Monitoring of Fouling and Clean-in-place (CIP) Using the Rotoscope and Microscopy and Monitoring of Trihalomethanes (THMs) Produced from Electro-Chemically Activated (ECA) Water during CIP

By

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"Most successful men have not achieved their distinction by having some new talent or opportunity presented to them. They have developed the opportunity that was at hand."
- Bruce Barton

"Flaming enthusiasm, backed by horse sense and persistence, is the quality that most frequently makes for success. "
-Dale Carnegie

"Success is to be measured not so much by the position that one has reached in life as by the obstacles which he has overcome."
-Booker T. Washington

"The ability to discipline yourself to delay gratification in the short term in order to enjoy greater rewards in the long term is the indispensable pre-requisite for success."
- Brian Tracy

"An exaggerated competitive attitude is inculcated into the student, who is trained to worship acquisitive success as a preparation"
-Albert Einstein

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DECLARATION

I, the undersigned, herewith declare, that this thesis, which I hereby submit for the degree of MSc (Microbiology) is my own original work and has not previously been submitted at this, or any other University.

Signed:

The 26th day of May 2011

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Summary

The unwanted occurrence of biofilms in various industries requires critical remedial action in order to prevent their detrimental effects which potentially result in huge economic losses. Adequate monitoring of biofilms is a powerful tool to aid their thorough understanding and ultimate control. The Rotoscope is an instrument based on the principle of light absorption and reflection that was used in this study to monitor and study biofilms. Biofilm development of cocci and bacilli species was monitored using the Rotoscope and microscopy. Light reflectance decreased over time as the biofilm developed. Information on the different stages of biofilm development could also be deduced from light reflectance assays of the Rotoscope. Microscopy validated results which were obtained from light reflectance assays. Information on the morphology of the bacteria, depth of the biofilm as well as the different stages of biofilm development was deduced from EM and CLSM images. The Rotoscope was an easy, effective, on-line monitoring device for the development of biofilms. It was also showed to be useful in collecting information to help characterize bacteria species present within a biofilm

The Rotoscope was integrated into a simulated soft drink (SD) production line to monitor biofouling and the efficacy of a clean-in-place (CIP) regime using electrochemically activated (ECA) water. During CIP the Catholyte and Anolyte (components of ECA) were effective as detergent and disinfectant respectively. This was indicated by results obtained from microbial analysis of removable slides from the MPD, microscopic analysis, as well as pH, ORP and EC analysis. The absence of microbial growth and soil on microscope slides from the Rotoscope were a good indication of the high efficacy of Catholyte and Anolyte (components of ECA) as detergent and disinfectant respectively in a CIP system. In addition, the relatively constant values reported for pH, ORP and EC before and after CIP suggests that the Catholyte and Anolyte were effective during CIP.

The effect of Anolyte on trihalomethane (THM) formation was observed because of the presence of chlorine compounds. Low levels of THMs were obtained from CIP effluent which provided a good indication that Anolyte is an environmentally friendly alternative disinfectant compared to conventional disinfectants currently used in CIP. Increased pH and the presence of bromide resulted in an overall increase of THMs in systems using dissolved organic carbon (DOC) models (Glucose, maltose and phenol). There was however variation in the amount of THM produced using the three DOC models. The differences were attributed to the composition of organic matter in particular the aromacity and the nature and position of the functional groups of the model DOCs.

Chapter 1: Introduction

Van Leeuwenhoek has been credited as the person to first establish the existence of biofilms, with the first observation on a tooth surface (Donlan, 2002). A biofilm can simply be defined as a community of microbes that have adhered to a surface. The microbes are embedded in a protective matrix called the exopolysaccharide (EPS). The EPS also acts as an adhesive to which the biofilm adheres to a surface (Costerton, 1999). Depending on the environment in which they form, different non-cellular materials can be found in the matrix of the biofilm (Donlan, 2002). Microbes which are associated with a biofilm are different from their planktonic counterparts in terms of the genes that they transcribe (Donlan, 2002). Biofilms can be detrimental to the surfaces on which they from, causing serious damage; this is termed biofouling (Flemming and Schaule, 1996; Klahre and Flemming, 2000).

Biofouling can occur on surfaces of heat exchangers, ship hulls and storage and distribution systems amongst other systems (Flemming, 2003). Biofouling damages cause huge economic losses which (Flemming, 2003). In many cases damages by biofouling are recognized when it is too late. A solution to avoiding huge economic losses is to recognize biofouling early. This can be achieved by monitoring systems.

There are a number of methods available which can be used to monitor systems for biofilm growth. Microscopy is a tool that is used to study biofilms. Improvements in microscopy over the years have greatly increased interest in the use of microscopy to study biofilms. Light microscopy, electron microscopy, confocal laser microscopy and atomic force microscopy are some the types of microscopy that are used to monitor biofilms (Nivens *et al.*, 1995). Spectroscopic techniques are also used to monitor biofilms. Unlike microscopy, spectroscopy can be implemented to study biofilms on-line (Nivens *et al.*, 1995). Another method of monitoring biofilms is by deposit measurements. Deposits are considered to be biofilms. Features of deposits that can be monitored include light absorption, heat transfer and pressure drop (Flemming, 2003). The Rotoscope was recently developed by Prof. T.E. Cloete in South Africa. The Rotoscope is based on the principle of light absorption and reflection. When light is emitted on a surface that has biofilm growth, the biofilm will absorb some of the light and reflect back less light. As liquid passes through the Rotoscope, biofilm/deposit development in a system is monitors by means of light reflection (Cloete and

Maluleke, 2005). Light reflection readings are automatically saved onto a data logger. The Rotoscope can be easily integrated into a system (Cloete and Maluleke, 2005)

Although there are several methods that can be used to monitor biofilms, a good quality method is of utmost importance. A good quality method for monitoring biofilms should be simple to use, rapid, accurate, have real time monitoring capabilities and should not be destructive and invasive to biofilms under study (Flemming, 2003).

Once biofilms have been detected in a system they have to be eradicated from the system. In industry, cleaning-in-place (CIP) is generally applied for cleaning and disinfection purposes (Chisti, 1999). CIP is a unit-operation process that does not require dismantling of equipment (Chisti, 1999). CIP can prevent further build up of biofilms as well as damage associated with biofilms. A number of factors must be taken into account when choosing a CIP system and chemicals. The CIP system must have low toxicological risk, low ecological risk, be economical and should be considerate of occupational health (Orth, 1998; Chisti, 1999).

Electro-chemical activation (ECA) of water is a green technology, which is economical (Thantsha and Cloete, 2006). The technique involves a saline solution being passed through an electrolytic cell to separate the anode solution from the cathode solution (Thantsha and Cloete, 2006). The products of ECA are called an Anolyte and a Catholyte. The Anolyte (Cl^-) is a disinfectant with a biocidal effect and the Catholyte (Na^+) has surfactant and antioxidant properties (Thantsha and Cloete, 2006). The major problem associated with the use of disinfectants that contain chlorine is the production of disinfection by-products (Thantsha and Cloete, 2006).

Disinfection by-products (DBPs) are formed when a disinfectant reacts with organic and/or inorganic matter (precursors) in water (Sadiq and Rodriguez; 2004). Trihalomethanes (THMs), haloacetic acids (HAAs), haloacetonitriles (HANs), haloketones (HKs), chloral hydrate and chloropicrin (CPN) are the major DBPs that exist (Peters *et al.*, 1990; Singer, 1994; Antoniou *et al.*, 2006). THMs and HAAs are said to be the most abundant DBPs that result from chlorination (Krasner *et al.*, 1989; Wiesel *et al.*, 1999). There are concerns about health risks that have been associated with THMs. Epidemiological studies have indicated that there is an association between chlorination and a number of adverse health effects such

as cancer and adverse reproductive outcome (Gopal *et al.*, 2007). The adverse health risks associated with THMs have necessitated strict regulatory measures by authorities; e.g. limitation on permissible concentration (µg/l) of DBPs in drinking water or any other liquids intended for human consumption (Gopal *et al.*, 2007). These strict regulations on THMs have influenced a lot of industries into using disinfectants that produce small amounts of THMs.

The main objectives of this study were:

- To test the sensitivity and rapidness of the Rotoscope as an instrument to detect and monitor biofouling development
- To validate results obtained from the Rotoscope by confocal laser scanning microscopy (CLSM) epifluoresence microscopy (EM).
- To Monitor the efficiency of Anolyte and Catholyte as disinfectant and detergent respectively in a clean-in-place (CIP) system by monitoring electric conductivity (EC), oxidation-reduction potential (ORP) and pH of the Anolyte and Catholyte.
- To detect trihalomethanes (THMs) in effluent produced from CIP using gas chromatography-mass spectrometry (GC-MS)
- To conduct THM formation potential studies of Anolyte and Catholyte

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Chapter 2: Literature review

2.1 Literature Review on Biofilm Monitoring

The tendency for bacteria to grow on surfaces was first recognized in the 17th century (Locy, 1923). In 1684, Antonie van Leeuwenhoek reported on "live animals" which he had scraped from his teeth (Locy, 1923) using a "primitive" microscope to make his observations (Locy, 1923; Costerton, 1999). Zobell and Allen 1935 and Zobell 1937 studied bacteria in the marine environment and concluded that bacteria are attracted to surfaces where they are sometimes attached to (Costerton, 1999). The biggest breakthrough was in 1964 when Ralph Mitchell and Kevin Marshall studied stages of biofilm development, where they differentiated reversible from irreversible adsorption (Costerton, 1999).

Microorganisms are faced with physical, chemical and biological challenges in their natural environment which can be of (Stephens, 2002). They survive these challenges by adhering to surfaces, which can lead to the development of biofilms (Stephens, 2002). Biofilms can be defined as durable communities of microbes that are embedded in a protective and adhesive polysaccharide matrix (Costerton *et al.*, 1995; Stephens, 2002), and can therefore be considered to be protective microbial microenvironments. A mature biofilm consists of aggregates of microbes encased in an exopolysaccharide separated from other microbial aggregates by water channels (Lewandowski, 2000; Donlan, 2000).

The occurrence of biofilms in industry can have detrimental effects. The existence of pathogenic microorganisms in a biofilm growing on a surface in a food processing industry can pose a threat on food safety (Chmielewski and Frank, 2003). In addition, the adverse effect of biofilms growing in heat exchange equipment can cause a reduction in heat transfer and the efficiency at which the equipment will operate (Kumar and Anand, 1998). Biofilms can also cause microbiologically-influenced corrosion (MIC) on metal surfaces which has been reported to in the oil industry and in power plants (Tribollet, 2003). It is clear from the above examples that biofilms potentially cause huge economic losses in industry and require critical remedial action. In order to control the damage caused by biofilms, biofilms need to be thoroughly understood; this can be achieved through biofilm monitoring. There are different methods which are used to monitor biofilms. These will be discussed in the present review.

Biofilm Development

It has been established that bacteria survive within hostile environments existing as adherent populations (sessile bacteria or biofilms) (Olson *et al.*, 2002). In an "ideal world" for bacteria, planktonic cells or "free swimming" can be viewed as bacteria in a translocation phase from one surface to another (Brown and Häse, 2001). Biofilms can be composed of either homogeneous or heterogeneous species of bacteria. Biofilm development occurs in four distinct phases namely: 1) initial reversible attachment, 2) irreversible attachment, 3) maturation, and 4) detachment (Stoodley *et al.*, 2002).

Initial reversible attachment

Prior to the attachment of cells to a surface, there are cell-surface and cell-cell interactions that occur (Palmer and White, 1997; O'Toole *et al.*, 2000). Planktonic cells approach a surface and explore it in a series of species-specific behaviours (Costerton, 1999). Cells of some species roll across the surface before they settle where they can initiate adhesion, others move very little before they initiate adhesion, while others start by aggregating resulting in the formation of microcolonies (Costerton, 1999). Following different species-specific behaviours of exploring surfaces, the cells slow down motility and then adhere to surfaces (Watnick and Kolter, 2000; Stephens, 2002). The first adherence stage is reversible because the bacteria form a temporary association with the surface while searching for an appropriate part of a surface to inhabit (Watnick and Kolter, 2000).

Irreversible attachment

Irreversible attachment occurs once the bacteria have settled (Stephens, 2002). As the bacteria divide and form microcolonies during irreversible attachment, close proximity to the surface causes physiological changes to occur (Stephens, 2002). One of the most important physiological changes that occur is the induction of exopolysaccahrides (EPS) production (Stephens, 2002). The EPS is made up of polysaccharides, proteins, nucleic acids and phospholipids (Stoodley *et al.*, 2002). The function of EPS is to anchor the bacteria to a surface (Habash and Reid, 1999) as well as to provide structure to biofilms (Stoodley *et al.*, 2002). The EPS has been reported to safeguard the biofilm against environmental stresses such as ultra-violet (UV) radiation, changes in pH, osmotic shock, and drying out (Flemming, 1993; Davey and O'Toole, 2000). The EPS also has the potential to prevent the entry of certain antimicrobials into the biofilm (Gilbert *et al.*, 1997; Davey and O'Toole, 2000).

Biofilm maturation

Once the bacteria have attached irreversibly to the surface and have produced EPS, the biofilm is referred to as mature (Stoodley *et al.*, 2002). Thus, the bacteria would have created a protective microenvironment for themselves (Habash and Reid, 1999). The roles of biofilms include: 1) safeguarding bacteria against changing environmental conditions, 2) providing easy access to nutrients by means of bulk transfer, 3) removing metabolic waste products; 4) providing an environment of synergy where by-products of certain bacteria become substrates for others (Habash and Reid, 1999).

Detachment

Detachment is a process whereby individual cells or groups of cells become released from the mature biofilm (Stoodley *et al.*, 2002). There are three modes of detachment, namely: 1) erosion (shearing), 2) sloughing, and 3) abrasion (Brading *et al.*, 1995; Donlan, 2002). Erosion (or shearing) involves the continuous removal of small segments of the biofilm from the mature biofilm (Brading *et al.*, 1995; Donlan, 2002). Sloughing occurs when massive segments of the biofilm are rapidly removed (Brading *et al.*, 1995; Donlan, 2002). Abrasion happens when particles in the bulk fluid collide with the biofilm and result in a detachment (Brading *et al.*, 1995; Donlan, 2002). Detachment of biofilms can be due to either physical, chemical, or biological factors.

Physical factors

Physical factors that influence biofilm detachment are generally in the bulk liquid enveloping the biofilm (Moore *et al.* 2000). Fluid velocity has been observed as a physical factor by researchers conducting experiments in a fluidized bed (Chang *et al.* 1991; Moore *et al.* 2000). Shear stress at the biofilm-liquid interface is another factor that causes detachment (Characklis, 1990; Moore *et al.*, 2000) and is affected by the magnitude as well as the frequency of change in shear stress (Stoodley *et al.*, 1999b; Moore *et al.*, 2000).

Chemical factors

The availability of nutrients can have an influence on biofilm detachment (Stoodley *et al.*, 1999a; Moore *et al.*, 2000). Sawyer and Hermanowicz (1998) found that nutrient limitation at the biofilm-liquid interface of *Aeromonas hydrophila* increased the rate of detachment (Moore *et al.*, 2000). In contrast, Characklis *et al.* (1990) found that an increase in nutrients can lead to an increase in the rate of biofilm detachment (Moore *et al.*, 2000).

Biological factors

Certain biochemicals that are produced by bacteria as they grow in the biofilm lead to biofilm detachment (Moore *et al.*, 2000). For instance, *Pseudomonas aeruginosa* biofilms produce an enzyme, alginate lyase that induces detachment by degrading alginate (Boyd and Chakrabarty, 1994; Moore *et al.*, 2000). Alginate is a polysaccharide that enhances attachment of cells to surfaces (Mai *et al.*, 1993; Moore *et al.*, 2000). In addition, another enzyme, surface protein releasing enzyme (SPRE) releases proteins from the surface of the cell (Moore *et al.*, 2000) whose activity increases bacterium detachment from the biofilm (Lee *et al.* 1996; Moore *et al.* 2000).

Biofilm Architecture

Although every microbial biofilm is unique (Donlan, 2000; Tolker-Nielsen and Molin, 2000), they all have a common structure that contains microcolonies encapsulated in EPS and are separated from other microcolonies by interstitial channels (Donlan, 2000; Lewandowski, 2000). Stoodley *et al.* (1994) have shown that there is a flow of liquid through these channels (Davey and O'Toole, 2000) which allows circulation of nutrients and oxygen (Donlan, 2002), as well as the elimination of toxic metabolites (Davey and O'Toole, 2000).

Variation in the architecture of biofilms is due to the location of the biofilm, the nature of microorganisms that make up the biofilm, as well as the nutrients present in the system (Stickler, 1999). The structure of a biofilm can vary from a patchy single layer of cells (Wimpenny *et al.*, 2000), confluent cell layers (Stickler, 1999), to stalks of cells projecting from a thin basal layer (Stickler, 1999).

Biofilm Distribution

Biofilm formation can occur on a wide spectrum of surfaces including human tissues (e.g., lung and skin) (Costerton *et al.*, 1999), medical devices (e.g., urinary catheter) (Habash and Reid, 1999), natural aquatic systems, and industrial pipes (Donlan, 2002). The present review however, focuses on biofilms in industry where they can be both beneficial and detrimental. There are three properties of biofilms that can affect industrial processes, namely, physical (structural), chemical (metabolic), and biological (living) (Ludensky, 2003).

Industry can benefit from biofilms by using their metabolic activities (Gilbert *et al.*, 2003) to manage wastewaters (Manem and Rittmann, 1992; Gilbert *et al.*, 2003), to treat sewage (Yu *et al.*, 1999; Gilbert *et al.*, 2003) and in biotechnological processes in a variety of solid-state fermentations (Kirimura *et al.*, 1999; Gilbert *et al.*, 2003). In contrast, biofilms also cause a lot of damage in industry which cause significant economic losses (Flemming, 2003). These include huge losses in efficiency, processing down-time, damage to equipment, obstruction of pipelines, and lowering the (Characklis, 1990; Little *et al.*, 1990; Gilbert *et al.*, 2003). EPS is an example of a structural property of biofilms which can cause problems in cooling water systems; energy losses occur because EPS increases frictional resistance of fluid as well as heat transfer resistance (Ludensky, 2003).

Biofilm Monitoring

Biofilm monitoring is critically important in effectively reducing problems associated with biofilms. However, monitoring biofilms needs reliable methods which are able to detect and quantify biofilms (Cloete, 2003). Biofilm monitoring can focus on one of the following: biofilm development, biofilm architecture, biofilm biochemical or microbial composition, biofilm physiological activities, and multicellular interactions (Denkhaus *et al.*, 2007). Different methods used for biofilm monitoring are summarized in Table 2.1 and will be discussed in detail in the present review.

Table 2.1: A summary of methods used to monitoring biofilms

Method	Information obtained	Advantages	Limitations	References
Microscopy				
• Light microscopy	Biomass appearance	Simple, rapid and no complex sample treatment required	Analysis limited to early phase of biofilm formation, removal of biofilm from substratum distorts results	Geesey and White (1990); Pierzo *et al.* (1994); Lazarova and Manem (1995); Wolf *et al.* (2002)
• Epifluorescence microscopy	Physiology of cells in relation to environment, bacterial counts	Simple, environment-physiology relationship	Limited to thin biofilms, not accurate in distinguishing dead cells from live cells	Porter and Feig (1980); Rodriguez *et al.* (1992); McFeters *et al.* (1995a); McFeters *et al.* (1995b); Saby *et al.* (1997); Wolf *et al.* (2002); Keevil, (2002); Morató *et al.* (2004)
• Electron microscopy	Biofilm development, internal structure of biofilm	High resolution	Complex and destructive sample preparations	Dawson *et al.* (1981); Kinner *et al.* (1983); Eighmy *et al.* (1983); Sreekrishnan *et al.* (1991); Lazarova and Manem (1995); Surman *et al.* (1996); Wolf *et al.* (2002); Lawrence *et al.* (1991)

• Confocal laser microscopy	3-dimensional structure of biofilm, cell morphology, metabolism, phylogeny, bacterial growth	Studies hydrated biofilms, penetrates thick specimens, *in vivo* and *in vitro* studies, non-destructive	High quality scanning takes a long time, overlapping of sample and probe fluorescence	Caldwell *et al.* (1992); de Beer *et al.* (1994); Costerton *et al.* (1995); de Beer and Stoodley, (1995); Davey and O'Toole, (2000); Morató *et al.* (2004); Wolf *et al.* (2002)
• Atomic force microscopy	Biofilm formation, chemistry of biofilm on metal surfaces	Resolution at atomic scale, *in situ*, no prior staining and fixation required.	Requires dehydration of sample	Surman *et al.* (1996); Telegdi *et al.* (1998); Gaboriaud and Dufrêne (2007)
Spectroscopic				
• Fourier transform infrared (FTIR) and Attenuated total reflection (ATR)-FTIR	Characterizing bacteria, studying the effects of biofilm on substrata	High analysis speed, high accuracy	Unable to distinguish dead from live biomass	Harrick (1967); Nivens *et al.* (1995); Schmitt and Flemming (1998); Wolf *et al.* (2002)
• Photoacoustic (PAS)	Biofilm development, changes in biofilm composition over time	On-line, no sample pre-treatment required	Complex technique	Schmid *et al.* (2001); Wolf *et al.* (2002); Schmid (2006)

• Nuclear magnetic resonance	Biofilm metabolites, substrate consumption, effect of environment on biofilm formation	Non-destructive, non-invasive	Acquiring data takes a long time	Nivens *et al.* (1995); Lewandowski *et al.* (1993); Stoodley *et al.* (1994); Mayer *et al.*(2001); Wolf *et al.* (2002)
Deposit measurements				
• Differential turbidity measurements (DTM)	Turbidity in systems due to biofilms by measuring absorption and scattering of light by biofilms	On-line, real time and non-destructive	Unable to detect biofilm less than 0.1 mm thick	Klahre and Flemming (2000); Janknecht and Melo (2003)
• Heat transfer resistance (HTR)	Restriction of heat transfer due to biofilms	Non-invasive, real-time	Inability to detect initial biofilm attachment	Ludensky (1998); Melo and Vieira (1999); Janknecht and Melo (2003)
• Pressure measurements	Pressure drop of liquid in pipes due to biofilms	Simple, cheap	Inability to differentiate between biotic and abiotic deposits	Janknecht and Melo (2003)
• Thickness	Biofilm thickness	Simple	Inability to differentiate between biotic and abiotic deposits	Pinheiro *et al.* (1988); Janknecht and Melo (2003)

Electric signals	Detection of biofilms and effect of biofilms on metals	Non-destructive, real-time	Complex method that requires an expert to operate	Nivens *et al.* (1995); Janknecht and Melo (2003); Tribollet (2003)
Vibration signals	Detection of biofilms on surfaces	On-line, real time	Readings affected by environmental temperature and liquid phase pressure detection limit of 3×10^5	Nivens *et al.* (1993); Bunde *et al.* (1998); Helle *et al.* (2000); Janknecht and Melo (2003); Kujundzic *et al.* (2007)
Other monitors				
• Rotoscope	Monitoring of microbial growth by means of light sensor	Real-time, non-destructive and on-line	Inability to differentiate between biotic and abiotic deposits	Cloete and Maluleke (2005)
• Roto Torque System	Morphological studies of heterogeneous biofilms	Non-destructive, parameters can be adjusted to be analogous to natural conditions	Inability to differentiate between biotic and abiotic deposits	Kuballa and Griebe (1995); Headley *et al.* (1998)
• Infrared monitor	Monitoring of biofilms by means of infrared absorption	Real-time	Only able to monitor through a transparent glass	Tinham and Bott (2003)

• Aquasim modeling system	Models biofilms in aquatic systems	Different biofilm models can be created from the program	Simplification of assumptions	Noguera and Morgenroth (2004); Wanner and Morgenroth, (2004); Reichert (1998a); Reichert (1998b)
• BioGeorge ™ on-line monitoring system	Detection of biofilms due to electrochemical changes	Real-time	Requires the assemblage of the hardware and loading of software, an expert is required for data analysis	Guezennec *et al.* (1991); Licina and Nekosa (1993); Nalepa *et al.* (1996); Bruijis *et al.* (2000)

2.1.1 Microscopy

Microscopy has played a significant role in the understanding of microorganisms and is a basic tool that allows detection of biofilms at an early stage (Nivens *et al.*, 1995; Wolf *et al.*, 2002). This includes the detection of changes in composition of microbial populations, structural complexes, biochemical as well as physiochemical changes (Wolf *et al.*, 2002). Over the years microscopy has been coupled with on-line image acquisition and has recently been upgraded for *in situ* analysis resulting in an increased interest in the usage microscopes (Wolf *et al.*, 2002). With microscopy automation there is an increased throughput of samples and a decrease in time required for observation (Nivens *et al.*, 1995; Wolf *et al.*, 2002). The present review focuses on a discussion of light microscopy, epifluorescence microscopy, electron microscopy, confocal laser microscopy, and atomic force microscopy.

2.1.1.1 Classical light microscopy

Classical light microscopy can be regarded as groundwork for biofilm studies as it mainly provides information on how the biomass appears (Wolf *et al.*, 2002). Mature biofilms have to be removed from where they have been attached and then be put onto a microscope slide prior to analysis (Wolf *et al.*, 2002). Removal of biofilms from where they are attached is limiting to biofilm analysis because the natural structure of the biofilm becomes distorted (Geesey and White, 1990; Lazarova *et al.*, 1994; Pierzo *et al.*, 1994; Lazarova and Manem, 1995; Wolf *et al.*, 2002). Light microscopy is also limited to early phases of biofilm formation when studying live biofilms (Wolf *et al.*, 2002). Despite its limitations, its simplicity, ability to provide rapid results, and its non-requirement of complex sample pre-treatment procedures render it quite useful in biofilm monitoring (Lazarova and Manem, 1995; Wolf *et al.*, 2002).

2.1.1.2 Epifluorescence microscopy

Fluorescence refers to light of one colour being absorbed at a certain wavelength soon after being emitted at a longer wavelength as light of a different colour (McCarthy 2007). Therefore, fluorescence occurs when molecules rise from their ground (S_0) to their excited (S_1) states. Although some cellular components are autofluorescent, they provide limited information leading to a need for cells to be stained with fluorochromes (fluorescent probes) (McCarthy, 2007).

The introduction of fluorochromes into fluorescence microscopy occurred in the early 20th century (Claxton *et al.*, 2008). They are designed to bind to either biological macromolecules (e.g., nucleic acids) or to specific localized structural regions (e.g. mitochondria) (Claxton *et al.*, 2008). Therefore when biological samples are stained with fluorochromes, they accumulate or bind to a specific structure or compound (Sudgen, 2004). Fluorochromes can provide information on physiological activities and cellular structures of microbes, detect particular chemicals in microbes and genetic mapping (Claxton *et al.*, 2008; Sudgen, 2004). Epifluorescence microscopy makes use of fluorochrome staining. Fluorochromes that are commonly used for direct staining of biofilms include acridine orange (AO), 5-cyano-2, 3-ditolyl tetrazolium chloride (CTC), and 6-diamidino-2-phenylindole (DAPI) (Wolf *et al.*, 2002; Morató *et al.*, 2004).

AO interacts with the DNA of cells and is used as an indicator for physiologically active microbes (McFeters *et al.*, 1995a; Wolf *et al.*, 2002). The disadvantage of using AO staining is that it cannot accurately differentiate between dead cells and actively growing cells because they both fluoresce at the same spectrum (Keevil, 2002; Wolf *et al.*, 2002). A red to orange fluorescence occurs between AO and single stranded DNA (ssDNA), whereas a green fluorescence occurs between AO and double stranded DNA (dsDNA) (McFeters *et al.*, 1995a). In physiological studies of cells, green fluorescent cells are considered to be active, whereas either red or orange cells are considered to be inactive (i.e., which is a similar fluorescence of ssDNA; McFeters *et al.* 1995a). However, it has been suggested that discrimination between inactive and active cells can be documented based on the ratio of RNA:DNA (McFeters *et al.* 1995a). Highly active cells have a high ratio of RNA:DNA compared to inactive cells which have a significantly lower ratio (McFeters *et al.* 1995a).

CTC is used to visualize respiring bacteria (McFeters *et al.*, 1995a). In active bacteria, during respiration, CTC fluoresces as it changes from its oxidized state to its reduced state emits a red fluorescence from being colourless (Rodriguez *et al.*, 1992; Wolf *et al.*, 2002). DAPI is highly specific to DNA (Porter and Feig, 1980; Saby *et al.*, 1997). When light of wavelength of 365 nm or more than 390 nm is applied to a sample, DNA-DAPI complex fluoresce bright blue and unbound DAPI or DAPI-non-DNA complex fluoresce a weak yellow (Porter and Feig, 1980). The difference in fluorescence colour makes it easy to distinguish bacteria from other matter (Porter and Feig, 1980). In biofilm studies, DAPI is used for bacterial count. As with light microscopy, the disadvantage of epifluorescence

microscopy is that it can only be applied to thin biofilms (McFeters *et al.*, 1995b; Wolf *et al.*, 2002).

2.1.1.3 Electron microscopy

Electron microscopy was developed in the 1930s and provides very high resolution (Lazarova and Manem 1995; Wolf *et al.*, 2002). The resulting enlarged image formed is due to illumination of electrons on the specimen. The present review focuses on scanning electron microscopy, transmission electron microscopy and environmental scanning electron microscopy.

Scanning electron microscopy (SEM) is usually used to study complex structure, whereas transmission electron microscopy (TEM) is used to study cross-sectional structures (Surman *et al.*, 1996; Wolf *et al.*, 2002). Lazarova and Manem (1995), Eighmy *et al.* (1983) and Sreekrishnan *et al.* (1991) used SEM to study different stages of anaerobic biofilm development using and found complementary results. TEM on the other hand, has been used to study the internal structures of the biofilm (Dawson *et al.*, 1981; Eighmy *et al.*, 1983; Kinner *et al.*, 1983).

SEM and TEM require that the biofilm be dehydrated, fixed and be prepared using complex methods which may result in shrinkage of the biofilm, damage of the biofilm, and biofilm loss (Wolf *et al.*, 2002). This suggests that SEM and TEM cannot be used to study intact biofilms *in vivo* or *in vitro* (Blenkinsopp and Costerton 1991; Surman *et al.*, 1996; Wolf *et al.*, 2002). Environmental scanning electron microscopy (ESEM) is a modification of SEM that has been developed. This method uses a low-vacuum which allows studies of hydrated biofilms (Surman *et al.*, 1996). Unlike the conventional SEM, ESEM does not require biofilm dehydration and sample fixation. This eliminates the risk of damage to and loss of the biofilm. ESEM also produces higher magnifications than conventional SEM (Wolf *et al.* 2002).

2.1.1.4 Confocal laser scanning microscopy

Confocal laser scanning microscopy (CLSM) is another technique that contributed significantly to the understanding of biofilms (Wolf *et al.*, 2002). CLSM greatly relies on fluorescence as a mode of imaging due to the high sensitivity and specificity of fluorescence (Claxton *et al.*, 2008). CLSM has a laser light source that can penetrate thick specimens

(Wolf *et al.*, 2002), and unlike conventional SEM and TEM, it allows the study of fully hydrated biofilms and *in vivo* and *in situ* biofilm studies (Wolf *et al.* 2002).

