shows that nitrates support and promote biofilm growth. As the nitrates increase, the biofilm percentage cover on the substrates also increases. The peak biofilm cover and the nitrates both peaked on day 9. There was a decrease in the nitrates and the biofilm cover after day 9. Other researchers have also confirmed this relationship between bacteria and nitrate (Chu, et al., 2005; Freitag, et al., 1987).

Shaharuddin & Abdul-Talib (2005) grew biofilms in municipal water and then tested the nitrate and nitrite utilization rate due to biofilms. They concluded that the utilization occurs in 2 distinct phases. In stage 1, nitrate is utilized with the accumulation of nitrite. In stage 2, nitrite is utilized after the depletion of nitrate. They also concluded that nitrite levels peak approximately at the time that nitrate is close to depletion. Nitrates, nitrites and biofilms are sensitive to changes in temperature, pH and other environmental conditions (Kerr, et al.,

2003). This could explain the variation between this research and other published research. Due to the instrument limitation, organic nitrogen was not measured. This research shows that the presence of inorganic nitrogen (nitrates, nitrites and ammonia) affects biofilm formation. Lab studies indicate that bacteria and phytoplankton both use organic and inorganic nitrogen (Brown 1980; Wheeler et al. 1974). Zweifel et al. (1993) carried out experiment using seawater cultures and monitored nutrient concentrations and bacterial growth. Their results suggest that over a period of days, bacteria preferentially used inorganic nitrogen and phosphorous over organic nitrogen to support growth.

The biofilms on the substrate and the nutrients present are co-dependent. Higher levels of nutrients stimulate more biofilm growth whilst greater biofilms and bacteria present release more ammonia through respiration and excretion (Conovera & Corner, 1968) and will ultimately act as a ‗food source' for bacteria and biofilms. At the peak on day 9, there was the large percentage of biofilm cover meant that the nutrients present were unable to provide ‗food' for all the microorganisms presents and that a very large portion of the nitrates present had been depleted by the large population of biofilms. This lead to the dying off or ‗crash' of biofilms due to there being insufficient nutrients. Dead biofilms lose their adhesive properties and are detached from the coupon substrate (Winstanley, et al., 2010). Decomposing dead organisms release ammonia to the water which will then be oxidized into nitrites and nitrates (Boyde, et al., 1998). Although not clear on Figure 4.16, there is a slight increase in the ammonia from day 9 to day 12, proving that the dead organic matter is releasing ammonia. The numerical values for the nitrogen levels and biofilms are shown in Appendix C.

4.2.1.5. Biofilm and phosphorous

Many researchers have demonstrated the importance of phosphorous for bacterial growth (Jiang, et al., 2011; Lehtola, et al., 2001; Smith & Prairie, 2004). Figure 4.17 below shows the relationships between biofilm growth and phosphate concentrations. As shown in ure 4.17, the phosphate concentrations are really low and are almost constant. With such low concentrations of phosphate in the test waters, a relationship between biofilms and phosphate concentrations and whether or not phosphorous is a limiting nutrient could not be established. However, it can be seen that as the biofilm covers increased between day 6 and day 9, the phosphorous levels decreased, showing that biofilms use phosphorous as a ‗food' source. Phosphorous being used as a ‗food' source for nutrients has been observed by other researchers as well (Boelee, et al., 2011; Gomes, et al., 2014; Lock, et al., 1990).

Miettinen, et al.(1997) carried out tests in a lab where different levels of nutrients were added to test waters. They found that addition of phosphorous increased the microbial growth in drinking water samples. Smith & Prairie (2004) inoculated lab samples at different phosphorous levels and assimiable organic carbon (AOC) levels to investigate their effects on bacterial growth. Their results showed that phosphorous promotes bacterial growth. At phosphorous concentrations below 0.7 µg/L, the bacteria found it difficult to obtain phosphorous and this lead to a very slow rate of biofilm growth. The approximate ratio of carbon:nitrogen:phosphorous for optimum growth of heterotrophic bacteria is 100:10:1, so after carbon and nitrogen, phosphorous is the most important nutrient for growth (LeChevallier, et al., 1991).

4.2.1.6. Ratios of nutrients present

Optimum growth conditions for biofilms occur when the 3 main nutrients occur in the C:N:P ratio is 100:10:1 (US Environmental Protection Agency, 1994; Van der Kooij, et al., 1982; Zhang & DiGiano, 2002). This shows that carbon is the controlling nutrient, followed by nitrogen and then phosphorous. Since the TOC readings returned null readings, the C:N:P ratio in the test waters could not be determined. However, the N:P ratio was found and is shown in Appendix C. The N:P ratios ranged from 16.9:1 to 67.1:1, showing that the concentration of inorganic nitrogen was far greater than the concentration of phosphorous, hence nitrogen had a greater influence on biofilms than phosphorous. Due to the large variation in the total inorganic nitrogen concentrations and the phosphorous concentrations having relatively less variation, the ‗pattern' or ‗trend' of the total inorganic nitrogen over the 12 day period is very similar to the ‗pattern' or ‗trend' of the N:P ratio over the 12 day period.

