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BIOFILM MONITORING AND CONTROL USING ELECTROCHEMICALLY ACTIVATED WATER AND CHLORINE DIOXIDE

By

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DECLARATION

I, the undersigned, certify the thesis hereby submitted to the University of Pretoria for the degree of Masters and the study contained herein is my original work and has not previously been submitted for any degree.

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Summary

Biofilms are important in nature and in engineered processes. Because of this, a fundamental understanding of their growth and behaviour is required. This work aimed at monitoring biofilm growth using a biological rotating reactor and the Rotoscope biofilm monitor. Both methods worked on the principle of a rotating circular disc that was semi-submerged in water and the light reflected of the area that was outside of the water. Light reflectance on the disc was taken three times a day and the average recorded as the daily reading. It was noticed that in both systems, growth of biofilms on the discs caused a decrease in the amount of light reflected. A decrease in light reflectance indicated an increase in biofilm thickness. The growth of biofilm was confirmed by scanning electron microscopy analysis. The addition of a biocide caused a slight increase in light reflectance indicating partial biofilm removal. The Rotoscope was very sensitive to changes in biofilm characteristics. Rotoscope met the requirements needed for an on-line, real-time and non-destructive biofilm monitoring system.

The aged anolyte was effective in killing both suspended and biofilm bacteria at a concentration of 1:10 irrespective of its age and storage conditions. Exposure of aerobic bacteria to different concentrations of sodium nitrite at different time intervals indicated that sodium nitrite had a limited, or no biocidal effect on these bacteria mostly encountered in biofilms. The ready to use chlorine dioxide was also used as the means of controlling biofilms. MIC for RTU ClO_2 was found to be 80ppm, which

in certain instances killed all bacteria immediately upon exposure while in other cases an exposure time of 1h was required. It was indicated that at this concentration, biofilms were removed. This was confirmed by scanning electron microscopy analysis. Proteins of suspended bacteria treated with 1:10 and 1:100 anolyte dilutions and the control were extracted and compared using SDS-PAGE. Protein bands of bacteria treated with 1:10 NaCl derived anolyte were fewer and fainter as compared to those from untreated cells. More bands were produced in cells treated with 1:100 NaCl derived anolyte as compared to the untreated cells. Cells treated with the non-halide anolyte, both 1:10 and 1:100 dilutions, produced more bands than in the untreated cells. Anolyte destroyed vital proteins for bacterial survival causing cell death or it caused fragmentation of proteins to small peptides, reducing the number of viable cells. $NaNO_2$ was ineffective as biocide while aged anolyte and RTU liquid ClO_2 were effective as biocides. SDS-PAGE indicated that anolyte killed bacteria by affecting their proteins.

List of abbreviations

EPS – extracellular polymeric substances

MIC – microbially induced corrosion

MIC – minimum inhibitory concentration

SRB – sulphate reducing bacteria

ECA – electrochemically activated water

SEM – scanning electron microscopy

CLSM – confocal laser scanning microscopy

AFM – atomic force microscopy

ATP – Adenosine Tryphosphate

MRD – modified Robbins device

RD – rectangular duct

PVDL – poly(vinylindele)fluoride

LPR – linear polarization resistance

EIS – electrochemical impedance spectroscopy

EN – electrochemical noise

EPA – Environmental Protection Agency

FISH – fluorescence in situ hybridization

DGGE – denaturing gradient gel electrophoresis

MAR – microautoradiography

NMR – nuclear magnetic resonance

NOM – natural organic matter

BDOC – biodegradable dissolved organic carbon

FOD – fiber optical device

ORP – Oxidation-reduction potential

EC – electrical conductivity

THMs – Trihalomethanes

DBPs – disinfectants-by-products

RTU – ready-to-use

SDS-PAGE – sodium dodecyl sulphate polyacrylamide gel electrophoresis

STB – sample treatment buffer

ddH_2O – double distilled water

HACCP – Hazard analysis critical control point

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CHAPTER 1

1. 1 INTRODUCTION

Surfaces that come in contact with water are conditioned by adsorption of organic materials and bacteria. Mobile waterborne bacteria migrate to surfaces and attach by excretion of exopolysaccharides (EPS), forming gel-like matrices in which the bacteria are enclosed. There are several reasons for this purpose. The surface could be a source of nutrients for adsorbed material, and the continuous flow of water across the surface also provides oxygen for aerobic species. The individual microorganisms are likely to be smaller than the crevices and roughness of the surface, so they are able to "hide" from the removal effects of flow across the surface (Melo and Bott, 1997). In other words, the surface provides shelter for microorganisms. Eventually areas will join together and form a continuous biofilm. The sticky EPS that provides protection to the microorganisms in a biofilm also produce a more occluded creviced environment which is more resistant to mechanical action (Bruijs *et al.,* 2003).

The biofilm mass is predominantly water (85-95% wet weight), bacteria (10^{11}-10^{12} cells/ml) and EPS (1-2% wet weight). Despite the small EPS content, most of the physical and physicochemical properties of biofilms such as water binding, sorption of dissolved substances and particles, and mechanical stability of the biofilm are caused by EPS (Schmid *et al*., 2002). Biofilms are quite diverse due to the wide range of contributing factors like, availability of nutrients and oxygen, microbial species, surface type, flow velocity of the surrounding liquid, etc. The type of bacteria found on biofilms depends on the rate and extent of their growth because they do not grow in homogeneous structures. They change their shape, size and other chemical/physical characteristics across any given unit area and across the whole system. Spatial distribution of biofilm is a major factor in determining the ease of detachment, which occurs through erosion, sloughing, abrasion and grazing (Meyer, 2003).

Biofilm develop virtually on any surface in natural soil and aquatic environments, on tissues of plants, animals and humans as well as in man-made technical systems. The industrial systems contain many sites and components susceptible to biofilm

formation. Biofilm formation can be technologically important e.g. when used in wastewater purification or as coatings on graft materials. Biofilms have significant influence in public health with regard to contamination of drinking water and bacterial infections of living tissues, teeth, prosthetic devices and contact lenses (Peyton, 1996). In industrial pipelines, biofilms cause accelerated corrosion of steel surfaces, increased pressure drops, and product contamination and spoilage (Stoodley *et al*., 2002). Studies have reported that biofilms play an important role in microbial growth and industrial fouling (Kim *et al.,* 2004).

Industrial fouling results in microbial influenced corrosion (MIC). MIC is the deterioration of a metal by corrosion processes that occur directly or indirectly, as a result of metabolic activity of microorganisms (Sarioglu *et al.,* 1997). Under anaerobic conditions, a diversity of microorganisms can have an effect on the corrosion of metallic iron. Sulphate-reducing bacteria (SRB) play a significant role in corrosion by accelerating the electrochemical processes whenever conditions are favorable (Brock and Madigan, 1991). In many environments, methanogenic bacteria, which are found near SRB, may also cause biocorrosion (Boopathy and Daniels, 1991).

Chemical treatment during corrosion is quite effective to microbes but is undesirable due to their impact on environmental quality. The effectiveness of various treatments for biofilm in actual field applications must be assessed in a meaningful, accurate and sensitive manner. Frequently other reacting components interfere with the intended control procedure for biofilm. Water quality and substratum composition must be considered in choosing a treatment program to minimize corrosion (Characklis and Marshall, 1990).

Biocides are chemicals used in water systems to prevent the build-up of microorganisms. These are single compounds (or a mixture of compounds) capable of killing or inhibiting microbial growth. Biocides can be inorganic such as chlorine, ozone, bromine etc. or organic including isothiazolines, quartenary ammonium compounds, aldehydes etc. Because some of these biocides are carcinogenic (e.g. chlorine's by-products), and toxic to humans (bromine) and some bacteria have

acquired resistance to some of them (e.g. quaternary ammonium compounds), the best biocide for each situation still has to be determined (Videla, 2002).

Biocides are applied, in hope that killing of the fouling organisms will solve the problem. It is commonly believed that dead bacteria cause no more problems, but this is not the case. In the first place, biofilm organisms are highly tolerant to biocides (LeChevallier *et al.,* 1998). Secondly, dead biomass will usually not be removed from the surface, and may serve as nutrients for the newly coming bacteria (Tamachkiarow and Flemming, 2003).

From the above, it can be clearly seen that many problems in technical water systems are caused by biofilms and not by planktonic cells. Thus, countermeasures against biofouling must be directed against surface-attached biofilms. These measures should include the detection, monitoring, removal, prevention and at least control of biofilm formation. A variety of sanitation measures for the treatment of biofouling exist such as regular cleaning using physical methods (e.g. rinsing, brushing, ultrasonic treatment), application of chemical agents (oxidants, alkali, surfactants, enzymes, complexing substances, dispersant) to kill and detach biofilm organisms, and limitation of nutrients to minimize microbial growth (Schulte *et al*., 2003). Because of the difficulties associated with the removal of existing biofilms, one strategy of dealing with biofilms is to prevent the formation of new biofilm (Schulte *et al*., 2004).

Research has proved that the use of existing antimicrobial chemicals has more disadvantages than advantages. This necessitates research for a more effective, simple and precise biocide.

The main objective of this study was to monitor biofilm growth using a laboratory Rotoscope. In addition the following were included:

- To monitor biofilm growth using light reflectance.
- To evaluate sodium nitrite as a possible biocide.
- To control bacteria and biofilm growth using ready to use chlorine dioxide and electrochemically activated water (ECA).

- To determine the biocidal effect of ECA on bacterial proteins by comparing the proteins of treated and untreated bacterial cells using sodium dodecyl sulphate (SDS-PAGE).

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CHAPTER 2

LITERATURE REVIEW

2.1 WHAT IS BIOFILM?

Biofilm can be defined as a microbially derived sessile community, characterized by cells that are irreversibly attached to a substratum or interface or to each other, and embedded in a matrix of extracellular polysaccharide substances (EPS) that they have produced, and exhibit an altered phenotype with respect to growth rate and gene transcription (Russell, 2003). Gilbert *et al.* (2003) described biofilm as microbial aggregates that form and persist at phase boundaries and may include heterogeneous populations of bacteria, fungi, algae and protozoa.

Biofilm develop virtually on any surface as well as in man-made technical systems. They can be found on stones in rivers, on pipes in industrial water systems, in paper and paint manufacturing plants and on tooth surfaces (Walker and Marsh, 2004).

2.1.1 Formation of biofilm

Attachment

Planktonic bacteria are bacteria that are free living in the fluid phase. Cells must reach a surface in order to become part of the biofilm. Microorganisms are able to attach firmly to almost every surface in our environment. They adhere in two ways, that is either by generic physical and chemical forces, or with the use of specific surface structures of the cell such as pilli, fimbriae or other appendages. In situations where adhesion is not directly expected to be a specific process, such as in soils, long-range interactions are always responsible for the first step in the attachment of bacteria (van Loosdrecht *et al.,* 1989). Apart from the possible “chemotaxis” to the surface, electrical forces may take part in the adhesion process. The charge on a microorganism may be different from that on the surface, giving rise to attraction (Melo and Bott, 1997). The initial adhesion of bacteria is suggested to be reversible and thus relatively weak, but after a period of time, the adhesion becomes more substantial and irreversible (van Loosdrecht *et al*., 1990).

Attached cells grow, reproduce and produce inert extracellular polymeric substances, which frequently extend from the cell, forming a tangled matrix of fibers that provide

structure to the assemblage. Attached cells attract those that are free-floating to form microcolony. Secondary colonizers attach to primary colonizers by co-adhesion and contribute to community development. Microbial attachment is a dynamic complex process, which can be affected by many variables including flow rate, surface roughness and hydrophobicity, and the presence and properties of conditioning film (the surface coated with molecules absorbed from the aqueous medium) (Walker and Marsh, 2004). During this period several patterns may arise, depending on the mode of attachment:

(i) Cells are reversibly adhered to the surface and to each other. Thus resulting in an equilibrium distribution between adhered and suspended cells.

(ii) Cells are irreversibly bound to the surface, but not to each other, resulting in the formation of a monolayer of cells on the surface.

(iii) Cells are irreversibly attached to the surface and to the each other, resulting in biofilm formation (van Loodsrecht *et al*., 1990).

Inside the conditioning film

Bacteria excrete toxins and EPS within the biofilm. EPS are primarily composed of polysaccharides, uronic acid, sugars and amino acids groups, which could be acidic and capable of binding ions (Fang *et al.,* 2002). EPS can contain adsorbed macromolecules from other origins. The biofilm mass is predominantly water (85-95% wet weight), bacteria (10^{11} - 10^{12} cells/ml) and EPS (1-2% wet weight). Despite the small EPS content, most of the physical and physicochemical properties of biofilms such as water binding, sorption of dissolved substances and particles, and mechanical stability of the biofilm are caused by the EPS (Schmid *et al*., 2002b). EPS also change the properties of the original bacterial cell. The attached cells must synthesize new exopolysaccharides material in order to 'cement' their adhesion to the surface and to other bacterial cells in the developing biofilm, to progress from the reversible attachment stage to the irrreversible adhesion phase of biofilm formation. Life inside the EPS matrix offers many advantages for bacteria such as stabilization of bacteria to surfaces, enhancement of biofilm resistance to environmental stress and antimicrobial agents, thereby giving biofilm protection (Zhang and Bishop, 2003).

Phenotypic variation

Biofilm cells are different from their planktonic cells. According to Walker and Marsh (2004), gene expression is regulated once the cell comes in contact with the surface, where in some organisms, up to 22% of genes were up-regulated in the biofilm state and 16% down regulated.

Gene transfer

A biofilm community provides an ideal niche for the exchange of DNA and plasmids. Cells in biofilms will exchange plasmids (conjugation) at a greater rate than cells in the planktonic phase (Walker and Marsh, 2004).

Quorum-sensing communication

Bacteria within a biofilm need to communicate and interact with each other under certain conditions, in order to perform certain activities. This is known as quorum sensing behaviour. The quorum sensing system can be divided into two types: LuxR/Lux1-type quorum-sensing in Gram-negative bacteria, which use N-acyl-homoserine lactones (AHLs) as the autoinducers (small, diffusible signaling molecules whose extracellular concentration is related to the population density of the producing organism) and oligopeptide two-component-type quorum-sensing in Gram-positive bacteria, which use oligopeptide as inducers. At high population densities, where the concentration of autoinducers reached the threshold, autoinducers penetrate intercellular and bond to receptors forming auto inducer-receptor complex, which binds to DNA and activates multiple targets genes involved in behavioural traits and in the production of further signals. Thus, cells respond quickly to a particular environmental condition such as altering its gene expression accordingly and respond to activities including conjugation, luminescence, virulence, swarming and antibiotic production (Morton *et al.,* 1998; Zhihua *et al*., 2004). At sufficient population densities, the cell signaling molecules, homeserine lactones in Gram-negative bacteria and peptides in Gram-positive bacteria, reach concentrations required for activation of genes involved in biofilm differentiation (Walker and Marsh, 2004).

Biofilm detachment

Biofilm detachment refers to the dissociation of biofilms into the surrounding environment. The detached bacteria provide an inoculum for growth of a non-attached

population and for colonization at new sites (Boyd and Chakrabarty, 1995). Detachment occurs if local shear and normal forces acting on the biofilm exceed the cohesiveness of the biofilm. This cohesiveness is influenced by the composition and structure of the polymeric matrix forming the biofilm, which in turn is determined by the history of the biofilm, growth conditions and developmental stage of the biofilm (Telgmann *et al*., 2004).

Different processes are responsible for detachment of biomass and four categories of detachment can be distinguished, that is abrasion, erosion, sloughing and predator grazing. Abrasion and erosion both refer to the removal of small groups of cells from the surface of the biofilm but are differentiated by their mechanism. Erosion is caused by shear forces of the moving fluid in contact with the biofilm surface, while abrasion is caused by the collision of the biofilm support particles, e.g. during back washing of fixed bed reactors. Sloughing refers to the detachment of relatively large portions of the biofilm whose characteristic size is comparable to or greater than the thickness of the biofilm itself, while grazing is the removal of biofilm due to its consumption by higher organisms such as protozoa (Morgenroth and Wilderer, 2000).