Information on the 3-dimensional (3D) structure of a biofilm can be obtained using CLSM (de Beer *et al.*, 1994; Costerton *et al.*, 1995; de Beer and Stoodley, 1995; Davey and O'Toole, 2000). When samples are treated with fluorescent molecular probes, information obtained from CLSM can be extended to cell morphology, metabolism and phylogeny, biofilm matrix structure and architecture (Costerton *et al.*, 1995; Wolf *et al.* 2002; Morató *et al.*, 2004). The limitations of CLSM include that the scanning of high quality images takes a long time, sample autofluorescence can overlap with the fluorescence of probes, and the presence of opaque objects below and above the specimen can cause appearance of shadows on the focal plane (Caldwell *et al.*, 1992; Wolf *et al.*, 2002).

2.1.1.5 Atomic force microscopy

Atomic force microscopy (AFM) is one of the most powerful techniques used for microbial studies (Telegdi *et al.*, 1998). The best attributes of AFM are that it provides very high resolution at nanoscale, in particular at atomic scale (Telegdi *et al.* 1998; Gaboriaud and Dufrêne, 2007), and does not require fixation and staining of the biofilm (Surman *et al.*, 1996). The disadvantage of AFM is that it requires dehydration of a biofilm sample (Surman *et al.*, 1996).

Telegdi *et al.* (1998) reported that AFM is one of the most effective methods that allows for on-line, high resolution observation in aqueous solution *in situ*. Telegdi *et al.* (1998) used AFM to study microbially influenced corrosion (MIC) where they were able to visualize biofilm formation by attached bacteria, exopolymer excretion as well as the resulting metal surface corrosion. From this, they deduced that biofilm formation and corrosion were related to bacterial action and the chemical character of the surface.

2.1.2 Spectroscopy

Spectroscopic techniques are a valuable tool used in the study of biofilms (Wolf *et al.*, 2002). Spectroscopic techniques can be used to study biofilm development, architecture, metabolic activity as well as to quantification. Spectroscopic techniques are suitable for on-line implementation and some of them can be used for *in situ* studies (Wolf *et al.*, 2002). However, off-line analysis using spectroscopic techniques can be invasive and may require

destruction of the biofilm (Gjaltema and Griebe, 1995; Wolf *et al.*, 2002). Commonly used spectroscopic techniques that will be discussed in this review include infrared spectroscopy, photoacoustic spectroscopy, and nuclear magnetic resonance spectroscopy.

2.1.2.1 Infrared (IR) spectroscopy

IR spectroscopy is based on the observation that different bacteria exhibit different IR spectra (Schmitt and Flemming, 1998). IR radiation is measured as frequency or wave number ranges (Nivens *et al.*, 1995). The IR region between 4000 and 500cm^{-1} has characteristic bands which are suitable for microorganism characterization (Schmitt and Flemming, 1998). The functional groups of microorganisms used for IR spectroscopy studies include peptide and protein structures, polysaccharides, phospholipids and nucleic acids (Schmitt and Flemming, 1998). The variation in quantity and distribution of different functional groups amongst different strains of bacteria give rise to different IR spectra patterns (Nivens *et al.*, 1995; Wolf *et al.*, 2002). The obtained patterns are then compared to those that already exist in a spectra library. For biofilms, the spectral fingerprints obtained are due to all functional groups that are in the sample (Nivens *et al.*, 1995; Wolf *et al.*, 2002). Fourier Transform Infrared (FTIR) and FTIR with Attenuated Total Reflection (ATR) will be discussed in this review.

FTIR Spectrometry

FTIR spectrometers measure all frequencies from the sample simultaneously and not sequentially like other dispersive instruments (Griffiths and Haseth, 1986; Nivens *et al.*, 1995). FTIR spectroscopy is used for studying aqueous biofilms (Nivens *et al.*, 1995). FTIR spectroscopy is characterised by high analysis speed, improved signal:noise ratios, better wave number accuracy and greater signal throughput.

ATR-FTIR Spectrometer

ATR spectroscopy is a technique used in FTIR spectrometry and is used for the examination of aqueous environments near the surface of an internal reflection element (Harrick, 1967; Nivens *et al.*, 1995). Several studies have demonstrated the effectiveness of ATR-FTIR spectrometry in understanding biofilm composition as well as the effects of biofilms on substrata (i.e., the inside out of biofilms (Nivens *et al.*, 1995). The limitation of ATR-FTIR spectrometry is its inability to distinguish between living and dead biomass (Nivens *et al.*, 1995).

2.1.2.2 Photoacoustic spectroscopy

Photoacoustic spectroscopy (PAS) is one of the on-line techniques used for the monitoring of biofilms (Ref). PAS is based on the absorption of electromagnetic radiation in a sample, where the absorbed energy is converted into heat (Wolf *et al.*, 2002). The heat from the sample causes a pressure wave and the pressure wave can be detected by microphones or piezoelectric transducers (Wolf *et al.*, 2002).

Schimd *et al.* (2001) used a photoacoustic sensor system for depth-resolved analysis. They investigated growth of biofilm on the surface of a sensor head. Absorption profiles were used to determine organic mass of a biofilm. An increase in the biofilm and changes in the biofilm composition was examined by analyzing photoacoustic signals at a particular wavelength. PAS does not require pre-treatment of biofilms (Schmid, 2006).

2.1.2.3 Nuclear magnetic resonance (NMR)

The basis of NMR is the absorption of radio frequencies in the presence of magnetic fields (Nivens *et al.*, 1995; Wolf *et al.*, 2002). NMR is a non-invasive and non-destructive technique (Lewandowski *et al.* 1993; As and Lens 2001; Schügerl 2001; Wolf *et al.*, 2002). However, it takes a long time to acquire data using NMR (Lewandowski *et al.*, 1993; Stoodley *et al.*, 1994; Mayer *et al.*, 2001; Wolf *et al.*, 2002). Substrate consumption and intracellular metabolites of biofilms have been studied by means of NMR (Wolf *et al.* 2002). NMR has also been used to study the effects of hydrodynamics of surrounding liquid on biofilm formation (Wolf *et al.* 2002). Mayer *et al.* (2001) used NMR to study structural information of biofilms.

2.1.3 Biofilm Monitoring by Means of Deposit Measurements

Deposits that occur on solid surfaces can be considered to be biofilms. Like any other deposits, biofilms have features that can be measured. The features include light absorption, thermal insulation and increased friction to moving fluids (Janknecht and Melo, 2003). Therefore monitoring of deposits can play an important role in the control of biofilms. Differential turbidity, heat transfer, pressure drop and biofilm thickness are features of biofilms which can be measured and will be discussed in detail in the present review.

2.1.3.1 Differential turbidity measurement (DTM) and Fiber optical device (FOS)

The basis of this method is the absorption and scattering of light by a biofilm (Klahre and Flemming 2000; Janknecht and Melo, 2003). According to Klahre and Flemming (2000) differential turbidity can be measured continuously, on-line, in real time and is non-destructive. Two turbidity probes were used in their study where one probe was cleaned regularly and the other was left unclean. The difference in light reflectance between the two probes was measured. Their results indicated that the increase in turbidity and decrease in light reflectance was due to biofilm growth. This method was not able to detect biofilm that was less than 0.1mm thick (Janknecht and Melo, 2003).

There is another technique that has a similar principle to DTM which makes use of a fiber optical device (FOS). In this technique, the light source and a light detector are put below the surface of where a biofilm is growing (Janknecht and Melo, 2003). The light detector detects how much light is reflected back by the biofilm (Klahre and Flemming, 2000). Tamachkiarow and Flemming (2003) applied the FOS in a brewery water pipeline. This method is unable to study thick biofilms (Janknecht and Melo, 2003).

2.1.3.2 Heat transfer resistance (HTR)

When a biofilm builds up on a surface, it increases the resistance of heat transfer of a surface. Data can be obtained by measuring temperature on a test section by inducing a heat load on a test section that has a given flow rate and monitoring the change in temperature (Ludensky, 1998). As biofilm deposits increase, the temperature of the test section will increase, indicating a restriction in heat transfer (Ludensky, 1998). The restricted heat transfer is due to the insulation of the surface by the biofilm (Ludensky, 1998).

Heat transfer monitors are suitable for monitoring biofilms in industrial cooling water systems and power plant condensers (Janknecht and Melo, 2003). Measuring of HTR is a non-invasive technique that can be applied during continuous operation (Melo and Vieira, 1999). This technique is unable to detect the initial attachment of microorganisms (Janknecht and Melo, 2003).

2.1.3.3 Pressure measurements

Biofilm attachment to surfaces of pipes increases the roughness of the surface which is in contact with the liquid and also reduces the cross-sectional area of the pipe where the

liquid flows (Janknecht and Melo, 2003). The two mentioned effects can be identified by measuring the changes in pressure of the liquid that flows through the pipe (Janknecht and Melo, 2003). Pressure measurements are suitable for biofilm monitoring in heat exchangers; because this method is not only simple but also cheap (Janknecht and Melo, 2003).

2.1.3.4 Thickness

According to Janknecht and Melo (2003), Pinheiro *et al.* (1988) measured biofilm thickness using a needle which was connected to a micrometer. Thickness was measured by subtracting the micrometer reading when the needle touched the base surface from the micrometer reading when the needle touched the liquid-biofilm interface.

2.1.4 Biofilm Monitoring by Means of Electric Signals

Acquiring an electric signal can be one way of detecting the presence of microorganisms. Most methods that make use of electric signals to detect microorganisms consist of two or more electrodes being brought in contact with an aqueous phase. A build up of electric potential in the electrode(s) having a passive response to application of voltage or current signals is an indication of the presence of microorganisms (Janknecht and Melo, 2003).

Electrodes are commonly used to monitor microbiologically influenced corrosion (MIC). MIC is due to the biofilm changing the electrochemistry of the metal-water interface. MIC appears on some metallic surfaces that are coated with biofilms. MIC on surfaces is usually observed as a localized corrosion which can develop into uniform corrosion (Tribollet, 2003). MIC is a great concern in the oil industry, water distribution and treatment sector, geothermal plants, power plants and in the waste management sector (Tribollet, 2003).

There are a number of available techniques used to monitor MIC. Open corrosion potential (OCP) is a technique used to measure potential difference between a metal probe and a reference electrode (Janknecht and Melo, 2003). Measurement of electrochemical noise (EN) detects fluctuations in potential or current (Janknecht and Melo, 2003). EN and OCP can be used as indicators of microbial presence as well as indicate microbial activity under a defined set of conditions (Nivens *et al.* 1995; Janknecht and Melo, 2003). Commercial electrochemical sensors have been developed and these include the BIOX® system and the BIO GEORGE sensor.

2.1.5 Biofilm Monitoring by Means of Vibration Signals

Vibration signals can be used to detect the presence of biofilms. There are two types of sensors used to detect vibration signals from biofilms; sensors on solid surfaces and sensors in liquid phase.

2.1.5.1 Sensors on solid surfaces

The most commonly used sensors in biofilm studies are the piezoelectric crystal sensors. The sensors consist of a crystal and two electrodes (Bunde *et al.*, 1998; Janknecht and Melo, 2003). When an electric signal is applied to the electrodes, a vibration signal is given off by the piezoelectric crystal sensor. There will be a higher vibration signal from the sensor if there is no microbial growth, compared to when there is growth. Janknecht and Melo (2003) compared the situation to knocking at similar glasses that contain different amounts of water; the glass with little water will give off a higher pitched sound compared to the glass with more water.

The piezo sensor used in biofilm studies is called a quartz crystal microbalance (QCM) (Nivens *et al.*, 1993; Janknecht and Melo, 2003). Other researchers have referred to it as the thickness-shear mode quartz (TSM) (Helle *et al.*, 2000; Janknecht and Melo, 2003). Nivens *et al.* (1993) used the QCM to monitor resonance frequency due to attachment and growth of *Pseudomonas cepacia* biofilm on the surface of the crystal. Limitations of QCM reported by Nivens *et al.* (1993) are detection limit of 3×10^5 cells cm^{-2} as well as the influence of environmental temperature and liquid phase pressure on QCM.

2.1.5.2 Sensors in liquid phase

Ultrasonic frequency-domain reflectometry (UFDR) is a technique used to monitor biofilms in liquid phase. UFDR can be used as a real-time tool to monitor biofilms (Kujundzic *et al.*, 2007). This technique is based on the principle of the difference in the echo created by smooth surfaces compared to attenuation of sound by biofilms that have grown on a surface (Janknecht and Melo, 2003).

Kujundzic *et al.*, (2007) used UFDR to monitor biofilm growth on polymeric surfaces. Their results indicated that UFDR has enough sensitivity to detect growth on dense and porous polymeric surfaces. They suggested that their approach may be used to monitor biofilms on other surfaces.

2.1.6 Other Methods

2.1.6.1 The Rotoscope

The Rotoscope (Figure 2.1) is a newly developed reactor that is used to monitor biofilms. It was developed by Prof. T. E. Cloete (South Africa). A Rotoscope consists of a water tank, a rotating disc and a modified Peterson device (Cloete and Maluleke, 2005). As water circulates through the Rotoscope, biofilms grow on the rotating disc and can be monitored by means of a light monitor. The Rotoscope is based on the principle of light absorption and reflection. When light is emitted on a surface that has biofilm growth, the biofilm will absorb some of the light and reflect back less light (Cloete and Maluleke, 2005). The Rotoscope has a monitor that measures light reflected back by the biofilm on the disc (Cloete and Maluleke, 2005). The modified Peterson device has removable glass slides on which biofilms also grow (Cloete and Maluleke, 2005). These glass slides can be removed and be used for microscopy. Cloete and Maluleke (2005) used the Rotoscope to study biofilms from a river water sample. They also evaluated growth on the glass slides from the modified Peterson device using SEM to validate the results they obtained from light reflection data. Their results indicated that the Rotoscope can be used as an on-line, real-time non-destructive monitor for biofilms.

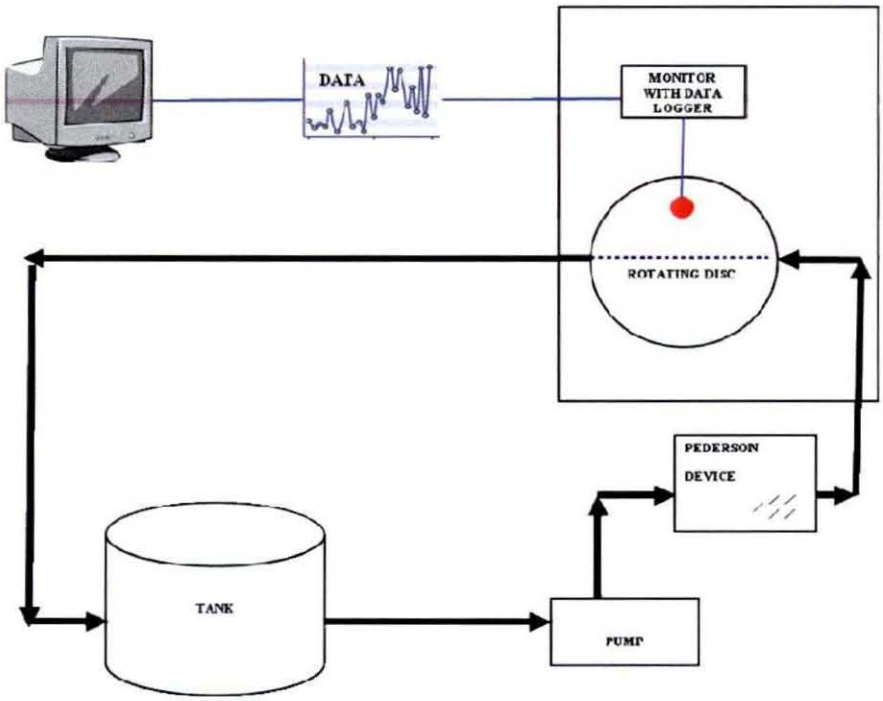

Figure 2.1: Schematic representation of the Rotoscope

2.1.6.2 AQUASIM modelling system

The AQUASIM modelling system is a computer program that models biofilms. Biofilm models have been developed in order to establish their suitability for solving typical biofilm problems (Noguera and Morgenroth, 2004; Wanner and Morgenroth, 2004). A biofilm reactor compartment with three zones (bulk fluid, biofilm solid matrix and biofilm pores for water) is simulated using the AQUASIM program (Wanner and Morgenroth, 2004). The program can be used to calculate biofilm development, microbial composition, biofilm thickness and substrate development in the three zones (Wanner and Morgenroth, 2004). The most apparent limitation to the model is simplifications of assumptions deduced from the model (Wanner and Morgenroth, 2004). This is because the AQUASIM program assumes that biofilms are "one dimensional in space" (Wanner and Morgenroth, 2004). This assumption has been contradicted by a study that illustrated that some biofilms have three-dimensional mushrooms-like structures (Wanner and Morgenroth, 2004).

2.1.6.3 BioGEORGE™ Probe

The BioGEORGE™ probe is a commercial electrochemical sensor used to monitor electrochemical activity of biofilms on-line. The probe is made up of two electrodes which are electrically isolated from each other (Bruijs *el al.*, 2000). When one of the electrodes is polarized, the polarization causes current known as "applied current" to flow from one electrode to another (Bruijs *el al.*, 2000). This current in turn produces slight changes in the environment. These changes favour microbial growth on the probes and induce biofilm formation (Guezennec *et al.*, 1991; Licina and Nekosa, 1993; Nalepa *et al.*, 1996; Bruijs *el al.*, 2000). When biofilm has grown on the probe, a more conductive path for the current is created i.e. significantly increasing current flow (Bruijs *el al.*, 2000). The continuous polarization of the probe creates chemical differences between the anode and the cathode (Bruijs *et al.*, 2000). These chemical differences in turn generate a current flow termed, "generated current" that can be detected, without any potential being applied (Bruijs *el al.*, 2000)

2.1.6.4 Infrared monitor

An infrared monitor measures biofilm accumulation in flowing systems; by means of infrared absorption. However, the section where accumulation is measured must be transparent (Tinham and Bott, 2003). This is because radiation from the infrared emitter must pass through the transparent section and be absorbed by the infrared sensor.

Differences between radiation emitted and absorbed are indications of biofilm accumulation (Tinham and Bott, 2003).

2.1.6.5 Roto Torque System

The Roto-Torque system is a biofilm reactor. The reactor consists of an outer drum that contains a rotating cylinder, an effluent port and an outlet port (Headley *et al*, 1998). The rotating cylinder has recirculation tubes that ensure optimal mixing of the system (Headley *et al*., 1998). Flow rate, nutrient concentration, shear force, pH and types of microorganism used are essential parameters that can be regulated and adjusted in the bioreactor to be analogous to natural conditions (Kuballa and Griebe, 1995; Headley *et al*, 1998). The regulation of these parameters provides great advantage to the system. The system also allows non-invasive observation of biofilms (Huang *et al*., 1992). The reactor was used by Gjaltema *et al*. (1994) for morphological studies on biofilm heterogeneity. Their study showed that biofilms are not homologous layers of biomass as previously thought to be. They revealed structures that looked like dunes and ridges.

2.1.7 Conclusions

Biofilms cause damage worth a lot of money annually in industry. This necessitates a need for damage control implementation. However damage control can only be implemented if biofilms are thoroughly understood. Thorough understanding of the physical, biological and chemical aspects of biofilms will lead to better understanding of the mechanisms behind biofilm formation (Shi and Zhu, 2009). In order to understand mechanisms of biofilm formation, the above mentioned aspects of biofilms need to be monitored. Better understanding of biofilms will facilitate the development of cleaning strategies and inhibition biofilm development and growth (Shi and Zhu, 2009).

This review has discussed methods which can be used to monitor biofilms. Very few of the discussed methods can be implemented in industry where they are needed the most. Collaborative efforts between industry and scientists can lead to development and implementation of monitoring devices that are suitable for industry use. The methods have to be effective, cheap, simple, real-time, and non-destructive to biofilm monitoring.

2.2 Literature Review on Electro-chemically Activated Water

2.2.1 The concept of electrochemical activation

Electrochemical activation of water was discovered in Russia by V. Bakhir in 1974. The raw materials for electro-chemically activated (ECA) water are water and saline (Marais and Brözel, 1999). The "activation" of the water occurs in an electrolytic cell where a dilute saline solution is passed through an electrolytic cell (figure 2). The cell contains a cathode and an anode which are separated by a diaphragm (Ayebah and Hung, 2005). During the process of activation electric current is passed through the entire volume of the liquid in the electrolytic cell (Ayebah and Hung, 2005). The process of electrochemical activation is a complex of electrochemical and electrophysical forces acting on water, and the ions and molecules of dissolved substances (Tomilov, 2002; Kirkpatrick, 2009). This process causes the salt solution to dissociate and transform into a metastable state (active state) via an electrochemical unipolar action at the cathode and anode in the electrochemical cell (Solovyeva and Dummer, 2000). The metastable state of the ECA solutions results in changes in viscosity, diffusivity, heat conduction, electrical conductivity, catalytic activity, oxidation-reduction (ORP or REDOX) balance and surface tension (Bakhir, 1999; Kirkpatrick, 2009). The ECA solutions remain in the metastable state for about 48 hours after production before they return to the inactive stable state (Marais and Williams, 2001). Two types of products are produced in this ECA process: the Anolyte and Catholyte which have oxidative and reductive properties respectively. Simplified reactions that occur at the anode and cathode chambers of the electrochemical cell are shown below (Bakhir, 1999; Kirkpatrick, 2009).

Anode

Oxidation of water: $2H_2O - 4e^- \rightarrow 4H^+ + O_2$

Formation of gaseous chlorine: $2Cl^- - 2e^- \rightarrow Cl_2$

Formation of highly active oxidants that include Cl_2O, ClO_2, ClO-, HClO, Cl-, O_2^-, O_3, HO_2, OH^-

Cathode

Reduction of water at the cathode: $2H_2O + 2e^- \rightarrow H_2 + 2OH^-$

Formation of highly active reductants that include OH^-, $H_3^-O_2^-$, H_2, HO_2^-, HO_2^-, O_2^-

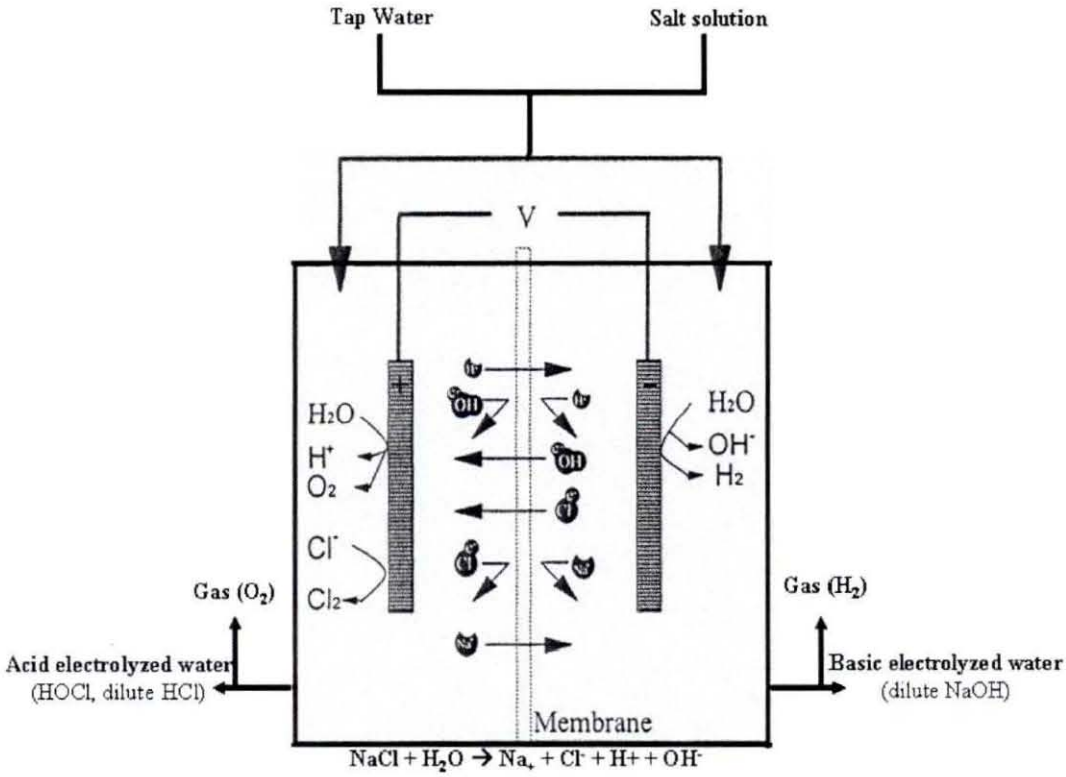

Figure 2.2: Electrolytic cell diagram (Al-haq *et al.*, 2005).

2.2.2 The Design of the ECA Device

The first technical devices used to carry out electrochemical activation of water were developed between 1974 and 1975 (Tomilov, 2002). After more than 20 years of research an electrochemical cell called the "flow-through electrochemical modular" (FEM) cell (figure 3) was created (Tomilov, 2002). The FEM cell is usually 210mm long and 16mm in diameter (Kirkpatrick, 2009). It consists of an anode and a cathode which are both made from titanium (Solovyeva and Dummer, 2000). The anode is a solid cylinder with special coating which fits coaxially inside a hollow cylinder. The cathode also has special coating (Solovyeva and Dummer, 2000). The two electrodes are separated from each other by a ceramic membrane (Solovyeva and Dummer, 2000) which has a low filtration capacity.

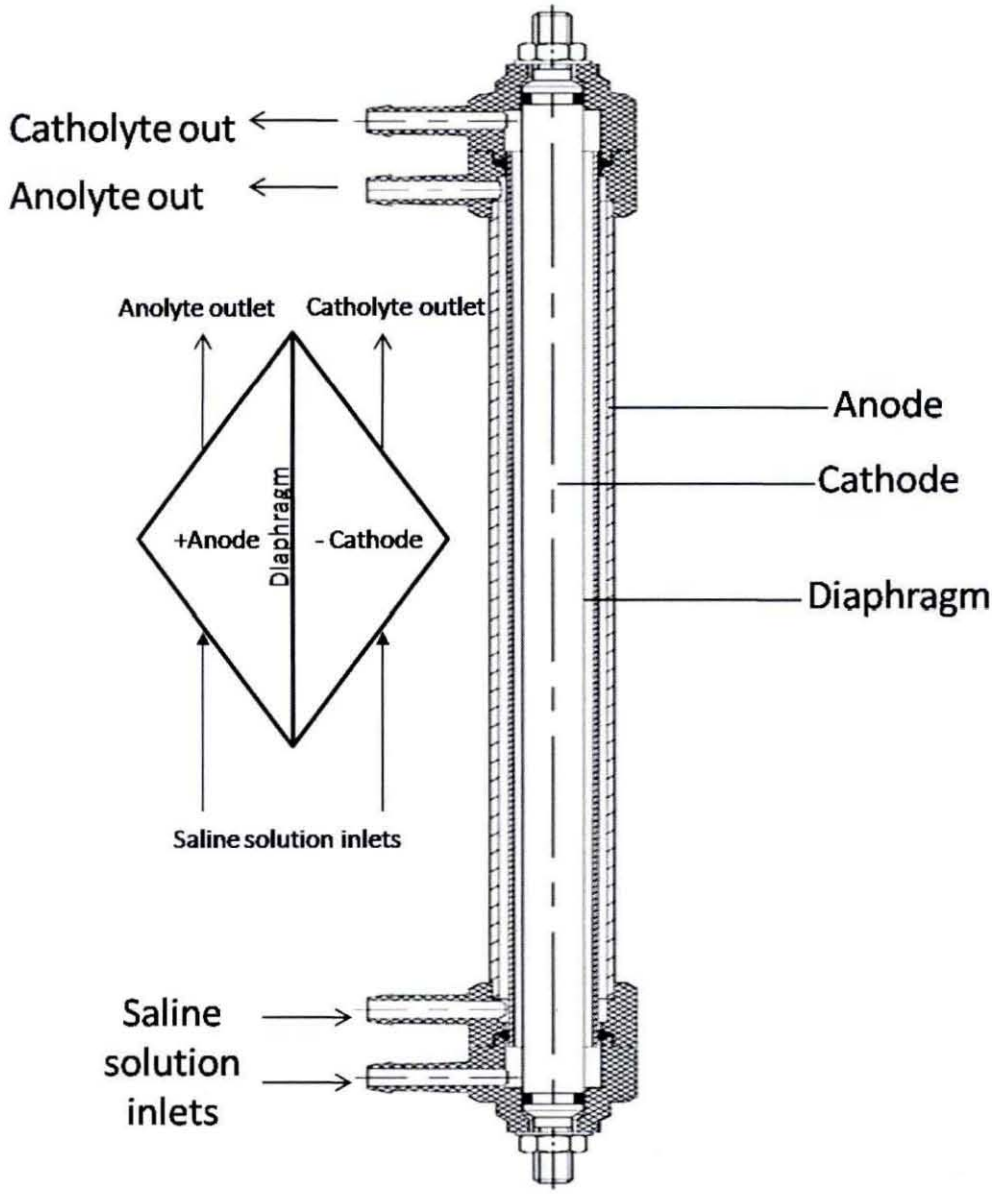

Figure 2.3: Diagram of the FEM cell (Adopted from: Bakhir and Zadorozhny , 1997).

The low filtration capacity of the membrane prevents solutions at the anode and cathode from mixing (Kirkpatrick, 2009). Electrochemical activation of the anode and the cathode occurs via activation at the diaphragm electrolyzer (Solovyeva and Dummer, 2000). Electrochemical activation results in the production of ECA solutions. To date more complex prototypes FEM cells have been created (Boyle *et al.*, 2010). Currently there are different ECA devices that are used for different applications including: devices for generating liquid

and gaseous chlorine e.g. AQUACHLOR-type; devices for generation electrochemically activated mixed oxidants solutions e.g. STEL-type and devices for the treatment of drinking water e.g. EMERALD-type (Bakhir, 1999; Kirkpatrick, 2009).

2.2.3 Classes of products produced during ECA of water

Three classes of products are produced in the process of electrochemical activation of water: stable products; highly reactive unstable products and quasi-stable structures (Cloete and Brözel, 2003).

2.2.3.1 Stable products

Stable products that are produced are acids and bases in the Anolyte and Catholyte respectively (Cloete and Brözel, 2003). The acids and bases influence the pH of the Anolyte and Catholyte as well as other active species in the ECA water (Cloete and Brözel, 2003).

2.2.3.2 Highly reactive unstable products

Highly reactive unstable products that are produced include active ion specie and free radicals (Cloete and Brözel, 2003). The function of these products is to enhance the oxidation-reduction potential (ORP) of the Anolyte (Cloete and Brözel, 2003). These products have a lifetime of less than 48 hours (Cloete and Brözel, 2003).

2.2.3.3 Quasi-stable products

Quasi-stable products are formed near or at the surface of the electrode; this is as a result of the extremely high voltage drop in those regions (Cloete and Brözel, 2003). They are structural complexes around ions, molecules, radicals and ions and they enhance diffusion, catalytic and biocatalytic properties of water (Cloete and Brözel, 2003).