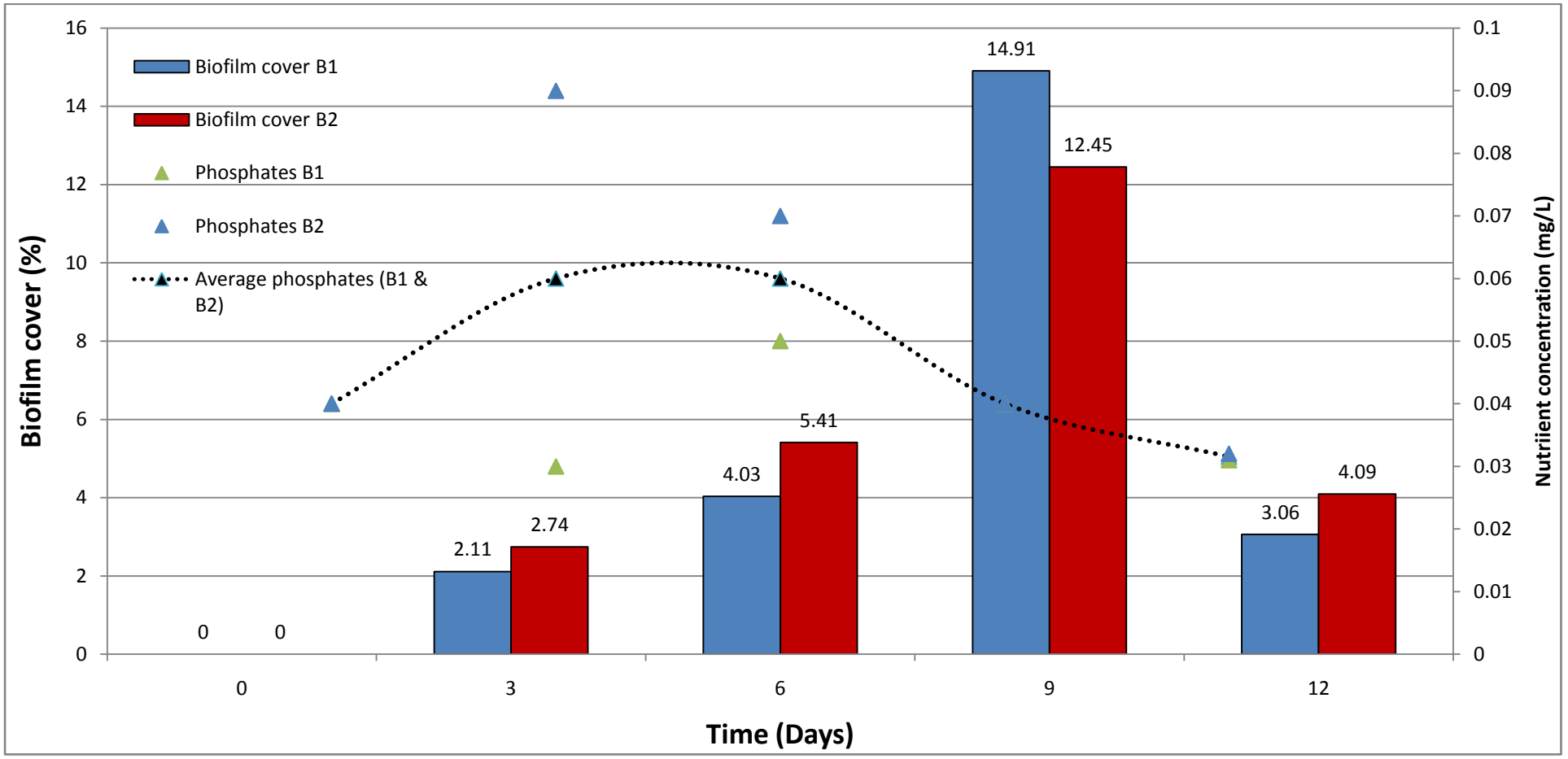


Figure 4.17: Biofilm growth and phosphorous in Beaker 1 (B2) and Beaker 2 (B2) for the 12 day lab tests. The green markers indicate Beaker 1 (B1) and the blue markers indicate Beaker 2 (B2). The dashed black lines are the average of beaker 1 and beaker 2.

The numerical values for Figure 4.17 are shown in Appendix C.

4.2.2. Qualitative analysis

As for Experiment A, only a visual analysis of the shape, structure and size of the biofilms were done. Identification and classification using biological methods (such as DNA extraction etc.) was not performed as this is outside the scope of this project.

The ‗jelly-bean' cell and the EPS matrix were the most common observed biofilm structures on the substrate as seen on the SEM images. These were the same as the biofilms observed from Experiment A. Figure 4.18 shows a typical ‗jelly-bean' cell and typical EPS matrix seen under SEM microscopy for Experiment B, which indicates the consistence with Experiment A. Also visible, are rod-shaped cells embedded in the EPS matrix.