Detachment may occur at anytime during biofilm development, resulting in the release and re-suspension of the microorganisms, which may include pathogenic species, from the biofilm to the planktonic phase of the system (van Loosdrecht *et al*., 1990).

2.1.2 Factors affecting growth of biofilm

The morphology and structure of biofilm is likely to change as the biofilm ages and will be dependent on external conditions, particularly with respect to nutrient availability and flow over the biofilm.

Nutrient availability

Nutrient and surface characterization have been shown to govern biofilm formation and growth. High levels of nutrients appear to produce open structure in the biofilm while lower concentrations tend to give a more compact structure. It is also interesting to note that, when a surface has been contaminated with bacteria and nutrients are available, the biofilm will continue to develop, even though there are no further

microorganisms in the aqueous phase. The structure of the biofilm has an effect on the availability of nutrients to the constituents' cells. For example, for aerobic bacteria, the availability of oxygen is necessary unless the particular microorganism can exist under oxygen-starved conditions (Melo and Bott, 1997). In low nutrient environments surface attached growth predominates over suspended growth. According to the research done by Soini *et al.* (2002), reduction in nutrient concentration decreases the biofilm density.

Temperature

Microbial activity is very sensitive to temperature. The optimum temperature for bacteria found in cooling water systems is about 40ºC. At this temperature, small changes in temperature are likely to produce substantial changes in biofilm growth because microbial activity is very sensitive to temperature (Melo and Bott, 1997).

Surface conditions

The formation of biofilm is dependent on the surface characteristics of the substratum, including metal surface, free energy, roughness and hydrophobicity. Thick biofilms are found on rough metals as compared to smooth metals. The biofilm formed under turbulent flow is homogenous and slimy, while those formed under laminar flow is scattered on the surface (Simões *et al.*, 2003), and an increase in the amount of biofilm formed under turbulent flow conditions has also been reported (Melo and Bott, 1997).

Presence of particles

The simultaneous depositions of small particles that are transported with the incoming water usually accompany biofilm formation. When the particles are of organic nature, they can act as substrates for the microorganisms and be degraded by them, contributing to the growth of the biomass. Apart from the effect that the clay particles seem to have on the physical structure of the microbial film, the particles could also contribute to the maintenance of a suitable pH value within the biofilm on account of their well-known adsorption and ion-exchange properties (Melo and Bott, 1997).

2.1.3 Up side and downside of biofilm

Bacteria which are associated with mammalian skin and mucosal surfaces protect the host from pathogenic bacteria whilst sessile communities in the human large intestine play important roles in the metabolic well-being of the animal (Gilbert *et al*., 2003). In natural water systems, surface-associated microorganisms degrade organic compounds and detoxify xenobiotics, thereby maintaining some aspects of water quality. The metabolic activities of microbial biofilms have been harnessed for wastewater management sewage treatment and in biotechnology for a variety of solid-state fermentation processes (Gilbert *et al.,* 2003).

Biofilms have significant influence in public health with regard to contamination of drinking water and bacterial infections of living tissue, teeth, prosthetic devices and contact lenses (Peyton, 1996). Biofilms formed on surfaces of industrial equipment cause serious problems such as decrease of heat transfer in heat exchangers, increase of the fluid friction resistance at the surface, increase of corrosion and product contamination (Stoodley *et al*., 2002). The formation of biofilms also results in undesirable effects in membrane processes including reduction of permeate flux, increase of soluble accumulation near membrane surfaces module differential pressure and biodegradation of membrane polymers (Azeredo *et al*., 2003; Ludensky, 2003).

2.2 BIOFILM MONITORING

Environmental biofilms are complicated assemblies of different cellular and polymeric constituents, which are associated with interfaces. As they are of high importance in the field of ecology, medicine and biotechnology, their development, structure and function under different cultivation conditions is of interest to a wide audience of scientists. Biofilms not planktonic cells cause many problems in technical water systems. Therefore countermeasures against biofouling must be directed to biofilms. The measures should include detection, monitoring, removal, prevention or control of biofilm (Flemming, 2003).

Monitoring the microbial water quality in a distribution system is part of the regulatory requirements that a water treatment plant must meet (Chang *et al*., 2003).

Monitoring is a colloidal term that helps to communicate ideas among biofilm researchers and practitioners. According to Lewandowski and Beyenal (2003), the suitable mathematical models that prevents extrapolating the monitored data are lacking, and the next best strategy will be to monitor the parameters that are evidently related to biofilm accumulation, or an effect of biofilm accumulation, and to select the intensity of the measured signal that triggers a warning system; if the readout exceeds a certain number, a biocide must be added.

Many biofilm monitoring systems follow such a preventive strategy, and they act as action triggers. The most popular parameters to monitor in biofilms are light intensity, heat transport resistance, electrical conductivity, torque pressure drop and frequency of oscillation of piezoelectric crystals (Lewandowski and Beyenal, 2003).

Biofilm thickness, the perpendicular distance from the substratum to the biofilm-bulk liquid interface, has been historically used to determine the distance through which substrates and nutrients must diffuse to fully penetrate a biofilm. Biofilm thickness negatively influences fluid frictional losses and heat transfer resistances in industrial process equipment, and is a primarily variable that determines the plugging of porous media for in situ bioremediation. Thickness has been primarily estimated by three methods: optical, volumetric displacement and electrical conductance. Volumetric displacement provides the average thickness over the measured area; whereas the optical and electrical methods give point measurements at specific thickness (Peyton, 1996).

Light intensity, dispersed or reflected, changes with biofilm thickness, but it can also depend on the concentration of particulate matter in the system, colour (biofilm has different colours), or chemical composition of the water. Monitoring light intensity or pressure drop may be quite useful, particularly when they are monitored for an extended period of time at the same location (Lewandowski and Beyenal, 2003).

Quantifying biofilm function is not necessary because biofilm vary in thickness, density and physical/chemical composition from point to point in any given process

of water system. Biofouling monitoring is a means of measuring and comparing specific parameters of biofilms in a specific process over a period of time.

2.2.1 Devices used to monitor biofilm growth

Light and Scanning electron Microscopies

Light microscopy has been useful as a preliminary step in biofilm studies supplying information on the general appearance of the fixed biomass. Better information about the location and spatial distribution of thin biofilms with active biomass can be obtained rapidly and effectively by binocular magnifier observations after staining with INT [(2-Cp-iodophenyl)-3-(p-nitro phenyl)-5-phenyl tetrazolium]. The main advantages of light microscopy are simplicity, rapidity and possibility to observe the biomass immediately without preliminary treatment (Lazarova and Manem, 1995).

The techniques of light and scanning electron microscopy has been and continues to be the basic method of biofilm structure investigations (Cloete *et al*., 1994). The scanning electron microscopy (SEM) provides a high magnification and image contrast. By means of SEM, some researchers have revealed some complementary information about the different stages of anaerobic biofilm development, demonstrating non-homogeneous biofilm spatial distribution. The major disadvantage of SEM is that it is slow and has complex sample preparation procedure, which may induce specimen damage, distortion or biofilm loss (Lazarova and Manem, 1995).

Confocal laser scanning microscopy

Confocal laser scanning microscopy (CLSM) has been described as a powerful technique to study biofilms. This technique extended the possibilities of in-depth visual observation of biofilm structure by means of 3-D images, which provide a bridge between light microscopy and electron microscopy (Lazarova and Manem, 1995). The technique requires the addition of fluorescent probes in order to record as much of the various biofilm constituents as possible (Staudt *et al*., 2003). The laser produces a high intensity illumination, and since the returning signal is processed point-by-point, even low levels of fluorescence can be imaged with a sensitive photomultiplier (Cloete *et al.,* 1998). This technique has been used to study nascent biofilms without affecting biofilm structure or architecture. The high sensitivity, and

the capability to observe samples *in situ*, renders CLSM suitable to demonstrate the presence and distribution of fluorescent molecules in biological material such as biofilms. The limitations of this technique are that it is expensive and it is not possible to observe motile bacteria and eukaryotic grazers within the biofilm (Cloete *et al.,* 1998).

Atomic Force Microscopy

Atomic force microscopy (AFM) is a method used for measuring surface topography on a scale from angstroms to microns. It allows imaging in any environment. It can be operated in aqueous solutions, without sample drying and this makes it possible to investigate biological samples under physiological conditions (Dufréne, 2001). AFM provides not only the opportunity for observing the specimens at molecular resolution, but also of quantifying surface feature information (Xu *et al.,* 2002). In situ AFM imaging procedure will allow the direct observation of biospecific interaction with biopolymers of bacterial cell, the destruction of bacteria by drugs, their growth and division in situ. Dried in ambient conditions for AFM analysis, bacteria remain alive and being returned to a culture medium can continue their shelf life (Bolshakova *et al*., 2001). The AFM imaging of live bacteria can sometimes be difficult due to a number of reasons. Firstly, bacteria often have flagella allowing them to move through the growth medium and if, they choose to attach to an AFM substrate, they can frequently detach and swim away. Secondly, if they chose to remain on the AFM substrate, they often synthesize extra polymeric substances, such as alginate, which obscure cell wall; and lastly, if cells are not motile, there will be reliance on Brownian motion to bring them into contact with AFM substrate. The initial adhesion forces after contact are reversible and weak (Yao *et al.,* 2002).

Pedersen's device

This device consists of a closed unit through which water can be diverted. Inside the unit is a coupon on which the undisturbed biofilm grows. Staining attached cells with DAPI (4,6-diamidino-z-phenylondole) follows biofilm development and counting the cells by epifluorescence microscopy (Jacobs *et al*., 1996).

Robbins Device

The Robbins device was first developed at the University of Calgary for monitoring biofilm in industrial systems and pipelines (Johnston and Jones, 1995), and later modified to low, perspex Robbins device (MRD) for the study of medical, environmental and industrial biofilm. The Robbins device is a ported biofilm sampler consisting of removable test surfaces, which are exposed to circulating fluids (Cloete *et al*., 1998). MRD can be attached to the chemostat, and control the flow rate of water, over the discs. The disc can then be microscopically monitored or cells can be removed from the disk and enumerated (Jackson, 2002). This device can also be used to determine the concentration of biocides and antibiotics that kill planktonic bacteria in bulk fluids (Cloete *et al*., 1998).

Rectangular duct

In order to evaluate optical methods for quantitative biofilm analysis Bakke *et al*. (2001) used the rectangular duct (RD) biofilm reactor. The reactor has thin, plane, transparent walls so that a microscope can be focused on any plane in the reactor parallel to the outside walls to obtain a visual image of the biofilms. These images are used to determine optical biofilm thickness without disturbing the biofilm. Biofilm optical density is measured by transmitting light through the biofilm and through the reactor. Light passes through glass, biofilm and the bulk water. Thus, the biofilm optical density is determined by subtracting the bulk water optical density from the total optical density (Bakke *et al*., 2001).

The on-line monitoring system BIoGEORGE™

The BIoGEORGE™ is an electrochemical biofilm activity monitoring system developed to monitor biofilm activity on-line (Bruijs *et al*., 2003). The probe consists of a series of metallic discs comprising two electrodes, which are electrically isolated from each other. When a biofilm forms on the probe of one electrode, it becomes polarized and provides a more conductive path for the applied current. As the probe is repeatedly polarized, it creates conditions over the cathode, which is chemically different from those over the anode. The generated current is monitored every ten minutes (Bruijs *et al.,* 2003).

Photoacoustic spectroscopy (PAS)

Photoacoustic spectroscopy (PAS) is based on the absorption of electromagnetic radiation inside a sample where non-radiative relaxation processes convert the absorbed energy into heat (Schmid *et al*., 2002a). PAS allows nondestructive investigation of biofilm. The photoacoustic poly(vinylindele)fluoride (PVDL) film is coupled to a transparent prim by a conductive epoxy. The prism allows monitoring of pH-value, flow conditions particulate, etc., at the different positions inside a flow channel (De Saravia and Lorenzo De Mele, 2003).

PAS combines features of optical spectroscopy and ultrasonic topography and allows in contrast to other spectroscopic techniques – a depth-resolved analysis of both optically and acoustically inhomogeneous media, which is not possible with other spectroscopic technique. Additionally, it allows optical absorption measurements even in strongly scattering or optical opaque media (Schmid *et al*., 2004).

ATP for the determination of the active biomass fraction in biofilms

ATP is an attractive parameter for determining the concentration of active biomass (biofilm) on water-exposed surfaces. It can also be used for determining the concentration of suspended biomass in treated water and in experimental systems. Collecting data with this parameter about biofilms present in water treatment, in distribution systems, in biofilm monitoring devices and in materials testing gives a framework for evaluation of the observed concentrations. The objectives of using ATP are to control biofilm formation in water treatment and to achieve biological stability in distribution system (Thantsha, 2002).

AQUASIM as modelling system

AQUASIM is a computer program for the identification and simulation of aquatic systems. It consists of three zones: “bulk fluid,” “biofilm solid matrix” and “biofilm pore water.” AQUASIM calculates the development of microbial species and substrates, as well as the biofilm thickness over time (Wanner and Morgenroth, 2003). Detachment and attachment of microbial cells at the biofilm surface can be monitored using this technique. AQUASIM’s limitation is that it only considers spatial gradients of substrates and microbial species in the biofilm in the direction perpendicular to the substratum (Wanner and Morgenroth, 2003).

Biofouling monitoring using an infrared monitor

The monitor is designed to measure biofilm accumulation in a tube through which microbial contaminated water is flowing. Infrared radiation from an emitter passes through the transparent glass wall, through any accumulated biofilm on the adjacent surface, through the flowing water, through the biofilm residing on the glass surface adjacent to the sensor and finally through the biofilm walls itself for the sensor. The emitter and the sensor are contained in a specially designed housing that fits round the glass tube. The difference in the radiation emitted to that collected, is the amount absorbed by the system, including the two glass walls, the flowing water and two biofilms. By "zeroing" the instrument with no biofilm present but with the water flowing as it would be during assessment, the absorption of radiation can be attributed to the presence of the two biofilms subsequently formed from the contaminated water (Thantsha, 2002).

The Roto Torque System

This is an excellent laboratory system for monitoring biofilm development because of its sensitivity, particularly to changes in fluid frictional resistance. It consists of a stationery outer cylinder and a rotational inner cylinder. An outer cylinder has removable glass slides that permit sampling of the biofilm so that thickness, mass and biofilm chemical and microbial composition can be determined (Characklis and Marshall, 1990).

Biofilm monitoring by Electrochemical Techniques

a. Linear polarization resistance

This is commonly used in commercial instrument corrosimeters to measure uniform corrosion. Linear polarization resistance (LPR) gives a direct measurement of current vs. potential within 10mV from the free corrosion potential, assuming that in this range, an applied current density is approximately linear with potential. The analysis with the LPR technique is valid if there are no other electrochemical reactions contributing to the response of the interface, which would result in a very complicated system to interpret (Christiani *et al*., 2002).

b. Electrochemical Impedance Spectroscopy

The use of electrochemical impedance spectroscopy (EIS) is especially favourable for non-conducting and semi conducting films or in media with low conductivity. EIS has been applied for MIC studies and monitoring with differing success. The technique is mainly applied in the laboratory experiments and provides better information than LPR, for mechanistic studies in particular. Limitation of this technique is that the data interpretation is not simple and requires specialist knowledge (Christiani *et al.,* 2002).

c. Electrochemical Noise

Electrochemical Noise (EN) is a non-destructive measure of potential or current fluctuations and can be conducted to measure open circuit potential without applying an external signal. The technique is able to detect the initiation of pitting and has been used for laboratory studies of the effect of SRB and other organisms on the formation of iron sulphide film contributing to pitting corrosion of reinforced steel and concrete (Christiani *et al*., 2002).

d. Redox potential

The reduction-oxidation reactions can be used mainly to establish if corrosion processes are developing in aerobic or anaerobic conditions, by evaluating the redox potential of the solution. The technique can be useful in combination with other electrochemical measurements (Christiani *et al*., 2002).