2.2.4 Types of ECA solutions that can be produced

There are five types of ECA solutions that can be produced. These five solutions are different from each other by physiochemical properties due to different technological processes used to generate those (Bakhir *et al.*, 2003). Each solution has specific pH values, ranges of oxidation-reduction potential (ORP) and active species (Kirkpatrick, 2009). The five solutions are: acidic Anolyte, neutral Anolyte, neutral-alkaline Anolyte, alkaline Catholyte and neutral Catholyte.

2.2.4.1 Products of Anolyte

Anolyte is an electrolyzed solution produced in the anode chamber. The Anolyte has a strong oxidizing potential (+400 to + 1200 millivolts) and is said to have antimicrobial properties (Marais, 2000; Ayebah and Hung, 2005). Anolyte can be produced to be acidic, neutral or neutral-alkaline (pH 2 to 9) (Marais, 2000; Kirkpatrick, 2009).

Acidic Anolyte (A)

Acidic Anolyte is a highly oxidizing solution with pH values <5.0 and REDOX values ≥ +1200mV (Kirkpatrick, 2009). Acidic Anolyte is produced when there is a high quantity of mineralisation (> 5g/l NaCl) and where no Catholyte is fed back or recirculated into the anode chamber (Kirkpatrick, 2009). The active species in the acidic Anolyte are Cl_2> HClO > HCl > HO^-_2 in the order of oxidizing potential (Kirkpatrick, 2009).

Neutral Anolyte (AN)

Neutral Anolyte has pH ranges between 5.0 and 7.0 and REDOX values between +700 and +800mV (Kirkpatrick, 2009). To generate neutral Anolyte some catholyte must be recirculated into the anode chamber and the mineralization is usually low (< 3g/l NaCl) (Kirkpatrick, 2009). The species produced are highly reactive and there is a predominance of unstable radical species (Kirkpatrick, 2009). The active species in the neutral Anolyte are HClO > ClO_2, ClO^-, O_3 > HO^-, HO^-_2, HO^-_2, H_2O_2, $^1O_2$, Cl^-, $HClO_2$, HO^-, O^- in the order of oxidizing potential (Kirkpatrick, 2009).

Neutral-alkaline Anolyte (ANK)

Neutral-alkaline Anolyte has pH ranges between 7.2 and 8.2 and REDOX values between +259 and 800mV (Kirkpatrick, 2009). To generate neutral-alkaline Anolyte the majority of Catholyte produced must be recirculated into the anode chamber (Kirkpatrick, 2009). Neutral-alkaline Anolyte has similar properties to neutral Anolyte; however it has a shorter activation period compared to neutral Anolyte (Kirkpatrick, 2009). The active species are ClO^- > HClO > HO^-_2, HO^-, H_2O_2, $^1O_2$, Cl^- in the order of oxidizing potential (Kirkpatrick, 2009).

2.2.4.2 Products of Catholyte

Catholyte is a reducing solution (-80 to -900 millivolts) produced at the cathode chamber. Catholyte solution can be alkaline or neutral (pH 7 to 12) (Kirkpatrick, 2009). Catholyte is said to have strong cleaning or detergent properties (Marais, 2000).

Alkaline Catholyte (K)

Alkaline Catholyte is a highly reducing solution with pH values > 9.0 and REDOX values between -700 and 820 mV (Kirkpatrick, 2009). Activation periods of alkaline Catholyte are significantly shorter than those of Anolyte solutions (Kirkpatrick, 2009). The active species are NaOH, O^{-}_{2}, HO^{-}_{2}, HO^{-}_{2}, OH^{-}, OH^{-}, HO_2^{-}, O^{2-}_{2} (Kirkpatrick, 2009).

Neutral Catholyte (KN)

Neutral Catholyte has pH values < 9.0 and REDOX values between -300 and -500mV (Kirkpatrick, 2009). The active species are O^{-}_{2}, HO^{-}_{2}, HO^{-}_{2}, H_2O_2, H^{-} and OH^{-} (Prilutsky and Bakhir, 1997; Kirkpatrick, 2009).

2.2.5 Antimicrobial Properties of ECA water

A number of studies have been conducted and they have indicated the efficacy of ECA water as antimicrobials on a variety of microorganisms (Table 2). The antimicrobial activity of ECA water is not yet fully understood (Suzuki and Watanabe, 2000; Al-haq *et al.*, 2005). There are theories that explain the antimicrobial activity of ECA water. Some researchers suggest that the antimicrobial activity of ECA water is due to the high ORP of the ECA water (Huang *et al.*, 2008). It has been suggested that the high ORP could cause metabolic instability in microbial cells and changes in ATP production in cells possibly due to change in electron flow in cell (Huang *et al.*, 2008). Other researchers have suggested that the low pH of ECA water may sensitize the outer membrane of cells to allow entry of HOCl (McPherson, 1993; Huang *et al.*, 2008). HOCL is one of the active compounds of ECA water and is said to be the most active of all chlorine compounds (Huang *et al.*, 2008). HOCl inactivates cells by inhibiting carbohydrate metabolism, disrupting protein synthesis, disrupting reactions of nucleic acids, inducing DNA lesions and creating chromosomal abnormalities (Marriott and Gravani, 2006; Huang *et al.*, 2008). Although there are theories that explain the efficacy of ECA water as an antimicrobial, it has been firmly established that ECA water has strong bactericidal and virucidal properties and moderate fungicidal properties (Al-haq *et al.*, 2005). It is worth noting that different researchers have different

names for ECA water including: Acid Oxidizing Water (AOW), Acidic Electrolyzed Water (AEW), Electrolyzed Oxidizing Water (EOW), Redox water, Sterilox water, Superoxide water, Strong Ionized Water, Electrolyzed Strong Acid Water (ESAW), Electrolyzed Strong Acid Aqueous Solution (ESAAS), Electronically generated chlorine water, Electrolyzed NaCl solution, Aqua oxidation water, Chlor-aqueous Solution, Aqua oxidation water (Al-haq *et al.*, 2005).

Table 2.2: Studies indicating antimicrobial efficacy of ECA water against different microorganisms (Al-haq *et al.*, 2005; Huang *et al.*, 2008)

Microorganism	**Reference**
Bacteria	
Listeria monocytogens	Fabrizio and Cutter, 2003; Park *et al.*, 2004
Salmonella typhimurium	Fabrizio and Cutter, 2003
Staphylococcus aureus	Park *et al.*, 2002b; Suzuki *et al.*, 2002
Streptococcus	Hotta *et al.*, 1994
Bacillus cereus	Len *et al.*, 2000; Sakashita *et al.*, 2002; Vorobjeva *et al.*, 2003
Enterobacter aerogenes	Park *et al.*, 2002b
Campylobacter jenuni	Park *et al.*, 2002a
Escherichia coli 0157:H7	Len *et al.*, 2000; Park *et al.*, 2004;
Mycobacterium tuberculosis	Iwasawa and Nakamura, 1993
Vibrio parahaemolyticus	Huang *et al.*, 2006; Kimura *et al.*, 2006
Xanthomonas	Lazarovits *et al.*, 2004
Fungi	
Fusarium	Grech and Rijkenberg, 1992
Aspergillus	Buck *et al.*, 2002; Suzuki *et al.*, 2002
Botrytis	Buck *et al.*, 2002
Penicillium	Buck *et al.*, 2002
Phytophthora	Grech and Rijkenberg, 1992
Botryosphaeria	Buck *et al.*, 2002
Cladosporium	Buck *et al.*, 2002

Virus	
Human immunodeficiency virus (HIV)	Kakimoto *et al.*, 1997; Morita *et al.*, 2000; Kitano *et al.*, 2003
Hepatitis B virus (HBV)	Morita *et al.*, 2000; Tawaga *et al.*, 2000; Sakurai *et al.*, 2003
Hepatitis C virus (HCV)	Morita *et al.*, 2000; Tawaga *et al.*, 2000; Sakurai *et al.*, 2003

2.2.6 Advantages and disadvantages of ECA technology

There are a number of reasons why ECA water is a better disinfectant compared to other conventional disinfectants such as chlorine. ECA water can be produced on-site using simple electrolysis using water and salt solution; this indicates that there are less adverse environmental effects from the production ECA water (Kim et al., 2000; Al-haq et al., 2005). On-site production reduces costs involved in handling, transportation and storage (Al-haq *et al.*, 2005). Operation costs of electrolysis apparatus are minimal (Bonde *et al.*, 1999; Al-haq *et al.*, 2005). ECA water can be modified during production to reduce the amount of chlorine concentration; which reduces health concerns concerning disinfection by-products produced from chlorinated compounds (Kim et al., 2000; Al-haq *et al.*, 2005). ECA solutions revert back to ordinary water after use which indicates that minimal harmful gases such as chlorine are produced (Bonde *et al.*, 1999; Al-haq *et al.*, 2005). There have been claims by researchers that state that microorganisms do not acquire resistance to ECA water (Al-haq et al., 2005). ECA technology is not a thermal technology, therefore it does not result in changes in ingredients, texture, flavour and scent which are usually brought about by thermal treatment (Yoshida 2003; Al-haq *et al.*, 2005). The cytotoxicity of ECA water is far less than that of conventional disinfectants (Cloete, 2003; Al-haq *et al.*, 2005). Thantsha and Cloete (2006) have indicated that not only is ECA water effective in inactivating sessile microbes but also biofilms.

Despite the advantages there are disadvantages to the use of ECA water. The major disadvantage of ECA water is that it loses its antimicrobial activity if the solutions are not being continuously supplied with the active compounds such as H^+, HOCl and Cl_2 by means of electrolysis (Kiura et al., 2002: Huang et al., 2008). The initial cost of purchasing and setting up ECA water equipment may be very high (Al-haq *et al.*, 2005; Hricova *et al.*, 2008).

There have been reports of corrosion on certain metals and synthetic resin degradation due to the strong acidity and free chlorine content of ECA water (Huang et al., 2008). Chlorine gas is emitted during the production of ECA water which necessitates the use of extractor fans (Huang et al., 2008).

2.2.7 Effect of pH and ORP on antimicrobial activity of ECA solutions

Many researchers believe that the antimicrobial activity of ECA water is quantitatively correlated to pH and ORP (Al-haq *et al.*, 2005). Therefore accurate monitoring and recording of pH and ORP during disinfection processes is important. There are probes with pH and ORP sensors that can monitor and track these parameters during disinfection (Suslow, 2004)

2.2.7.1 ORP

ORP is the potential at which oxidation occurs at the anode and reduction occurs at the cathode" (Suslow, 2004). ORP therefore reflects the antimicrobial potential of a solution. Monitoring of ORP can provide "real-time" monitoring and recording of ECA water disinfection potential (Suslow, 2004). This will provide the operator with rapid assessment of disinfection potential of ECA water (Suslow, 2004). Higher ORP levels of solutions have been associated with high antimicrobial strength which makes it desirable to keep ORP levels high (Cloete *et al.*, 2009).

2.2.7.2 pH

Chlorine species that are found in ECA water include HOCl, Cl_2 and OCl^- (Hricova *et al.*, 2008). HOCl is the most active of the chlorine species (Huang *et al.*, 2008). Len *et al.* (2000) have indicated that the value of pH determines the equilibrium of HOCl and OCl^- in solution ECA water (Yang *et al.*, 2003). At pH 6.0 to 9.0 the dominant chlorine species in ECA water are HOCl and OCl^- (Len *et al.*, 2002). When pH is slightly reduced and is between 4.0 and 5.0 HOCl constitutes more than 97% of the total chlorine in ECA water (Park *et al.*, 2004). At more acid pH levels, (lower than 4.0) Cl_2 is the most dominant, whilst OCl^- dominates at higher pH levels (higher than 5.0) in ECA water (Park *et al.*, 2004; Hricova *et al.*, 2008).

High ORP and low pH act in synergy with HOCl to inactivate microorganisms (Hricova *et al.*, 2008). It would be ideal to have high ORP and low pH where HOCl

dominates to ensure the disinfectant functions optimally. Research conducted has indicated that light, temperature and agitation also influence antimicrobial activity of ECA water (Hricova *et al.*, 2008). Exposing ECA water to harsh light increases the rate of decomposition of chlorine and reduces antimicrobial activity of ECA water (Al-haq *et al.*, 2005). To maintain high antimicrobial activity levels it would be ideal to keep ECA solutions in dark environments to lower the rate of ECA solution decomposition (Al-had *et al.*, 2005). Storing of ECA solutions in cool temperatures is said to stabilize the residual chlorine and ORP of the solution (Fabrizio *et al.*, 2002; Hricova *et al.*, 2008).

2.2.8Applications of ECA water

2.2.8.1 Food applications

Studies have been conducted on the practical applications of ECA water in the food industry. These studies include the use of ECA water to disinfect food processing surfaces, inactivation of pathogens from fruits, vegetables, seafood, meat and poultry (Huang *et al.*, 2008; Hricova *et al.*, 2008). Huang *et al.* (2008) and Hricova *et al.* (2008) have compiled extensive reviews about the applications of ECA water in the food industry.

Studies on the use of ECA water in food processing surfaces have been conducted (Huang *et al.*, 2008). Microbial contamination of food processing surfaces resulting in food spoilage and transmission of diseases has been extensively reported (Abrishami *et al.*, 1994; Helke *et al.*, 1995; Blackman and Frank, 1996; Zhao *et al.*, 1998; Al-haq *et al.*, 2005). Therefore an effective sanitizer that will reduce and eliminate microbes is vital. ECA water has been reported to be effective in the removal of food-borne pathogens from cutting boards (Venkitanarayanan *et al.*, 1999; Huang *et al.*, 2008). There have also been reports on the use of ECA water in pipelines of a milking system (Walker *et al.*, 2005a, 2005b; Huang *et al.*, 2008). ECA water was used in a clean-in-place (CIP) set up and it great efficacy in removing bacteria from the milking system pipelines. Lin and Su (2006) reported the effectiveness of ECA water on the removal of *Listeria monocytogens* from reusable and disposable gloves (Hricova *et al.*, 2008). ECA water has also been reported to be effective in reducing *Enterobacter aerogenes* from surfaces of glass, stainless steel, glazed ceramic tile, unglazed ceramic tile and vitreous china; which are commonly used surfaces in the food industry (Park *et al.*, 2002b; Huang *et al.*, 2008).

The market for fruits and vegetables has undergone rapid growth within the food industry (Rico *et al.*, 2008). Postharvest decay of fruits and vegetables is a common problem and causes economic loss in industry. Disinfection and decontamination are vital and inevitable steps in ensuring safety and extending shelf-life of food (Rico *et al.*, 2008). ECA water has been reported to be effective in inactivating pathogens on fruits and vegetables. Izumi (1999) reported that ECA water was effective for disinfecting fresh-cut carrots, bell peppers, spinach, potatoes, Japanese radish and tomatoes (Hricova *et al.*, 2008; Huang *et al.*, 2008). Disinfection was achieved by dipping, rinsing or blowing the vegetables with ECA water. It was also reported that there was no visible effect on the physical appearance of the vegetables.(Izumi, 1999; Huang *et al.*, 2008). Park *et al.* (2001) reported the effectiveness of ECA water on reducing bacteria on lettuce. Park *et al.* (2001) obtained similar to results to Izumi (1999) where they also reported that there was no change in the general appearance and no discolouration occurred on the lettuce (Huang *et al.*, 2008). Al-haq *et al.* (2001) reported that ECA water can be used as an alternative to liquid sterilants that are used to sterilize the surfaces of fruits with smooth surface such as peaches (Huang *et al.*, 2008). Different results were obtained on strawberries; although there was a reduction of bacteria, the reduction was less compared to that obtained in fruits with smooth surfaces (Huang *et al.*, 2008). These results were attributed to the uneven and complex surface structure of the strawberries (Koseki *et al.*, 2004; Huang *et al.*, 2008). The above mentioned studies indicate that the efficacy of ECA water depends on the surface structure of the fruits being treated (Huang *et al.*, 2008).

Bacteria on the surface of eggs due to faecal contamination in the nesting place can serve as a vehicle for transmission of human pathogens (Huang *et al.*, 2008). Russell (2003) reported that ECA water was effective in completely eliminating *Listeria monocytogens*, *Salmonella typhimurium,* and *Staphylococcus aureus* on eggshells (Huang *et al.*, 2008). Fabrizio *et al.* (2002) reported that treatment of chicken carcasses with ECA water was effective in inactivating aerobic bacteria, total coliforms, *Escherichia coli* and *Salmonella typhimurium* (Hricova *et al.*, 2008). Hinton *et al.* (2007) suggested that ECA treatment of poultry extended their shelf life of refrigeration (Hricova *et al.*, 2008). Kruger and Buys (1998) reported that ECA water was effective in reducing bacteria on beef carcasses (Thantsha, 2002).

Studies have been conducted on the use of ECA water for inactivating bacteria in raw seafood have been reported (Huang *et al.*, 2008). Treatment of raw salmon with ECA water was found to be effective in inactivating *Escherichia coli* and *Listeria monocytogens* (Ozer and Demirci, 2006; Huang *et al.*, 2008). The treatment of tuna with ECA water and CO gas resulted in prolonged shelf life during refrigeration and frozen storage (Huang *et al.*, 2006; Huang *et al.*, 2008). ECA water was effective in reducing the amount of *Vibrio parahaemolyticus* and *Vibrio vulnificus* from oysters (Ren and Su, 2006; Hricova *et al.*, 2008).

2.2.8.2 Medical applications

ECA water is widely used for disinfection purposes in the medical field. Selkon *et al.* (1999) conducted a study on the efficacy of ECA water as a disinfectant of endoscopes. Studies were conducted on a variety of bacteria, bacterial spores, fungi and viruses. ECA water was shown to be effective as a disinfectant of endoscopes against mentioned variety of microbes. 99.999% reduction in microbes was reported after exposing endoscopes to ECA water for two minutes or less.

Microorganisms are responsible for a large percentage of root canal infections (Varghese *et al.*, 2010). Endodontic treatment is ideal and aims to eradicate all microorganisms in the root canal; thus preventing infections of the root canal (Varghese *et al.*, 2010). Hata *et al.* (1996) reported that ECA water was effective as a root canal irrigant during endodontic treatment (Marais and Williams, 2001).

ECA solutions are reportedly being used for the treatment of topical disease conditions, wounds and chronic ulcers (Selkon, 2007; Kirkpatrick, 2009). Selkon (2007) patented a wound therapy system called Vashe™ (Kirkpatrick, 2009). Vashe™ is an ECA water based technology (Kirkpatrick, 2009). Vashe™ is said to be a safe, non-invasive, easy-to-use, wound management process that promotes healing by reducing wound bioburden and promoting tissue repair (Kirkpatrick, 2009).

2.2.9 Conclusions

The process of electrochemical activation (ECA) leads to the formation of a detergent called Catholyte and a disinfectant called Anolyte. ECA water can be produced on-site using simple electrolysis using water and salt solution and there are less adverse environmental effects from the production ECA water. The products of ECA have proven to be highly effective against microbe. They are currently being used in the food and beverage industry, agriculture and in medical industry.

2.3 Literature Review on Trihalomethanes (THMs)

Since its first application in 1908 (Pavón *et al.*, 2008), chlorination has been the most widely applied water disinfection system. Low cost, high oxidizing potential, low residual levels and protection against microbial contamination are factors which have contributed to the extensive use of chlorine and its compounds (Sadiq and Rodrssiguez, 2004; Gopal *et al.*, 2007). A significant disadvantage of chlorination is the production of disinfection by-products (DBPs). DBPs are products of a reaction between chlorine and dissolved organic matter (DOM) (Sun *et al.*, 2009). Over 300 types of DPBs have been identified (Becher, 1999; Gopal *et al.*, 2007). The most commonly studied DBPs have been categorized into three classes by the United States Environmental Protection Agency (USEPA) in 1999 (Mbonimpa, 2007) (Table 3). This review will focus on the most common DBPs; which are trihalomethanes.

Trihalomethanes (THMs) are halogenated organic by-products of chlorination (Mbonimpa, 2007). THMs were first identified by Rook as DBPs in chlorinated water in the 1970s (Rook, 1974; Serrano and Gallego, 2007). Chloroform ($CHCl_3$), bromoform ($CHBr_3$), bromodichloromethane ($CHCl_2Br$) and dibromochloromethane ($CHBr_2Cl$) are four species of THMs (Golfinopoulos *et al.*, 1998). The process of the formation of trihalomethanes is indicated in Figure 2.4. Epidemiological studies have linked THMs to bladder and renal cancer (Craun, 1991; Simpson and Hayes, 1998; Golfinopoulos, 2000), colon cancer (Itoh and Matsuoka, 1996; Gopal *et al.*, 2007), adverse reproductive outcomes, neurotoxicity, nephrotoxixity (Table 2.4)

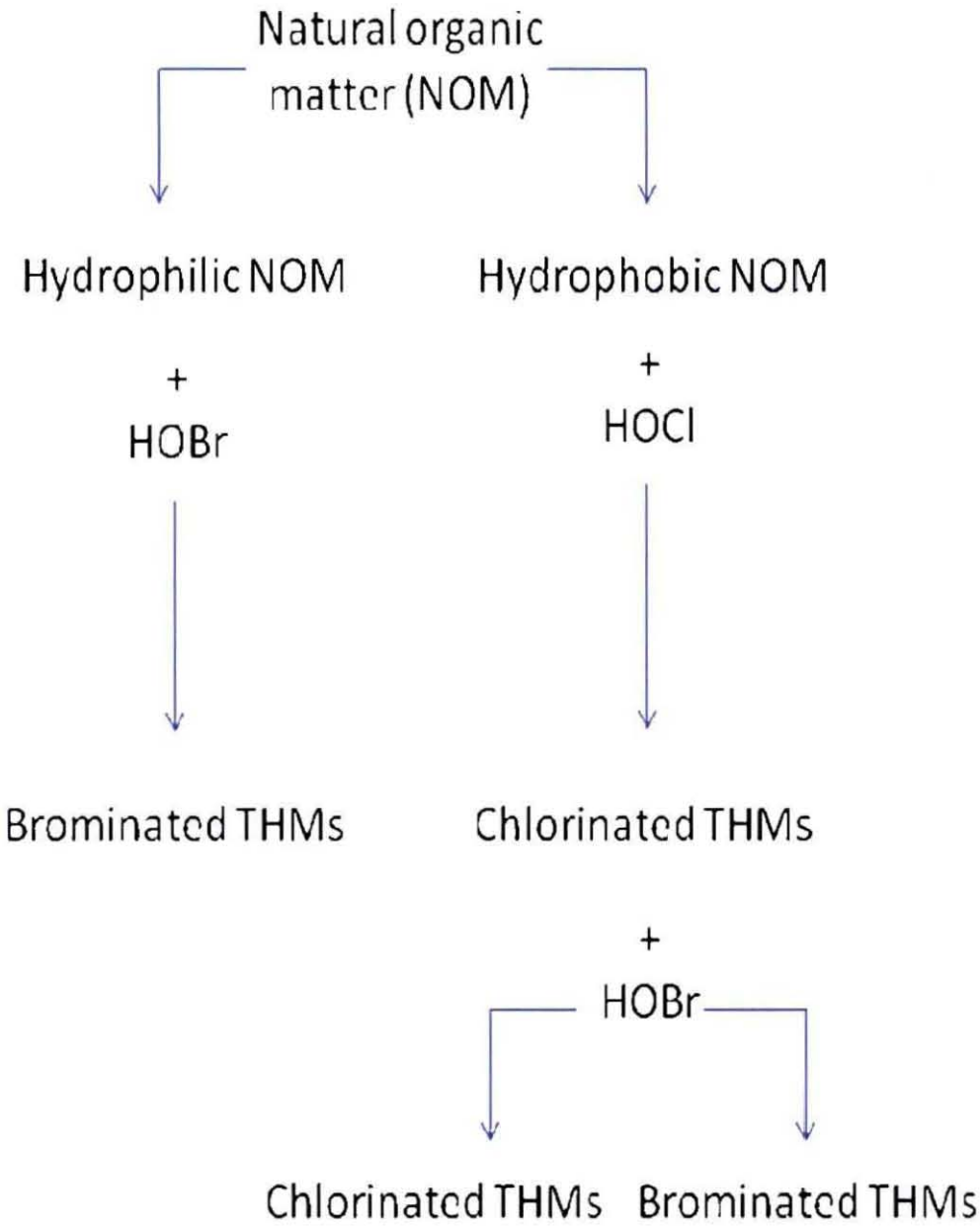

Figure 2.4: The process of trihalomethanes formation (Adopted from Chowdhury, 2009).

Table 2.3: Disinfection By-Products adapted from USEPA (Mbonimpa, 2007).

Halogenated Organic by-Products	**Organic Oxygenated by-Products**	**Inorganic by-Products**
Trihalomethanes • Chloroform • Bromodichloromethane • Dibromochloromethane • Bromoform Haloacetic acids • Monochloroacetic acid • Dichloroacetic acid • Trichloroacetic acid • Dibromoacetic acid Haloacetonitriles • Dichloroacetonitrile • Bromochloroacetonitrile • Dibromoacetonitrile • Trichloroacetonitrile Haloketones • 1,1-Dichloropropanone • 1,1,1-Trichloropropanone Chlorophenols • 2-Chlorophenol • 2,4-Dichlorophenol • 2,4,6-Trichlorophenol Chloropicin Chloral hydrate Cyanogen chloride Organic chloramines MX (3-Chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone	Aldehydes • Formaldehyde (methanol) • Acetaldehyde (43thanol) • Pyruvaldehyde (oxopropanal) • Other aliphatic aldehydes Carboxylic acids • Acetic acid • Other aliphatic monocarboxylic acids • Oxalic (ethanedioic) acid Ketoacids • Glyoxilic (oxoethanoic) acid • Pyruvic (oxopropanoic) acid • Ketomalonic (oxoprpanedioic) acid	Chlorate ion Chlorite ion Bromate ion

Table 2.4: Health effects associated with Trihalomethanes (Waller *et al.*, 1998; Chen *et al.*, 2004; Gopal *et al.*, 2007; Richardson *et al.*, 2007).

Compound	Health effects
Chloroform	Carcinogenic, hepatoxic, increased risk of spontaneous abortion and still birth
Dibromochloromethane	Neurotoxic, hepatoxic, increased risk of spontaneous abortion and still birth
Bromodichloromethane	Hepatoxic and nephrotoxic, increased risk of spontaneous abortion and still birth
Bromoform	Carcinogenic, increased risk of spontaneous abortion and still birth

Concerns over adverse health effects caused by trihalomethanes have lead to several countries setting up maximum contaminant levels (MCL) of THMs in finished drinking water in order to regulate the amount of THM exposure to human beings (Gopal *et al.*, 2007). In 1979 the Environmental Protection Agency (EPA) in the USA initiated a regulatory standard for THMs to be at 100µg/l (Gopal *et al.*, 2007). Similarly the European Communities (EC) also set their standard drinking water to contain THMs at a maximum level of 100µg/l; however level of THMs is currently under review (Boorman *et al.*, 1999; Gopal *et al.*, 2007). The World Health Organization (WHO) has also set up guidelines for MCL of the four species of THMs in drinking water (Table 5).

Table 2.5: World Health Organization guidelines for maximum contaminant levels of the four species of THMs in drinking water (Richardson, 2005)

Trihalomethanes	**WHO guideline value (µg/l)**
Chloroform	200
Dibromochloromethane	100
Bromodichloromethane	60
Bromoform	100

2.3.1 Factors affecting THMs formation

According to Amy *et al.* (1987) factors that affect THM formation include chlorine dose, concentration, organic matter, reaction time, pH, temperature of the water and the occurrence and concentration of bromide ions (Gopal *et al.*, 2007).

2.3.1.1 Organic matter

Organic matter has long been recognized as a factor in the formation of THMs during water treatment using chlorine (Liang and Singer, 2003; Rodrigues *et al.*, 2007). Studies have shown the effects that characteristics of organic matter such as size, structure, functionality and aromacity have on the formation of THMs (Liang and Singer, 2003; Rodrigues *et al.*, 2007). Reckhow *et al.* (1990) showed that halogenated DBP formation including THMs increases with the activated (electron-rich) aromatic content of organic matter (Liang and Singer, 2003). UV absorbance at 254 nm (SUVA254) is used as an indicator for characterizing organic matter (Parsons *et al.*, 2007). The ability to characterize organic matter can be used as a guideline to predict the DBP formation potential of water because SUVA254 correlates well with the aromatic content of organic matter (Chin *et al.*, 1994; Croué *et al.*, 1999; Liang and Singer, 2003)

Aromatic carbon content = SUVA254 x 100 / [DOC] **equation 1**

Edzwald and Tobiason (1999) have developed guidelines using SUVA254 to determine the nature of organic matter (Table 2.6) (Parsons *et al.*, 2007).

Table 2.6: Guidelines for the nature of organic matter based on SUVA254 (Edzwald and Tobiason, 1999; Parsons *et al.*, 2007).

SUVA254 (L/m-mg C)	Composition of organic matter in water
> 4	High hydrophobicity, high molecular weight
2 - 4	Mixture of hydrophobic and hydrophilic organic matter, intermediate molecular weight
< 2	Low hydrophobicity, low molecular weight

The distribution of hydrophobic and hydrophilic organic material also has an influence on the formation of THMs in chlorinated water (Liang and Singer, 2003). Hydrophobic organic material is rich in aromatic material (Liang and Singer, 2003). Liang and Singer (2003) established that hydrophobic fractions of organic matter were more significant precursors of THMs compared to hydrophilic fractions. Similar results were obtained by Leenheer and Croué (2003). Other researchers obtained different results; for example results obtained by Marhaba *et al.* (2006) and Croué *et al.* (1999) suggest that hydrophobic fractions of organic matter are major precursors of THMs compared to hydrophilic fractions. Differences between results obtained might be attributed to differences in the nature of water sources used. The nature and distribution of hydrophobic and hydrophilic materials differ depending on the source of the water and the biogeochemical processes involved in the recycling of carbon within terrestrial and aquatic systems (Liang and Singer, 2003)

2.3.1.2 pH

Studies have indicated that there is a correlation between pH and THM formation (Chowdhury and Champagne, 2008). THM formation occurs by means of base-catalysed hydrolysis of reactive functional groups (Sohn *et al.*, 2006). Increasing pH will lead to an increase in base compounds. This leads to an increase in base-catalysed hydrolysis of functional groups, which ultimately retults in an increase in THM formation (Sohn *et al.*, 2006). Several studies have reported that a significant increase in THM formation with an increase in pH (Stevens *et al.*, 1976; Oliver and Lawrence, 1979; Kim *et al.*, 2002; Chowdhury and Champagne, 2008). Oliver and Lawrence (1979) indicated that there was an increase of between 30% and 50% when the pH of solution was increased from pH 7 to pH 11 (Chowdhury and Champagne, 2008). The high pH values create an alkaline environment; this causes to fast chlorination and fast substitution and oxidation of organic matter, eventually leading to an increase in the formation of THMs (Garcia and Moreno, 2006). When pH is ≤ 6, the acidic environment causes the THM formation pathway to become too slow to form end products of THMs; only intermediates are formed.

pH has an effect on the fate of chlorine that ends up as THMs during chlorination (Navalon *et al.*, 2008). Navalon *et al.* (2008) indicated that pH 8 represents the maximum on the percentage of chlorine that forms THMs. pH lower or higher than 8 leads to lower

chlorine forming THMs i.e. disfavours chlorine reaction pathways involved in THM formation (Navalon *et al.*, 2008).

pH has different effects on species of THMs that form during chlorination. Although there is an overall increase in the amount of THMs when pH is increased; there are different effects on the four species of THMs. Rathbun (1996) indicated that of the four species of THM, the species that are most affected by an increase in pH are brominated THMs. Similar results were observed by Lange and Kawczynski (1978). Bromide incorporation at high pH occurs due to the higher activation energy of HOBr reaction compared to HOCl reaction (Sohn *et al.*, 2006).