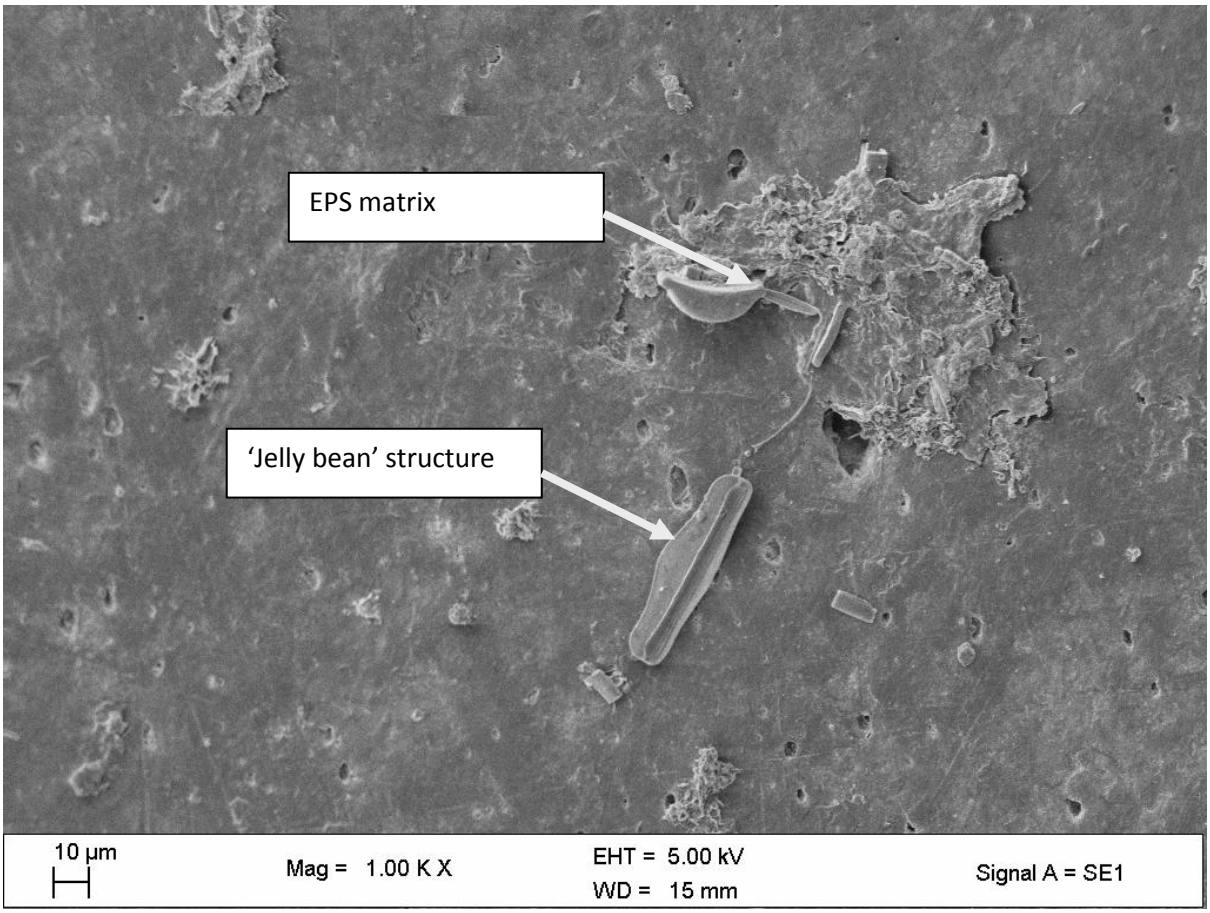


Figure 4.18: 'Jelly-bean' shaped biofilm and EPS matrix on plastic coupon used in Experiment B at 500X magnification.

4.2.3. Water quality of test waters

The concentrations of the nutrients tested during the 12 days of the experimental run was compared to the maximum nutrient limits set by DWAF, EPA and WHO to determine whether the test water could be classified as drinking water, even though they were inoculated with pond water. Table 4.3 shows the nutrients tested and the respective limits set by the EPA, DWAF and WHO. This allowed for comparison with local and international drinking water standards.

Table 4.3: Maximum observed results from drinking water tests carried out regularly during Experiment B.

Parameter (max) (Expressed as nitrogen)	**Beaker 1**	**Beaker 2**	**WHO limits***	**EPA limits***	**DWAF limits***
Nitrates (mg/L)	3.1	3.1	11	10	11
Nitrites (mg/L)	0.85	0.91	1.1	1	0.9
Nitrates & nitrites (mg/L)	3.34	3.13	-	10	10
Ammonia (mg/L)	1.2	1.2	1.5	-	1.5

Table 4.3 shows that the drinking water tests carried out for all test waters that were inoculated with pond water for beakers 1 and 2 of the experimental run were within the water quality limits set by the US EPA (2013), WHO (2011) and DWAF (2011). The test waters can be deemed fit to be used for human consumption as the test results all fell within local (DWAF) and international (US, EPA and WHO) drinking water regulations.

4.2.4. Error and Accuracy

Since the analyses for water quality and biofilm quantification are same for Experiment A and Experiment B, the error and accuracy in the results will be the same for both set of experiments. Section 4.1.5 which shows the level of accuracy and error for Experiment A will also apply for Experiment B.

The tests for Experiment B were run in duplicate. The closeness of results for biofilm cover on substrate and for biofilm cover indicates that these experiments are consistent and repeatable.

4.2.5. Comparison between Experiment A and Experiment B

The biofilm growth and collapse pattern observed during Experiment A and Experiment B is very similar. Both experiments had biofilm cover on substrates increasing until it peaked, thereafter collapsing. With Experiment A, the disinfectant did not have much effect on the growth of biofilms as the disinfectants were allowed to decay. This decay meant that the very low levels of disinfectants were ineffective, as the growth observed for coupons place in chlorine and chloramines test waters were very similar to the coupons placed in distilled water. Constant flow through pipelines in a real water distribution network means that there will always be a reasonably high dosage of disinfectant present.