The use of the "Biowatch" system for biofouling monitoring

The "Biowatch" system is available through ONDEO – Nalco and is based on a rotating transparent disc with a diameter of approximately 30cm, where one half of the disc is exposed to the system water, in a chamber which houses the disc. Rotating the disc out of the water and taking a photometer reading by transmitting a light beam through the transparent disc measures biofouling. The quantity of light transmitted then gives an indication of the severity of fouling. This is one of the most practical commercially available on-line monitors currently available.

Fluorescence in situ hybridization

Fluorescence in situ hybridization (FISH) is highly effective for detecting specific bacteria and analyzing the spatial organization of a complex microbial community, due to the possibility of detecting specific bacterial cells at the single-cell level by in situ hybridization using phylogenetic markers, labelled with a fluorescent compound. FISH is one of the most powerful tools and has become reliable and commonly used method. The spatial organization of unknown and unculturable bacteria has been analyzed by the combined use of FISH and denaturing gradient gel electrophoresis (DGGE). This enables the design of an oligonucleotide probe for FISH following the determination of target bacterial species and their 16S rDNA sequences (Aoi, 2002). Samples are usually hybridized on glass slides and specifically stained cells are detected with epifluorescence microscopy or flow cytometry after stringent washing (Ivanov *et al*., 2003). When FISH was combined with CLSM in characterizing colonization processes in intact biofilms on sand grains in a laboratory column inoculated with an aquifer enrichment culture, Letsiou and Hausner (2004) realized that this combination, offers insight into biofilm structure and dynamics of communities, and some cells were detected further away from the sand grain which served as surface.

LIVE/DEAD® BacLight™

The commercially available viability staining kit LIVE/DEAD BacLight™ has become a popular tool for interrogating the viability of bacteria in biofilms. The application of this kit to biofilms depends on the assumption that the stains adequately permeate throughout the biofilm. The kit contains a common stain combination that is used for viability testing of bacterial cells. SYTO 9 and Propidium iodide (PI). SYTO 9 is a green-fluorescent nucleic acid stain, which is designed to stain every cell. Propidium iodide is a red-fluorescent nucleic acid stain which is intended to stain any cell with a compromised cell membrane, while displacing any SYTO 9 that may be in those cells (Davison *et al*., 2004; GrayMerod *et al.*, 2004). In the experiment performed by Davison *et al.* (2004) on *S. epidermidis* and *P.aeruginosa*, SYTO 9 did not diffuse as quickly or as deeply as PI through biofilm during traditionally accepted staining periods. SYTO 9 did not contact every biofilm cell and stain them green as it was supposed to, but PI appeared to stain the

entire biofilm red. They then concluded that a reaction might be occurring that destroyed SYTO 9, as it diffuses in, limiting its penetration (GrayMerod *et al.*, 2004).

Microautoradiography

Microautoradiography (MAR) is a technique, which enables a direct visualization of active microorganisms and their metabolic capabilities without prior enrichment or cultivation. The method is based on the fact that radiolabelled substrate taken up by individual prokaryotic cells can be visualized by a radiation-sensitive silver halide emulsion, which is placed over the radiolabelled organisms and subsequently, processed by standard photographic procedures. The radiotracers used in microbial ecology are typically the soft beta, ^{3}H, ^{14}C, ^{33}P and in a few cases, the stronger beta emitter ^{32}P. MAR has been used in combination with various simple staining techniques and with micro-electrodes, to characterize microbial communities. When MAR is combined with FISH, it is possible to link valuable information about identity to the physiology of specific bacteria (Nielsen and Nielsen, 2004).

MAR-FISH procedure includes biofilm sampling, incubation under selected conditions such as selection of radiotracer (type and amount), incubation time, biomass concentration, temperature, and presence of inhibitors. After incubation, the samples are fixed, washed, and hybridized with relevant FISH probes or other stains. Subsequently, the liquid radiosensitive film is placed on top of the sample and exposed for typically 3-6 days before being developed and ready for examination. The stained MAR-positive bacteria can be examined by a combination of bright field or phase-contrast and epifluorescence microscopy or laser scanning microscopy (Aoi, 2002; Kjellerup *et al.*, 2003; Nielsen and Nielsen, 2004). In a study related to biocorrosion, Nielsen and Nielsen (2004) were able to observe MAR-positive bacteria in thin biofilms directly on corroding metal surfaces and they investigated the relative number of aerobic and anaerobic bacteria, which were able to consume labeled acetate and bicarbonate. This made it possible to locate the bacteria around the corroding pits, thus revealing possible corroding microorganisms and possible corrosion mechanisms (Nielsen and Nielsen, 2004).

Nuclear Magnetic Resonance Imaging

Nuclear magnetic resonance (NMR) imaging showed that biofilms grow in micro colonies embedded in an exopolysaccharide matrix structure, which is interspersed with less dense regions containing permeable water channels (Wolf *et al.,* 2003). NMR has lower resolution, but provides a wealth of non-invasive information for living samples. For live cells, NMR provides metabolite content, metabolic pathways and flux information, convective and diffusive mass transport, water compartmentation and does not suffer opacity losses and scattering effects. The most interesting fact with NMR imaging techniques is that they can be implemented with temporal and/or spatial resolution (Majors *et al*., 2004).

2.3 BIOFILM CONTROL

Microbial adhesion is the first step in the formation of a biofilm, which can be detrimental to both human life and industrial processes, causing infection and contamination by pathogens and dental decay, but which can be also beneficial to some environmental bioprocesses and to agriculture. Therefore control of microbial adhesion is important for the inhibition and utilization of biofilms (Ishii *et al*., 2004).

Controlling biofilm is a precondition in all stages of water supply, aiming at optimal use on one side and effective limitation on the other. Biofilm control can be divided into two methods; mechanical and chemical methods. Mechanical includes the physical removal of biofilm from the surface. Such technique can be applied “online” when the equipment is operating or “offline” when the equipment is shut down (Melo and Bott, 1997). Chemical methods involve using a disinfectant usually called a biocide. Applications may be used on a continuous basis or intermittently, depending on the severity of the problem and cost (Melo and Bott, 1997).

Dispersants are employed to maintain the cells in suspension, thereby reducing the opportunity to stick to solid surfaces. Surfactants have been intensively used to control biofilm formation in industrial equipment, especially in the food industry and membrane processes. The most commonly used surfactants are cationic and anionic. These types have a dual role in biofilm control. They can inactivate living cells and alter the surface properties of the attachment substratum, thereby either preventing attachment or promoting detachments of the adhering cells (Azeredo *et al.,* 2003).

Disinfectants and antiseptics are biocides or products that are primarily used to inhibit or destroy hygienically relevant microorganisms. Disinfectants usually leave dead biomass in the system that accumulates and promotes re-growth of the organisms by using the dead biomass as a nutrient source (Schulte *et al*., 2003).

According to Cloete *et al.* (2003), biofilm controls currently follow the following five mitigations:

- Bacteria are chemically killed by application of bactericidal compounds, termed biocides at lethal doses,
- Biofilms are dispersed by dispersant,
- Biofilms are removed physically by a variety of processes,
- Biofilm structure is weakened by enzymes or chelants and
- Planktonic bacterial numbers are controlled by ultraviolet light.

2.3.1 Biocides

These are inorganic or synthetic organic molecules used to disinfect, sanitize, or sterilize objects and surfaces, and to preserve materials or processes from microbiological degradation (Chapman, 2003). In order to kill or remove biofilm, biocide must penetrate the EPS and gain access to the microbial cell. Removal of microbes and EPS is important in practice to reduce attachment of new microbes to the surface and new biofilm formation. The action of biocides can be bactericidal, fungicidal or algicidal and their effectiveness depends on the nature of the microorganism to be eliminated and the operating conditions of the system to be treated (Cloete *et al*., 2003).

The mechanisms of biocides action can be divided into four main categories (Fig. 2.1). The oxidants include rapid speed of kill agents such as chlorine and peroxides that oxidize organic material. Electrophile agents include inorganic ions such as silver, copper and mercury, and formaldehyde and isothiazolines, which react covalently with cellular nucleophiles to inactivate enzymes. Alcohols such as phenoxyethanol destabilize membranes leading to rapid cell lyses. Weak acids e.g.

ascorbic and benzoic acids interfere with the ability of the cell membrane, resulting in disruption of metabolism (Chapman, 2003).

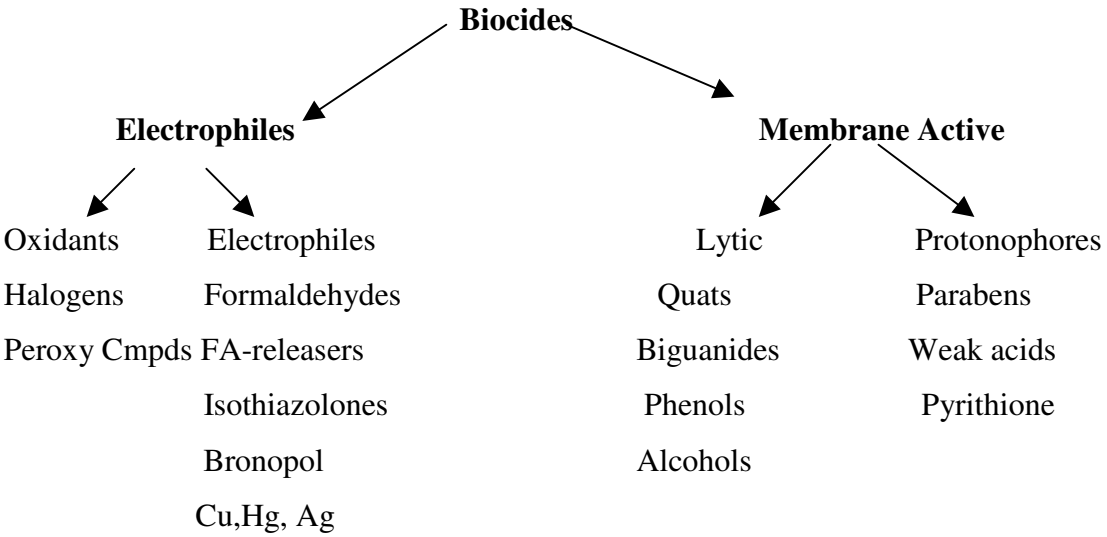

Figure 2.1 Mechanisms action of biocides (Chapman, 2003)

An industrial biocide should have the following characteristics:

- Selectivity against target microorganisms,
- Capability to maintain inhibitory effect in the presence of different properties of other compounds and under the operating conditions of the system,
- Lack corrosives and
- Should not be biodegradable.

In addition to the above, the following characteristics are also necessary: The biocide must act in a reasonable length of time, as temperature or pH changes, be nontoxic, readily available, be safe and easy to handle and apply, easy to determine proper concentrations, able to provide residual protection, capable of being applied continually and the pathogenic organisms must be more sensitive to the biocide than nonpathogens, and must not add unpleasant taste or odour (Spellman, 1999).

There are some important aspects to be followed when using a biocide:

- The use of chemical additives must not affect the use of other treatment chemicals such as corrosion inhibitors and descalants.

- Some oxidizing agents are toxic and can have an impact on the environment they are being discarded. Therefore legal requirements in respect of usage and discharge must be fully considered before employing any chemical additive to an aqueous system.

In the process of disinfection, the disinfectant attempts to disrupt the normal life processes of the organism. This is done by penetrating the cell wall of the organism and upsetting the natural life cycle processes or altering the enzymes. With the cycle so disrupted, organism die or the species cannot reproduce. Target structures of biocides include cell walls, cytoplasmic membranes, and ribosome of vegetative cells, the coat and cortex of bacterial spores. They disrupt the cell membranes leading to leakage of intracellular components and destruction of many cellular functions such as replication, transcription, etc. (Cloete *et al*., 1998).

2.3.1.1 Enzymes

Enzymes and detergents have been used as synergists to boost disinfectant efficiency. Lysozyme has enzymatic activity against the (1-4) glycosidic linkages between N-acetylmuramic acid and N-acetylglucosamine of cell wall peptidoglycan. Gram-positive bacteria are sensitive to lysozyme. The mode of action involves the electrostatic attraction of the positively charged lysozyme to the negatively charged phospholipids on the cell surface. In the lytic mode of action, lysozyme hydrolyses the peptidoglycan layer and causes cell lysis. In the non-lytic mode, cell death is the result of membrane perturbation, not of cell lysis. Gram-negative bacteria are generally resistant to lysozyme due to their outer membrane shield (Gill and Holley, 2003).

Nisin has also shown to alter the cell membrane of sensitive organisms resulting in leakage of low molecular weight cytoplasmic components and the destruction of the proton motive force. The specific mode of action makes it difficult, however to find enzymes that are effective against all different types of biofilms. According to Meyer (2003), formulations containing several different enzymes may be necessary to be successful. Chelators such as EDTA can destabilize the cell membranes of bacteria by complexing the divalent cations, which act as salt bridges between membrane macromolecules such as lipopolysaccharides (Gill and Holley, 2003).

2.3.1.2 Treatment with oxidizing biocides

a. Oxidizing halogens

Chlorine compounds

Chlorine is available in three common forms: liquid (sodium hypo chlorite – NaOCL), powder (Calcium hypo chlorite – $CaOCL_2$) and liquefied compressed gas (CL_2). Chlorine is one of the oxidants used to treat industrial process water in cooling towers, in pulp and paper industries and as a disinfectant in health, food and consumer industries (Chapman, 2003). Chlorinating is an age-old, inexpensive, efficient and effective biocide process for drinking water disinfection. The normally used concentration of chlorine is 1mg/L. When added in water, chemical reaction occurs between the molecules of chlorine and water. Chlorine reacts rapidly with water to form hypochlorous acid (reaction 1) and hypochlorite ion (reaction 2) and some of it remains as free chlorine.

$$CL + H_2O \leftrightarrow HOCL + H^+ + CL^- \qquad \text{(reaction 1)}$$

$$HOL \leftrightarrow OCL^- + H^+ \qquad \text{(reaction 2)}$$

Chlorine in the form of hypochlorous acid is very effective against bacteria and viruses (Momba, 1997).

Chlorination kills microorganisms through a series of mechanisms including interference with cell permeability and damage to bacterial nucleic acids, and enzymes. Generally, this will cause cell damage. The cells that are injured in this way generally fail to re-grow. Unfortunately some seem able to recover in the small intestine of animals and so can regenerate their pathogenicity. The effectiveness of bacterial kill can be improved by increasing the concentration of the biocide but this may exacerbate some of the problems associated with chlorine. (Duckhouse *et al.*, 2004). In water treatment, chlorine is more effective in eliminating *Legionella* biofilms than nonoxidative biocides such as quaternary ammonium compounds (Meyer, 2003).

The main advantage of disinfecting with chlorine is the development of an easily measured residual, which remains in the water after disinfection, and protects water in storage and in the distribution systems. According to Dalvi *et al.* (2000), chlorination is a good servant but a bad master in the sense that it is very economical and effective, but if not controlled properly, it forms disinfectant by-product (DBPs). DBPs depend

upon many factors including high dose and contact time of chlorine and other particles. The important precaution when using chlorine is to control pH, as low pH minimizes the formation of trihalomethanes (THMs) while high pH minimizes haloacetic acids (HAAs) and other DBPs (Meyer, 2003).