2.3.1.3 Reaction/ Contact Time

According to Lou and Chang (1994) reaction time is one of the dominant factors that affect the formation and concentration of THMs of a system that has undergone chlorination. Pourmoghaddas (1995) found that 70% of THMs were formed within 6 hours of reaction time for varied experimental conditions that they used. They also noticed that no significant differences were observed between 48 and 168 hours in terms of the THMs that formed. This trend has also been confirmed by other researchers (USEPA, 1981; Pourmoghaddas, 1995).

2.3.1.4 Chorine dose

Studies have indicated that an increase in chlorine dose leads to an increase in the amount of THMs produced (Amy *et al.*, 1987; Rathbun, 1996; Garcia and Moreno, 2006; Rodriguez *et al.*, 2007). The increased chlorine dosage provokes more halogenations and opening of aromatic structures of the still available organic matter (Garcia and Moreno, 2006). The substitution and oxidation of organic matter structures by chlorine leads to an increase in the formation of THMs (Garcia and Moreno, 2006).

A point is reached where equilibrium of DBP formation is reached regardless of the increase in chlorine dose (Fleischacker and Randtke, 1983; Bougeard, 2009). Fleischacker and Randtke, (1983) indicated that after 96 hours of contact time, a dose less than 6mg free chlorine/ mg dissolved organic carbon (DOC), THMs form in proportion to increasing chlorine. However a dose greater than 6mg free chlorine/ mg DOC, slight additional THMs form as free chlorine increases (Fleischacker and Randtke, 1983; Bougeard, 2009). It is however thought that it is the nature of the organic matter that determines the effect that

chlorine has on THM formation; different types of organic matter are affected differently by chlorine dosage (Bougeard, 2009).

2.3.1.5 Bromide ions

During chlorination, the bromide ion found in natural water gets oxidised by chlorine to form a weak acid called hypobromous acid (HOBr). HOBr then reacts with organic materials to form brominated species of THMs (Rodriguez *et al.*, 2004; Pavelic *et al.*, 2005; Sorlini and Collivignarelli, 2005; Serrano and Gallego, 2007). Waters containing significant concentrations of bromide have an abundance of brominated species of THMs (bromodichloromethane, dibromochloromethane and bromoform) and they represent 60-80% of total THM; however, if waters contain negligible amount of bromide, then chloroform will be the main THM and it will represent 60-80% of the total THM (Serrano and Gallego, 2007). A higher concentration of bromide in a disinfection solution results in a higher concentration of brominated THM, and associated with a lower concentration of chloroform (Rodrigues *et al.*, 2007). The shift towards brominated THMs is attributed to the formation of HOBr, which is a more effective halogen substituting agent compared to HOCl (Sohn *et al.*, 2006).

2.3.1.6 Temperature

Using varied temperature conditions (3, 25 and 40°C) at constant pH (7), Stevens *et al.* (1976) established that THMs increased by between 1.5 to 2 times with each stage of temperature change (Chowdhury and Champagne, 2008). Similar results were obtained by El-Shahat *et al.* (2001) and Hellur-Grossman *et al.* (2001) where comparative studies on THM concentrations in winter and summer months showed an increase in THM concentration during summer months (Chowdhury and Champagne, 2008). Engerholm and Amy (1983) estimated that every increase of 10°C is associated with an increase between 25 and50% in THM concentration (Chowdhury and Champagne, 2008).

2.3.2 Methods of detecting THMs

Adverse health effects associated with THMs have also lead to the need for simple, fast and reliable analytical methods for their determination (Serrano and Gallego, 2007). The development and optimisation of reliable, sensitive, rapid and simple analytical methods is essential for proper monitoring of THMs in water distribution systems (Pavon et al, 2008). This will lead to better understanding of THM formation and their removal from water

distribution systems; their removal will lead to the reduction in their associated health risks (Pavon et al, 2008). THMs are mainly detected using gas chromatography (GC) followed by electron capture detection (ECD) or mass spectroscopy (MS) (Pavon et al, 2008). The concentration of THMs is measured in the order of ng/l to µg/l (Pavon et al, 2008).

Samples have to be prepared prior to analysis. The step of sample analysis separates compounds of interest from the surrounding medium (Pavon et al, 2008). These compounds are then preconcentrated to improve selectivity, sensitivity, reliability, accuracy and reproducibility of analysis (Hyötylänen and Riekkola, 2004; Pavon et al, 2008). An ideal sample preparation method should be fast, very selective and use low amounts of solvents and reagents (Somenath and Mitra, 2003; Demeestere *et al.*, 2007; Hyötylänen and Riekkola, 2008; Pavon et al, 2008). Currently the trend in analytical chemistry is to use "green chemistry", i.e. using minimal solvent or using solvent free sample preparation methods (Pavon et al, 2008). Such methods include microextraction, membrane extraction and headspace techniques (Pavon et al, 2008). Other methods include liquid-liquid extraction and direct aqueous extraction (Pavon et al, 2008).

Following sample preparation, the samples are separated using chromatography (Pavon et al, 2008). Chromatographic columns are used for separation of samples prior to determining the THMs samples using detectors (Pavon et al, 2008). Chromatographic separation is achieved by separating the components of a mixture between two phases; a stationary phase and a mobile phase (Scott, 2003). The stationary phase, where the sample is placed consists of a column coated or bonded with particular matter (Scott, 2003). The mobile phase can either be gas or liquid. The mobile phase passes through the column under pressure and it elutes the sample from the column and separates it into different components (Scott, 2003). The separation process is as a result of different forces between each molecular type and the stationary phase whereby each solute is retained to a different extent (Scott, 2003). The more weakly held will elute first and the more strongly held elute last (Scott, 2003). It usually takes approximately between 15 and 35 minutes to separate the four THMs by conventional gas chromatography (Pavon et al, 2008).

The eluted samples have to be detected for identification. Detector most widely used for THM analysis is mass spectrometers (MS) (Pavon *et al.*, 2008). The MS is a powerful detector that allows rapid qualitative identification of analytes (Pavon *et al.*, 2008). MS

separates analytes by comparing their mass spectra with those in a spectra library of known compounds (Pavon *et al.*, 2008). Other detectors include the electron capture detectors (ECD), pulsed discharge photoionization detector (PDPID), dry electrolytic conductivity detector (DELCD) and atomic emission detector (AED) (Pavon *et al.*, 2008). An extensive literature on sample preparation, sample separation and detection has been published by Pavon *et al.* (2008).

2.3.3 Conclusions

Chlorination has been shown to contribute to the formation of THMs during disinfection process. Depending on their concentration, THMs cause adverse health effects, and this has necessitated the development of regulatory measures on THMs. Factors such as pH, chlorine dose, temperature, organic matter, contact time and bromide ion concentration have an effect on THM formation and can be used to control the amount of THMs that can be formed. Considering the negative implications of disinfection by products, there is an opportunity for the development and application of alternative disinfectants.

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Chapter 3: Biofilm monitoring of Water Samples using the Rotoscope, Epifluoresence and Confocal Laser Scanning Microscopy

3.1 Introduction

Biofilms can be defined as "microorganisms immobilised in a heterogeneous matrix containing extracellular polymeric substances (EPS), water and absorbed substances, interspersed with interstitial voids" (Wolf *et al.*, 2002). A biofilm can be comprised of single and/or multiple species. Biofilm development involves four distinct phases: 1) initial reversible attachment, 2) irreversible attachment, 3) maturation, and 4) detachment (Figure 3.1) (Stoodley *et al.*, 2002).

During phase 1, cells attach to a surface. During this stage, attachment to the surface is reversible because the bacteria have a weak interaction with the surface, thus the bacteria must continue grow and produce a slime-like matrix called the extracellular polymeric substance (EPS); i.e. EPS provides structural support for the biofilm (Flemming *et al.*, 2000; Stoodley *et al.*, 2002). During phase 2 there is a transition from reversible to irreversible attachment; during this stage bacteria rigidly become "cemented" onto the surface, this is frequently mediated by the presence of EPS (Stoodley *et al.*, 2002). Phase 3 is the phase of biofilm maturation. At this phase the biofilm is a complex structure that has architecture of the biofilm is complex that consists of channels and pore; the bacteria also become redistributed away from the surface (Davies *et al.*, 1998; Stoodley *et al.*, 2002). Biofilm detachment occurs during Phase 4; this is where individual cells or groups of cells become released from the biofilm (Stoodley *et al.*, 2002).

Within a biofilm, microbes are able to have increased access to nutrients which become concentrated onto the substrata. The "flow system" created by the biofilm also facilitates an increased availability of dissolved nutrients. Microniches (e.g. anaerobic sites within an aerobic environment) provide a degree of protection to microbes within the biofilm from unfavourable environmental factors (Nivens *et al.*, 1995).

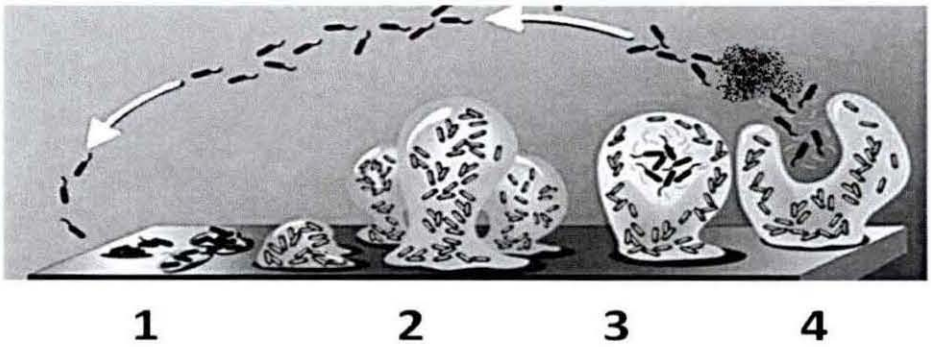

Figure 3.1: Diagram indicating the four stages of biofilm development (Stoodley *et al.*, 2002).

The growth of biofilms can cause undesired effects on surfaces such as heat exchangers, ship hulls, drinking and process water treatment, food processing, storage and distribution systems which result in huge economic losses (Flemming, 1996; Flemming, 2002a, 2002b; Flemming, 2003). Proper control of biofilm depends on information that is obtained from the biofilm via monitoring. Monitors that are currently being used to monitor biofilms include devices that monitor light reflectance and microscopy techniques (Flemming,2003).

Light reflectance assays are often used to monitor biofilm development. When light is transmitted onto a surface, the light that is reflected back will depend on deposits that are on the surface. The presence of a biofilm on a surface will absorb some of the light that is being transmitted onto the surface. This will result in less light being reflected. Flemming *et al.* (1998) designed a light reflection fiber optic sensor (FOS) which was used to monitor biofilms. The Rotoscope is an instrument that has also been used to monitor biofilm development. The Rotoscope was used by Maluleke (2006) as a real-time, on-line and non-destructive monitoring instrument for biofilms (Figure 3.2). The Rotoscope is a device that also uses the principle of light reflection. There is a light transmitter (Figure 3.3C) that transmits light onto the rotating disc (Figure 3.4A). There is a data logger (Figure 3.3B) that monitors light reflected back by the biofilm on the rotating disc. The data logger has a universal serial bus (USB) interface that is used to transfer data to a computer for viewing and analysis (Figure 3.3B). There is modified Peterson device (MPD) attached to the Rotoscope (Figure 3.2). The MPD has removable slides (Figure 3.4B) on which biofilm can grow and can be used for microscopy (Cloete and Maluleke, 2004).

Microscopy plays an important role in studies about microbial processes including the process of biofilm development (Wolf *et al.*, 2002). Microscopy has developed over the years and it now has the ability to be coupled with automated on-line digital image acquisition (Wolf *et al.*, 2002). This has contributed to the ability for researchers to acquire a more comprehensive and clearer picture about the development of biofilms. Other features such as biofilm function, speciation, bacteria interaction and quantification can now be easily studied using microscopy (Wolf *et al.*, 2002). CLSM and EM are microscopy techniques that make use of fluorescent probes. The fluorescent probes are designed to bind to biological macromolecules or specific localized structural regions (Claxton *et al.*, 2008). Fluorochromes can provide information on physiological activities and cellular structures of microbes and detect particular chemicals in microbes and genetic mapping (Claxton *et al.*, 2008). Both EM and CLSM are recommended for monitoring biofilm development because they are non-invasive and non-destructive to the biofilms (Wolf *et al.*, 2002; Xavier *et al.*, 2003). EM and CLSM can be used to study the characteristics of biofilms *in vivo* in their naturally hydrated form (Wolf *et al.*, 2002; Xavier *et al.*, 2003). The formation of biofilms can also be observed in detail by scanning the same area repeatedly (Xavier *et al.*, 2003).

The aim of this chapter was to monitor biofilm development from a mixed culture of bacillus and streptococci using the Rotoscope. Epifluoresence microscopy (EM) and confocal laser scanning microscopy (CLSM) were performed to confirm results obtained from light reflectance assays of the Rotoscope.

3.2 Materials and Methods

3.2.1 Light reflection assays using the Rotoscope

Two known Bacillus and Streptococci species cultures were used. Colonies were removed from Nutrient Agar plates and were placed into Nutrient Broth (NB). The NB with the cultures was incubated overnight at 37°C in a shaking incubator. Bacterial growth of the overnight culture was verified using a spectrometer at a wavelength of 620nm. The reading on the spectrometer was 0.9; this indicated that the bacteria had grown to a satisfactory level. 10ml of the overnight culture was placed into 100ml of nutrient broth. This mixture was then placed into 20l of tap water in the sample tank of the Rotoscope (Figure 3.2). This was circulated through the Rotoscope for three consecutive days. As the water circulated through the Rotoscope it passed through the rotating disc which rotates mechanically. Biofilm

growing on the rotating disc was monitored by a light reflection sensor which was set to take readings at one hour intervals. Light reflection (mV) was plotted against time (hr) in a graph using MS Excel (2007).

3.2.2 Epifluourescence microscopy and confocal laser scanning microscopy

The slides were removed from the MPD after 24 h, 48 h and 76 h. Slides were placed in Ringers solution (Merck) to preserve the biofilm growth prior to epifluoresence microscopy (EM) and confocal laser scanning microscopy (CLSM). Microscope fields were randomly chosen on the slides. These were stained with Syto 9 (Invitrogen; excitation wavelength 480nm; emission wavelength 500nm). After staining samples were incubated in the dark for 15 minutes. Imaging was carried out using a Fluorescence microscope, Zeiss Axiovert 200, Germany microscope. The model of CLSM which was used for sample analysis was Zeiss LSM 510, Zeiss SMT, Jena Germany was used for image analysis. The CLSM system was equipped with a Plan-neofluar 40x/1.4 Oil DIC objective lens which was used for analysis. Zeiss LSM image browser (Zeiss SMT, Jena, Germany) was used to analyse images obtained from CLSM. 3-D images were generated using Zeiss LSM image browser (Zeiss SMT, Jena, Germany).

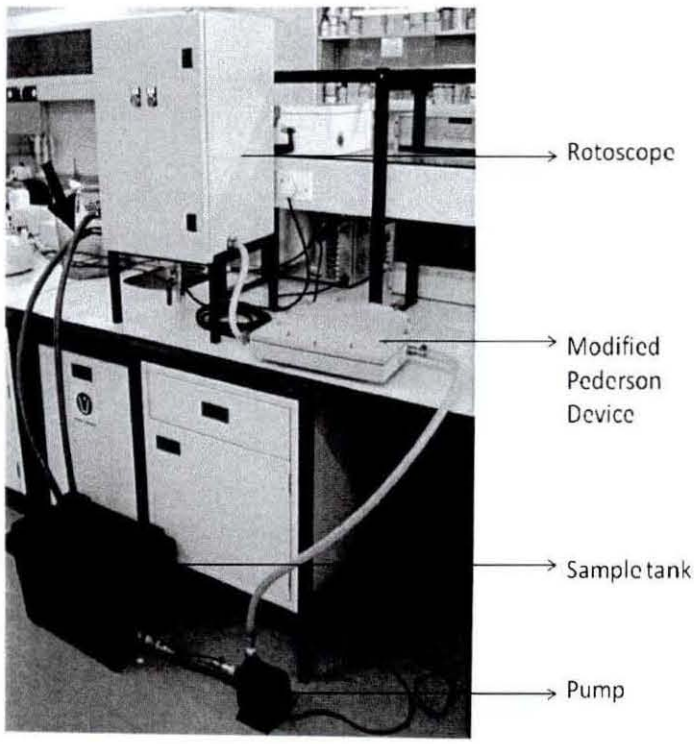

Figure 3.2: A diagram of the Rotoscope.

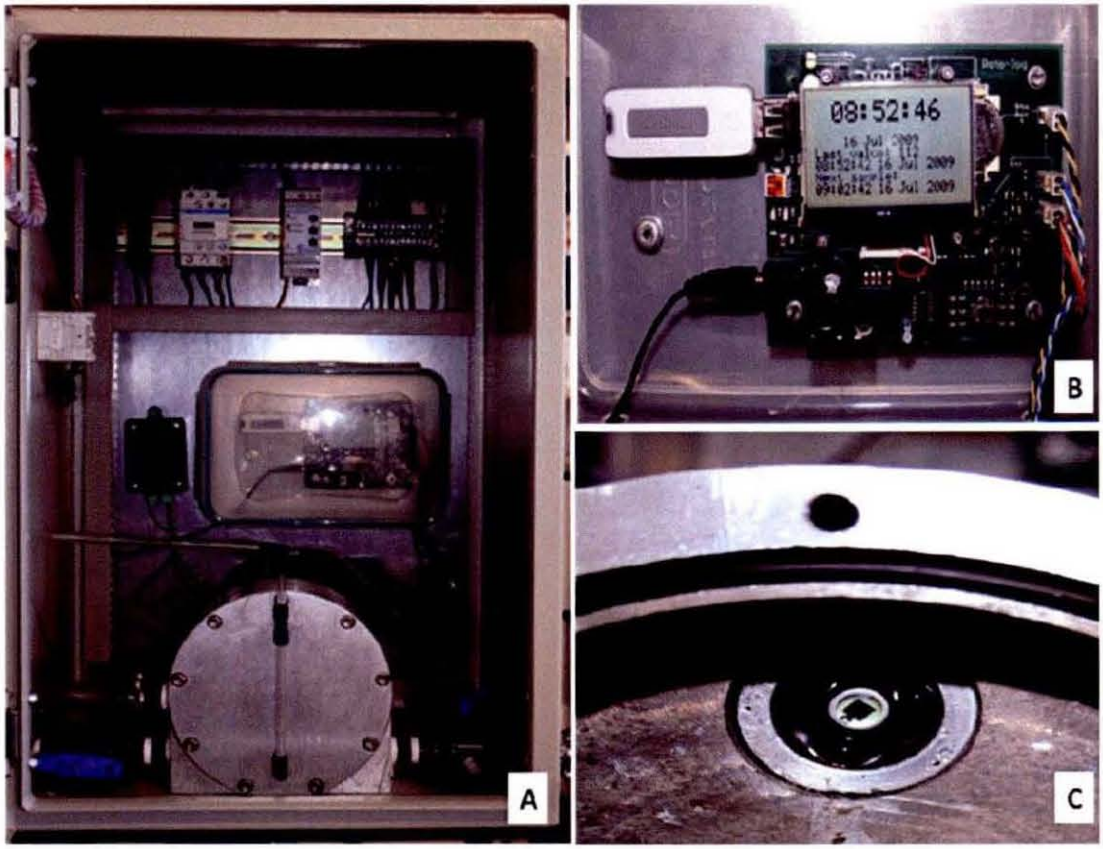

Figure 3.3: A: The interior of the Rotoscope. B: The data logger. C: The light transmitter.

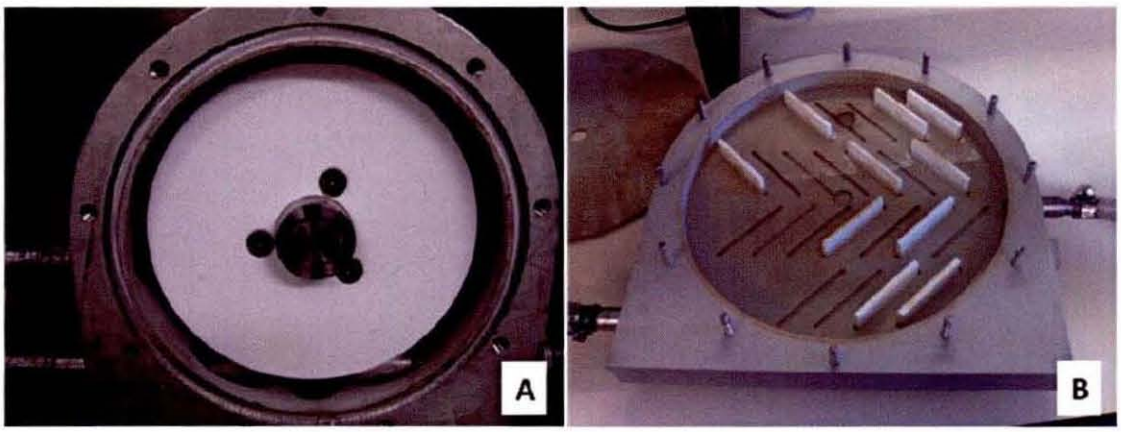

Figure 3.4: A: The rotating disc. B: The Modified Pederson Device (MPD) with removable slides.

3.3. Results and Discussion

3.3.1 Light reflectance assays from the Rotoscope

After three days the rotating disc was removed from the Rotoscope. Slime was visible on the rotating disc (Figure 3.5). This was an indication that attachment of bacterial cells occurred and they developed into biofilm on the rotating disc over the 76 h. Another indication of biofilm formation was shown by the data of light reflection (mV) over time (hr) (Figure 3.6). There was a decrease in light reflection over time; as clearly indicated by the linear trendline of the graph (Figure 3.6). Although the trendline indicated a decline in light reflection; the peaks on the graph of light reflection (mV) over time (hr) were irregular. This is because light reflectance readings were obtained from different places on the rotating disc; this is a clear indication that biofilm distribution is not uniform of a surface. According to Clement (2003), distribution of biofilm on surfaces is not uniform. Light reflectance changes according to the thickness of a biofilm. Areas with thick biofilm development will reflect less light than areas with thin biofilm development.

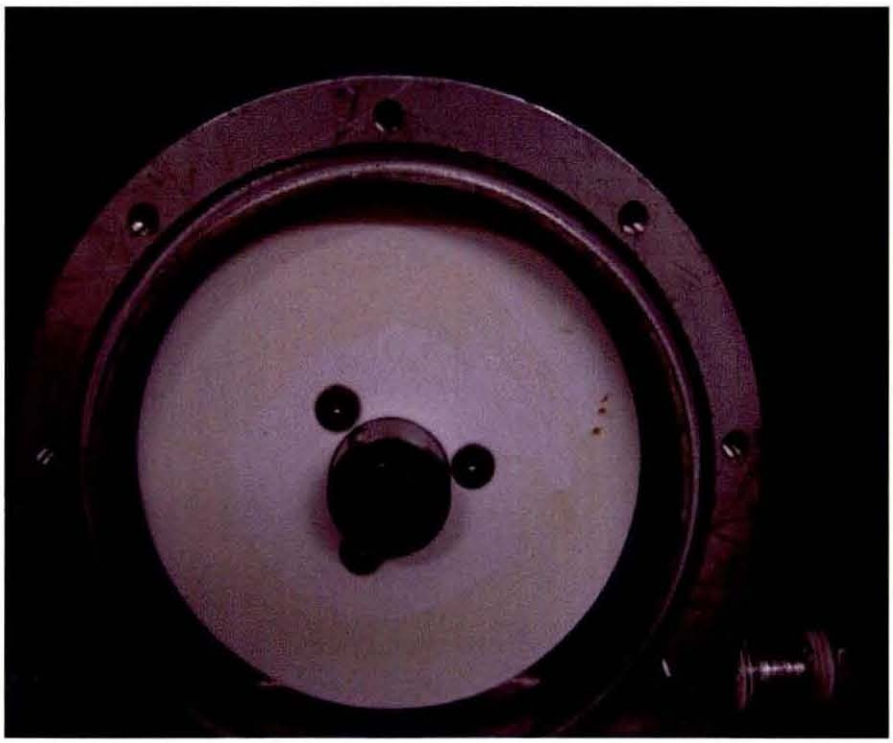

Figure 3.5: Slime on the rotating disc after three days of biofilm growth

The four distinct phases (Phases 1, 2, 3 and 4) of biofilm development that were evident from the light reflectance assay (Figure 3.6). During phase 1, light reflection decreased and increased shortly thereafter. This was an indication that there was initial attachment that occurred which was shortly followed by detachment. This can be interpreted as the reversible attachment phase of biofilm development. Attachment to the surface at this stage is reversible because the bacteria have a weak interaction with the surface. The bacteria must continue grow and produce EPS which will provide the structural support for the biofilm (Flemming *et al.*, 2000; Stoodley *et al.*, 2002). During phase 2 there was a sharp and steady decrease in light reflection over time. This suggested that the EPS had been produced by the bacteria and it provided structural support for the biofilm resulting in irreversible attachment of bacteria to the biofilm. During this phase the bacteria becomes cemented onto the surface and this is mediated by the presence of EPS (Stoodley *et al.*, 2002). It is also probable that the nutrient levels were still high enough to support the biofilm to develop into the irreversible stage. During phase 3, there was no apparent overall decrease in light reflection over time. This stage can be interpreted as the stage of biofilm maturity. At this stage the biofilm is said to have a stable yet complex architecture with channels and pores and the bacteria is also redistributed away from the surface (Davies *et al.*, 1998; Stoodley *et al.*, 2002; Kroupkamp *et al.*, 2010). This plateau stage of biofilm development is also known as the steady stage (Kroupkamp *et al.*, 2010). During stage 4, light reflection slightly decreased and shortly thereafter it increased. The increase in light reflection can be interpreted as the stage where detachment of cells occurs. At this stage individual cells or groups of cells are released from the biofilm (Stoodley *et al.*, 2002). This indicates that more light will was reflected as there was less biofilm absorbing the light. Depletion of nutrients may have lead to the stage of detachment. The process of detachment is physiologically regulated (Stoodley *et al.*, 2001; Stoodley *et al.*, 2002). It has been hypothesized by O'Toole *et al* (2000) that starvation may lead to detachment which allows bacteria to search for alternative nutrient-rich environments, (Stoodley *et al.*, 2002).

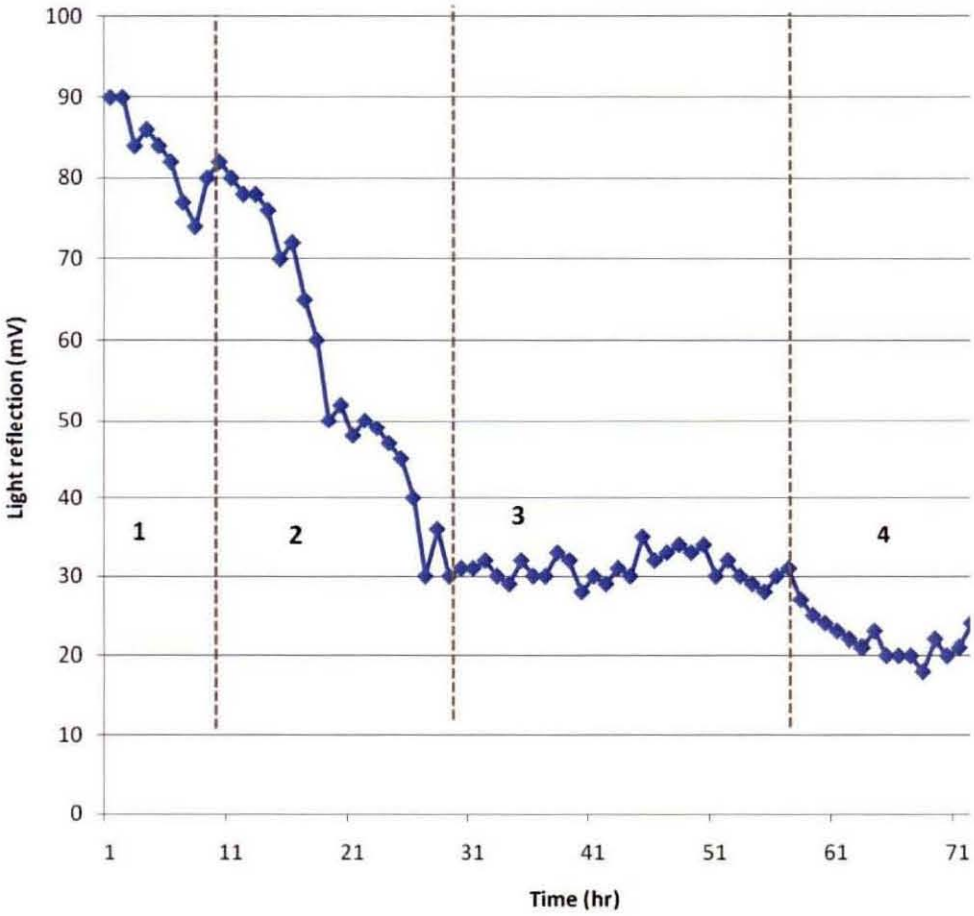

Figure 3.6: Effect of biofilm development on light reflection over a 76 h period. 1, 2, 4 and 4 indicate the four different stages of biofilm development.

Cloete and Maluleke (2005) and Tamachkiarow and Flemming (2003) also monitored biofilm development by using light reflection assays. Cloete and Maluleke (2005) used the Rotoscope to monitor biofilm development of a river sample. Tamachkiarow and Flemming (2003) used a FOD to monitor biofilm formation in a brewery pipeline system. Similar to the results obtained in this study the data of Cloete and Maluleke (2005) and Tamachkiarow and Flemming (2003) indicated that light reflectance does decrease over time as the biofilm develops. This is a clear indication that light reflection is a simple variable to interpret that can be used to monitor biofilm development. Overall the data obtained in this study indicated that the Rotoscope was sensitive to changes that occurred during biofilm development. It

also indicated that the Rotoscope can be used as a real-time, on-line and non-destructive monitor of biofilms.

3.3.2 Epifluourescence microscopy and confocal laser scanning microscopy

Although it has been indicated that the Rotoscope can be used as a real-time, on-line and non-destructive monitor of biofilms, it cannot distinguish between biotic and abiotic matter (Flemming, 2003). The reason why epifluoresence microscopy (EM) and confocal laser scanning microscopy (CLSM) analysis was to confirm that results obtained from light reflectance assays using the Rotoscope were indeed as a result of biofilm growth. Slime was visible in the MPD and on the removable slides (Figure 3.7 A and B).