Although nutrients concentrations were not monitored in Experiment A, Experiment B proves that the collapse of nutrients in Experiment B was due to the decline in nutrients present in the test water. The decrease occurred after the biofilms had peaked. With increased biofilms present, the nutrients were consumed by the bacteria present much quicker. This lead to a decline in the nutrients present which ultimately resulted in the death of a substantial amount of micro-organisms present. The similar pattern and trend observed for biofilm cover shows that the experiment consistent and is repeatable.

4.3. Chapter Summary

Due to the relatively quick decay of chlorine and chloramines disinfectants for Experiment A, a comprehensive comparison between both these disinfectants and their influence on biofilm cover on coupons could not be made. However, Experiment A shows that chloramines is a more stable disinfectant and has a slower rate of decay. This experiment demonstrates the importance of regularly dosing standing water (for example: tanks and reservoirs) with disinfectant.

Experiment B looked at TOC, the inorganic species of nitrogen and phosphorous and compared them to biofilm present. Unfortunately, the TOC results were not useful enough to be used for analyses. The results showed that ammonia present ultimately oxidizes into nitrates and nitrates that promote biofilm growth. The phosphorous levels were too low to have an impact on biofilms.

Experiment A looked at the relationship between biofilms and disinfectants and Experiment B looked at the relationship between nutrients and biofilms. These experiments were independent of each other. Hence a connection between disinfectant decay and nutrients present could not be established.

5. Conclusions

This chapter is the summary of the laboratory investigation and findings and provides further recommendations on conducting future research in this field.

5.1. Conclusions

- The aim of this research was to find the relationship between biofilm and disinfectant (chlorine and monochloramine), biofilm and pipe material (plastic pipes and mortar pipes) and biofilm and nutrients (carbon, nitrogen and phosphorous).

- Over the 60-day period during which the testing was carried out, the influence of the disinfectant on the biofilm growth could not be determined as the disinfectant residuals were not maintained and were allowed to decay.

- Pipe materials do not have an effect on the disinfectant residuals. The UV spectrophotometer results show that the pipe materials and disinfectant residuals are independent of each other. The similar decay using plastic coupons and mortar coupons is also due to there being a very small surface area of each pipe material being exposed to the test water.

- It was not possible to compare the difference in biofilm growth between plastic coupons and mortar coupons as mortar coupons became totally covered in a crystal structure and no biofilm could be observed under SEM on the mortar substrate.

- Qualitative analysis showed that there is microbial activity in tap water which forms primary colonizers in pipelines. The primary colonizers found in tap water were not found in pond water or distilled water from the lab.

- The tests were run over 60 days, which wasn‘t sufficient to allow enough biofilm growth to have a significant effect on the hydraulic transmissivity.

- The reduction in effective diameter due to biofilm growth is proportional to the diameter of the pipe. For a given thickness of biofilm, the reduction of diameter will be proportionally higher in a small diameter pipeline when compared to a large diameter

pipeline. However, the changes in head loss and/or flow in a pipeline occur mainly due to the increase in surface roughness that is caused by the presence of biofilms.

- There is a strong relationship between inorganic nitrogen and biofilms. The nitrogen cycle that takes place in the test waters has an effect on the growth of biofilms.

- No relationship was found between TOC and biofilms as the TOC values were below detection limits. The phosphorous concentrations are much lower than the nitrogen concentrations, and at such low concentrations, it is not evident if phosphorous is a limiting nutrient or not.

5.2. Recommendations for further research

- Disinfectant residuals should be constant for each disinfectant by changing the water regularly or adding in disinfectant to see how maintaining constant residuals (at different concentrations) would have on biofilms. In a pipe network, water is being continuously pumped from the water treatment works; a constant residual of disinfectant is maintained. Analyzing the biofilm growth over a range of disinfectant residuals will also enable the researchers to determine the optimum dosages for disinfection.

- The experiment should be carried out with pipe coupons placed in a flow channel, and biofilm analyses should be carried out at different velocities to see the effect of shear stresses on biofilm formation.

- For Experiment A, Over the 60 day period, more SEM images should have been taken to ensure more accurate results. About 2-3 sets of images should have been taken per week. This will allow for better trends to be established.

- For the sake of uniformity, in Experiment A, all tests should be over the same time period, rather than having experiments in phase 1 over a 40 day period and experiments in phase 2 over a 60 day period.

- Biofilms should be identified through methods that are currently being used in biology/microbiology such as DNA extraction and analysis to see if there are different strains that grow under different disinfectant conditions.

- Other physic-chemical factors that contribute to biofilm growth such as biological oxygen on demand, chemical oxygen on demand, etc. should be investigated as well.

- Taste and odour tests should be determined to see the effect that the different disinfectants has on downstream consumers.

- Together with SEM, atomic force microscopy (AFM) should be used for biofilm analyses. AFM provides a roughness profile by running a diamond probe over the substrate. Roughness influences the head loss in pipelines and a roughness profile will be able to provide thicknesses of biofilms, especially when biofilms are ‗stacked' on top of one another.

- A more sterile environment should be used to prevent contamination. The lab which the tests is conducted in should be free from any other experiments having microbial activity (although great care was taken to prevent any biological interference).

- Inoculation for the water samples should be done using known cultures to have as much uniformity as possible in the test waters. The biological activity in pond water is dynamic, and varies according to temperature, UV light, depth at which water is taken, season amongst other factors.