Chlorine reacts with organic carbon and creates difficulties in maintaining chlorine residuals (Melo and Bott, 1997; Momba, 1997). The loss in disinfectant residuals generally leads to an increase in disinfectant application with outcomes being increased operating costs and the likelihood of unacceptable disinfection by-products formation (Chandy and Angels, 2001). The use of chlorine is limited by the fact that it can be consumed by thick biofilm structure before it can react with cell constituents (Massi *et al.,* 2003), and chlorine concentrations of as little as 1-5mg/L may not penetrate biofilm or inactivate bacteria (Momba, 1997). These limit the use of chlorine.

Chlorine can be combined with ammonia to form chloramines. Monochloramine is the predominant species in chloraminated water. It enters the cell and reacts with proteins and nucleic acids, tryptophan and sulfur-containing amino acids (LeChevallier *et al*., 1998). Recent studies have shown that chloramines have greater biofilm penetration, less reactive and provide less formation of by-products. Chloramines have low corrositivity and less noticeable taste and odours when compared to chlorine. The use of monochloramines in water systems provides a longer residual effect in controlling biofilm cells before regrowth (Momba *et al*., 1998).

Developing biofilm causes chloramines decay in drinking water systems. This decay is also facilitated by biofilm, which are able to convert non-reactive organic carbon species into those able to react with chloramines (Chandy and Angels, 2001).

Chlorine dioxide

This is an explosive water-soluble gas, which is always produced on-site and dissolved immediately in water. Its boiling point is 11ºC and the melting point is -59ºC. It has an unpleasant odour and is irritating to the respiratory tract. Chlorine dioxide (ClO_2) is generated from sodium chlorite and either gaseous chlorine or

hydrochloric acid in a solution of low pH (Connell, 1997; Vigneswaran and Visvanathan, 1995). Chlorine dioxide is a powerful oxidizing agent and biocide, with broad-spectrum efficacy against bacteria, fungi, algae, viruses and protozoa. ClO_2 is used as a substitute of gaseous chlorine in water treatment and as a sanitizing rinse for fruits and vegetables (Rossmore, 1995). It cleaves the bonds between the EPS, which are responsible for the attachment of biomass. Purchasing of chlorine dioxide is expensive while generating it on site requires time, which adds to labor cost and it is hazardous (Rossmore, 1995).

The basic properties of chlorine dioxide that differentiate it from other oxidizing biocides are:

- Does not react with water to form hypochlorus acid, or free chlorine.
- Chlorine dioxide possesses broad-spectrum anti-microbial capabilities (Vischetti *et al*., 2004).
- It is not sensitive to system pH.
- It readily dissolves.
- It has limited tendency for formation of THMs and other by-products that occur with chlorine and chloramine.
- Chlorine dioxide does not react with ammonia to form the potentially toxic chloramines.
- It is active in water for at least 48hours as a bactericide, and it may have a longer period of effectiveness than that of chlorine.
- Chlorine dioxide has been found to be active against pathogens that are resistant to chlorine (Vigneswaran and Visvanathan, 1995).

Like any other disinfectant, chlorine dioxide has disadvantages:

- Chemical and equipment costs are substantially high compared with chlorine and it must be produced on-site.
- The by-products chlorate and chlorite may be toxic.
- Chlorine dioxide itself is dangerous because two dangerous gases (chlorine and sodium chlorite) must be handled.
- The gaseous chlorine dioxide is unstable and explosive and it is therefore generated on-site (Spellman, 1999).

- In some situations, ClO_2 may react with other organic vapours to produce new odours, for example ClO_2 can vaporize from tap water and produce a mousy odour with volatile organics from household furnishing such as new carpets (Stevenson, 1997).

A summary of the effect of chlorine dioxide on various drinking water components (Spellman, 1999) is shown in Table 2.1

Table 2.1 Summary of the effect of chlorine dioxide on drinking water components.

Constituents	**Reaction**
Selected natural and synthetic organics	Can react to form chlorite
Iron and manganese	Oxidized
Colour	Removed
THMs	Lowered
Organics	Oxidized
Phenols	Reacts to form chlorinated phenols and quinones

Ready-to-use CLO_2

The newly developed SABRE Chlorine Dioxide Generators

The generator (Figure 2.2) is built from a block of chemically resistant Schedule 80PVC plastic. Many of the chemicals feed lines and check valves are incorporated directly into the block. The Integral Component Block Mounted (Solid State) design eliminates the need for the maze of external tubes and valves and their associated spare parts required by other generators. The results are greater efficiency, improved performance and little maintenance. The Tuned Reaction Column™ (patent pending) eliminates clogging and insures a steady stream of pre-cursor chemicals. This allows the unit to operate efficiently at lower water flows without variation in meter reading and with a greater turndown ratio. There is simply no need for frequent flushing, disassembly or cleaning. Chlorine dioxide is generated at a minimum of 95% efficiency with no more than 5% excess chlorine.

Figure 2.2 The SABRE ready to use chlorine dioxide generator

These systems generate 2g/L solution of chlorine dioxide from three precursor chemicals, namely sodium chlorite, sodium hypochlorite and hydrochloric acid. Chlorine dioxide is generated at a concentration of 1 000 – 3 000ppm concentration. In order to differentiate it from the gas chlorine dioxide, which was produced on-site, this liquid is called ready-to-use chlorine dioxide. It has the stability of four weeks at room temperature if stored below 25°C out of sunlight (in a UV stabilized tank).

In addition to the properties of chlorine dioxide above, this chemical has been approved by the EPA for drinking water disinfection, it is significantly less corrosive than chlorine, is 100 – 1 000 times more effective at removing/preventing biofilm than chlorine and this chemical can be easily, simply and cost effectively measured in water to meet any HACCP regulations (Personal communication).[1]

The ready-to-use chlorine dioxide has been approved for use in the following applications:

[1] Mashiloane D (2004) BTC Products and Services CC, Randburg, South Africa

- Private area and public health area disinfectants
- Veterinary hygiene biocidal product
- Food and feed area disinfectants
- Drinking water disinfectants
- Preservative for liquid cooling and processing systems
- Preservative for food or feedstuffs.

Bromine

Bromine can be used in water treatment, slime control, pulp and paper refinement, etc. (Jackson, 2002). Biocidal action of the bromine compounds has been reported (by Videla, 2002) as more effective at a wider pH range than hypochlorous. Unlike chlorine, bromine's popularity increased because of its superior biocidal activity in the presence of ammonia. Because of its toxicity to humans, bromine's use as antimicrobial agent is limited (Penny, 1991).

Bromine can be added to chlorine to produce bromine chloride (BrCl), with a sharp, harsh, penetrating odour. When added to water, BrCl hydrolyzes to hypobromous acid (HOBr) very rapidly and forms monobromamines and diabromamines when added to water containing ammonia-nitrogen. BrCl is effective at higher pH levels, and can be applied at lower dosages than chlorine to give same pathogen kill. It has been proved to be a disinfectant with significant bacteriocidal effectiveness over a broad range of pathogens including viruses, cysts and bacteria. The disadvantages of BrCl includes severe burns that occur as a result of contact with skin, and other tissues, its corrosiveness that requires special handling and safety precautions and technical difficulties. These limit the use of BrCl as a disinfectant (Spellman, 1999).

Peroxygens

Hydrogen peroxide (H_2O_2) and peracetic acid are the most widely used peroxygens biocides. H_2O_2 is bactericidal against numerous Gram-negative and Gram-positive bacteria and many viruses. The high concentrations of H_2O_2 are sporicidal. The bactericidal activity of hydrogen peroxide is based on the production of highly reactive hydroxyl radicals that can attack membranes (lipids, proteins, DNA, etc.).

Peracetic acids are widely used in clinical environments for decontamination of surgical endoscopes and associated equipments. Peracetic acid containing products are commonly used in dairy and beverage industries. They have broad-spectrum microbial action and good degradability. The use of peracetic acid in drinking water and wastewater biofilms has been reported to have a relatively fast and effective inactivation, without removal of biomass. This can lead to re-growth of the bacteria. Suggestions have been made that peracetic acid can be used in combination with hydrogen peroxide since hydrogen peroxide has the capability to detach biofilm.

Ozone

Ozone is a gas, which comprises three atoms. It is a strong disinfectant but still it is also not free of all problems. It eliminates planktonic bacteria (at very low concentrations of 0.01 and 0.05mg/L) more quickly than chlorine and bromine. Ozone can detach sessile bacteria from surface metals and can also disrupt organic materials (Viera *et al.,* 1999).

Ozone offers several advantages as compared to other biocides:

(a) Minimal on-site chemical inventory since it is used as generated.
(b) Non-oxidant discharged since its rapid decomposition minimizes downstream toxicity risks.
(c) Reduction of water discharge (Viera *et al*., 1999).

The corrosiveness of ozone on metals depends on its concentration levels. In ozonated water treatment, the pH levels stay between 8.5 and 9.0 where the corrosion rate of iron and steel approaches zero. Therefore its use as a substitute of conventional water treatments would be only limited by its high oxidation power, which could facilitate corrosion of the structural metals (Viera *et al*., 1999). Ozone reacts with natural organic substances to produce low-molecular-weight by-products that are more biodegradable than their precursors; it therefore requires special generators, which are costly and consume much power. In addition to these limitations, it can also react with organics in water to form epoxides, which are environmental problems (Characklis and Marshall, 1990).

b. Treatment with Non-oxidizing biocides

Quaternary ammonium compounds

Quaternary ammonium compounds (QACs) interfere with respiratory and ATP synthesis in bacterial cells. If added in required concentration, they cause membrane leakage, release of cellular constituents and even cell death. Although they have been widely used, they are no longer in use because some bacteria are resistant to them (Massi *et al.,* 2003).

Aldehydes-type biocides

Gluteraldehydes and formaldehydes are the mostly used non-oxidizing agents in food industries. They are also used to control microorganisms in water systems including cooling systems in dairies (Rossmore, 1995). These biocides are effective against bacterial spores. The biocidal rate of gluteraldehyde is very much dependent on the pH of the environment. Under a neutral or alkaline condition, its ability to kill and its antimicrobial activity rate is very high. It is predominantly used because it is non-corrosive to stainless steel, soft metals, rubber and glass (Laopaiboon *et al.,* 2003). Formaldehyde damages the transport properties of membrane porins, decreasing the rate of proline uptake and of enzyme synthesis. It is active over a wide pH spectrum (3.0-10.0), and is sporicidal. The disadvantages of these aldehydes are that they are less toxic to microorganisms, irritating to the skin and have offensive odours (Cloete *et al.,* 1998).

Thiol oxidizing biocides

Isothiazolines contain sulfur, nitrogen and oxygen. Isothiazolines can be used over a broad pH range and they are compatible with other water treatment chemicals. The problem with isothiazolines is that when discharged via the tank drain, they exceed acceptable concentrations for the marine life which is 81-124ppm and they also have superior anti-fungal activity but less anti-bacterial activity. They are also deactivated by H_2S and are therefore not expected to be effective in environments containing hydrogen sulfide (Videla, 2002).

Carbamates

Carbamates are used to inhibit mycelial proliferation in fuel. Because these classes of chemicals have better anti-fungal activity than antibacterial activity, and are often

formulated with other active agents such as dimethyl sulphoxide, they are no longer in use (Rossmoore, 1995).

Ions

Copper and silver ions have been used to disinfect cold and hot water systems at hospitals against *Legionella* bacteria (Kim *et al*., 2002). Copper and silver ions for inactivation in cooling water tower are usually used at concentrations of approximately 0.2 – 0.4mg/L respectively (Kim *et al*., 2004a). These ions react with cellular nucleophiles to inactivate enzymes involved in cellular respiration and bind to DNA at specific sites. They also initiate the formation of intracellular free radicals, which contribute to their lethal action (Chapman, 2003). Although these ions have no residual and corrosion problems, their efficacy against *Legionella* is not good at high pH and scaling on electrodes should be washed regularly, when using silver and copper ionization system. Excessive high ion concentrations might also turn the water colour to black (Kim *et al*., 2004a; Kim *et al*., 2004b).

Surface-bound biocides and activated surfaces

Biocides can be bound to surfaces in order to prevent the development of biofilms, e.g. anti-fouling on ships and boats. Paint is used for protection against growth of bacteria, algae, mussels and other invertebrates (Konstantinou and Albanis, 2004) and decoration of surfaces, but deterioration can defeat these functions. The spoilage occurs where temperature and humidity levels are suited for microbial growth i.e. high humidity favours growth of fungi while low humidity allows bacterial growth. For many years tributylin (TBT) compounds were the most widely used active ingredients in paint formulations (Kim *et al*., 2002; Konstantinou and Albanis, 2004).

A serious problem faced with painting is that if cells attach to a surface impregnated with biocides, and is actually killed; they do not leave the surface. Therefore the surface will soon be covered with dead bacteria and loose its efficacy. Another disadvantage of painting is that after a certain period of time, they will be released into the environment and unfold harmful effects to marine organisms (Thomas *et al*., 2003).

UV Irradiation

This is a non-chemical form of disinfection. Research has developed a more efficient and reliable UV light source for disinfection of drinking water by UV rays. In this form of disinfection, the killing of microorganisms depends on its morphology. Short wave UV is known to have biocidal effect with maximum kill at 254nm and acts by producing thymine dimers, which hamper DNA replication. It has been widely used in wastewater disinfection (Kim *et al*., 2002) because it leaves no residuals to provide protection against potential downstream contamination (Richter *et al*., 2002). The efficiency of UV-irradiation also depends on the quality of water, especially in relation to the turbidity of water, agglomeration of microorganisms and organic and inorganic dissolved substances. Suspended particles in water absorb UV-rays, and thus the effective dosage is reduced (Cloete *et al*., 1998). Bacteria with thick cell enclosures are more difficult to destroy than bacteria with thin cell enclosures. In addition to the above disadvantage, not all microorganisms exposed to UV are inactivated or killed immediately, but a portion of the actual live quantity is inactivated at certain time intervals (Momba, 1997).

2.3.2 Electrochemically activated water

Electrochemical activation (ECA) is based on the generation of activated solutions featuring extra-ordinary physico-chemical and catalytic activity, using special electrochemical systems. The main material used is ordinary, mineral, natural, tap or potable water to which a small amount of various salts, sodium chloride or sodium bicarbonate is added. Water is passed through a special electrochemical cell or cells, consisting of negative electrode (anode) and a positive electrode (cathode). When the anode and the cathode are placed in water and direct current is applied, electrolysis of water occurs at the poles, leading to the breakdown of water into its constituent's elements, producing gaseous oxygen and hydrogen. If sodium chloride (NaCl) or table salt is used as a solution, the dominant electrolysis end product is hypochlorite, a chlorine based reagent, which is commonly used to treat water to kill microorganisms (Thantsha, 2002). Most of the compounds are formed in the anolyte chamber, and are acidic in nature and very strong oxidizing compounds. Reactive species formed in the catholyte chamber tend to be basic and are strong reducing agents. As a result, the anolyte is acidic (pH 2.4-4) while the catholyte is basic (pH 10 -12) relative to the neutral pH of the starting NaCl solution.

(http://www.camwell.net/electrochemical_activation.htm). Most of the reagents used in electrochemical activation technology are presented below.

Table 2.2: Reactive ions and free radicals formed in the anolyte and catholyte solutions by electrochemical activation (Thantsha, 2002).

	Anolyte	**Catholyte**
Reactive molecules	O_3, O_2, H_2O_2, ClO_2, HClO, Cl_2, HCl, $HClO_3$	H_2O_2, NaOH, H_2
Reactive ions	H^+, H_3O^+, OH^-, ClO^-	
Reactive free radicals	HO, OH_2, O_2, O, ClO, Cl	O_2, H_3O_2

The molecules of water in the anolyte and the catholyte acquire special properties that cannot be reproduced by other means. This electrochemical treatment results in the creation of anolyte and catholyte solutions whose pH; oxidation-reduction potential (ORP) and other physico-chemical properties lie outside of the range, which can be achieved by conventional chemical means. ECA solutions (anolyte and catholyte) are clear and colourless aqueous solutions with a faint clean smell of sterilants and disinfectants. ECA devices are mostly used in potable water systems. Anolytes are used for disinfection and sterilization, while catholytes are used for life support and enhancement and to modify viscosity and surface activity.