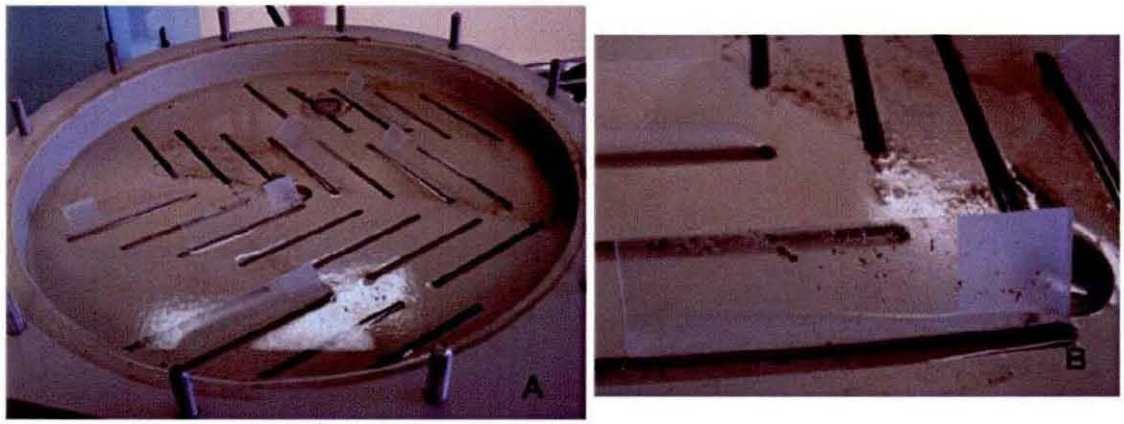

Figure 3.7: Slime in the MPD (A) and on the removable slides (B)

A cell permeable, nucleic acid binding dye Syto 9 was used to stain the removable slides prior to both EM and CLSM analysis. Once Syto 9 has bound to the nucleic acid of the bacteria in the biofilm it will stain the cells green. Once the stain is bound the samples can be viewed using EM and the CLSM. After 24 h a mass of cells had formed and EPS was also visible (Figures 3.8 A and 3.9 D) indicating that at this stage, irreversible attachment of biofilm to the removable slides had occurred. Development of biofilm did increase over time (Figures 3.8 B, C, D and Figure 3.9 A, B, C) as show by an increase in the volume of the biomass. It can be said that at this stage the biofilm had reached its maturation stage. The stage where the biomass detaches from the biofilm is easily visible in Figure 3.8 C. The obtained images from EM and CLSM are an indication that light reflectance data obtained from the Rotoscope was indeed due to biofilm formation.

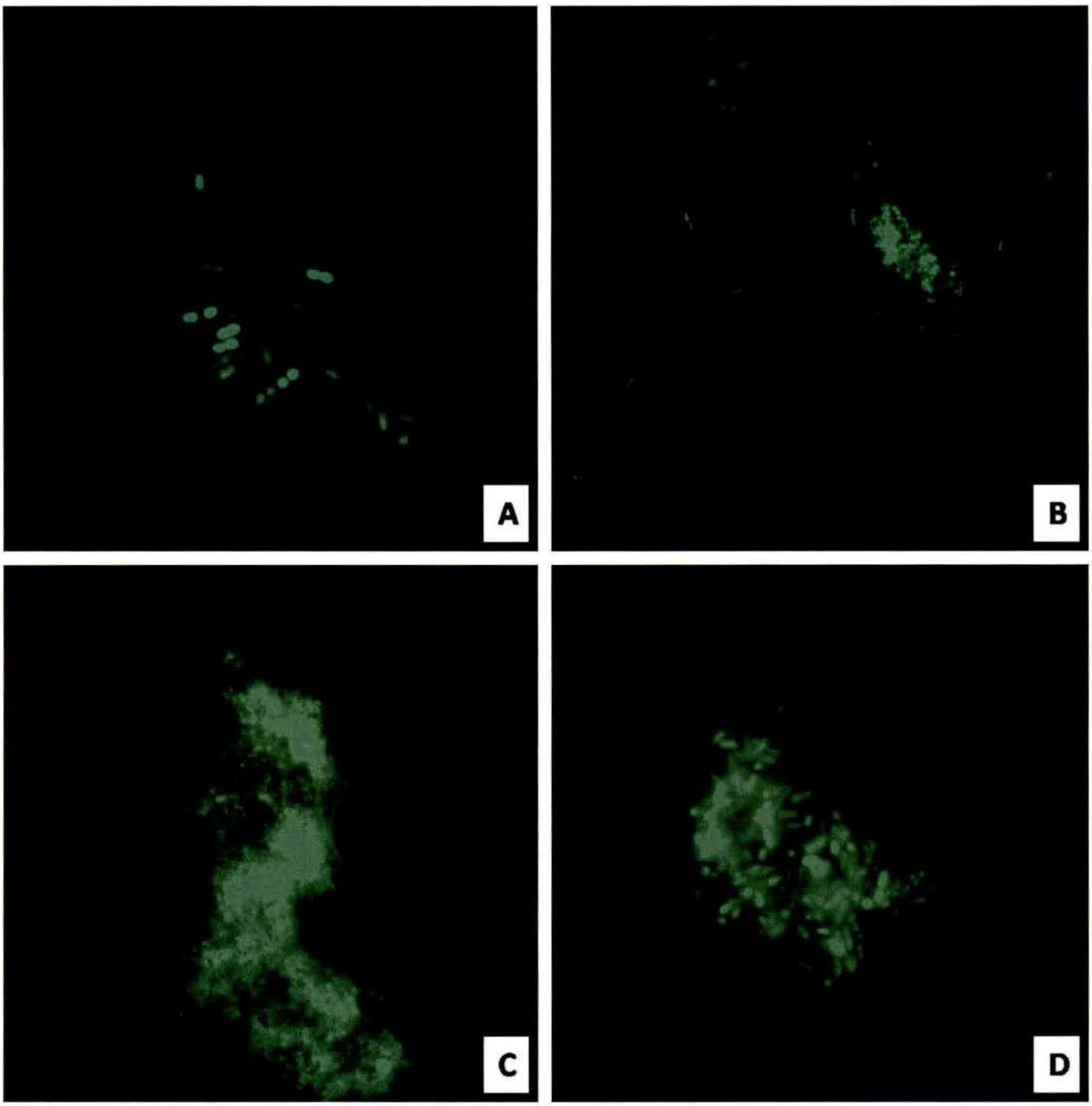

Figure 3.8: Micrographs of biofilm development obtained from epifluoresence microscopy. A: 24h, morphology of the bacteria; bacilli and cocci were easily visible; B: 48h and C and D: 76h old biofilm

Just like any method EM and CLSM have their advantages and disadvantages. When comparing EM to CLSM; EM was quicker when processing images compared to CLSM. EM processed images in a quarter of the time it took for CLSM to process images. The number of Z-stacks required per image to enhance the depth of focus for EM is less than that of CLSM, even at the same magnification; therefore it will take less time to acquire an image for EM compared to CLSM (Peighambardoust *et al.*, 2010). During the processing of images by CLSM photobleaching of samples occurred. Photobleaching is the photochemical damage of a fluorochrome which leads to the fluorochrome permanently losing the ability to fluoresce (Rost, 1990). Photobleaching of samples is a major setback that is associated with the slow scanning action of the CLSM. This problem of photobleaching has also been reported by Ono *et al.*, (2001) and Halbhuber and König, (2003).

Enhancement of image analysis using a computer is a powerful tool for analysis of micrographs. It enables automation of image analysis routines (Wolf *et al.*, 2002). In this study it was used in EM for morphological distinctions of the mixed culture (Figure 3.8A). The morphology of the cultures (cocci and bacilli shape of the bacteria) was easily visible from the EM micrographs (Figure 3.8). In CLSM computer enhanced image analysis was used to construct a three-dimensional view of the biofilm and to determine the depth of the biofilm (Figure 3.9). The 3D image (Figure 3.10) from the CLSM did show that indeed the distribution of biofilm on a surface is not uniform (Clement, 2003). The computer enhanced image analysis also provided depth of the biofilm; the depth of the biofilm after three days was found to be 146µm. Overall both EM and CLSM were satisfactory at demonstrating biofilm growth on the removable slides.

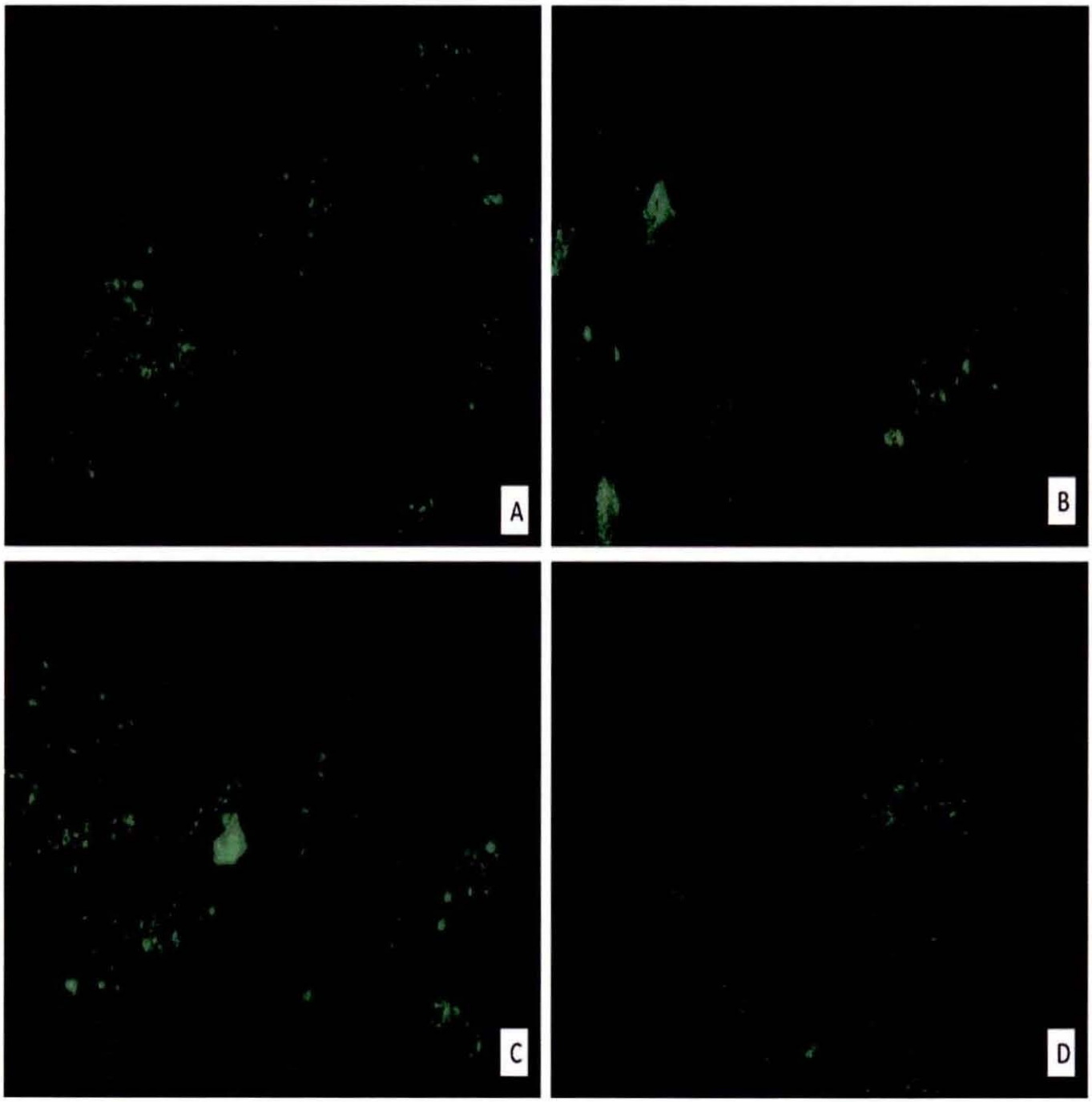

Figure 3.9: Micrographs from CLSM of biofilm development. A, B, C and D indicate the times at which analysis were conducted. A: 24h, B: 48h; C and D: 76h of biofilm formation.

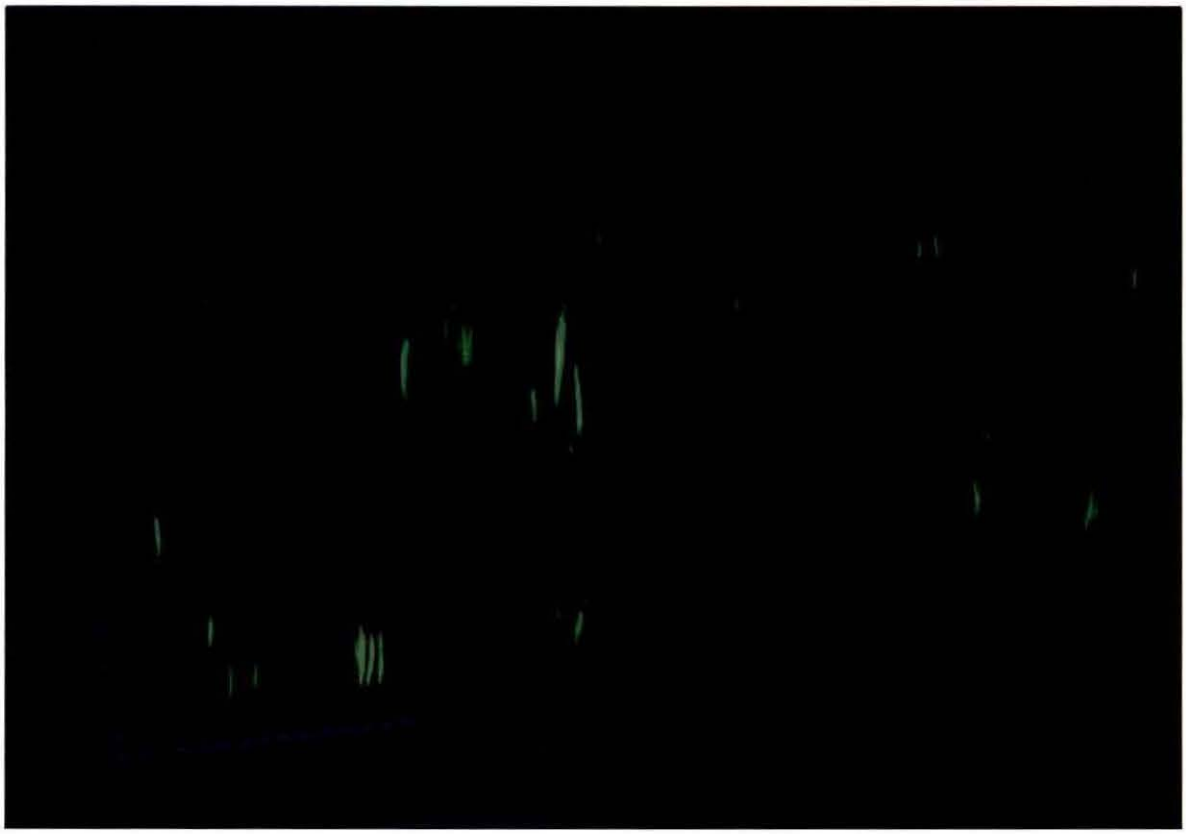

Figure 3.10: 3D structure of the biofilm obtained from CLSM using computer enhanced image analysis. Biofilm thickness was 146µm thick.

3.4 Conclusions

The Rotoscope has shown to be an easy, effective, on-line monitoring device for the development of biofilms. Light reflectance decreased over time as the biofilm developed. Information on the different stages of biofilm development could also be deduced from light reflectance assays of the Rotoscope. Microscopy validated results which were obtained from light reflectance assays. Both EM and CLSM provided insightful information about the development of the biofilm. Information on the morphology of the bacteria, depth of the biofilm as well as the different stages of biofilm development was deduced from EM and CLSM images.

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Chapter 4: Monitoring of the Efficacy of Electro-Chemically Activated Water in a Simulated Soft Drink CIP System

4.1 Introduction

Soft drinks are a category of non-alcoholic beverages that can either be carbonated or non-carbonated. Soft drinks have been developed and to date, they have diversified into products such as: purified, mineral and spring waters, flavoured or enhanced waters, colas, fruit flavoured sodas, sports and energy drinks, fruit juices, ready to drink (RTD) teas and coffees and milk-based drinks (The Coca-Cola Company, 2004; Short, 2005; Lawlor *et al.*, 2009). Of all beverages on the global market, soft drinks are the most dominant. According to Hirsheimer (1998), soft drinks ranked 1st among manufactured beverages; this was still the case a decade later (The beverage World, 2008). In 2005, the global revenue that soft drinks generated was USD$ 319.2 billion (Lai, 2007). Studies have indicated a forecast of positive growth in the revenue of soft drinks for the period of 2005 to 2010 (Datamonitor, 2006; Lai, 2007). To maintain such high sales, soft drink products always have to be of good quality and they must also be microbiologically safe. Such standards can only be obtained and maintained by having good hygiene practices. Typical beverage manufacturing scheme is indicated in Figure 4.1.

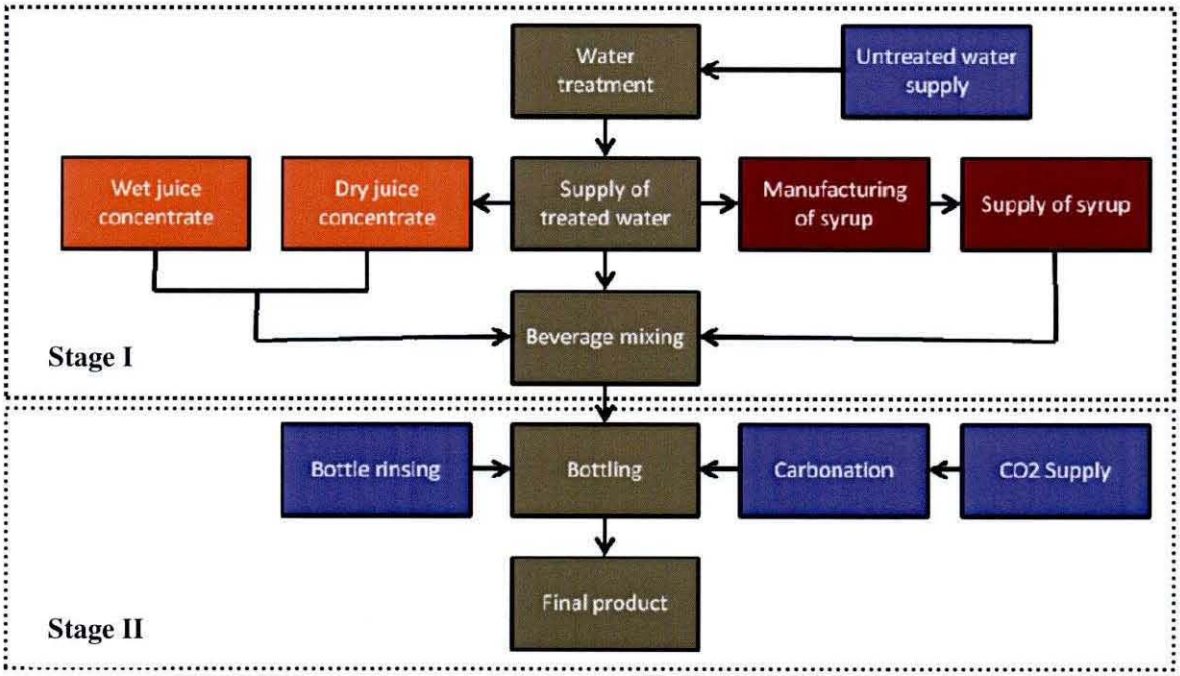

Figure 4.1: Scheme of soft beverage production (Ferreira *et al.*, 2009).

There are two main stages of beverage production; stage I is the liquid flavour (concentrate or syrup) preparation and stage II is liquid bottling (Ferreira *et al.*, 2009). Stage II occurs at filling lines. Filling lines consist of a conveyer belt machine that washes bottles and fills them with liquid flavour and carbonated or non-carbonated water, a sealer for capping the bottles and labeller for labelling the bottles (Ferreira *et al.*, 2009). Filling lines can be adjusted to give soft drinks of a given flavour in a given bottle size (Ferreira *et al.*, 2009) and shape.

Good hygiene practices are of utmost importance in the food industry. There is an abundance of exposed areas in processing environments. Microorganisms can use food residues in these exposed areas as culture systems to attach, grow and develop into biofilms if good hygiene is not practiced (Mattila-Sandholm and Wirtanen, 1992; Holah *et al.*, 1994; Sutherland, 1997; Willcock *et al.*, 2000). The formation of biofilms in food industry can have undesired and detrimental effects. The presence of microorganisms can lead to product contamination by spoilage and/or pathogenic microorganisms which ultimately result in reduced shelf-life and disease transmission (Chmielewski and Frank, 2003).

Biofilm formation in distribution systems leads to a reduction liquid velocity and pipe carrying capacity; congestion of piping, increased energy usage and an overall reduction in operation efficiency (Ridgeway and Olson, 1981; LeChevalier *et al.*, 1987; Kumar and Anand, 1998). In heat exchangers and cooling towers, development of biofilms leads to a reduction in heat transfer. This results in decreased operating efficiency of which food processing equipment (Lehmann *et al.*, 1992; Mattila-Sandholm and Wirtanen, 1992; Bott, 1992; Kumar and Anand, 1998). Biofilms that are composed of sulphate-reducing or acid-producing bacteria catalyze chemical and biological reactions which cause corrosion of metal surfaces (Costerton and Lappin-Scott, 1989; Kumar and Anand, 1998; Chmielewski and Frank, 2003). From the discussed effects that the presence of biofilms has in food processing environments, it is evident that their presence can lead to immense economic losses annually. In order to prevent such huge economic losses, hygienic conditions of equipment and processing surfaces should be controlled by applying cleansing and disinfection systems that will aid in the elimination and control of biofilms (Mosteller and Bishop, 1993; Wilcock *et al.*, 2000). Cleaning-in-place (CIP) is a vital unit-operation that is generally applied in the food industry (Van Asselt *et al.*, 2002) for cleaning and disinfection of systems.

According to the NDA Chemical Safety Code of 1985, CIP can be defined as "the cleaning of complete items of plant or pipeline circuits without dismantling or opening the equipment and with little or no manual involvement of the operator automated method of cleaning in unit-operation" (Palmowski *et al.* (2005). Early research on CIP occurred between the 1940s and the 1950s (Herreid and Luetscher, 1962). This was followed by a widespread interest CIP installation in the 1950s in dairy industry (Herreid and Luetscher, 1962); an industry where CIP originated (Chisti, 1999). A CIP system would typically include piping for circulating cleaning agents, tanks and reservoirs for cleaning solutions, spray heads and devices such as valves, sensors, gauges, recording devise that are used to manage flow of cleaning solutions (Chisti, 1999). From the above mentioned components of a CIP system, it is evident that to set up a CIP system requires a significant amount of capital investment. The long term economic benefits of CIP far outweigh the benefits of manual cleaning (Chisti, 1999). The use of CIP is said have many advantages compared to manual cleaning including: reduced downtime (absence of dismantling of equipment); elimination of product contamination (consistent cleaning) and improved Occupational Health and Safety (OH&S) (Chisti, 1999; DEH, 2003; Palmowski *et al.*, 2005).

A typical CIP cycle is characterized by the following sequence: product flush, pre-rinsing, main cleaning step, final rinsing, disinfection step and post rinsing (Romney, 1990a; Australian Standards, 2001; Palmowski *et al.*, 2005). There are several cleaning and disinfection agents that are used in the food industry. Sodium hydroxide or caustic soda (NaOH) and nitric acid are common detergents that are widely used in the food and beverage industry (Palmowski *et al.*, 2005). Chlorine based disinfectants are the most common biocides used in the food and beverage industry. Due to the negative environmental effects associated with the use of traditional CIP cleaning and disinfection agents, companies in the food and beverage industry are becoming interested in the use of cleaning and disinfection agents in CIP which have minimal environmental impact. Other factors that are taken into account when choosing cleaning and disinfection agents are low toxicological risk and good economical application (Orth, 1998). This interest has led to research being conducted to find alternative cleaning and disinfection agents with the above mentioned properties. One of the outcomes of the research has been the development of Electro-Chemical-Activation (ECA) of water.

ECA technology was developed in Russia in the early 1970s (Leonov, 1997; Bakhir, 1997; Marais and Williams, 2001); the Russians own over 300 patents from this technology (Marais and Williams, 2001). This technology was recently introduced to South Africa (Cloete, 2002). Radical Waters is a South African based company that is one of the leaders of ECA technology. During electro chemical activation of water, a dilute saline solution is "activated" by being passing through an electrolytic cell that has anodic and cathodic chambers which are separated by a permeable membrane (Cloete, 2002). The two main resulting products are called Anolyte and Catholyte. The Anolyte is an oxidizing agent with an antimicrobial effect and the Catholyte is a reducing agent with surfactant and anti-oxidizing effects (Cloete, 2002). Using ECA technology reduces average CIP contact time by 70% compared to conventional chemicals used in CIP (Radical Waters, 2010). In addition, substantial energy savings can be achieved because ECA chemicals are operational at ambient temperature (Radical Waters, 2010). Changing from conventional chemicals to ECA technology can lead to cost savings of 90% (Radical Waters, 2010). Anolyte and Catholyte can be reclaimed and re-used prior to discarding; this is based on the ORP value, the ORP value must be within the specified ranges for Catholyte and Anolyte (Radical Waters, 2010). There is a reduction in toxic effluent produced (Radical Waters, 2010).

The Anolyte has an ORP of between +400mV to +1200mV and the Catholyte has an ORP of -80mV to -900mV (Marais and Williams, 2001). ORP is an indication of a solution's reducing and oxidizing activity (Banhidi, 1999; Cloete *et al.*, 2009). A disinfectant (oxidizing agent), will draw electrons away from the cell membrane resulting in cell destabilization and leakage (Suslow, 2004). This will ultimately lead to metabolic inactivity or even cell death (Suslow, 2004). The reducing agent (Catholyte) has strong surfactant properties (Cloete and Brözel, 2003). Anolyte can be operated in acidic, neutral and alkaline form, with pH ranges of 2-9; whereas the Catholyte is an alkaline solution of pH 7-12 (Marais and Williams, 2001). Electric conductivity (EC) is the most accurate way of measuring dilution of ECA solutions (Kirkpatrick, 2009). EC is used to determine whether ECA solutions are still functioning at their optimal levels. From the above mentioned information it is apparent that monitoring ORP, pH and EC can give an indication of the efficacy of the Anolyte and Catholyte.

Various monitoring devices can be used to frequently and effectively monitor and manage the efficacy of a CIP system. Differential turbidity measurement (DTM) device (Klahre and Flemming, 2000; Janknecht and Melo, 2003) and the fiber optical device (FOS) (Tamachikiarow and Flemming, 2003; Janknecht and Melo, 2003) are some of the devices that are used to monitor fouling in industry. The Rotoscope is another device that can be used to monitor fouling. It was originally designed to monitor biofilm formation. The Rotoscope's operational principle is based on light reflection and absorption. Light reflection, whether reflected or dispersed, is one of the most popular parameters of monitoring deposits (Lewandowski and Beyenal; 2003). Light density changes as the thickness of deposits changes (Lewandowski and Beyenal; 2003). When light is emitted onto a reflective surface that has biofilm growth, there will be a reduced amount of light reflected depending on the biofilm thickness. The Rotoscope has a data logger that monitors that measures light reflected back by the biofilm on the disc. It is also equipped with a Peterson device that has removable slides on which biofilm also grows. These slides can be removed and viewed under a microscope.

The main aims of this chapter were to use the Rotoscope to simulate a mini production line as well as to monitor the development of fouling due to circulation of soft beverage products based on the principle of light reflection and the efficacy of the Anolyte as disinfectant and Catholyte as a detergent during CIP based on physico-chemical properties (pH, ORP and EC) of the solutions. Light microscopy was also used to determine the efficacy of ECA solutions during CIP.

4.2 Materials and Methods

4.2.1 Materials

Six products were supplied by ABI (Pretoria, South Africa): Minute Maid fruit juice (breakfast blend), Just juice (orange and breakfast blend), Nestle iced tea (lemon), Powerade syrup and coke syrup. Catholyte and Anolyte were supplied by Radical Waters (Pty.) Ltd. (Midrand, South Africa).

4.2.2 Monitoring of fouling and CIP using the Rotoscope

For each trial, samples were placed in the Rotoscope tank and circulated throughout the Rotoscope for at least 1hr but no more than 1.5hrs to emulate a mini soft drink (SD) production line. As the product circulated through the Rotoscope, it passed through a rotating disc. Behind the rotating disc was a light reflection monitor; that monitored light reflection and stored data in the Data Logger. The light reflection monitor was set to take readings at every five minutes.

Following SD product circulation, a 4-step CIP 20 min regime was conducted to clean the system. Firstly water was circulated through the Rotoscope for 1 min to flush product from the lines. Secondly a clean-up cycle was conducted to remove soil; it involved circulation of the Catholyte through the Rotoscope for 10 min. Thirdly a disinfection cycle followed; this involved the circulation of the Anolyte through the Rotoscope for 10 min. The fourth and final step involved circulation of water for 1 min through the Rotoscope to flush out any ECA solution that may be remaining in the lines.

4.2.3 Statistical analysis of light reflection data from the Rotoscope

Light reflection data that was collected by the data logger was analysed using regression analysis. Graphs were plotted for each of the 6 trials that were conducted. Regression analysis was used to measure the relationship between a dependent variable (light reflection) and the independent variable (time) during product circulation. The statistics were performed at 95% confidence. Light reflection data was also collected for CIP; however regression analysis was not performed. For each CIP trial an average value was calculated for the light reflection data that was obtained. These average light reflection values were compared with the final light reflection value obtained during product circulation. This was conducted to determine the efficacy of CIP based on light reflection. 5% Anolyte was circulated for 24hrs through the Rotoscope as a control.

4.2.4 Physico-chemical analysis of CIP by monitoring pH, EC and ORP

The efficacy of the Anolyte and Catholyte was analysed by monitoring the pH, EC and ORP before and after circulation through the Rotoscope. pH, EC and ORP were monitored using probes supplied by Radical Waters, (Pty.) Ltd. (Midrand, South Africa).

4.2.5 Microbial analysis after CIP

4.2.5.1Analysis of removable slides

Slides were removed from the MPD and were placed onto Nutrient Agar (NA) (Merck (Pty.) Ltd City, country) plates and Potato Dextrose Agar (PDA) (Merck (Pty.) Ltd, City, country) plates. The plates were incubated at 37°C for 24 hours.

4.2.5.2 Analysis of the final rinsing water effluent

Effluent from the final rinsing tap water was sampled. From the sampled water serial dilutions from 10^{-1} to 10^{-5} were made using Ringers solution (Merck (Pty.) Ltd, Johannesburg, South Africa). From each of the five serial dilutions, 100µl was removed were plated onto NA (Merck (Pty.) Ltd Johannesburg, South Africa) plates and PDA (Merck (Pty.) Ltd Johannesburg, South Africa) plates. The plates were incubated at 37°C for 24 hours.