- The testing should combine disinfectant decay and nutrient concentrations. They should not be run independent of one another. The results of this will be able to relate disinfectant decay to nutrient availability.

- Organic nitrogen should also be monitored during the course of the experiment to relate the biofilm growth to the total nitrogen present and to the Total Kjehdahl nitrogen (TKN).

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Appendix A

Calculation of standard errors for construction of error bars (Experiment A)

Table A1: combined average and standard error computation of biofilm cover from 4 beakers of chlorine disinfected water

	Percentage Biofilm Cover on coupons						
Day	Beaker 1	Beaker 2	Beaker 3	Beaker 4	ave	std dev	std error
0	0	0	0	0	0	0	0
1	0.17	0.43	0.44	0.56	0.4	0.17	0.083
4	3	2.5	4	2.7	3	0.64	0.32
7	3.9	2.2	5.3	4.8	4.1	1.4	0.68
10	-	-	8.2	7.9	8.1	0.22	0.15
14	32	28	13	12	21	0.52	0.37
21	1.6	1.8	1.5	0.72	1.4	0.47	0.23
28	4	4.2	5.4	2.9	4.1	1	0.51
40	1.7	1.4	2.5	2.8	2.1	0.67	0.34
50	-	-	6.2	6.3	6.2	0.073	0.052
60	-	-	8.5	7.6	8.1	0.63	0.45

Table A2: combined average and standard error computation of biofilm cover from 4 beakers of chloramine disinfected water

	Percentage Biofilm Cover on coupons						
Day	Beaker 1	Beaker 2	Beaker 3	Beaker 4	ave	std dev	std error
0	0	0	0	0	0	0	0
1	0.83	1.9	0.68	0.36	0.95	0.68	0.34
4	3.1	4.7	7	3.2	4.5	1.8	0.91
7	2.7	4.7	9.2	5.5	5.5	2.7	1.4
10	-	-	8	6.2	7.1	1.3	0.93
14	1.7	1.3	2.5	2.8	2.1	0.73	0.36
21	1.4	1.3	2	2.6	1.8	0.61	0.31
28	1.4	4	3.7	4.6	3.4	1.4	0.69
40	0.92	1.1	1.8	5.7	2.4	2.2	1.1
50	-	-	7.6	7.6	7.6	0.038	0.027
60	-	-	8.2	6.7	7.4	1.1	0.75

Table A3: combined average and standard error computation of biofilm cover from 4 beakers of distilled water

	Percentage Biofilm Cover on coupons						
Day	Beaker 1	Beaker 2	Beaker 3	Beaker 4	ave	std dev	std error
0	0	0	0	0	0	0	0
1	1.8	1.4	1.5	1.1	1.5	0.3	0.15
4	0.34	0.92	2.1	2.6	1.5	1	0.52
7	4.2	60	4.6	3.1	3.7	0.87	0.43
10	-	-	6.4	5.3	5.8	0.79	0.56
14	8.6	8.2	7.4	6.8	7.8	0.81	0.4
21	0.77	2.9	2.1	2.4	2	0.91	0.45
28	6.2	7.1	5.3	4.3	5.7	1.2	0.6
40	3.1	5.1	4.7	5.1	4.5	0.96	0.48
50	-	-	6.1	6.1	6.1	0.031	0.022
60	-	-	8	7.2	7.6	0.57	0.4

Table A4: combined average and standard error computation of biofilm cover from 2 beakers of tap water

	Percentage Biofilm Cover on coupons				
Day	Beaker 1	Beaker 2	ave	std dev	std error
0	0	0	0	0	0
1	1.6	2.0	1.8	0.26	0.18
4	4.4	3.7	4.1	0.48	0.34
7	7.5	6.6	7.1	0.67	0.47
10	10	9.9	10	0.61	0.42
14	4.6	4.8	4.7	0.13	0.091
21	6.3	5.4	5.9	0.61	0.43
28	5.9	6.1	7.5	0.93	0.66
40	8.6	7.9	8.3	0.93	0.26

Appendix B

Computation of forecasting of biofilm cover on coupons and calculation of prediction intervals (Experiment A)