ECA has applications in most situations where water is used and also in many situations where oxidation and reduction reactions occur. In Russia, where it was first discovered, their use is broad nowadays. The extensive uses range from drilling in petroleum exploration, medical sterilization, prevention and treatment of diseases to pests control and other agricultural applications, food preservation, water decontamination, etc.

Neutral anolyte in chlorine containing agent and its basic parameters is similar to routine preparations used for disinfection containing chlorine as an active ingredient.

Oral introduction of neutral anolyte does not cause death of animals. All types of investigated anolytes had no sensitizing ability and does not cause allergic reactions at active chlorine dose equivalent to 150mg/L. Neutral anolyte demonstrated a high decontamination ability of the water, particularly, swimming–pool water and household sewage water liquids. When tested on viability of sanitary-indicative bacteria, neutral anolyte of active concentration starting with 150mg/L possessed strong bactericidal effect on sanitary-indicative bacteria. The absence of pathogenic germs and dysbacteriosis were confirmed when the test was done on the intestinal microflora of calves (htt://www.izumrud.co.ru/eng/articles/echa 3 1.php).

In Botswana boreholes, an undiluted anolyte was introduced in the bore and the gravel pack. It was discovered that the anolyte killed the bacteria that were suspended in the water, and those attached to borehole walls. When compared with most chemicals used for well rehabilitation, the biocidal efficiency of anolyte has been reported to be superior. Anolytes were extremely effective in removing both suspended and biofilm cells. The Botswana people also practiced electrochemically activated water technology on human safety. It has been proven nontoxic and totally safe for use in humans, and this level of safety cannot be achieved with existing chemicals and other electrochemical technologies. No protective clothing or masks for handling and storage of anolyte are required (http://www.wcp.net/column.cfm?T=W&ID=1823).

Characteristics of electrochemical activated solutions

1. The pH, the Oxidation Reduction Potential (ORP) and other physicochemical properties of the activated solutions, lie outside the range that can be achieved by conventional chemical means.
2. Activated solutions can be used in their undiluted form for purposes of high-level disinfection, or the solutions can be used in varying dilutions, such as for bulk water disinfection and as a spray or mist for decontamination.
3. If not used for approximately two days, they begin to degrade back to the relaxed state of benign water, retaining some attributes of the activated solutions, such as altered conductivity and surface tension

4. Activated solutions are extremely effective in killing and controlling harmful microorganisms, but remain harmless to humans, animals and are environmentally friendly (http://www.justick.co/aqvator/tech-ewa2.htm/).

Electrochemical activation technology is unique. It can be applied separately to maximum effect. Recent advances have further minimized the size of the electrolytic cell and associated equipment, as well as increased the efficiency of anolyte and catholyte separation (Table 2.3).

Table 2.3: Properties of Anolyte and Catholyte Solutions

Anolyte	**Catholyte**
Positively charged solution	Negatively charged solution
Powerful mixed oxidant solution	Powerful antioxidant solution
Microcidal----extremely	Negatively charged surfactant properties Effective disinfectant
Anionic properties	Cationic properties
Generated at pH 2.0 to 8.5	Generated at pH range of 8.0 to 13.0

The anolytes advantages include:

- Safety and simplicity
- Ease of use
- Cost effectiveness
- Efficiency and environmental acceptability, as they return to a stable, inactive state within 48hours, i.e. pure water.

2.4 BACTERIAL RESISTANCE

The response of microorganisms to biocides depends on the type of organism, the biocide itself, and the concentration of the biocide. Resistance is defined as the ability of a microorganism to grow in the presence of elevated levels of an antimicrobial substance or to survive the treatment with an antimicrobial substance. Resistance of biofilm microorganisms has serious economic and environmental implications in many applications like cooling water, papermaking, medical implants, drinking-water

distribution, secondary oil recovery, metal working and food processing (Cloete, 2003).

Bacteria within biofilms tolerate higher levels of antibiotics than comparable planktonic cells do. The difficulty in treating biofilm-related infections on catheters and medical implants are thought to be due to the increased antibiotic resistance of biofilm bacteria. Although biofilms have some properties in common, their structure and composition depends on the component microorganisms and environmental conditions (Brözel and Cloete, 1993). Thus, in different situations, the level of antibiotic resistance may vary and the factors that give rise to the increased resistance may differ (Czechowski and Stoodley, 2002). Different groups of bacteria vary in their susceptibility to biocides, with bacterial spores being the most resistant, followed by mycobacteria, then Gram-negative organisms, with cocci generally being the most sensitive (Russell, 1998).

Two major types of microbial resistance can be distinguished: intrinsic and acquired resistance. Intrinsic resistance refers to a natural chromosomally controlled property, including physiological adaptation that is specific for a certain type of microorganism (Morton *et al.,* 1998). This type of resistance is found with bacterial spores, mycobacteria and Gram-negative bacteria. In some instances, this resistance is associated with constitutive degradative enzymes, but in reality is more closely linked to cellular impermeability (Russell, 1998). The biocide may be unable to reach its target site in sufficiently high concentrations to achieve a lethal effect.

Acquired resistance may be due to mutations with subsequent selection of resistant mutants from the population which has been exposed to the biocide, or it may results from the uptake of plasmids or transposons which confer resistance to biocides (Morton *et al*., 1998).

2.4.1 Abiotic factors affecting biocide activity

Biocide activity can be affected by several factors – notably concentration, period of contact, pH, temperature, the presence of organic matter or other interfering or enhancing materials or compounds and the nature, numbers, location and condition of

the microorganism (bacteria, spores, yeasts and moulds, protozoa) or entities (prions, viruses) (Russell, 2003).

Biocides such as isothiazolines are only bactericidal at high concentrations (Cloete *et al.,* 1998). At low concentrations bacteriocides often act bacteriostatically and are only bacteriocidal at higher concentrations. For a bacteriocide to be effective, it must attain a sufficiently high concentration at the target site in order to exert its antimicrobial action (Cloete, 2003). It is widely accepted that planktonic bacteria are more sensitive to biocides than sessile bacteria. Therefore, applications of large amounts or high concentrations of biocide are required to overcome biofilm formation in industries where biofilm is a major problem. Thick biofilms require more biocide concentration and more time.

Increase in concentrations will lead to environmental, ecological and toxicological problems when water contaminated with the biocide is discharged directly to natural water or to municipal effluent treatment plants. As the legislation becomes more restrictive, and the effect of biocides on secondary wastewater treatment plants is of concern, it may be necessary to treat wastewater-containing biocides before discharge (Laopaiboon *et al*., 2003). Most biocides, especially oxidizing agents, react with substances contained in water, decreasing the available concentration (Cloete *et al*., 1998).

Poor penetration of antimicrobials into a biofilm will provide for gradient of effective antimicrobial activity with respect to biofilm depth (Hunt *et al*., 2004). Certain deeper lying biofilm community members may be exposed to sub-lethal levels of antimicrobial over a prolonged scale. These organisms will be subjected to selection pressures for increased drug resistance (Gilbert *et al*., 2003).

Environmental pH may cause changes in the molecule of a biocide. The change in pH, may result from changes in the cell surface, the number of negatively charged groups on the bacterial cell surface increases as the pH increases, with the result that positively charged molecules have an enhanced degree of binding (Cloete *et al*., 1998; Thantsha, 2002). During chlorine disinfections, the low pH minimizes the formation of Trihalomethanes (THMs) and on the other hand, promotes the formation of

haloacetic acids (HAAs) and other disinfectants-by-products. The high pH values have the opposite results. Gluteraldehyde is stable in acid solution but only active at pH 7.5 – 8.5, so it must be alkalinified before application. A 2% solution at the correct pH is ten times more bactericidal than a 4% solution of formaldehyde (Cloete *et al*., 1998). Ozonation is greatly affected by pH change having a half-life of less than one hour at pH 8. Temperature also has an effect on the activity of a biocide. It has been reported that the reactivity of gluteraldehyde is related to temperature; a 2% solution kills spores of *Bacillus anthracis* in 15 min at 20ºC, whereas it requires only 2 min at 40ºC. Therefore the higher the temperature the more effective is gluteraldehyde (Viera *et al*., 1999).

Another parameter influencing the activity of biocide is the presence of natural organic matter (NOM). The fraction of NOM available to biofilm as organic carbon is defined as biodegradable dissolved organic carbon (BDOC) (Butterfield *et al*., 2002). The BDOC fraction is variable depending on substrate type and environmental conditions. The BDOC in water systems provides nutrients for biofilms in a distribution system and reacts with disinfectants. Amino acids are only a small fraction of the natural organic matter, but they represent a high regrowth potential and are highly reactive with chlorine. A variety of processes including the application of powered activated carbon; biological filtration and membrane processes have been used to control BDOC in water processes (Volk and LeChevallier, 1999). These processes are only partially effective in removal of higher molecular-weight humic substances and this allows the compounds to make their way into water distribution system and serve as biodegradable substrates for biofilm growth (Butterfield *et al*., 2002).

Ozone reacts with natural organic substances to produce low-molecular-weight oxygenated by-products that generally are more biodegradable than their precursors (Cloete *et al*., 1998). Chlorine may react with organic or inorganic compounds in the bulk water or with substratum material, which will result in substantial chlorine consumption before it can react with cellular constituents. This is a more intimate example of the “chlorine demand”, a phenomenon accounted for by generations of water treatment specialists (Cloete *et al.,* 1998).

The nature of the surface and of the microorganisms present in the biofilm can also affect biocide activity. Formation of the biofilm is dependent on the surface characteristics of the substratum; including metal surface, free energy, roughness and hydrophobicity. Because cells on surface have different growth rates and nutritional requirements than planktonic cells of the same species, they can hide in crevices of the substratum. Although biofilms have some properties in common, their structure and composition depends on the component microorganisms and environmental conditions. Thus, in different situations, the level of antibiotic resistance may vary and the factors that give rise to the increased resistance may differ (Hogan and Kolter, 2002). Laboratory studies have shown that microorganisms within the biofilm are protected from the lethal effects of biocides. EPS matrix presents a potential barrier, which delay or prevent biocides from penetration into the biofilm and from reaching target microorganisms in all parts of a biofilm (Elvers *et al*., 2002; Hogan and Koltex, 2002). A morphological state of an organism such as a bacterial spore is resistant to many chemical and physical treatments. In this way Gram-negative bacteria are more resistant to antimicrobial agents because of their extra outer membrane, unlike Gram-positive bacteria that are very sensitive to biocides (Hogan and Koltex, 2002).

Physiological heterogeneity develops within biofilms from nutrient and oxygen gradients and accumulation of waste products. Thus cells within the biofilm encounter different microenvironments, responding with alterations in growth rate or by changes in gene expression that infers a biofilm-specific phenotype and increased resistance to biocides (Elvers *et al*., 2002).

In some industries, the amount of biocide is increased in order to overcome the problem of bacterial resistance. This is likely to lead to environment ecological and toxicological problems when water contaminated with the biocide is discharged directly to natural water or to municipal effluent treatment plants (Laopaiboon *et al.,* 2003).

2.4.2 Role of persister cells

Damaged cells undergo a programmed cell death (apoptosis), while a small population of cells, which are defective in their suicide response (persister cells), would survive the exposure to the antimicrobial agent in protected niches within the

biofilm. When biocide treatment is discontinued, the persisters would start to multiply, producing a new biofilm population consisting mostly of biocide-sensitive cells and again only a minor fraction of new persisters. Persisters can react immediately and survive a sudden challenge by a biocide (Hunt *et al*., 2004). There is an indication that biofilm populations may always contain a sub-set of organisms, which ensure survival of the species by the ability to adopt transiently a biocide-resistant phenotype (Chambless and Stewart, 2004; GrayMerod *et al*., 2004; Schulte *et al.,* 2003).

Methods for determination of resistance

The minimum inhibitory concentration (MIC) is commonly used in screening of strains for resistance, or comparing the efficiency of antibacterial agents or studying synergy effects. The main advantages of the method are that it is easy to perform and many attains or antibacterial agents can be tested in the same experiment. But this applicability is limited because many commonly used disinfectants cannot be tested because of too high or low pH for growth or precipitation of the disinfectant in the nutrient broth. Bactericidal tests are therefore often used for determining resistance. The main advantages of these methods are that all types of disinfectants can be tested and the effects of temperature and interfering substances may be included. Bactericidal tests are easy to perform, but more time-consuming and less reproducible than the MIC test, therefore bactericidal test are mostly used when investigating a few strains and a small number of antibacterial agents (Langsrud *et al*., 2003).

Bacteria isolated from disinfectant solutions or disinfection equipment can lose their resistance rapidly in laboratory conditions and may not survive exposure to the disinfectants in laboratory tests. The reason is that under natural conditions, the microorganisms grow at surfaces whereas they have been cultivated in nutrient broth prior to exposure to the disinfectant in the laboratory tests (Langsrud *et al.,* 2003; Russell, 1998).

According to Brözel *et al.* (1993) bacteria do acquire resistance to water treatment biocides, during long-term exposure to sub-inhibitory concentrations. In most cases, such resistance is due to a process of adaptation and not to transfer of genetic

information. The long-term treatment of a system with any-single biocide will therefore result in a bacterial community resistant to the biocide.

Disinfectant adaptation

Exposure of bacteria to sub-lethal concentrations of disinfectant result in stable higher resistance. In the laboratory, adaptation is studied by exposing microorganisms to gradually higher concentration of disinfectant (Russell, 2004). It can occur in niches with poor rinsing leaving low concentrations of disinfectant on the surface. In an adaptation experiment with *L. monocytogenes*, it was not possible to adapt a resistant strain to higher concentrations of benzalkonium chloride (BC); but a sensitive isolate could adapt to grow in higher concentrations up to the resistance level of the initially resistant strain, but not higher (Langsrud *et al*., 2003).

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CHAPTER 3

BIOFILM MONITORING USING LIGHT REFLECTANCE

Abstract

Biofilms develop virtually on any surface. They commonly adhere to a solid surface at solid-water interfaces, but can also be found at water-air interfaces. Monitoring of biofilm formation in water distribution systems is a major challenge to researchers, as the techniques required has to be based on early warning signs which allow for timely, precisely directed and optimized countermeasures. The present work details the design of light reflectance for monitoring biofilm growth. A rotating biological contactor, which works on the principle of a rotating circular disc that was semi-submerged in flowing water, was used to grow biofilm. Water collected from a natural stream was used to grow biofilm. Biofilm growing on the disc was measured daily using light sensor. It was observed that the amount of light reflectance decreased with time, indicating an increase in biofilm thickness. The biofilm thickness was confirmed by scanning electron microscopy analysis (SEM), which indicated an increase of bacterial deposit on the slides. It was therefore concluded that in addition to laboratory use, light reflectance could be used to monitor biofilm formation in water systems.

Keywords: biofilm monitoring**,** light reflectance, rotating biological contactor, biofilm thickness

3.1 INTRODUCTION

A layer of microorganisms, forming a biofilm, quickly covers any surface submerged in water. Biofilm formation is initiated by formation of a conditioning layer composed of organic molecules attached on solid surfaces (Aoi, 2002).

In engineered systems such as wastewater treatment systems, various types of microorganisms usually exist as biofilms on supporting materials, flocs in activated sludge and anaerobic reactor granules (Aoi, 2002). Biological bioreactors have been

developed, where biofilm formation is promoted on different materials. According to Helle *et al*. (2000) hostile environments with low availability of nutrients, unfavourable pH, or toxic compounds, e.g. biocides, favours the formation of biofilms at the substratum surface. Cells grow at the expense of substrate and nutrients in bulk water increasing biofilm cell numbers (Aoi, 2002).