4.2.6 Light Microscopy

Slides that were removed from the MPD after 1.5hours of product circulation were designated unclean. After the 4-step CIP, more slides were removed from the MPD and were designated as clean. Cover slips were placed on top of the slides removed from the MPD and viewed under a light microscope. A Nikon Optiphot light microscope fitted with a DXM1200 Nikon digital camera was used to view slides and capture micrographs respectively. Figure 4.2 is a schematic representation of the protocol used.

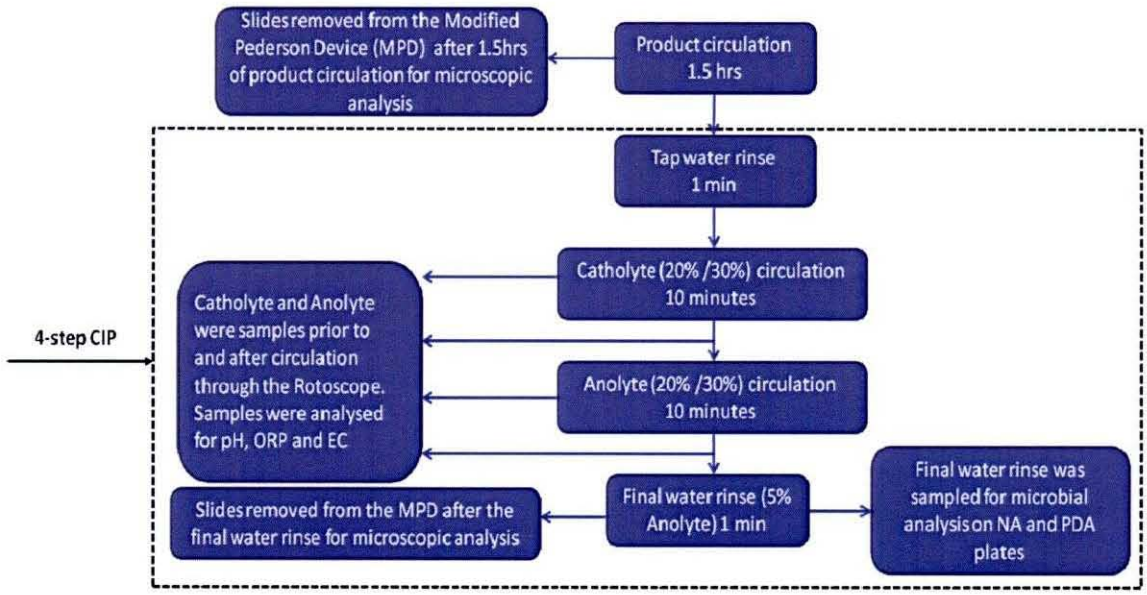

Figure 4.2: Schematic representation of the Protocol used

4.3 Results and Discussion

4.3.1 Relationship between light reflection and fouling during product circulation through the Rotoscope

The material that the slides and the rotating disc of the Rotoscope were made from is Perspex; this was done so that both the rotating disc and the slides are of the same material. Perspex is a hydrophobic synthetic material (Sekar *et al.*, 2004). Research conducted by several groups has established that microorganisms attach more rapidly to hydrophobic, non-polar surfaces such as Teflon and other plastics compared to hydrophilic materials such as glass and metals (Fletcher and Loeb, 1979; Pringle and Fletcher, 1983; Bendinger *et al.*, 1993; Donlan, 2002). The reason Perspex was chosen was to emulate "the worst case scenario"; i.e. fouling will occur more readily on perspex compared to stainless steel (which is mostly the material of choice in beverage plants).

Figures 4.3 to 4.8 show the effect of the progression of time on light reflection after more 1hr of product circulation through the Rotoscope. In all six trials conducted a similar trend (decline in light reflection) was observed for all the samples. The progressive decrease of light reflection could be attributed to the accumulation of deposits on the Perspex rotating disc of the Rotoscope. Cloete and Maluleke (2005) observed a similar trend when monitoring biofilm growth in river water using a Rotoscope. Regression analysis was conducted on the obtained data to analyse the relationship between the light reflection (mV) and time.

All the results obtained from the regression analysis of the six trials indicated that all models (light reflection over time) were statistically significant; this is indicated by the F value. The F and R^2 values obtained were as follows: coke syrup (F: 0.363 and R^2 0.016); minute maid (F: 3.868 and R^2 0.169); Powerade (F: 9.060 and R^2 0.268); iced tea (F: 12.102 and R^2 0.430); just juice breakfast blend (F: 5.459 and R^2 0.233) and just juice orange (F: 0.407 and R^2 0.023). The F and R^2 values obtained for each trial were different. This could be expected because the soft drinks used were different from each other in terms of the ingredients used to make them. The R^2 values indicated the percentage at which the model was explained. None of the models were explained 100%; i.e. no R^2 value came close to the value of 1. This may be an indication that there are other factors/variables that affect light reflection during fouling other than time. Melo and Bott (1997) have identified temperature,

pH, particles in a system, velocity and turbulence as factors that affect fouling include. These factors can be taken into account for future studies and multiple regression analysis can be conducted to determine their effect on light reflection over time during fouling until the model is explained almost 100%. Once the model has been explained almost 100% equations can be modelled to predict fouling using the Rotoscope.

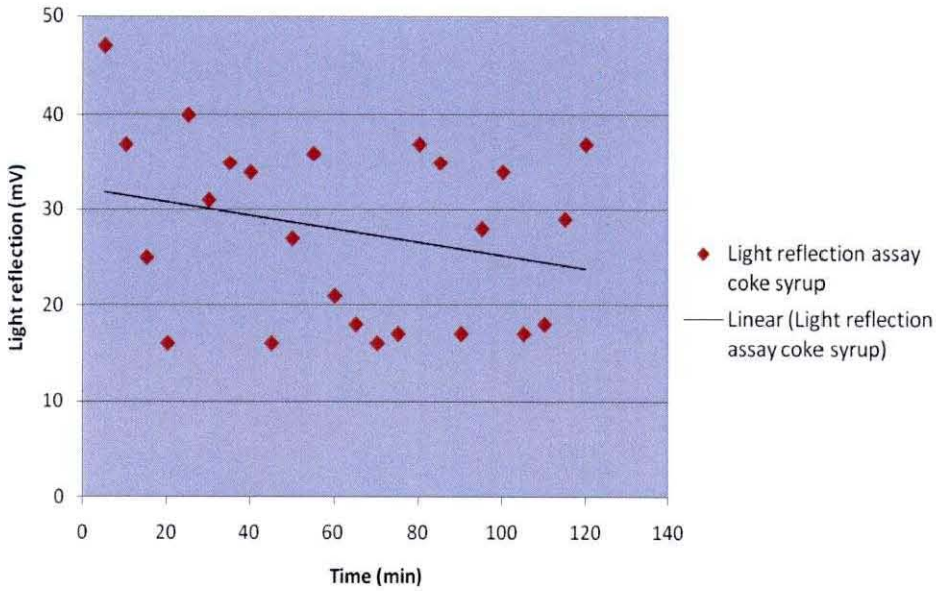

Figure 4.3: Light reflection assay of coke syrup. R^2: 0.016, F: 0.363

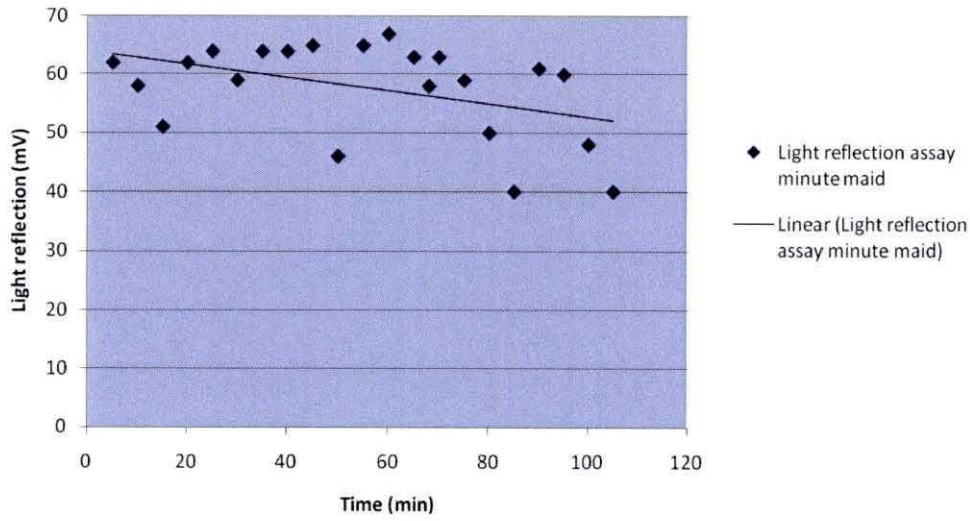

Figure 4.4: Light reflection assay for minute maid juice, R^2: 0.169, F: 3.868

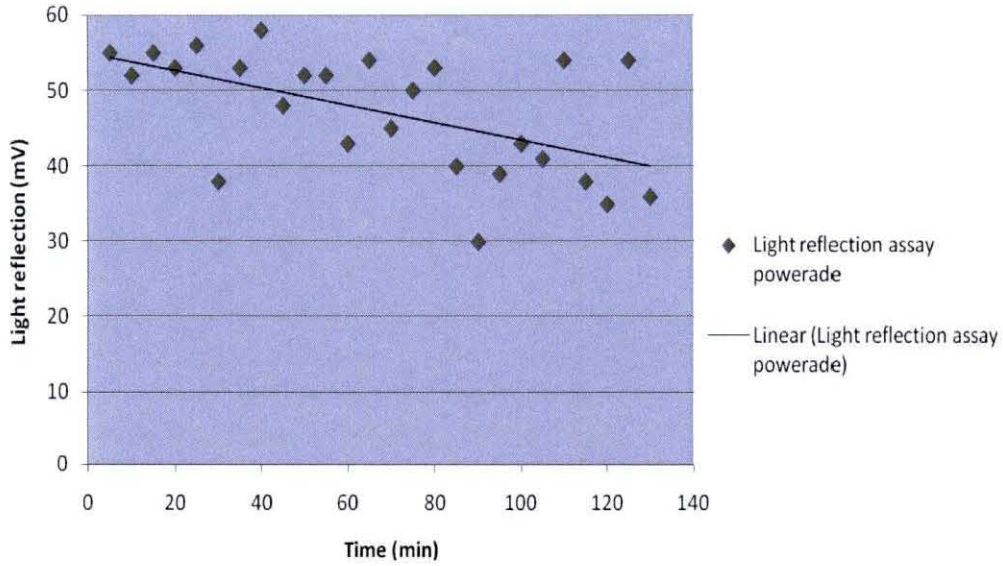

Figure 4.5: Light reflection assay for powerade. R^2: 0.283; F: 9.060

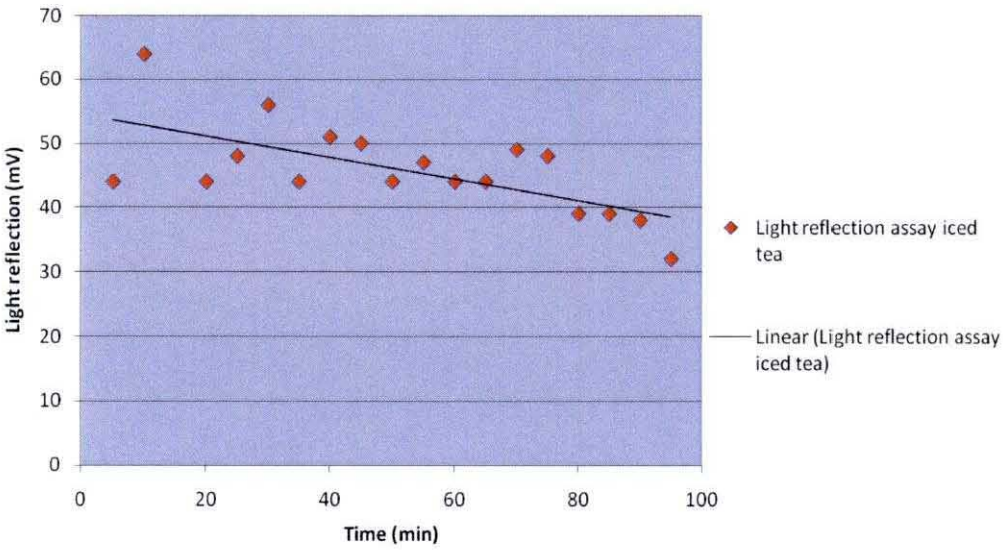

Figure 4.6: Light reflection assay for iced tea. R^2: 0.430; F: 12.102

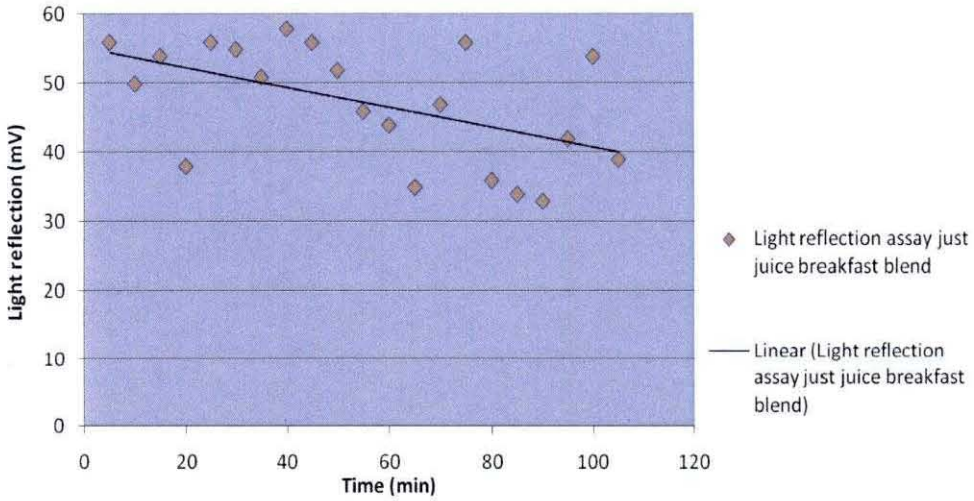

Figure 4.7: Light reflection assay for just juice breakfast blend. R^2: 0.233; F: 5.459

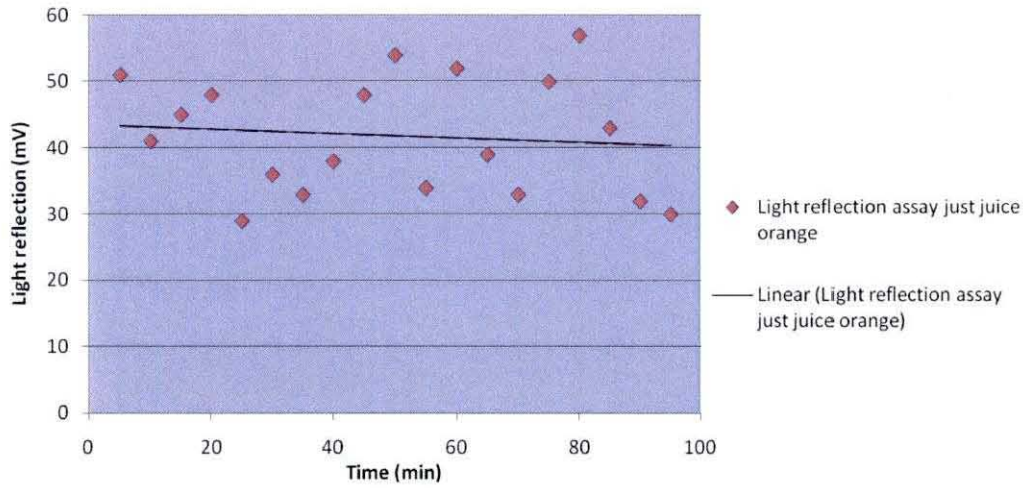

Figure 4.8: Light reflection assay for just juice orange. R^2: 0.023; F: 0.407

4.3.2 Light reflection results after the ECA 4-step CIP

After product circulation, a field-proven 4-step method for ECA CIP of CSD lines was followed (figure 4.2). During the CIP, the logger of the Rotoscope was set to take readings every minute. Light reflection increased; indicating the efficacy of the ECA 4-step CIP, in particular the Catholyte as a detergent and the Anolyte as a disinfectant. This proves that the ECA solution did indeed remove deposits from the simulated SD production line. The increase in light reflection was determined by comparing the final light reflection value

after product circulation with the average of the values obtained during the 4-step CIP for each trial as indicated in table 4.1.

Table 4.1: Values of light reflection (mV) before and after CIP

Samples	Light reflection before CIP (mV)	Light reflection after CIP (mV)
Coke syrup	37	56
Minute maid	40	53
Powerade	36	54
Iced tea	32	57
Just juice breakfast blend	39	55
Just juice orange	30	58
Control (5% Anolyte)	58	58

4.3.3 Analysis for microbial growth after CIP from the removable slides of the Pederson device and final rinsing water effluent

No growth was observed on NA and PDA plates (Table. 4.2). Catholyte and Anolyte were used at 20% concentration for the Coke syrup and 30% for the rest of the beverage products. As these concentrations are said to have been proven to work, microbial growth was not expected to occur on either the NA or PDA plates. However there was no growth on both the NA and PDA plates (Table 4.2). This was another way of indicating that Catholyte and Anolyte were indeed efficient as detergent and disinfectant respectively during CIP.

Table 4.2: Microbial growth studies obtained from removable slides of the Pederson device and from the final rinsing water of CIP plated onto NA and PDA plates incubated overnight at 37°C (- indicates no growth)

Samples	Growth from removable slides	
	NA	PDA
Minute Maid	-	-
Just Juice	-	-
Just Juice (orange)	-	-
Powerade	-	-
Iced tea syrup	-	-
Coke syrup	-	-

4.3.4 Analysis of removable slides after CIP

Microscopy was also used to analyse the efficacy of the 4-step CIP using ECA solutions. Sugar crystals were detected on slides designated as unclean (figure 4.9 A, B and C); whilst they were absent on the slides designated clean (Fig 4.9 D). The absence of sugar crystals were an indication of the efficacy of the Catholyte as a detergent. No microbes were detected on either the clean or unclean slides. This is because the Rotoscope was cleaned by circulating 3% Anolyte after each experiment; indicating the efficacy of the Anolyte as a disinfectant.

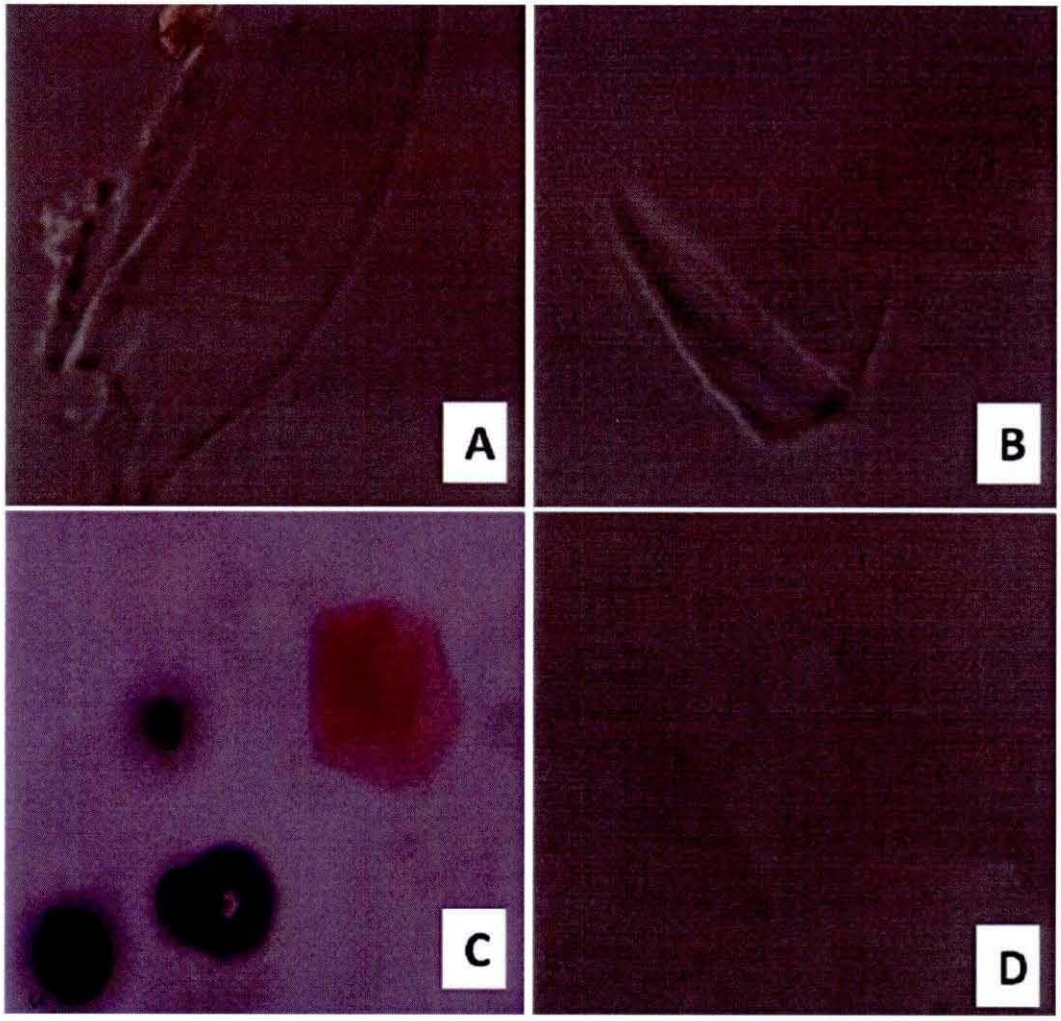

Figure 4.9: Micrographs from light microscopy of unclean (A, B and C) and clean (D) slides; slides after the 4-step CIP.

4.3.3. Analysis of the efficacy of Anolyte and Catholyte during CIP by monitoring Electric conductivity (EC), Oxidative-reduction potential (ORP) and pH

EC, ORP and pH levels of the Catholyte and Anolyte were monitored prior to circulation and after circulation through the Rotoscope (Table 4.3).

Table 4.3: EC (mV), pH and ORP (mS) readings of Catholyte and Anolyte prior to circulation and after circulation through the Rotoscope during CIP

Sample		Catholyte before circulation	Catholyte after circulation	Anolyte before circulation	Anolyte after circulation	Final rinsing water
Minute Maid	EC	1.96	2.03	1.72	1.73	0.27
	pH	10.8	10.9	7.3	7.4	
	ORP	161	255	868	844	
Just Juice (Breakfast blend)	EC	1.81	1.84	1.75	1.77	0.29
	pH	10.4	10.5	7.3	7.4	
	ORP	146	227	836	833	
Just Juice (Orange)	EC	1.97	2.09	1.91	1.93	0.30
	pH	10.8	10.8	7.3	7.5	
	ORP	103	114	819	824	
Powerade	EC	2.00	2.00	1.86	1.84	0.24
	pH	10.6	10.8	7.4	7.4	
	ORP	119	185	831	831	
Nestea Iced Tea	EC	1.91	1.91	1.86	1.92	0.27
	pH	10.9	10.8	7.4	7.5	
	ORP	170	190	843	861	
Coke syrup	EC	1.28	1.25	1.23	1.21	0.33
	pH	10.1	10.0	7.6	7.4	
	ORP	200	230	916	840	

The pH was monitored before and after CIP to determine whether the Catholyte or Anolyte were still functioning at their optimal pH. The pH ranges of Catholyte and Anolyte are (10-11.5) and (2-9) respectively (Cloete and Brözel, 2003; Huang *et al.*, 2008). The pH values of Catholyte for all the six trials conducted before circulation through the Rotoscope for CIP were in the range of 10 (Table 4.3). The pH values remained relatively the same after circulation through the Rotoscope; still in the range of 10 (Table 4.3). The pH values for the Anolyte for all the six trials conducted before circulation through the Rotoscope for CIP were in the range of 7; they remained relatively the same, still in the range of 7, after circulation through the Rotoscope (Table 4.3). Based on the observations, it is apparent that both the Catholyte and Anolyte were functioning at their optimal pH ranges.

ORP values of Anolyte in all CIP trials conducted were always above 800mV (Table 4.3). Even after circulation through the Rotoscope, the ORP values remained above 800mV. This gave an indication that the circulated Anolyte could be re-used again for another cycle of CIP. ORP monitoring allows for real-time assessment of the activity of the biocidal treatment that is being used (Suslow, 2004; Kirkpatrick, 2009). A high ORP value is an indication of a high antimicrobial efficacy (Kim *et al.* 2000; Huang *et al.*, 2008). ORP values higher than 500mV are said to be effective in the elimination of many types of pathogenic bacteria (Envirolyte, 2010). Throughout the CIP trials conducted, the ORP values of the Catholyte were between 103 and 200 mV prior to circulation and 114 – 260 mV after circulations through the Rotoscope. This was an indication that the Catholyte still had its reducing capabilities and that it did indeed remove the soil in the Rotoscope system.

Electric conductivity (EC) is the most accurate way of measuring dilution of ECA solutions and it follows a linear trend (Kirkpatrick, 2009). The EC values were measured for both the Catholyte and Anolyte prior to and after circulation through the Rotoscope during CIP. In order to ensure that the diluted ECA solutions remained at the correct concentration during CIP, the values before and after circulation through the Rotoscope should be similar. In all trials conducted the EC values for both the Catholyte and Anolyte prior to and after circulation through the Rotoscope showed negligible differences (Table 4.3). Catholyte was circulated through the Rotoscope following product circulation and the first water rinse. The consistency of EC values for the Catholyte indicated that there was no product or water from the first rinse that entered the "Catholyte system" to change its concentration. The Anolyte was circulated after the Catholyte cycle. Similar to the situation with the Catholyte, the

consistency in the EC values indicated that there was no Catholyte that entered the "Anolyte system" to change its concentration. This is an indication that the Catholyte and the Anolyte were effective during CIP and that they can be re-used again.

4.4 Conclusions

From the results obtained it shows that the Rotoscope can be able to be incorporated into an SD line to monitor fouling and CIP. During CIP the Catholyte and Anolyte were shown to be effective as detergent and disinfectant respectively. This was indicated by results obtained from microbial analysis of removable slides from the MPD, microscopic analysis, as well as pH, ORP and EC analysis as well as. No microbial growth was detected after CIP; microscopic analysis indicated no soil or microbial growth from slides that were analysed after CIP and values of pH, ORP and EC prior to and after CIP remained relatively the same, this indicated that the Catholyte and Anolyte were effective during CIP.

Results obtained during regression analysis between light reflection and time can be further studied by incorporating factors including pH, temperature velocity and turbulence. Multiple regression analysis can be conducted to determine their effect on light reflection over time during fouling until the model is explained close to 100%. Once the model has been explained close to 100% equations can be modelled to predict fouling using the Rotoscope.

The Rotoscope has potential of being used to monitor CIP and fouling in industry; however, this can only be confirmed once pilot plant trials have been conducted to study fouling and CIP using the Rotoscope.

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Chapter 5: Detection and Speciation of Trihalomethanes (THMs) produced during CIP Treatment Using Electro-Chemically Activated (ECA) Water using Gas Chromatography-Mass Spectroscopy (GC-MS)

5.1 Introduction

There is always a high and constant requirement for water in the beverage production process and in other related processes (CIP systems, crate washing, bottle washing, and general sanitation). These processes cannot rely on raw water and require a constant supply of treated water. Disinfection is carried out to eradicate and/or to inactivate pathogens from water by destructing the organization of the cell structure, interfering with energy yielding metabolism, biosynthesis and growth of microbes (Sadiq and Rodriguez; 2004). For many years chlorine and its associated compounds have been commonly used as disinfectants for water treatment because of its strong oxidizing potential (Sadiq and Rodriguez; 2004). One of the most crucial disadvantages of the use of chlorine is the formation of disinfection by-products (DPBs). Although much research has been conducted on DBPs; people only became more aware of them in the 1970s when Rook identified them in 1974 (Richardson, 2003).

Disinfection by-products (DBPs) are formed when a disinfectant reacts with organic and/or inorganic matter (precursors) in water (Sadiq and Rodriguez; 2004). Trihalomethanes (THMs), haloacetic acids (HAAs), haloacetonitriles (HANs), haloketones (HKs), chloral hydrate and chloropicrin (CPN) are the major DBPs that exist (Peters *et al.*, 1990; Singer, 1994; Antoniou *et al.*, 2006). THMs and HAAs are said to be the most abundant DBPs that result from chlorination (Krasner *et al.*, 1989; Wiesel *et al.*, 1999). Trihalomethanes are chemical compounds where three of the four hydrogen compounds of methane (CH_4) are replaced by halogen atoms (Clement and Hao, 2010). Halogen atoms include chlorine (Cl), iodine (I), fluorine, (F) and bromide (Br). There are four species of THMs, namely: chloroform ($CHCl_3$), dichlorobromothane ($CHCl_2Br$), chlorodibromethane ($CHClBr_2$) and bromoform ($CHBr_3$) (Antoniou *et al.*, 2006).

There are concerns about health risks that have been associated with THMs. Epidemiological studies have indicated that there is an association between chlorination (which gives rise to DBPs) and increased risk of cancer of the urinary and gastrointestinal

tracts (Cantor, 1997; Koivusalo and Vartiainen, 1997; Monarca, 2004). Other health risks associated with chlorination include spontaneous abortion and other reproductive and developmental effects (Waller *et al.*, 1998; Hwang and Jakkola, 2003; Monarca, 2004). The adverse health risks associated with THMs have necessitated the development of strict regulatory policies and measures. The United States Environmental Protection Agency (EPA) issued a regulation to restrict the amount of THMs in finished drinking water to $100\mu gl^{-1}$ in 1979, which was further reduced to $80\mu gl^{-1}$ in 1998 (Richardson, 2003; Serrano and Gallego, 2007). These strict regulations on THMs have influenced industry to change from using chlorine to using alternative disinfectants. Electro-chemically activated (ECA) water is a green technology that produces a detergent called Catholyte and a disinfectant called Anolyte (Radical Waters, 2010). These can be used as alternatives for detergents and disinfectants that are currently being used.

Studies have been conducted on THMs, e.g. studies concerning the amount of THMs generated in natural water treated with electrochemically generated mixed oxidants (Venczel *et al.*, 1997; Kerwick *et al.*, 2005). These studies have reported more than 50% reduction in total THMs (Venczel *et al.*, 1997; Kerwick *et al.*, 2005). There have been no documented studies on the generation of THMs caused by the treatment of systems with ECA water in the food and beverage industry.

THMs are mainly detected using gas chromatography (GC) followed by mass spectroscopy (MS) (Pavon et al, 2008). GC-MS is considered to be an analytical tool that provides conclusive proof of identity of organic compounds in complex mixtures (Douglas, 2010). The GC device separates compounds in complex mixtures prior to analysis with the MS instrument; and the MS instrument identifies compounds by determining their molecular mass (Douglas, 2010). There are existing libraries of molecular masses of different compounds; thus for identification purposes the obtained mass is compared with molecular masses that are in the existing libraries.