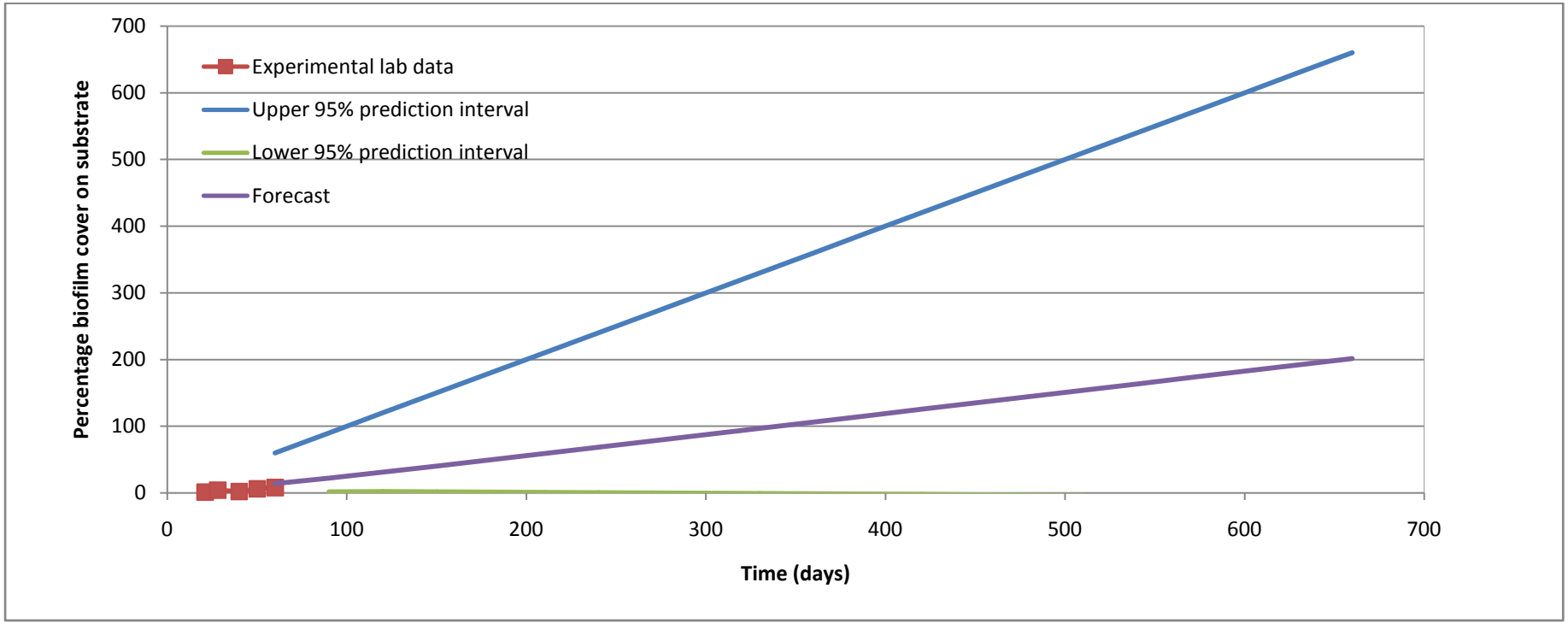

Figure A1: Forecast of biofilm cover on coupons placed in chlorine disinfected water until a 100% biofilm cover is achieved. The solid squares indicate data obtained from image analysis (red) and forecasted values (purple).

Table A1: Regression statistics for percentage biofilm cover of coupons placed in chlorine disinfected water

Multiple R	0.86
R Square	0.73
Adjusted R Square	0.64
Standard Error	1.1
Observations	5

Table A2: Predicted biofilm cover on coupons placed in chlorinated water, showing the standard error and prediction intervals

Time (Days)	60	90	120	150	180	210	240	270	300	330	360
Predicted Biofilm cover (%)	7.40	12.00	16.00	21.00	25.00	30.00	34.00	39.00	44.00	48.00	53.00
Standard Error	2.10	3.20	4.60	6.10	7.60	9.10	11.00	12.00	14.00	15.00	17.00
Lower 95% prediction interval	0.70	1.70	1.80	1.70	1.30	0.96	0.54	0.11	0	0	0
upper 95% prediction interval	14.00	22.00	31.00	40.00	50.00	59.00	68.00	78.00	87.00	97.00	110.00

Table A2 (continued)

Time(Days) (Cont)	390	420	450	480	510	540	570	600	630	660
Predicted Biofilm cover (%)	57.00	62.00	66.00	71.00	75.00	80.00	84.00	89.00	93.00	98.00
Standard Error	18.00	20.00	22.00	23.00	25.00	26.00	28.00	29.00	31.00	33.00
Lower 95% prediction interval	0	0	0	0	0	0	0	0	0	0
upper 95% prediction interval	120.00	130.00	130.00	140.00	150.00	160.00	170.00	180.00	190.00	200.00