Some microbial cells are capable of producing exopolymers (EPS) that compose of polysaccharides and glycoprotein. This external slime captures other bacteria, which live and grow off the waste products produced by the primary colonizers. The polysaccharides produced hold the biofilm together and serves as protection. Although the presence of biofilms in natural and engineered microbial systems was recognized long ago, the nature of biofilms, their structure and effects are still not fully understood. The nature of colonies may provide protection for themselves or by growing in microcolonies, the outer cells protect the inner cells, leaving them to grow and multiply (Aoi, 2002; Hermanowicz, 2003; Leon, 2003).

Unfavourable conditions such as shortage of nutrients lead to detachment of bacteria. The detached bacteria provide an inoculum for the growth of a non-attached population and for colonization at new sites. Erosion, sloughing, abrasion, human intervention and predator grazing can cause detachment. Turbulence in the surrounding environment may cause bacteria to detach from a solid surface. If biofilms are allowed to grow, undisturbed with little or no shear, they may grow to millimeter-scale loosely, adhered slimes containing more dead and inactive cells than thinner biofilms (Boyd and Chakrabarty, 1995).

Microorganisms in the sessile phase form biofilms that causes deleterious effects in cooling water systems and should therefore be monitored. In order to effectively control the growth of biofilms, it is necessary to investigate the structure of biofilms grown under different conditions. Information about the nature of a deposit, its quantity, thickness and distribution of formation and removal of microbial deposit is needed. This brings about the necessity of biofilm monitoring (Cloete *et al.,* 1998).

The most popular parameters to monitor in biofilms are light density, heat transport resistance, electrical conductivity, pressure drop and frequency of oscillation of

piezoelectric crystals (Lewandowski and Beyenal, 2003). Because the planktonic population does not properly reflect the type and number of organisms living in the biofilm causing biodeterioration hazard, monitoring methods adopted must provide information of sessile biofilms (Videla, 2002). The importance of monitoring devices is that they can help the system operator to take preventative measures such as addition of biocide and an increase in dosage when needed. Therefore monitoring of biofilm development, which will give information that can be recorded on-line, in real time, in situ and non-destructively without requiring sample removal, would be ideal (Jackson *et al*., 2001; Schmid *et al*., 2004; Schmitt and Flemming, 1998).

In this experiment, a biofilm monitor using a rotating disc and light reflectance was evaluated, based on total plate counts, scanning electron microscopy and the light reflected.

3.2 MATERIALS AND METHODS

3.2.1 Experimental setup

A laboratory rotating biological contactor (Fig 3.1) was used. It consisted of two tanks, each capable of carrying 40L of water when full. The tank on the left contained a water pump to assist in the circulation of water and the tank on the right contained a rotating disk, which was semi-submerged in water. Biofilm was allowed to grow on the rotating disk, which was used to take the measurement. The disc was fitted on an axle, which in turn was connected to a motor with a belt, enabling the disk to turn at a constant speed. The disk was calibrated (A – H) Fig 3.1 to enable the operator to take readings from the same area.

3.2.2 Measurement of biofilm growth

The light sensor was connected to a multimeter, which translated the amount of light reflected into millivolts (mV). To prevent any outside light from interfering, the sensor was rested against the disc when taking measurements. Water collected from a natural stream was used as feed water. 2 x 2cm Perspex disks for biofilm attachment were placed on the inside of the tank.

3.2.3 Scanning electron Microscopy analysis

Biofilm formation in the tank was monitored by removing one Perspex disc on a daily basis. The removed Perspex was aseptically placed into a sterile bottle containing 10ml of the fixing solution (2.5% gluteraldehyde in 0.0075 M phosphate buffer) until prepared for SEM viewing. Samples were washed 3 times with 0.075M $NaPO_4$ buffer (diluted 1:1) for 15min to remove all the gluteraldehyde. This was followed by dehydration steps, which were done by washing in a series of increasing concentrations of ethanol as follows: 1 x 15min 50%, 1 x 15min 70%, 1 x 15min 90% and lastly, 3 x 15min 100% ethanol. Perspex were dried for overnight with a Critical drying Point and coated with gold.

Figure 3.1 A biological rotating contactor used to monitor light reflectance

3.2.4 Total plate count

Total plate counts were done on Nutrient Agar plates using the spread plate method. A sample was taken from the back of the disk with a sterile swab and suspended in 10ml

of ¼ strength Ringer's solution. Serial dilutions from 10^{-1} to 10^{-8} were made and 100µl were plated out on NA plates. Plates were incubated at 37ºC for 24h.

3.3 RESULTS AND DISCUSSION

3.3.1 Light reflectance

The readings recorded in Table 3.1 are averages of 3 readings taken per day. The initial readings of the green light were high and no readings were generated using the infrared light. This meant that the wavelength used were too high for the infrared light. Light reflectance decreased with time (Fig 3.2). The findings of this study were in correlation with previous studies. Lewandowski and Beyenal (2003) discovered that light reflectance changes with biofilm thickness. The thicker the biofilm, the less light is reflected because biofilm thickness indicates the spatial dimensions of the biofilm (Heydorn *et al*., 2000). An increase of backscattered light was also observed when the sensor of a fiber optical device (FOD) was installed in a piping system for 8 months (Tamachkiarow and Flemming, 2003). In that experiment, the data acquired during a further year, reflected the development of biofilm in the system, and the efficacy of cleaning measures. Light reflectance is the most common variable monitored in biofilm literature probably because of its simple interpretation and the fact that it can be measured without the use of microscopy (Heydorn *et al*., 2000).

Table 3.1 Light readings reflected by biofilm formation

Time (d)	Green light (mV)	Infrared light (mV)
0	110.0	0.00
1	105.0	0.00
2	102.4	0.00
3	100.2	0.00
5	97.6	0.00
6	93.6	0.00
7	80.3	0.00
8	60.5	0.00
9	52.1	0.00

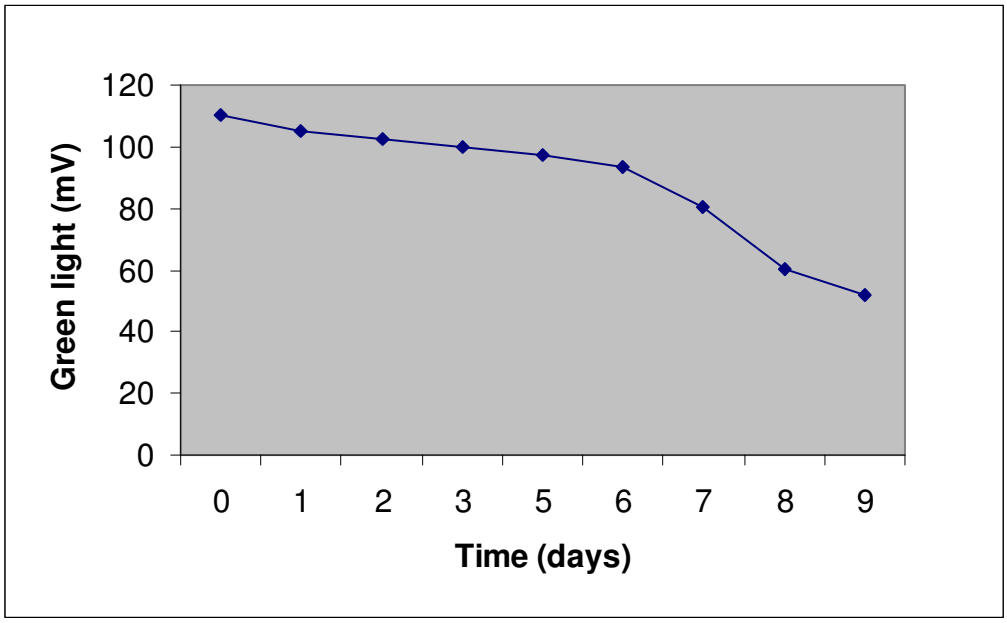


Figure 3.2 Average green light readings over time

3.3.2 Total plate count

Bacterial cells increased from $4.20x10^2$ cfu/ml to $9.00x10^2$ after one day. The number increased over time during the experiment. The cells attached to the disc increased as a result of the free space present (Table 3. 2 and Figure 3.3).

Table 3.2. Number of colonies formed per day

Time (d)	Total counts (cfu/ml)
0	$4.20x10^2$
1	$9.00x10^2$
2	$4.40x10^2$
3	$2.80x10^4$
4	$1.85x10^5$
5	$2.80x10^5$
6	$1.50x10^7$
7	$1.50x10^7$
8	$6.00x10^8$

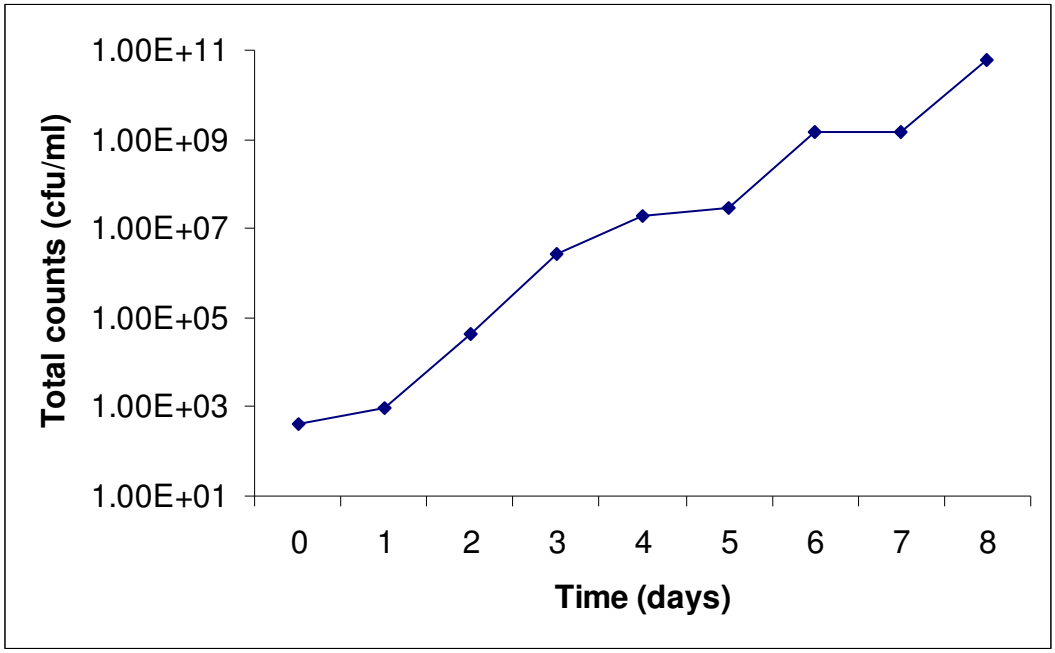
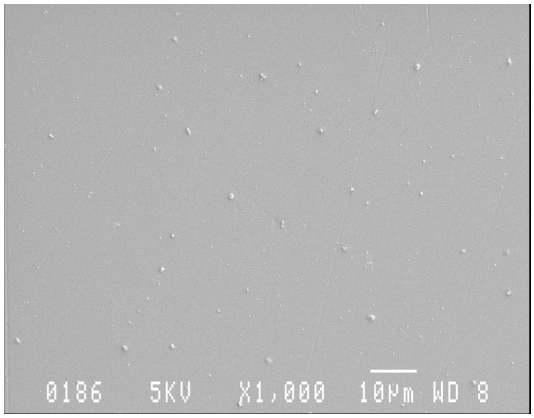


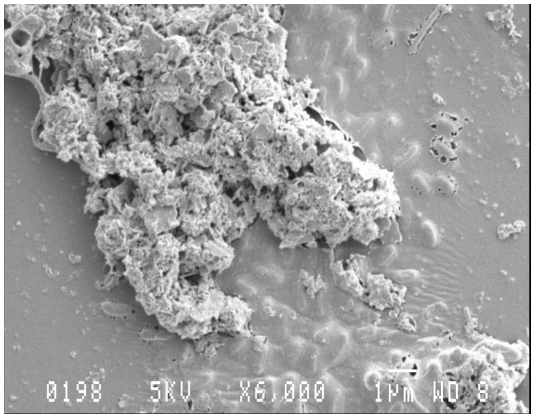
Figure 3.3 Bacterial colonies formed per day on the surface of the disc.

3.3.3 Scanning electron microscope (SEM)

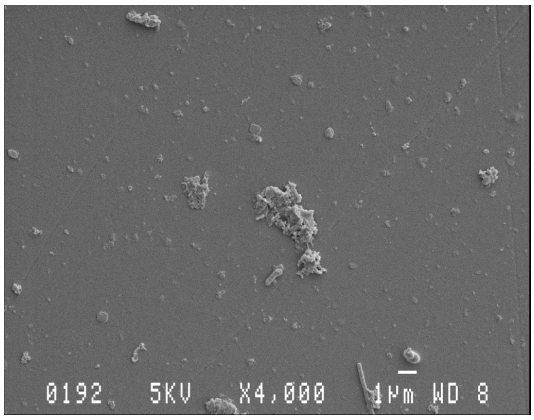
Bacteria colonized the Perspex discs after 24h and started forming microcolonies. During the first day of the experiment, only few bacteria cells were visible. The EPS became visible after 48h. The size of the microcolonies and the amount of EPS increased with time. The initial lag time of biofilm growth, varied from hours to days. The initial adherence phase, with slight biofilm growth occurring within 24h and the formation of EPS and much larger biofilm developing within 48h, causing the whole surface to be covered with biofilm (Figure 3.4).



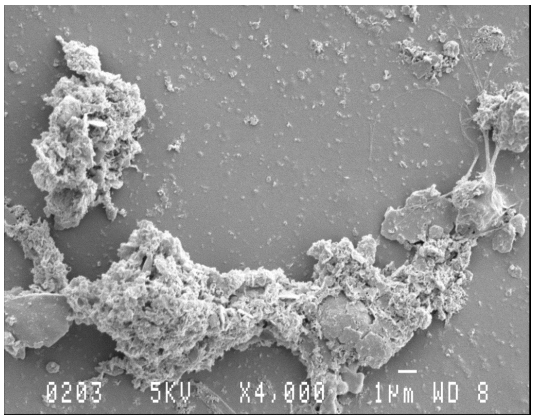
control



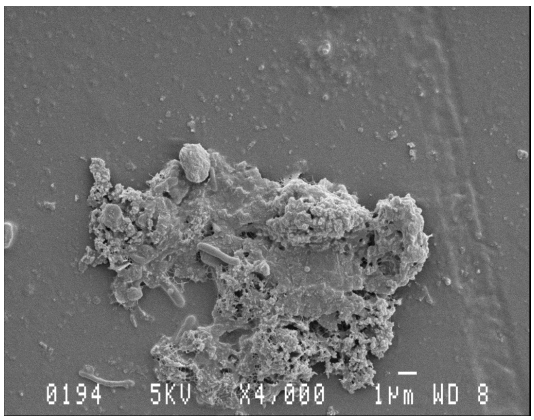
after 3d



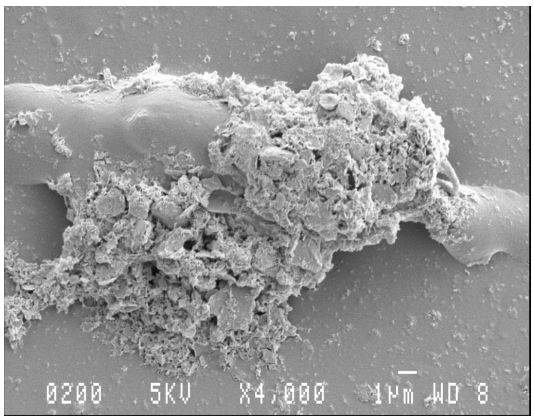
after 24h



after 4d



after 48h



after 5d

Figure 3.4 SEM of biofilm growth over a 5d period

3.4 CONCLUSIONS

- Biofilm formation could be successfully monitored using light reflectance method.
- Light reflectance changed with biofilm thickness. A decrease in light reflected indicated an increase in biofilm thickness.