The aims of this study were to determine the total THMs (TTHM) generated during CIP using Catholyte and Anolyte as well as to determine which species of THMs are present from CIP.

5.2 Materials and Methods

5.2.1 Five-step CIP and collection of effluent

A five-step CIP was conducted using the Rotoscope (figure 5.1). Coke syrup (CS) and two different juice samples (Minute Maid juice orange (MMO) and Minute Maid juice breakfast blend (MMBB)) were used to run three separate trials for the detection and speciation of THMs formed during CIP using ECA water.

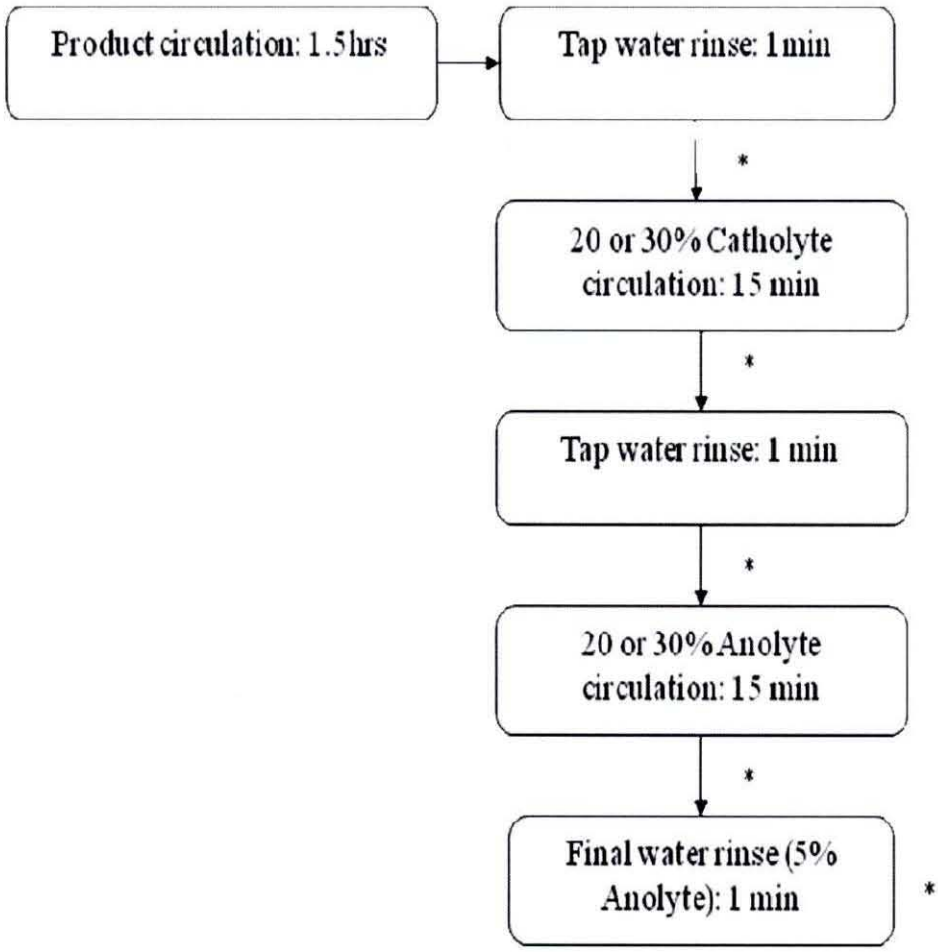

Figure 5.1: A five-step CIP was conducted using the Rotoscope.

Effluent was collected in 100ml glass vials after each CIP step; from the first step to the fifth step. This was done as follows: Soft drink (SD) product was run through the Rotoscope for 1.5 h. This was followed by tap water rinse for 1 min. Effluent from the tap water rinse was sampled. 30% Catholyte was circulated through the Rotoscope for 15 min;

the resulting effluent was sampled. The second 1minute tap water rinse followed and effluent was sampled. This was followed by a 30% Anolyte circulation through the Rotoscope for 15 min; the resulting effluent was sampled. 5% Anolyte was used for the final rinsing of the Rotoscope; effluent was also collected. A diagram of the five-step CIP and the five collection points are indicated by asterisks in figure 5.1. The glass vials were filled to just overflowing to preventing trapping of air bubbles in the bottles.

5.2.2 Detection and speciation of effluent for Trihalomethanes

All samples were analysed for THMs at Organic Analysis Laboratory (Queenswood, Pretoria, Republic of South Africa) using gas chromatography-mass spectrometry (GC-MS). Analysis for THMs of chemicals that were used during CIP was also conducted; this was done to determine their THMs standards. The chemicals are 30% Anolyte, 30% Catholyte rinsing tap water and the final rinsing water (5% Anolyte). This was done so that the values can be compared with those of the effluents (standards + organic matter) that were sampled during the trials.

5.3 Results and Discussion

5.3.1 THM Concentration and speciation of the six chemicals used for CIP

The six chemicals used for CIP were: 100% tap water, 20% Catholyte, 30% Catholyte, 20% Anolyte, 30% Anolyte and 5% Anolyte. The 20% Anolyte and Catholyte were used for the coke syrup trial and the 30% Anolyte and Catholyte were used for the juice trials. Tap water instead of treated water was used to dilute the Catholyte and Anolyte due to the unavailability of treated water. All the six chemicals used for CIP did contain THMs. It was expected that all chemicals used for CIP will contain THMs; because untreated tap water was used. Commercially, only treated water is used in beverage manufacturing. Obtained results of THM concentrations for the chemicals used for CIP are indicated in Table 5.1. All the four species of THMs; chloroform ($CHCl_3$), dichlorobromothane ($CHCl_2Br$), chlorodibromethane ($CHClBr_2$) and bromoform ($CHBr_3$) were detected in all the six chemicals used for CIP and their concentrations were determined.

Table 5.1: THMs concentrations (µg/l) and speciation of the six chemicals used for CIP

	CHCl3	CHBrCl2	CHBr2Cl	CHBr3	Total THM
Tap water	9	6	1	<1	16
30% Anolyte	53	11	2	<1	66
20% Anolyte	45	11	5	<1	61
30% Catholyte	6	4	1	<1	11
20% Catholyte	6	9	2	<1	17
5% Anolyte	31	15	4	<1	50

Total THM concentration in the tap water which was used for CIP was found to be 16µg/l (Table 5.1). These results are in agreement with those of van Steenderen *et al.* (1991) which revealed a low THM concentration (45µg/l) in South African tap water. Compared to maximum levels set for the United States (80µg/l), WHO (200µg/l for chloroform and 100µg/l for bromoform), this latter value of 45µg/l is comparatively low (Richardson, 2005).

Total THM concentrations for 30% and 20% Anolyte were 66µg/l and 61µg/l respectively (Table 5.1). 20% Anolyte was expected to have a lower THM concentration compared to 30% Anolyte because of the lower concentration of oxidative species. Anolyte predominantly comprises of ClO^-; HOCl; O_3 and Cl_2 (Kirkpatrick, 2009). The ClO^- converts to HOCl when the Anolyte is diluted in water resulting in HOCl being the predominant oxidant in dilute aqueous solution. The oxidative power of the species follows $HOCl>Cl_2>OCl^-$ (Kirkpatrick, 2009). This means that in the Anolyte HOCl will be the first species to oxidise compounds during disinfection and Cl_2 remains as the abundant form of free available chlorine (FAC). In contrast a conventional chlorine disinfectant such as sodium hypochlorite breaks down to form ^-OCl; i.e. FAC will be in the form of ^-OCl. A study has been conducted to compare the amount of THMs produced by Anolyte compared to other chlorine based disinfectants. According to Fenner *et al.* (2009), 50% less chloroform was formed when water containing natural organics was

treated with Eurostel Anolyte compared to water treated with similar doses of hypochlorite. This is because ^{-}OCl is the active agent for THM formation, and it represents less than 12% of the FAC in Anolyte solutions and 88% of FAC occurs in the form of Cl_2 (Fenner *et al.*, 2009).

Total THM concentration of 30% and 20% Catholyte were as expected lower than those of the Anolyte, at concentrations 11µg/l and 17µg/l respectively (Table 5.1). Diluted Catholyte is comprised mainly of NaOH as the active reducing agent. There are no chlorine compounds in the Catholyte. The presence of THMs in the Catholyte was ascribed to the tap water which was used for dilution when preparing the Catholyte dilution.

The six chemicals which were used for CIP were tested for the four species of THMs, i.e. chloroform ($CHCl_3$), dichlorobromothane ($CHCl_2Br$), chlorodibromethane ($CHClBr_2$) and bromoform ($CHBr_3$). $CHCl_3$, $CHCl_2Br$ and $CHClBr_2$ were detected in all the six chemicals (Table 5.1). The levels of $CHBr_3$ were negligible; i.e. less than 1(µg/l) in all the six chemicals (Table 5.1). $CHCl_3$ was the most dominant species followed by $CHCl_2Br$ and $CHClBr_2$. These results are a clear indication of the low bromide levels in tap water. According to Krasner *et al.* (1994) the levels of bromide will determine the speciation of THMs. If bromide levels are high THM speciation will shift towards brominated-THMs and if bromide levels are low THM speciation will shift towards chlorine-dominant-THMs (Krasner *et al.*, 1994).

5.3.2 THM occurrence and speciation of the six chemicals used for CIP

Results obtained from the chemicals used in the five-step CIP step were compared to those of the sampled effluent that was produced from each CIP step. All the effluent samples which were collected from the five-step CIP for all three trials contained THMs (Figures 5.2 – 5.8).

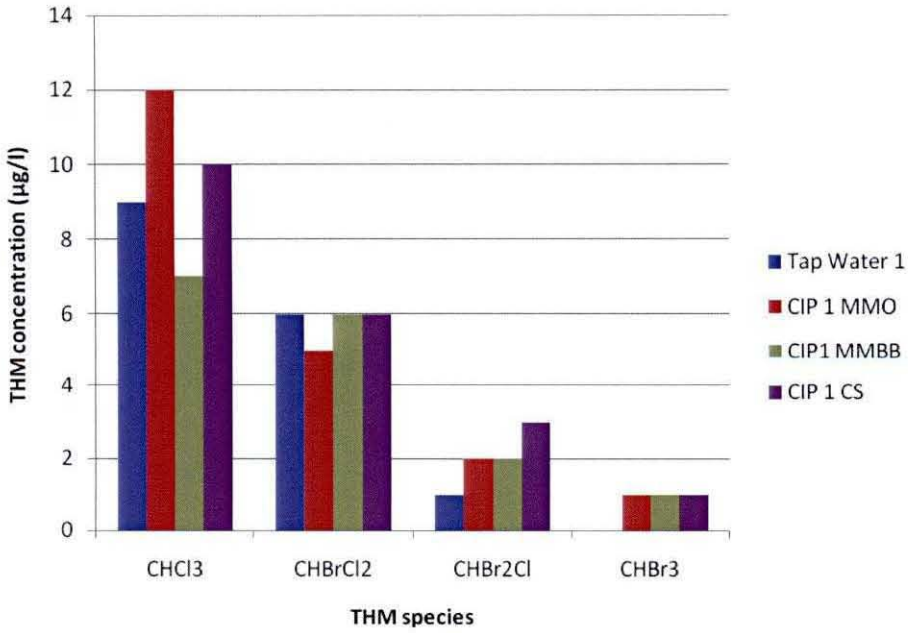

Figure 5.2: Total THM concentration (µg/l) and THM speciation for MMO, MMBB and CS at CIP step 1

Chloroform was the dominant species of THMs in the effluent that was sampled from the all 5 steps of CIP from the three trials (Figures 5.2 – 5.6). On average Chloroform accounted for 60% of all the THM species from effluent collected during CIP for all three trials conducted (Figures 5.2 – 5.6). Bromodichloromethane was the second most abundant with an average of 29% (Figures 5.2 – 5.6). The third most abundant THM was chlorodibromomethane with an average of 10% (Figures 5.2 – 5.6). The least occurring THM was bromoform with an average of 1% (Figures 5.2 – 5.6). The low levels of brominated-THMs compared to chloroform are a clear indication of the levels of bromide in tap water and the chemicals used for CIP. Studies have indicated that when levels of bromide are low in water species of THMs with fewer atoms of bromide; i.e. brominated-THMs get formed during chlorination (Krasner *et al.*, 1994; Nikolaou *et al.*, 2003; Mbonimpa, 2007). This explains the pattern at each speciation of THMs occurred from this study; less brominated-THMs were formed compared to chloroform.

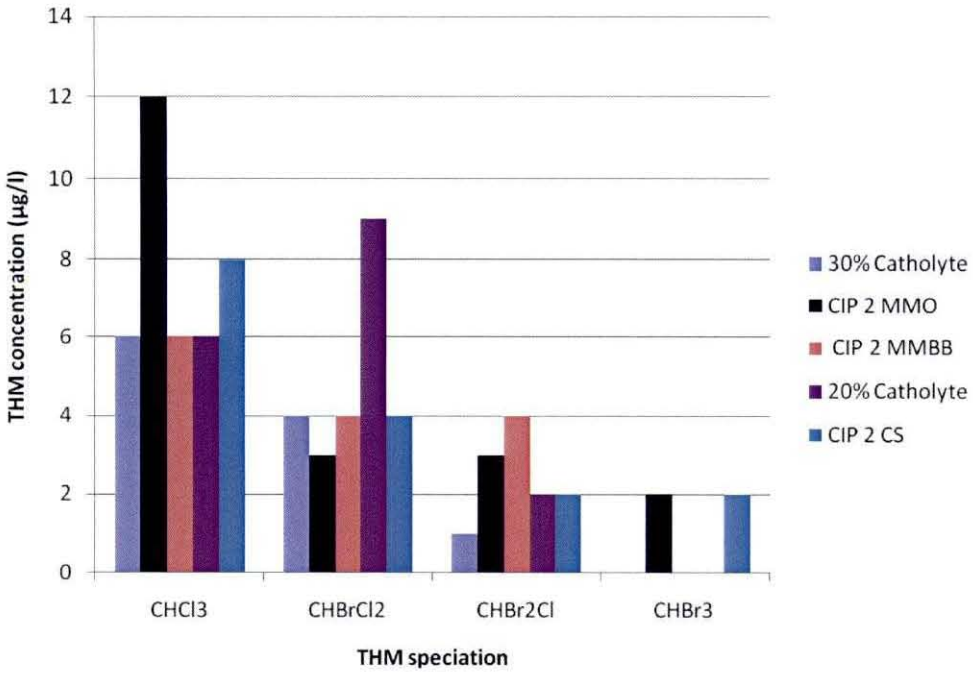

Figure 5.3: Comparison of TTHM concentration (µg/l) and THM speciation for MMO, MMBB and CS at CIP step 2

For CIP step 1 and 3 in all three trials conducted, THM levels were low; this was expected because tap water was used as a rinsing agent in the 1st and 3rd steps of CIP (Figure 5.2 and Figure 5.4). Low THM levels were a result of the absence of additional chlorine compounds (no Anolyte was added) to react with the organic matter from the tap water and from the juices and the syrup. Similar results were expected from the 2nd CIP step (Figure 5.3), where Catholyte was used as a rinsing agent. 100% Catholyte does not contain chlorine compounds; the active compound is NaOH. The only chlorine compounds from the Catholyte were from the tap water which was used to dilute the Catholyte to 20 and 20%. The averages for total THM concentrations for all three trials in the 1st, 2nd and 3rd steps of CIP were 18µg/l, 16µg/l and 20µg/l respectively.

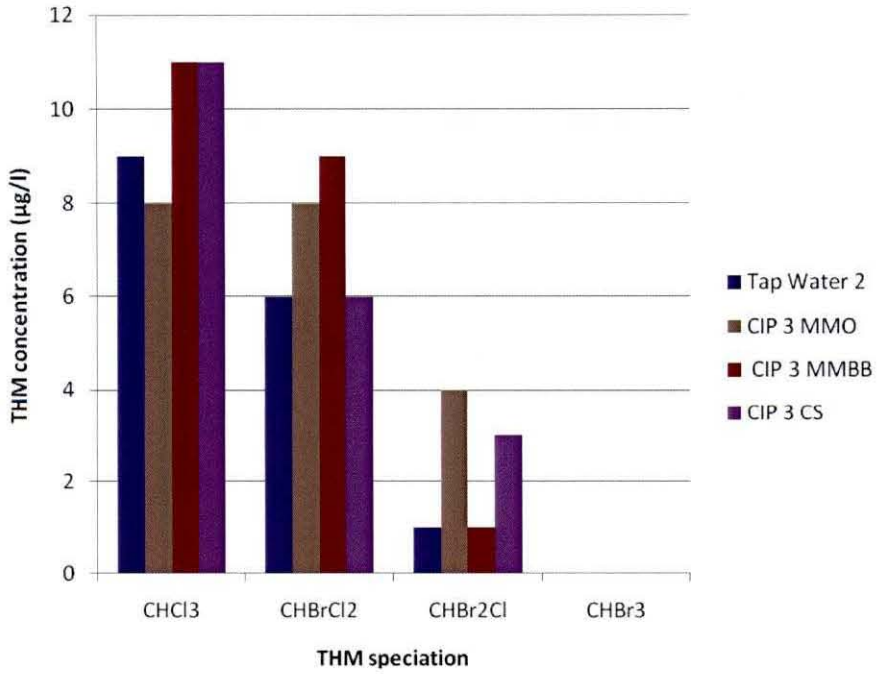

Figure 5.4: Comparison of TTHM concentration (µg/l) and THM speciation for MMO, MMBB and CS at CIP step 3

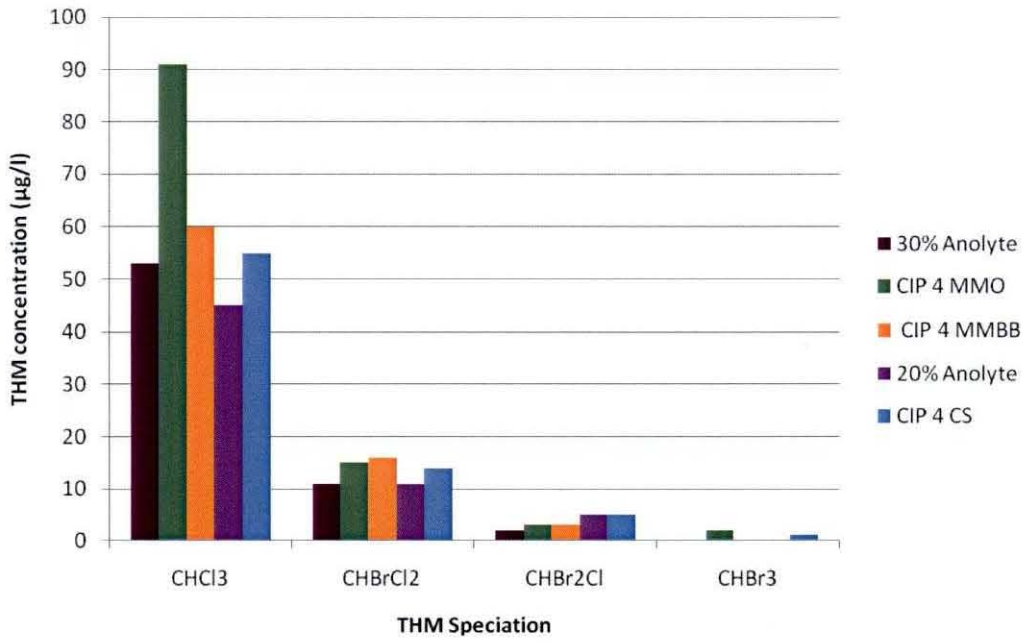

Figure 5.5: Comparison of TTHM concentration (µg/l) and THM speciation for MMO, MMBB and CS at CIP step 4

The highest concentrations of THM were obtained in the 4th and 5th steps of CIP (Figure 5.5 and Figure 5.6). The average THM concentrations for all three trials were 88µg/l and 38µg/l for CIP step 4 and 5 respectively. This was expected because Anolyte was used as a rinsing agent for the 4th and 5th steps of CIP. Anolyte contains ClO^-; HOCl and Cl_2 which provide free available chlorine that will react with organic matter to form THMs (Hricova *et al.*, 2008).

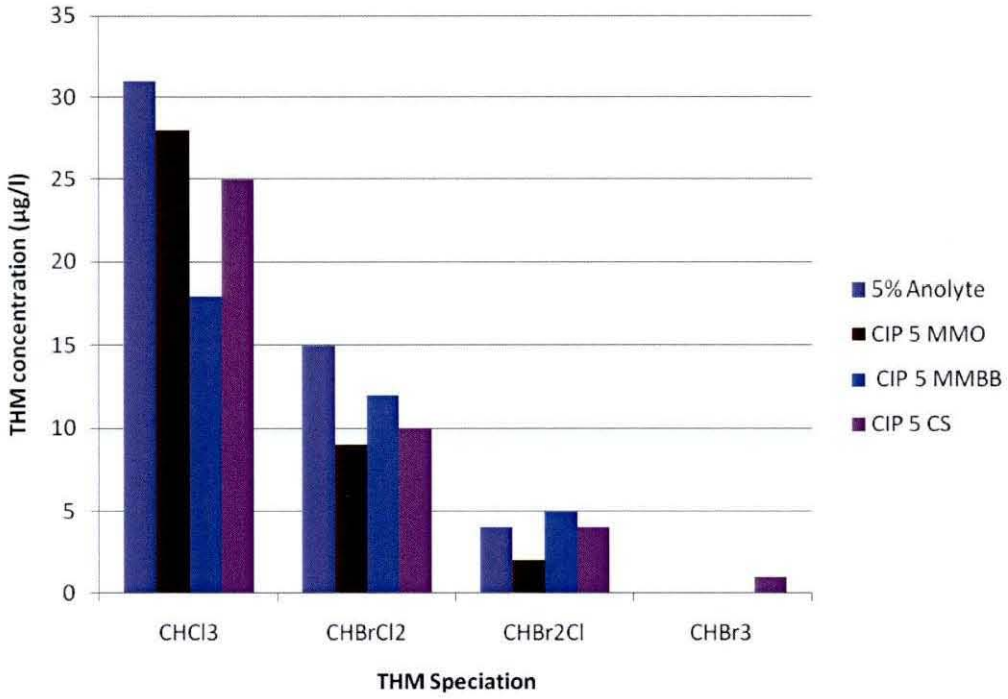

Figure5. 6: Comparison of TTHM concentration (µg/l) and THM speciation for MMO and MMBB at CIP step

Total Trihalomethane (TTHM) concentrations for effluent from the five-step CIP were determined for all three trials (Table 5.2). The TTHM concentrations in all trials were relatively low for CIP effluent; they were 210µg/l (MMO), 161µg/l (MMBB) and 171µg/l (CS). These low levels indicate that very little treatment will be required for the treatment of CIP effluent prior to it being discarded into public streams and lakes. This is beneficial in terms of saving costs and it is environmentally friendly.

The MMO trial had the highest concentration of TTHM (Table 5.2). Orange juice contains significant levels of citric acid which is an organic compound. Citric acid has been discovered to be a precursor of THM formation and it enhances the formation of chloroform

(Navalon *et al.*, 2009). The results in this study are in agreement with findings of Navalon *et al.* (2009). MMO had the highest concentration of chloroform in all 5 steps of CIP compared to MMBB and CS (Figures 5.2 – 5.6).

Table 5.2: Total THM (TTHM) concentrations (µg/l) obtained for the MMO, MMBB and CS CIP trials

	TTHM in CIP step 1	**TTHM in CIP step 2**	**TTHM in CIP step 3**	**TTHM in CIP step 4**	**TTHM in CIP step 5**	**[TTHM] per 5 step CIP**
MMO	20	20	20	111	39	210
MMBB	15	11	21	79	35	161
CS	20	16	20	75	40	171

Kirkpatrick (2009) has patented the use of electrochemically activated water in beverage manufacturing, processing and dispensing. Figure 5.7 is a schematic representation of an improved fruit juice production, processing and packaging. The schematic representation shows the incorporation of an ECA device into a beverage manufacturing system. The device can be used to produce Catholyte and Anolyte. On the diagrams it is also indicated which systems and subsystems can make use of the Catholyte and Anolyte; these include bottle washing, cap preparations, crate washing and CIP systems. Based on results obtained in this study ECA technology is indeed a green technology and is a better alternative to conventional chlorination and can be incorporated into a soft drink production line.

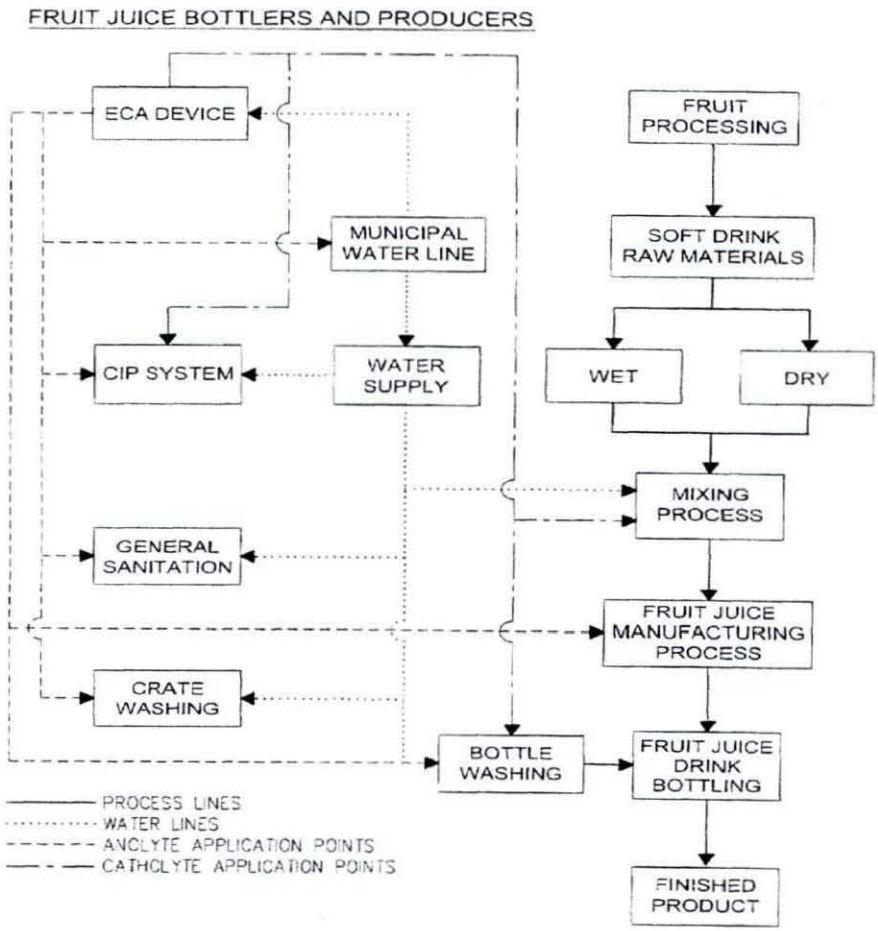

Figure 5.7: Schematic representation indicating the incorporation of ECA device into a soft drink manufacturing plant (Kirkpatrick, 2009).

5.4 Conclusions

The beverage production process requires big volumes of water which require disinfection prior to use. The big volumes of water required make it desirable to have cost effective methods for water disinfection, e.g. equipment that can produce a disinfectant and is located in house. ECA technology is one such technology that can be placed in house for the production of Catholyte (detergent) and Anolyte (disinfectant) during soft drink (SD) production. Although all four species of THMs were present from CIP effluent, low levels of THMs were obtained. The results obtained in this study indicate that ECA technology is indeed a green technology and is a better alternative to conventional chlorination and can be incorporated into a soft drink production line

5.5 References

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Chapter 6: The Influence of pH and Br^- on Trihalomethane Formation Potential of Carbohydrates and Phenol Treated with Anolyte

6.1 Introduction

Chlorination is the most widely used form of disinfection (Antoniou *et al.*, 2006). The major disadvantage of chlorination is the formation of disinfection by-products (Antoniou *et al.*, 2006). Trihalomethanes (THMs) are one of the most common disinfection by-products formed by chlorination. THMs are formed from a reaction of organic matter or humic acids and chlorine (Uppeegadoo *et al.*, 1999). Four types of trihalomethanes are: chloroform ($CHCl_3$), dichlorobromometane ($CHClBr_2$), dibromochloromethane ($CHBr_2Cl$) and bromoform ($CHBr_3$). Several studies conducted on mammals have revealed that THMs cause unpleasant effects on renal, nervous and reproductive systems (Chu *et al.*, 1980; WHO, 1998; Bielmeier *et al.*, 2001; Navalon *et al.*, 2008). Due to the detrimental health effects caused by THMs, control measures have been taken by limiting THM content in drinking water (Navalon *et al.*, 2008). Concerns over THMs have lead to studies being undertaken concerning mechanism behind THM formation (Navalon *et al.*, 2008).

Studies on the mechanisms of THM formation involve predicting THM formation and this is termed "trihalomethane formation potential" (THMFP). Studies on THMFP involve the treatment of different organic compounds with different concentrations of chlorine-containing disinfectants and measuring the resulting THM levels. THMFP studies are used to determine the maximum amount of THMs that can be formed in the system. Studies have shown that pH, bromide and chlorine concentration, temperature and total organic carbon can have an influence on THM formation (Beller *et al.*, 1974; Clark and Lykins, 1994; Nobukawa and Sanukida, 2001; Golfinopoulos and Arhonditsis, 2002; Navalon *et al.*, 2008). These factors therefore have to be considered when studying THMFP. Information obtained from THMFP studies will allow researchers to have knowledge about trends between THM precursors and THMFP; this information can be used to put in place unit processes that are capable of reducing overall THM levels in a system. Studies have been conducted on THMFP of model compounds of humic acids treated with chlorine containing disinfectants (Amy *et al.*, 1984; Mbonimpa, 2007; Fooladvand *et al.*, 2010). Humic acids are complex structures of natural origin (figure 6.1). They are variable macromolecules that contain

carbohydrates, resorcinol and phenolic compounds (Navalon *et al.*, 2008). Based on the complexity of the structure of humic acids, studies on THMFP have focused on model compounds (model dissolved organic carbon (DOC)) such as phenolic and resorcinol compounds (Venosa and Ram, 1984; Marhaba *et al.*, 2005; Bond *et al.*, 2009). Navalon *et al.* (2008) is the only group that has conducted studies on THMFP focusing on carbohydrates. There is limited knowledge on THMFP focused on the role of carbohydrates and more studies need to be conducted.