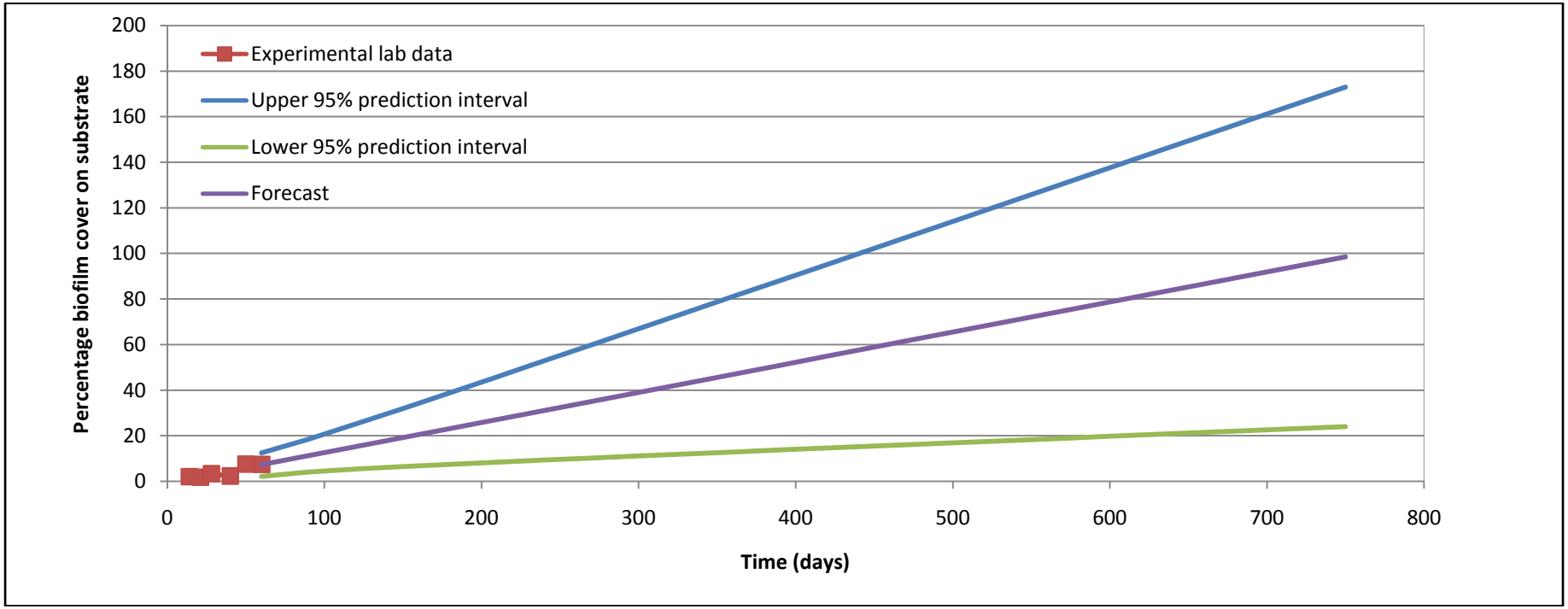

Figure A2: Forecast of biofilm cover on coupons placed in chloramine disinfected water until a 100% biofilm cover is achieved. The solid squares indicate data obtained from image analysis (red) and forecasted values (purple).

Table A3: Regression statistics for percentage biofilm cover of coupons placed in chloramine disinfected water

Multiple R	0.86
R Square	0.75
Adjusted R Square	0.69
Standard Error	1.48
Observations	6

Table A4: Predicted biofilm cover on coupons placed in chloramines disinfected water, showing the standard error and prediction intervals

Time(Days)	60	90	120	150	180	210	240	270	300	330	360	390
Predicted Biofilm cover (%)	7.40	11.00	15.00	19.00	23.00	27.00	31.00	35.00	39.00	43.00	47.00	51.00
Standard Error	1.80	2.60	3.50	4.60	5.60	6.70	7.80	8.90	10.00	11.00	12.00	13.00
Lower 95% prediction interval	2.20	4.10	5.40	6.50	7.50	8.50	9.40	10.00	11.00	12.00	13.00	14.00
upper 95% prediction interval	12.00	19.00	25.00	32.00	39.00	46.00	53.00	60.00	67.00	74.00	81.00	88.00

Table A4 (continued)

Time(Days) (Cont)	420	450	480	510	540	570	600	630	660	690	720
Predicted Biofilm cover (%)	55.00	59.00	63.00	67.00	71.00	75.00	79.00	83.00	87.00	91.00	95.00
Standard Error	15.00	16.00	17.00	18.00	19.00	20.00	21.00	22.00	23.00	25.00	26.00
Lower 95% prediction interval	15.00	16.00	16.00	17.00	18.00	19.00	20.00	21.00	21.00	22.00	23.00
upper 95% prediction interval	95.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00

Table A4 (continued)

Time(Days)	750
Predicted Biofilm cover (%)	99.00
Standard Error	27.00
Lower 95% prediction interval	24.00
upper 95% prediction interval	100.00