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CHAPTER 4

THE USE OF THE ROTOSCOPE AS AN ONLINE, REAL-TIME, NON-DESTRUCTIVE BIOFILM MONITOR

Abstract

Biofilms are involved in all kinds of biofouling and cause a significant economic loss of billions of dollars annually, worldwide. In order to effectively control the growth of biofilms, it is necessary to investigate the structure of biofilms grown under different conditions. Several methods are available to monitor biofilm progression, but their applications are limited by low intensity, high labour intensity, intrusive sampling, and long time lags from sampling to results. The goal of this research was to monitor biofilm growth using a biological rotating contact disc, based on scanning electron microscopy and the light reflection. The biocidal effect of the aged anolyte on isolated bacteria and biofilm was also evaluated. Light reflected changed with biofilm thickness and the thicker the biofilm, the less light was reflected. Addition of NaCl anolyte to the Rotoscope caused some detachment of the microbial cells. This was indicated by a slight increase in light reflection and was supported by SEM micrographs. Rotoscope proved to be sensitive to slight changes in biofilm characteristics. Anolyte samples used at a 10% dosage resulted in a 100% kill after 6h against all the bacterial cells used.

Key words

Biofilm monitoring, biofouling, biocorrosion, biocides

4.1 INTRODUCTION

Bacterial cells present in the fluid contact the substratum by a variety of transport mechanisms, and once at the substratum, cells can adsorb either reversibly or irreversibly. If the cells remain at the surface for a sufficient time, they secrete extracellular polymers that serve to attach them tenaciously to the substratum. Attached cells metabolise, grow, replicate and produce insoluble extracellular

polysaccharides, thus accumulating an initial viable biofilm community (Morton *et al*., 1998). Bacterial cells of the same or different species continue to be recruited from the fluid and incorporated into biofilm community. Biofilms develop on any surface in natural soil and aquatic environments, on tissues of plants, animals and humans as well as in man-made systems (Schulte *et al*., 2004).

Biofilms are involved in all kinds of biofouling and cause a significant economic loss of billions of dollars annually, world wide (Gilbert *et al*., 2003). When they develop on ship hulls, or in industrial pipe systems, they will increase frictional resistance that will lead to substantial pressure drop (Stoodley *et al.,* 2002) and an increase in energy consumption or to a reduction of speed of vessels. In cooling water systems, they cause increase in resistance to heat energy transfer. This growth reduces the water quality, increases the pressure differentials in membrane processes, and reduces the efficiency of heat exchangers (Schmid *et al*., 2004). In some cases, biofilms result not only in the unwanted accumulation of biological material on surfaces, but also promote the precipitation of minerals, such as carbonate; which leads to mixed biological and non-biological deposits that are particularly difficult to remove (Schulte *et al*., 2004)

Unwanted growth of biofilms in technical processes is a natural phenomenon, due to the favourable conditions of nutrients, temperature and availability of microorganisms (Giao *et al*., 2003). Five mitigation approaches are currently followed (a) Biofilms are killed by biocides at lethal doses (b) Biofilms are dispersed by dispersants (c) Biofilms are removed physically by a variety of processes (d) Biofilms are weakened by enzymes or chelates (e) Ultraviolet light can also be used to control bacterial numbers (Cloete *et al*., 1998).

In order to effectively control the growth of biofilms, it is necessary to investigate the structure of biofilms grown under different conditions (Staudt *et a*l., 2003). Several methods are available to monitor biofilm progression (Table 4.1), but their

applications are limited by low intensity, high labour intensity, intrusive sampling, and long time lags from sampling to results (Bakke *et al*., 2001).

Table 4.1 Devices used to monitor biofilm growth

Biofilm monitoring technique	Reference
Electron microscopy	Cloete *et al.,* 1998; Lazarova and Manem, 1995
Confocal laser scanning microscopy	Staudt *et al*., 2003
Pedersen's device	Jacobs *et al.,* 1996
Robbins Device	Johnston and Jones *et al.,* 1995
Rectangular duct biofilm reactor	Bakke *et al*., 2001
BIoGEORGE ™	Bruij *et al*., 2003
Photo-acoustic spectroscopy	Schmid *et al*., 2004
AQUASIM	Wanner and Morgenroth, 2003
The Roto – Torque System	Characklis and Marshall, 1985
Linear polarization resistance	Christiani *et al.,* 2002
Electrochemical Impedance Spectroscopy	Christiani *et al.,* 2002
Electrochemical Noise	Christiani *et al.,* 2002
Biowatch	Ondeo-Nalco
Atomic force microscope	Hilal and Bowen, 2002
Fibre optical device	Tamachkiarow and Flemming, 2003

Monitoring of parameters that are evidently related to biofilm accumulation or an effect of biofilm accumulation can help to select the intensity of the measured signal that triggers a warning system, e.g. if the readout exceeds a certain number, (it indicates the presence of biofilm) then add a biocide (Lewandowski and Beyenal, 2003). Many biofilm monitoring systems follow such a preventive strategy, and they act as action triggers.

The main objective of this research was to evaluate the Rotoscope for biofilm monitoring.

4.2 MATERIALS AND METHODS

4.2.1 Biofilm reactor

A schematic diagram of the biofilm Rotoscope reactor used in this study is shown in Figure 4.1. The system consists of a tank, which is capable of carrying 20L of water when it is full. A rotating plastic wheel (disc) is moved by the water that is pumped from the tank through the discharge side (pipe) back to the tank. The speed of the pump is 4000L/H. The suction side sucks water from the tank, through the pump to the discharge side. The biofilm growing on the rotating disc can be measured on a frequency using the light monitor or sensor. The light monitor measures the light reflected by the biofilm on the disc. This allows one to measure the kinetics of the biofilm deposit and compare it to the biofilm level after it has been subjected to a biocide. The flowing water passes through the Modified Peterson Device in which removable coupons e.g. glass slides are placed for the attachment of microorganisms.

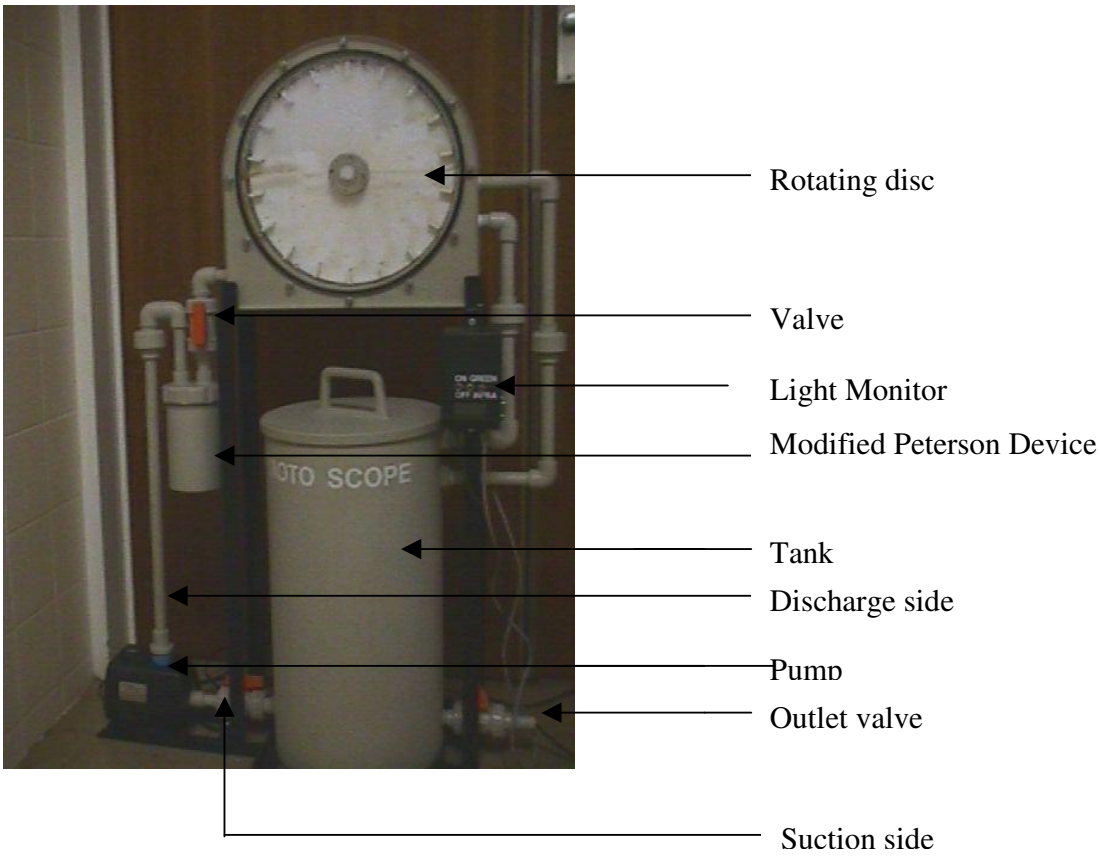


Figure 4.1 Laboratory Rotoscope used to monitor biofilm growth

4.2.2 Biofilm growth

Water was collected from LC dam at the University of the Pretoria (SA). Eighteen liters of water was used to grow the biofilm. Biofilm was allowed to grow on the rotating discs and on the removable glass slides, which were placed inside the Modified Peterson Device. Slides were removed daily and fixed in 10mL fixing solution (2.5% Gluteraldehyde in 0.0075M Phosphate buffer) for SEM. For SEM slides were washed 3x with 0.0075M Na_2PO_4 buffer (diluted 1:1 with distilled water) to remove all the gluteraldehyde. This was followed by the dehydration steps, which were done by washing in a series of increasing concentrations of ethanol as follows: 1 x 15min 50%, 1 x15min 70%, 1 x 15min 90% and lastly, 3 x 15min 100% ethanol. The slides were dried using a Critical drying Point for overnight and coated with gold. Light reflected were taken daily, and on the 19th day, an anolyte derived from NaCl (1:10 dilution) was added to the tank. Samples for the SEM were again taken and the light reflected noted.

4.2.3 Biocide evaluation

Two samples detailed as (1) STEDS Activated solution No.1, containing 0.15% $NaHCO_3$, batch No: 11/10 03 and (2) STEDS Activated solution No.3 (Root canal sterilizing solution), approximately pH – 7.0, hypotonic, batch No: 11/10/03, were received on the 28th May 2004. The packaging of the solutions was in accordance with medical grade polyethylene bottles, sealed with a tamper proof, rachet-type, and screw on cap. According to the manufacturer, the solutions had been retained indoors at ambient temperatures and with direct daily exposure to sunlight.

4.2.4 Kill test

For kill tests, four cultures were used: *B. subtilis*, *P. aeruginosa, E. coli* and *S. aureus.* These cultures were obtained from the culture collection, in the Department of Microbiology and Plant Pathology, University of Pretoria, South Africa. The cultures were tested for purity by Gram staining technique before use. A loopful of each culture was suspended in a 100ml conical flask containing 40mL of sterile tap water.

Ten milliliters of an anolyte derived from NaCl (1:10 dilution) were added to the experimental flasks. The control flasks contained 40mL of bacterial suspension and 10mL of sterile distilled water. Flasks were incubated for 30m, 1h, 6h and 24h at 37ºC. Samples (10mL) were taken at the end of incubation for serial dilutions (from 10^{-1} to 10^{-5}) and 100µl was spread plated on Nutrient Agar. Plates were incubated at 37ºC for 24h. The same experiment was repeated using 0.15% $NaHCO_3$ anolyte.

Oxidation – Reduction Potential (ORP) analysis

A Waterproof ORPScan (Double Junction) with replaceable Double Junction Electrode and 1mV Resolution was conditioned by immersing the electrode in tap water for 30m before use. A measure of 5ml of sample from the suspension was taken out to measure ORP.

Conductivity measurement

WP ECScan High with Waterproof and Floats, Replaceable Electrode, Temperature Display, Small Size and 0.01 mS resolution was used to measure electrical conductivity (EC) and temperature of the sample.

pH

A waterproof pHScan 2 tester was also used to measure the pH for every sample, including those from the reactor. ORP, pH and EC measurements from the reactor were also taken after addition of the anolyte NaCl.

4.3 RESULTS AND DISCUSSION

4.3.1 Biofilm growth measured with the Rotoscope

The decrease reflected light as a result of biofilm growth on the rotating disc is illustrated in Figure 4.2. Light reflected started at 60mV and decreased over time. The addition of an anolyte as indicated by an arrow (Figure 4.2) caused light reflectance to increase for a short while, indicating the partial removal of the biofilm, after which it decreased as a result of bacterial regrowth.

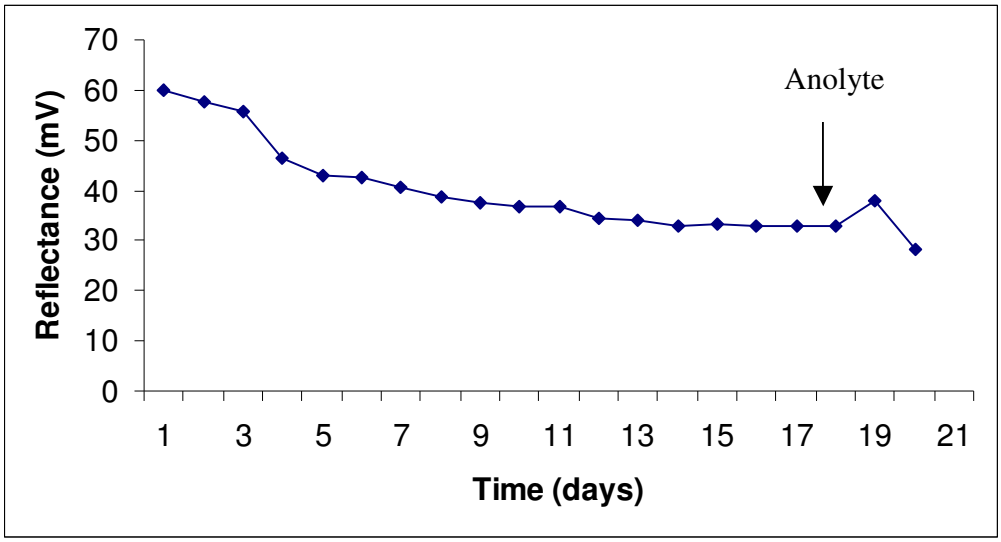
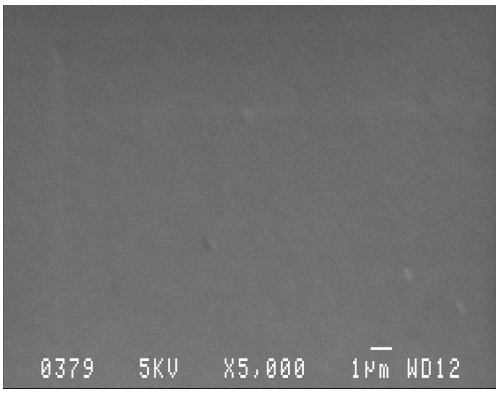


Figure 4.2 The average reflectance readings as affected by the biofilm on the rotating disc. An arrow indicates the time anolyte was added.

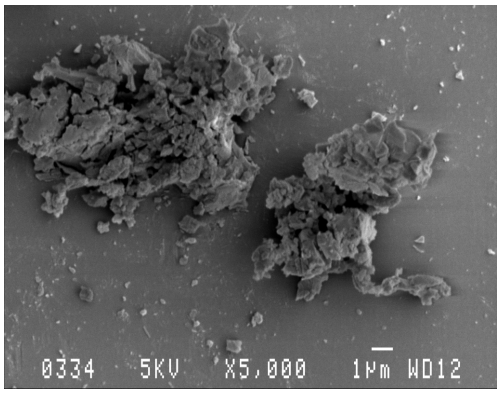
Table 4.2 shows the measurement of ORP, pH and EC from the tank after addition of the NaCl anolyte. The initial value of 371mV was taken just before the addition of anolyte. It can be seen by the decrease of this value that the anolyte was effective. The increasing EC values indicated total dissolved salts present in the tank. The water in the tank was changing to alkalinity because of the presence of anolyte.