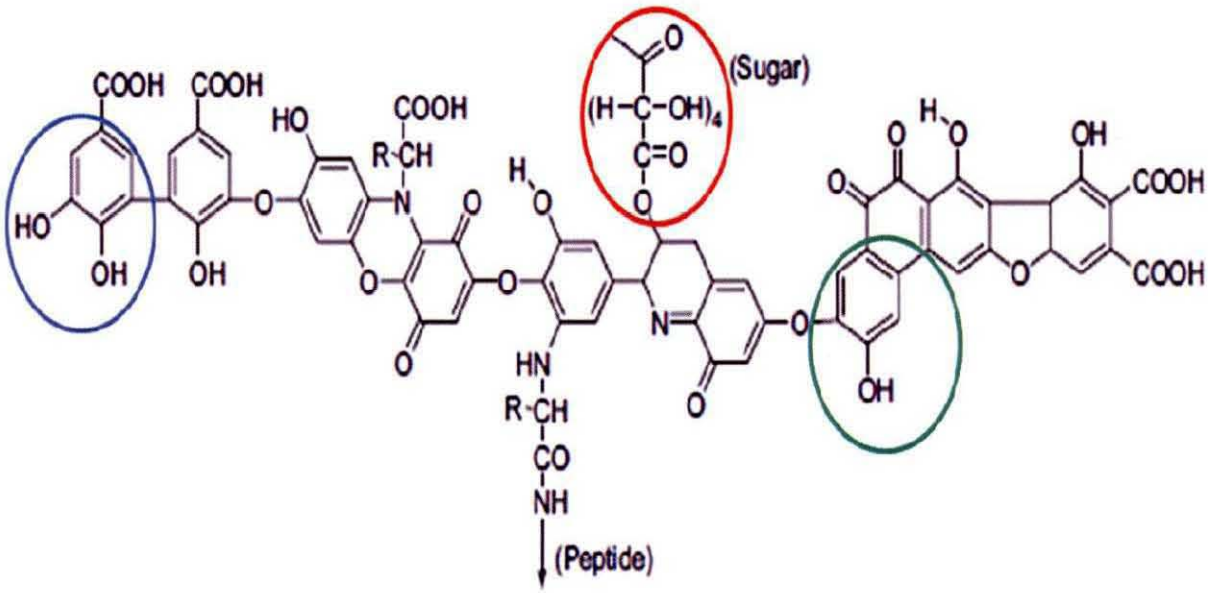

Figure 6.1: Structure of a humic acid. Blue circle indicates resorcinol compound, red circle indicates a carbohydrate (sugar) and green circle indicates phenol compound. Navalon *et al.* (2008).

The main aim of this study was to conduct THMFP studies on carbohydrates and phenol treated with Anolyte. The effect of pH, bromide and organic compounds concentration on THM formation were monitored. Several studies have been conducted on Anolyte as a disinfectant; however there is no documentation on THMFP of organic compounds treated with Anolyte.

6.2 Materials and Methods

6.2.1 Preparations of materials used

Model dissolved organic carbon (DOC) used in this experiment were glucose, maltose and phenol. 2 ppm was chosen as the concentration for the model DOCs were prepared (Navalon *et al.*, 2008). NaBr was used at 50 ppm. 10% Anolyte (100 ppm) was used in the experiments. Calculations used to prepare the NaBr, Anolyte and DOC models are indicated in appendix 5.5. The pH of the model solutions were buffered at 5 and 8 using KH_2PO_4 and K_2HPO_4. All solutions were prepared using deionised water. NaBr, KH_2PO_4, K_2HPO_4, glucose, maltose and phenol were all supplied by Sigma-Aldrich (Johannesburg, South Africa). Anolyte was supplied by Radical Waters (Midrand South Africa).

6.2.2 Trihalomethane Analysis

Model solutions of glucose, maltose and phenol each consisted of: 2 ppm DOC, 10% Anolyte and 0ppb or 50ppb of NaBr. KH_2PO_4 and K_2HPO_4 were used to adjust the pH of solutions to 5 and 8 respectively used. A pH meter (name and model) was used to monitor the pH. Vials were completely filled with mixed solutions of Anolyte, model DOC and NaBr leaving no headspace. Solutions were placed in a water bath at 20°C and ncubated for 72 h. At the end of the 72 h sodium chlorite was added to each sample to neutralize free residual chlorine and to end the process of THM formation. Samples were stored at 4°C and analyzed within 5 d. The solutions were analysed at Organic Analysis Laboratory, Queenswood, Pretoria, South Africa. The Method used to analyse the samples was GC-MS. An outline of the experiment protocol is outlined in Figure 6.2.

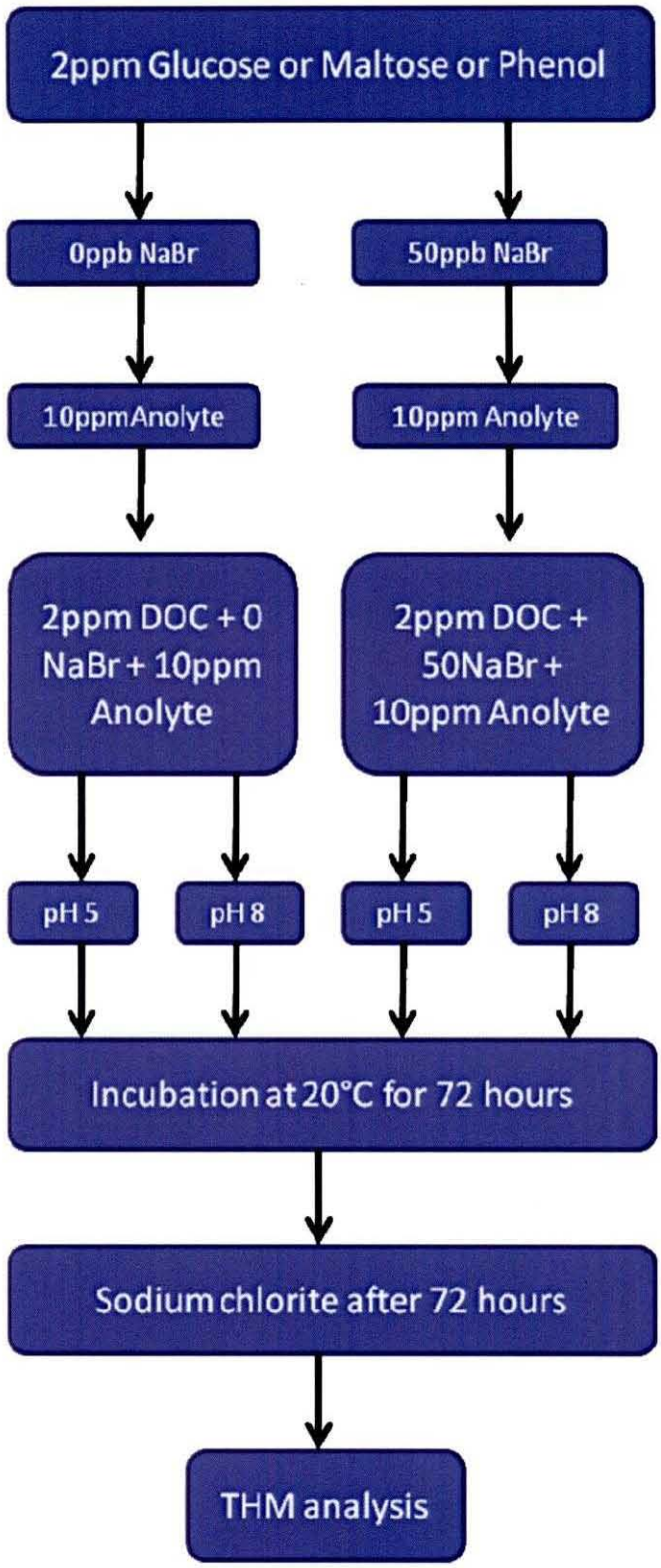

Figure 6.2: Outline of the THMFP protocol

6.3 Results and Discussion

6.3.1 Effect of pH on trihalomethane formation

The pH levels chosen for this study were 5 and 8. Similar to experiments were conducted by Navalon *et al.* (2008). They chose pH of 5 was chosen to show the effect of lower pH on THM formation; and pH 8 as a reference point. pH 8 was chose as a reference point because pH levels near 8 are said common in hard waters (Navalon *et al.*, 2008). The results for the effect of pH on THM formation are shown in figure 6.3. From the results it can be deduced that acidic pH levels caused a decrease in Total THM (TTHM) formation. THM concentrations for maltose, glucose and phenol at pH 5 were 4, 5 and 4 (µg/l) respectively. At pH 8 the THM concentrations were 6, 7 and 5 (µg/l) for maltose, glucose and phenol respectively. These results are similar to those that were obtained by Navalon *et al.* (2008). According to Navalon *et al.* (2008); Clark and Lykins (1994) and Wang and Huang (2006) also obtained similar results on the effects of pH on THM formation potential.

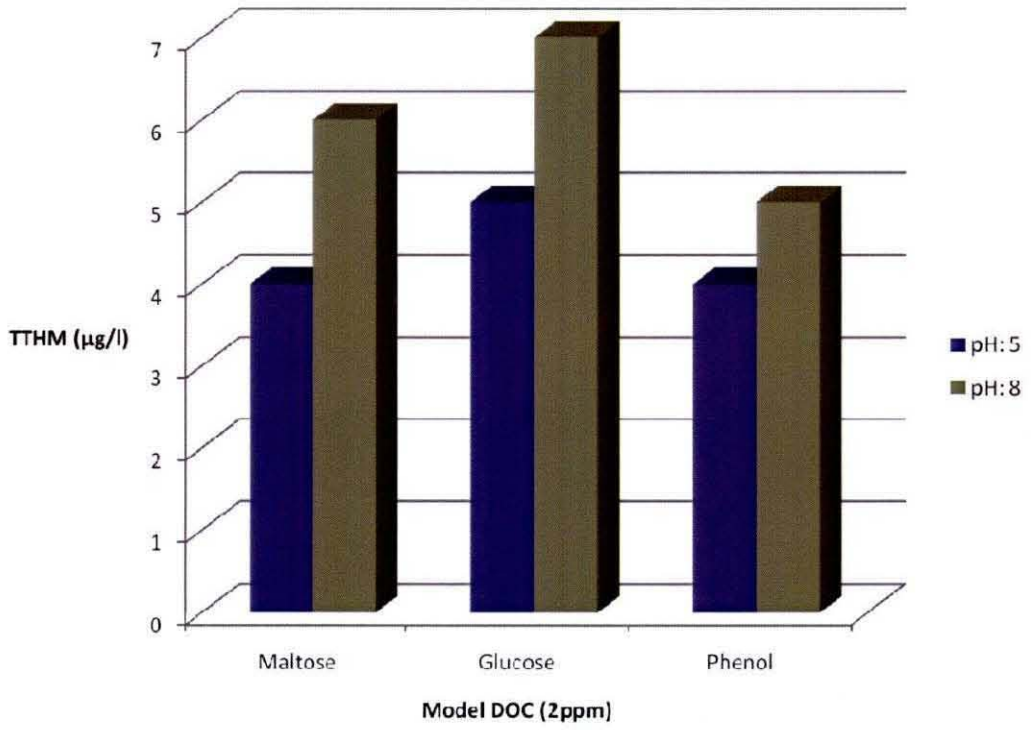

Figure 6.3: Effect of pH on THM formation (Experimental conditions: Temperature: 20°C; [Bromide]: 0ppb and pH: 8).

THM formation occurs by means of base-catalysed hydrolysis of reactive functional groups (Sohn *et al.*, 2006). Increasing pH will lead to an increase in base compounds and this will lead to an increase in base-catalysed hydrolysis of functional groups which will in turn lead to an increase in THM formation. Figure 6.4 shows a simplified model of the effect of pH on THM formation. According to literature pH is also said to increase the stability of carbanions (anions of carbons that have unshared electrons) (Reckhow *et al.*, 1990; Reckhow and Singer, 1990). The increase in THMs from experiments due to an increase in pH may therefore be explained by the two above mentioned statements. Detailed results of the distribution of trihalomethane species of the model organic compounds are indicated in Table 6.1.

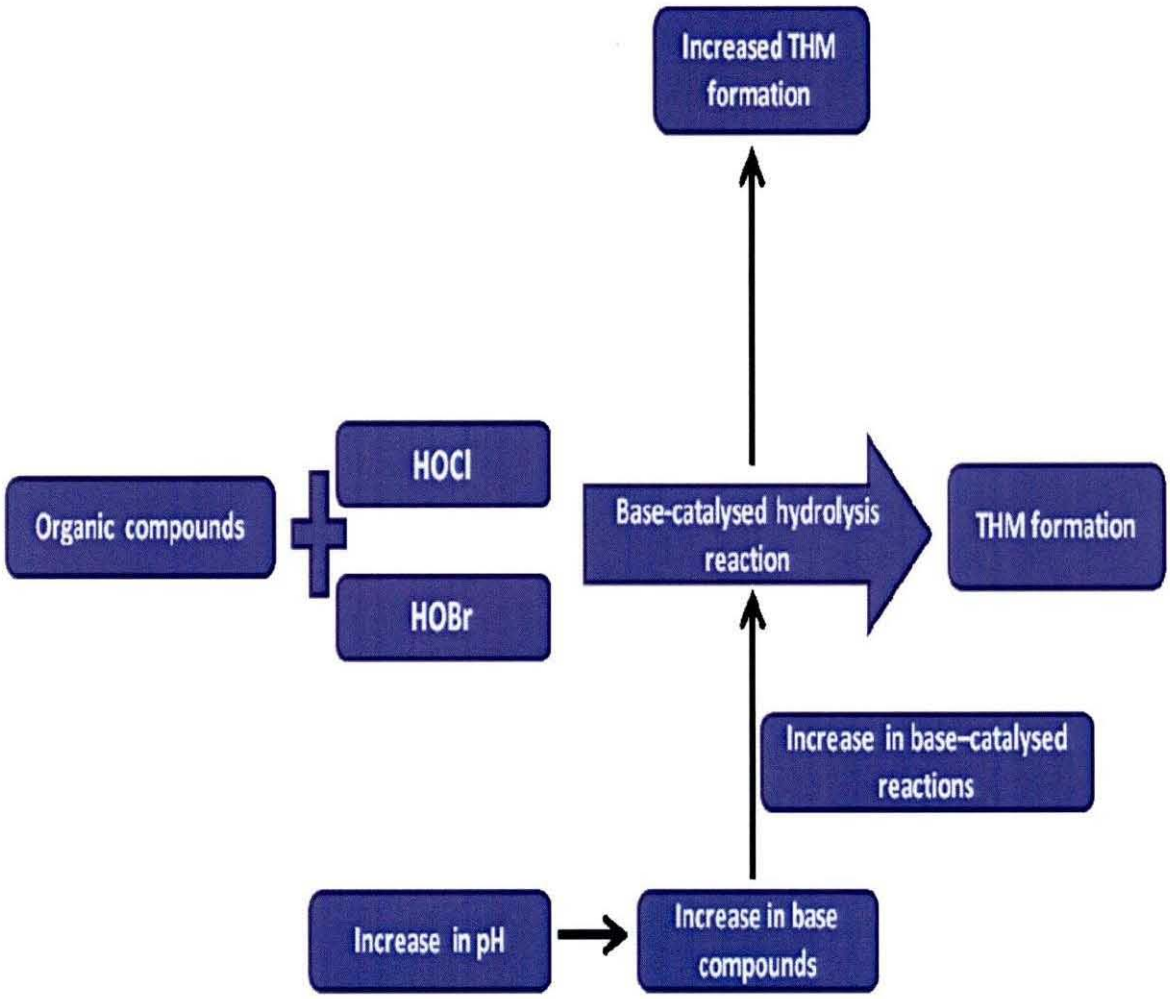

Figure 6.4: Simple Model explaining the effect of pH on THM formation

With reference to speciation of THMs; as indicated in table 1, chloroform and all 3 brominated-THMs were present at both pH 5 and pH 8 for all three models of DOC (maltose, glucose and phenol). The results indicated a pattern of slight increase in the concentration of brominated THMs with increased pH levels (i.e. pH 5 to pH 8); similar results were obtained for chloroform. The percentage increase for brominated species was more than that of chloroform. Chloroform and brominated species increased by an average of 25% and 30% respectively. These results are similar to those reported by Rathbun (1996). Ruthbun (1996) studied the effect of pH on THM speciation. It was observed that there was a slight increase in chloroform and brominated-THMs and brominated THMs increased more than chloroform (Ruthbun 1996). Research has indicated that hypobromous acid (HOBr) is a more powerful halogenating agent than hypochlorous acid (HOCl) (Morris, 1978; Nokes *et al.*, 1999). This means that reactions incorporating bromide into natural organic matter (NOM) during THM formation will be faster than those incorporating chlorine (Symons *et al.*, 1993; Nokes *et al.*, 1999). This provides a possible reason for a higher increase in brominated species compared to chloroform at pH 8. It is also worth noting that bromide incorporation at high pH occurs due to the higher activation energy of HOBr reaction compared to HOCl reaction (Sohn *et al.*, 2006).

Table 6.1: Distribution of trihalomethane species of the three model dissolved organic compounds at pH 5 and at pH 8. Experimental conditions: DOC (2ppm); Br: 0ppb

Model DOC	pH 5					pH 8				
	Chloroform (µg/l)	% distribution of chloroform	Brominated -THMs (µg/l)	% distribution of brominated THMs	TTHM at pH 5 (µg/l)	Chloroform (µg/l)	% distribution of chloroform	Brominated-THMs (µg/l)	% distribution of brominated THMs	TTHM at pH 8 (µg/l)
Maltose	1	33	3	66	4	2	33	4	66	6
Glucose	3	60	2	40	5	3	43	4	57	7
Phenol	2	50	2	50	4	3	60	2	40	5

6.3.2 Effect of bromide on trihalomethane concentration

Bromide is known to have an effect on THM formation (Bellar *et al.*, 1974; Cooper *et al.*, 1985; Krasner *et al.*, 1994; Navalon *et al.*, 2008). Bromide contribution towards THM formation occurs in the following manner: bromide becomes oxidised by chlorine (HOCl) to form hypobromous acid (HOBr); once HOBr is formed it becomes capable of participating in reactions similar to chlorine; as halogens both HOCl and HOBr compete for substitution with carbon atoms of organic matter to form DBP which contain either only chlorine or bromide or a mixture of both (Wong and Davidson, 1997; Nokes *et al.*, 1999).

Studies on the effects of bromide on THMFP were conducted at bromide concentrations of 0 and 50ppb and pH levels of 5 and 8. According to Navalon *et al.* (2008) bromide levels of 50 ppb are common in natural waters. Results obtained for the effect of bromide on THM formation are shown in figure 6.5

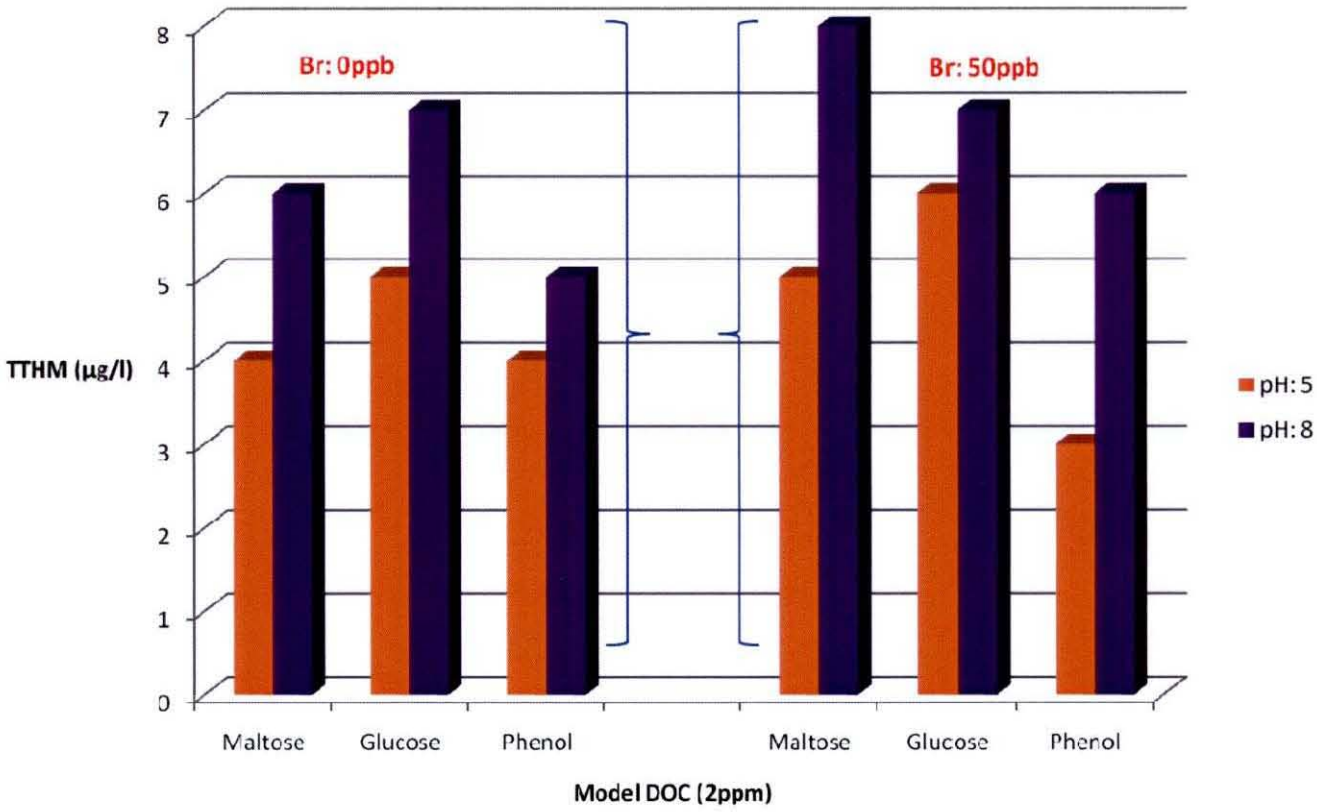

Figure 6.5: The effect of bromide on THM formation.

An increase in bromide concentration from 0ppb to 50ppb resulted in a slight increase in the amount of THM formation. Detailed concentrations are indicated in table 6.2. These results are similar to those that were obtained by Navalon *et al.* (2008). It has been reported that an increase in bromide content results in a concomitant increase in the hydrolysis rate of trihalomethane intermediates (Luong *et al.*, Aizawa *et al.*, 1989; Nokes *et al.*, 1999). This explains the increase of THMs with the increase of bromide concentration from 0ppb to 50ppb. An increase in pH from 5 to 8 resulted in an increase in the amount of THMs that were formed. According to Sohn *et al.* (2006) pH is one of the most significant factors that influence bromide incorporation during DBP formation. An increase in pH levels will increase bromide incorporation for chlorination DBP formation thus increasing the amount of DBP that will be formed.

Results of speciation of THMs that were obtained due to the effect of bromide and pH on THM formation are indicated in table 6.2. The results indicated that an increase in pH and bromide concentration has an effect on brominated-THMs. There was an increase in chloro-brominated ($CHCl_2Br$ and $CHClBr_2$) and brominated ($CHBr_3$) THM species. The increase in higher order brominated species ($CHClBr_2$ and $CHBr_3$) was higher compared to the other two species ($CHCl_3$ and $CHCl_2Br$). This indicated that the increase in the formation of brominated-THMs was related to the concentration of bromide in solution. These results are in agreement with those that were obtained by Stuart *et al.* (2001); Uyak and Toroz (2007) and Navalon *et al.* (2008). Compared to all four species of THMs, $CHCl_2Br$ was found to be the major component of the THM mix; these results are similar to those obtained by Navalon *et al.* (2008). This can be rationalized by the atomic ratio between chlorine and bromide (Morris, 1978; Symons *et al.*, 1993; Navalon *et al.*, 2008). Bromide has a higher atomic weight compared to chlorine and as previously stated bromide is reacts faster compared to chlorine during THM formation (Morris, 1978; Symons *et al.*, 1993; Navalon *et al.*, 2008).

Table 6.2: THM speciation at bromide concentrations of 0ppb and 50ppb studied under pH levels 5 and 8

Model DOC	Br: 0ppb								Br: 50ppb							
	THM speciation at pH 5 (µg/l)				THM speciation at pH 8 (µg/l)				THM speciation at pH 5 (µg/l)				THM speciation at pH 8 (µg/l)			
	$CHCl_3$	$CHBrCl_2$	$CHBr_2Cl$	$CHBr_3$	$CHCl_3$	$CHBrCl_2$	$CHBr_2Cl$	$CHBr_3$	$CHCl_3$	$CHBrCl_2$	$CHBr_2Cl$	$CHBr_3$	$CHCl_3$	$CHBrCl_2$	$CHBr_2Cl$	$CHBr_3$
Maltose	1	2	-	1	2	3	-	1	2	2	-	1	2	3	2	1
Glucose	3	1	-	1	3	2	1	1	2	1	1	2	2	1	1	3
Phenol	2	1	-	1	3	1	-	1	2	1	-	-	3	2	1	-
TTHM (µg/l)	13				18				14				19			

6.4 Conclusions

Although there is an overall increase in THM formation from the three trials due to increase in pH and bromide ; the results were variable for the three DOC models; according to literature the variation in results may be to differences between reactions of HOBr and HOCl with organic compounds (Sohn *et al.*, 2006; Chowdhury *et al.*, 2010). According to literature, the composition of organic matter, in particular the aromacity and the nature and position of the functional groups in the have an influence on the amount of chlorine and bromide that will be incorporated into the organic matter to form DBPs (Rook, 1980; Boyce and Horning, 1983; Heller-Grossman *et al.*, 1993; Nokes *et al.*, 1999). This may explain the differences in the amount of THMs which were formed by the three model compounds that were used for experiments. Further studies may be conducted to determine their influence on bromide and chlorine incorporation to form THMs from disinfection by Anolyte.

Navalon *et al.* (2008) went further in their experiments and studies the effects of basic pH on the formation of THM formation. They discovered that basic pH had an opposite effect to acidic pH; it increased the amount of THM formation. These studies may be conducted in future to determine the effect of extreme basic pH levels on the amount of THMs formed from disinfection by Anolyte.

Overall, the TTHMs produced by Anolyte in this study where extremely low; the average was +/-7(ppb) due to treament of Anolyte is extremely low; way below the recommended WHO standards of 100ppb. Similar studies conducted by Navalon *et al.* (2008) THMFP studies using chlorine produced an average of 102ppb; anolyte only produced less than 10% of what chlorine produced. This is a clear indication that Anolyte is indeed a green technology and it can be used as an alternative disinfectant to chlorine.

There are several models that have been devoloped to determine THMFP of solution. Sohn *et al.* (2004) developed a model that determines THMFP and it incorporates bromide, chlorine, pH, temperature (T), DOC and time (t) (Chowdhury

and Champagne, 2008); similar to parameters which were used in this study. The equation for this model is as follows:

$$TTHM = 10^{-1.385}(DOC)^{1.098}(Cl_2)^{0.152}(Br^-)^{0.068}(T)^{0.609}(pH)^{1.601}(t)^{0.263}$$

For future studies this model can be tested to see if it does fit with the results that were obtained in this study.

6.5 Appendix

DOC Model calculations

Amount required for DOC model = %Carbon in DOC model/ Molecular weight of DOC model

%Carbon = number of carbons x atomic number

Glucose: 2ppm

Glucose = $C_6H_{12}O_6$

% Carbon = 6 x 12

= 72

Molecular weight = 180

Amount required = 72/180

= 0.4 of a solution of 2ppm DOC

2ppm = 2mg/l

Mass of glucose = 2/0.4

= 5mg in a liter of water

Maltose: 2ppm

Maltose = $C_{12}H_{22}O_{11}$

% Carbon = 12 x 12

= 144

Molecular weight = 342

Amount required = 144/342

= 0.4 of a solution of 2ppm DOC

2ppm = 2mg/l

Mass of maltose = 2/0.4

= 5mg in a liter of water

Phenol: 2ppm

Phenol = C_6H_5OH

% Carbon = 6 x 12

= 72

Molecular weight = 94

Amount required = 72/94

= 0.8 of a solution of 2ppm DOC

2ppm = 2mg/l

Mass of phenol = 2/0.8

= 2.5mg in a liter of water

Preparations of other materials used

Bromide: 50ppb

50ppb = 50µg/l

50µg/l = 0.5mg/l of dry NaBr in a liter of water.

Anolyte: 100ppm

100ppm = 100ml/l

100ml/l = 100ml of Anolyte in a liter of water

= 10% Anolyte

6.6 References

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Chapter 7: Conclusions and Recommendations

The Rotoscope was used to monitor biofilm development of cocci and bacilli species over 72 hrs. Light reflectance decreased over time as the biofilm developed. The four distinct phases of biofilm development (1) initial reversible attachment, 2) irreversible attachment, 3) maturation, and 4) detachment) were evident from the light reflectance assay data that was obtained from the Rotoscope. The Rotoscope has shown to be an easy, effective, on-line monitoring device for the development of biofilms Epifluorescence microscopy (EM) and confocal laser scanning microscopy (CLSM) were used to validate results obtained from light reflectance assay data. Both EM and CLSM provided insightful information about the development of the biofilm. Information on the morphology of the bacteria, depth of the biofilm as well as the different stages of biofilm development was deduced from EM and CLSM images.

The Rotoscope was also used to monitor fouling and clean-in-place (CIP) in a simulated soft-drink (SD) production line. Electrochemically activated (ECA) solutions Anolyte and Catholyte were used during CIP. There was a progressive decrease of light reflection in the Rotoscope; this could be attributed to the accumulation of deposits on the Perspex rotating disc of the Rotoscope. During CIP the Catholyte and Anolyte were shown to be effective as detergent and disinfectant respectively. This was indicated by an increase in light reflection in the Rotoscope after CIP. Efficacy of the Anolyte and Catholyte were further validated by results obtained from microbial analysis of removable slides from the MPD, microscopic analysis, as well as pH, ORP and EC analysis as well as. No microbial growth was detected after CIP; microscopic analysis indicated no soil or microbial growth from slides that were analysed after CIP and values of pH, ORP and EC prior to and after CIP remained relatively the same.

A study of trihalomethanes (THMs) generated during CIP using Catholyte and Anolyte was conducted. Species of THMs that are present from CIP were also determined. Low levels of TTHM were obtained from CIP effluent which provided a good indication that Anolyte is an environmentally friendly alternative disinfectant. The results in this study are a good indication that ECA technology can be embodied into a beverage plant to improve production and other processes.

THMFP studies on carbohydrates and phenol treated with Anolyte were conducted. The effect of pH, bromide and organic compounds concentration on THM formation were monitored. Overall, the TTHMs produced by Anolyte in this study where extremely low; way below the recommended WHO standards of 100ppb. Although there is an overall increase in THM formation from the three trials due to increase in pH and bromide; the results were variable for the three DOC models. The variation in results may be to differences between reactions of the composition of organic matter, in particular the aromacity and the nature and position of the functional groups in the have an influence on the amount of chlorine and bromide that will be incorporated into the organic matter to form DBPs.

For future research in depth study of contents of the beverages and syrups being used for the experiments should be conducted. This will give a clear understanding of the chemical interactions between the beverages or syrups and those of the CIP chemicals (Anolyte and Catholyte in particular). Results obtained will be provide further understanding of the chemical interactions that occur between the organic compounds in the juices or syrups with ECA chemicals during CIP that lead to THM formation.

Pilot plant trials should be conducted for all experiments that have been performed in the laboratory. The results obtained from pilot plant trials will provide an idea of the up-scaling that will be required for the industrialization of the CIP using ECA technology as well as to determine the amount of THMs that are produced during CIP at an industrial scale.