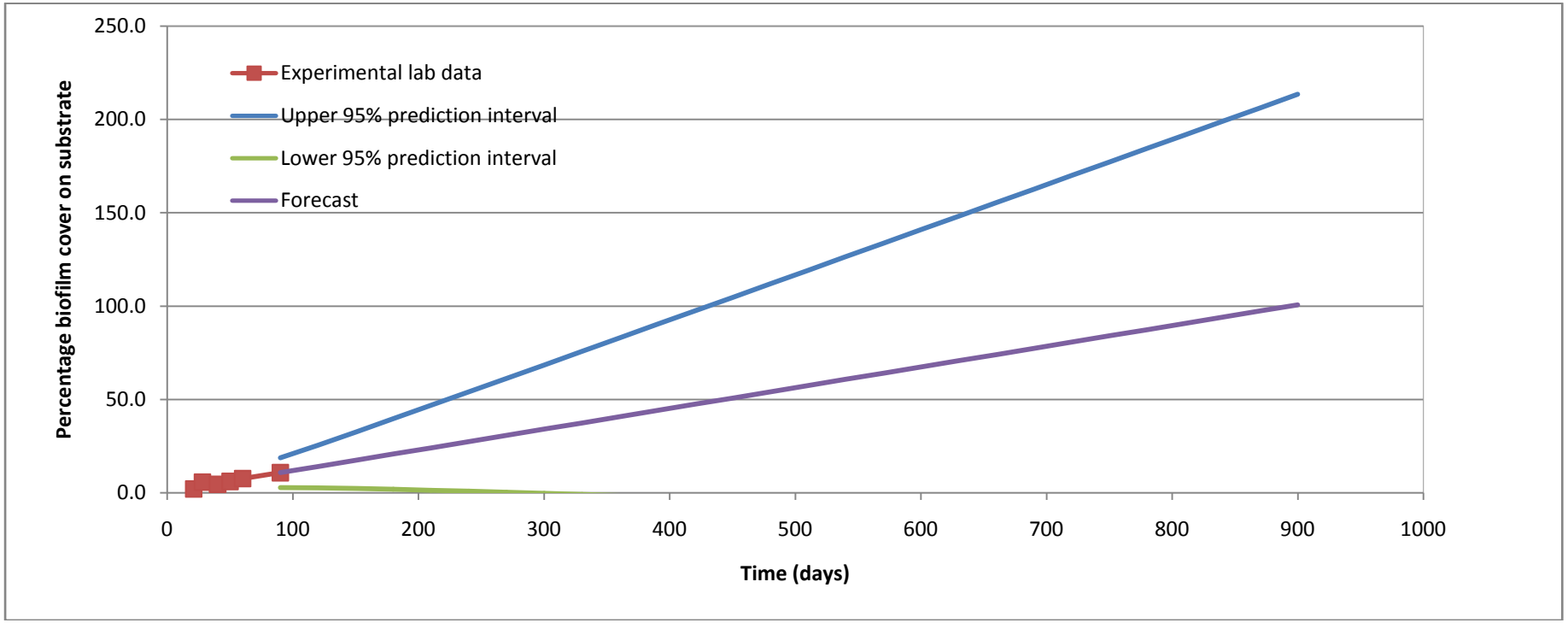

Figure A3: Forecast of biofilm cover on coupons placed in distilled water until a 100% biofilm cover is achieved. The solid squares indicate data obtained from image analysis (red) and forecasted values (purple).

Table A5: Regression statistics for percentage biofilm cover of coupons placed in distilled water

Multiple R	0.84
R Square	0.7
Adjusted R Square	0.61
Standard Error	1.3048475
Observations	5

Table A6: Predicted biofilm cover on coupons placed in distilled water, showing the standard error and prediction intervals

Time(Days)	90	120	150	180	210	240	270	300	330	360	390	420
Predicted Biofilm cover (%)	11.00	14.00	17.00	21.00	24.00	27.00	31.00	34.00	37.00	41.00	44.00	47.00
Standard Error	2.50	3.60	4.80	5.90	7.20	8.40	9.60	11.00	12.00	13.00	14.00	16.00
Lower 95% prediction interval	2.80	2.70	2.30	1.80	1.30	0.81	0.26	0	0	0	0	0
upper 95% prediction interval	19.00	26.00	33.00	40.00	47.00	54.00	61.00	68.00	76.00	83.00	90.00	97.00

Table A6 (continued)

Time(Days) (Cont)	450	480	510	540	570	600	630	660	690	720	750	780
Predicted Biofilm cover (%)	51.00	54.00	57.00	61.00	64.00	67.00	71.00	74.00	77.00	81.00	84.00	87.00
Standard Error	17.00	18.00	19.00	21.00	22.00	23.00	24.00	26.00	27.00	28.00	29.00	31.00
Lower 95% prediction interval	0	0	0	0	0	0	0	0	0	0	0	0
upper 95% prediction interval	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00

Table A6 (continued)

Time(Days) (Cont)	810	840	870	900	810	840	870	900	810	840	870	900
Predicted Biofilm cover (%)	91.00	94.00	97.00	100.00	91.00	94.00	97.00	100.00	91.00	94.00	97.00	100.00
Standard Error	32.00	33.00	34.00	35.00	32.00	33.00	34.00	35.00	32.00	33.00	34.00	35.00
Lower 95% prediction interval	0	0	0	0	0	0	0	0	0	0	0	0
upper 95% prediction interval	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00

The following equation was used for calculating the prediction interval:

$$IntervalEstimate = \hat{Y}_i \pm t_{n-2} \bullet S_{yx} \sqrt{1 + \frac{1}{n} + \frac{(X_i - \overline{X})^2}{\sum_{i=1}^{n} (X_i - \overline{X})^2}}$$

Where

S_{yx}=standard error of the estimate

t is the critical value from the t distribution critical values table.

with degrees of freedom=n-2, and 0.95 confidence, t=5-2=3, and the critical value chosen for t=3.182

Appendix C

Nutrient concentrations for test waters (Experiment B)

Table A7: Nutrient concentrations and N:P ratios in beaker 1 and beaker 2 in test waters used during Experiment B. B1 represents beaker 1 and B2 represents beaker 2

	TOC (mg/L)			Nitrates (mg/L)			Nitrites (mg/L)			Ammonia (mg/L)			Phosphorous (mg/L)			N:P ratio		
Day	B1	B2	Ave	B1	B2	Ave	B1	B2	Ave	B1	B2	Ave	B1	B2	Ave	B1	B2	Ave
0	0	0	0	0.2	0.2	0.2	0.74	0.7	0.72	1.2	1.2	1.2	0.04	0.04	0.04	67.1	67.1	67.1
3	0	0	0	0.3	0.3	0.3	0.85	0.91	0.88	0.08	0.09	0.085	0.03	0.09	0.06	67.4	23.3	45.3
6	0	0	0	1.2	1	1.1	0.44	0.39	0.415	0.05	0.05	0.05	0.05	0.07	0.06	36.1	23.8	30.0
9	0	0	0	3.3	3.1	3.2	0.04	0.03	0.035	0.04	0.03	0.035	0.02	0.04	0.03	38.0	33.8	35.9
12	0	0	0	0.7	0.7	0.7	0.09	0.08	0.085	0.14	0.09	0.115	0.031	0.032	0.032	21.8	21.4	21.6