Table 4.2 ORP, pH and EC measurement from the Rotoscope tank

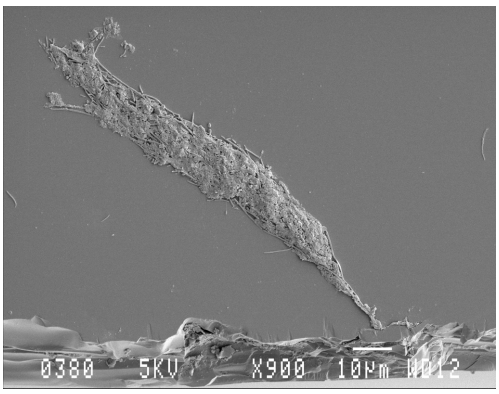
Day	Time	ORP (mV)	pH	EC(mS)	Temp (˚C)
1	13:00	371	8.4	0.25	22.1
	13:30	369	8.5	0.27	22.3
	14:00	367	8.5	0.35	22.9
	16:00	362	8.5	0.40	23.9
2	08:00	355	8.5	0.67	24.4
	12:00	350	8.2	0.71	25.0
	14:00	307	8.4	0.70	24.1



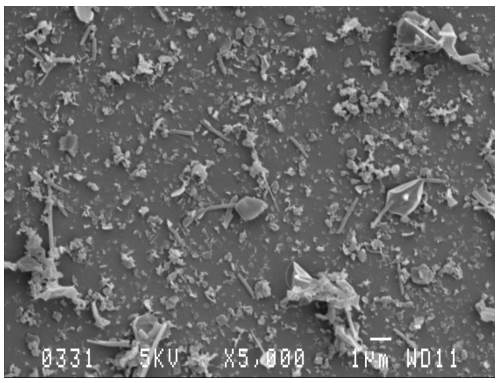
control (clean slide)



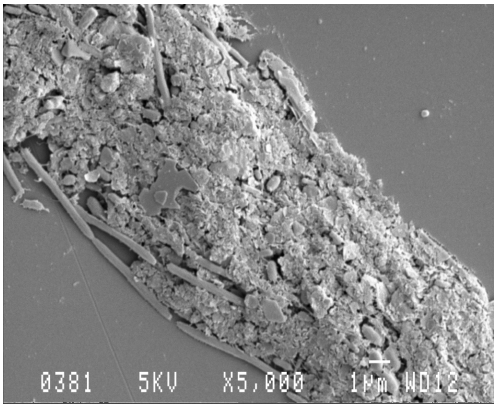
after 48h



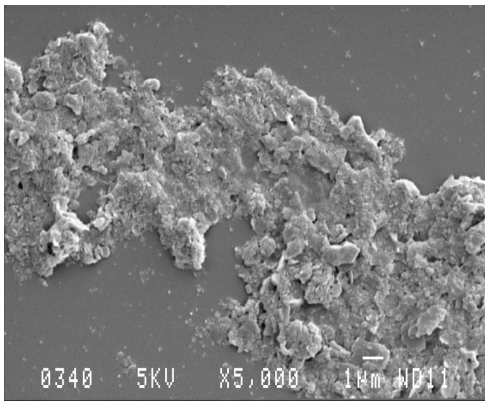
after 24h



after 72h

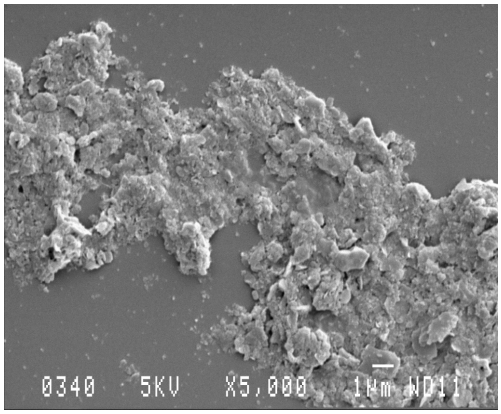


after 24h

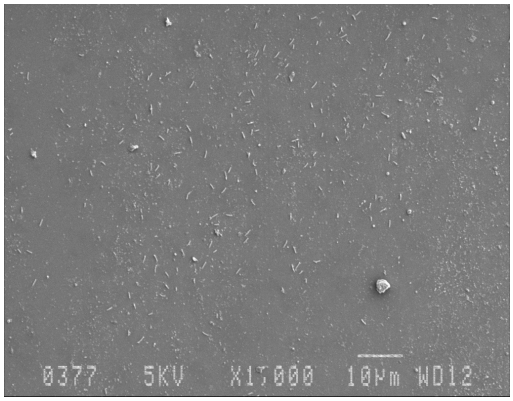


after 120h

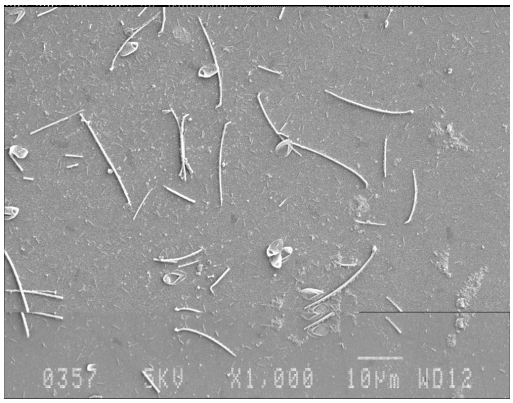
Figure 4.3 SEM micrographs of biofilm growth on glass over first 120h



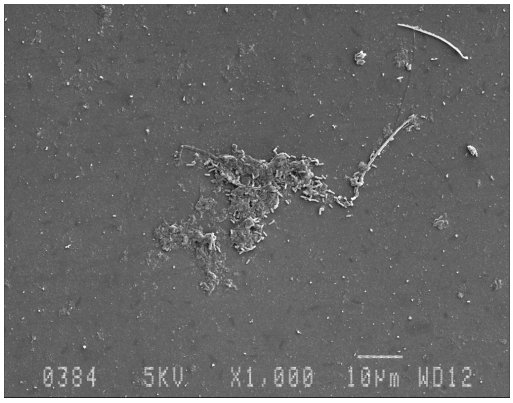
control (120h old biofilm)



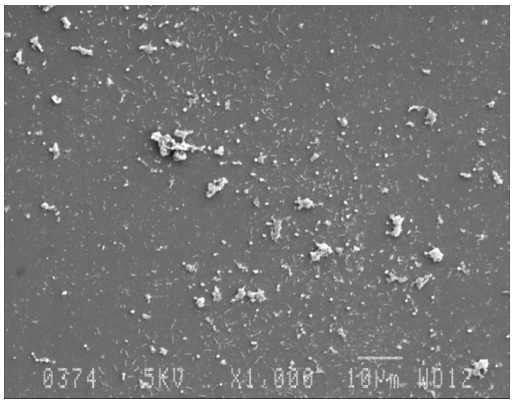
19h after treatment



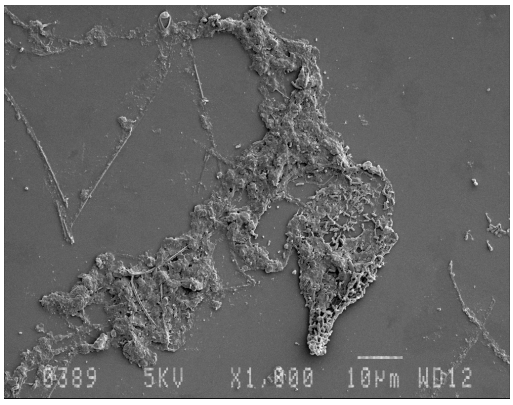
5min after treatment



27h after treatment



30min after treatment



45h after treatment

Figure 4.4 SEM micrographs of biofilm after addition of 1:10 dilution of NaCl anolyte

Bacterial cells attached on the glass slide and increased with time. Bacteria colonies formed on the slide within 24 hours forming a visible EPS. Cells move towards each other until they covered almost the whole surface (Fig 4.3).

The growth of biofilm was monitored using a laboratory Rotoscope reactor. In this study, as the disc was rotating in water, the microorganisms in the water attached to the disc, accumulated with time and lead to the formation of biofilm. This resulted in a decrease of the light reflected by the disc. The decrease in light reflectance was due to the attachment of microbial cells and production of EPS molecules on the surface of the rotating disc (Fig. 4.2). Light reflectance changed with biofilm thickness and the thicker the biofilm, the less light was reflected. Light reflectance is the most common variable monitored in biofilm literature probably because of its simple interpretation and the fact that it can be measured without the use of microscopy (Heydorn *et al*., 2000). These results are in agreement with a previous study showing that the changes in light reflectance were caused by biofilm thickness (Lewandowski and Beyenal, 2003). The thicker the biofilm, the less light was reflected because biofilm thickness affected the spatial dimensions of the biofilm (Heydorn *et al*., 2000). A decrease of backscatter light was also observed when a fiber optical device (FOD) was installed in a piping system to indicate the formation of biofilm and the efficacy of cleaning measures (Tamachkiarow and Flemming, 2003). As biofilm thickness increased with time, a critical thickness stage known as the steady-stage was reached. A relatively linear line from day 11 in this study indicated that this was happening (Fig. 4.2). Once this stage was reached, microbes covered the entire surface area. This phenomenon results in the underlying region of the biofilm becoming oxygen deficient (anaerobic), allowing the detachment (sloughing) of some microorganisms. At this stage, a steady state between attachment and detachment of cells is reached. In this study, the latter was also observed and supported by the SEM results. SEM indicated that attachment of microorganisms was not uniform. Few cells were initially attached. After 24h, a large number of microbial cells and EPS were visible. These increased with time until the entire surface area was covered.

4.3.2 Exposure of cells to the anolyte

Table 4.3a: *B. subtilis* numbers after treatment with a 1:10 dilution NaCl derived anolyte

Time (Hours)	Control	Treated	pH	ORP (mV)	EC (mS)	Temp(°C)
	Bacterial counts (cfu/ml)					
0.5	$9.80x10^6$	$8.70x10^5$	7.6	340	0.46	23.3
1	$1.04x10^7$	$1.01x10^4$	7.6	350	0.47	22.7
6	$9.60x10^7$	0	8.5	308	0.50	23.0
24	$1.42x10^8$	0	8.8	177	0.50	25.4

cfu = colony forming units

The control count for *B. subtilis* was $9.80x10^6$cfu/ml, after 30min and increased to $1.42x10^8$cfu/ml after 24h (Table 4.3a). The *B subtilis* numbers in the treated sample decreased from $8.7x10^5$cfu/ml after 30min, to $1.01x10^4$cfu/ml after 1h and were completely eliminated after 6h (Table 4.2a).

The pH increased from 7.6 to 8.8 during the 24h experimental period. The ORP remained constant (ca 340mV) between 0 and 6h decreasing to 177mV after 24h.
The temperature fluctuated between 23.3 and 25.4ºC over the 24h experimental period.

Table 4.3b: *E.coli* numbers after treatment with a 1:10 dilution of NaCl derived anolyte

Time (Hours)	Control	Treated	pH	ORP (mV)	EC(mS)	Temp (˚C)
	Bacterial counts (cfu/ml)					
0.5	$7.80x10^6$	$4.40x10^5$	7.7	320	0.51	23.1
1	$7.60x10^6$	$2.80x10^2$	7.6	345	0.48	23.0
6	$6.00x10^6$	0	8.5	315	0.52	23.1
24	$3.60x10^7$	0	9.0	183	0.50	25.5

cfu = colony forming units

The control count for *E. coli* was $7.80x10^6$cfu/ml, after 30min and increased to $3.60x10^7$cfu/ml after 24h (Table 4.3b). The *E. coli* numbers in the treated sample decreased from $4.4x10^5$cfu/ml after 30min, to $2.8x10^2$cfu/ml after 1h and were completely eliminated after 6h (Table 4.3b).

The pH increased from 7.7 to 9.0 during the 24h experimental period. The ORP remained constant (ca 3200mV) between 0 and 6h decreasing to 183mV after 24h.
The temperature fluctuated between 23.1 and 25.5˚C over the 24h experimental period.

Table 4.3c: *P. aeruginosa* numbers after treatment with a 1:10 dilution of NaCl derived anolyte

Time (Hours)	Control	Treated	pH	ORP (mV)	EC (mS)	Temp (˚C)
	Bacterial counts (cfu/ml)					
0.5	$7.30x10^5$	$3.60x10^4$	7.7	328	0.47	22.0
1	$6.20x10^6$	$1.30x10^2$	7.6	350	0.53	22.6
6	$7.40x10^7$	0	8.5	317	0.48	23.4
24	$1.13x10^8$	0	8.7	191	0.51	27.1

cfu = colony forming units

The control count for *P. aeruginosa* was $7.30x10^6$cfu/ml, after 30min and increased to $1.13x10^8$ cfu/ml after 24h (Table 4.3c). The *P. aeruginosa* numbers in the treated sample decreased from $3.6x10^4$cfu/ml after 30min, to $1.3x10^2$cfu/ml after 1h and were completely eliminated after 6h (Table 4.3c).

The pH increased from 7.7 to 8.9 during the 24h experimental period. The ORP remained constant (ca 328mV) between 0 and 6h decreasing to 180mV after 24h.
The temperature fluctuated between 21.3 and 27.8˚C over the 24h experimental period.

Table 4.3d: *S. aureus* numbers after treatment with a 1:10 dilution of NaCl derived anolyte

Time (Hours)	Control	Treated	pH	ORP (mV)	EC(mS)	Temp (˚C)
	Bacterial counts (cfu/ml)					
0.5	$4.90x10^6$	$4.80x10^4$	7.7	328	0.47	21.3
1	$2.00x10^6$	$2.80x10^3$	7.6	353	0.43	22.4
6	$5.60x10^7$	0	8.5	310	0.50	23.3
24	$7.30x10^7$	0	8.9	180	0.53	27.8

cfu = colony forming units

The control count for *S. aureus* was $4.90x10^6$cfu/ml, after 30min and increased to $7.30x10^7$cfu/ml after 24h (Table 5.3d). The *S. aureus* numbers in the treated sample decreased from $4.8x10^4$cfu/ml after 30min, to $2.8x10^3$cfu/ml after 1h and were completely eliminated after 6h (Table 4.3d).

The pH increased from 7.7 to 8.9 during the 24h experimental period. The ORP remained constant (ca 328mV) between 0 and 6h decreasing to 180mV after 24h. The temperature fluctuated between 21.3 and 27.8˚C over the 24h experimental period.

Table 4.4a: *B. subtilis* after treatment with 0.15% $NaHCO_3$ derived anolyte

Time (Hours)	Control	Treated	pH	ORP (mV)	EC(mS)	Temp(ºC)
	Bacterial counts (cfu/ml)					
0.5	$5.50x10^6$	$5.30x10^5$	9.0	223	0.36	20.0
1	$4.00x10^6$	$4.90x10^4$	9.0	281	0.33	20.1
6	$1.12x10^7$	0	9.1	231	0.30	21,7
24	$1.07x10^8$	0	9.2	220	0.31	23.7

cfu = colony forming units

The control count for *B. subtilis* was 5.50x106cfu/ml, after 30min and increased to $1.07x10^8$cfu/ml after 24h (Table 4.4a). The *B. subtilis* numbers in the treated sample

decreased from $5.3x10^5$cfu/ml after 30min, to $4.0x10^4$cfu/ml after 1h and were completely eliminated after 6h (Table 4.4a).

The pH increased from 9.0 to 9.2 during the 24h experimental period. The ORP remained constant (ca 223mV) between 0 and 6h decreasing to 220mV after 24h. The temperature fluctuated between 20.0 and 23.7˚C over the 24h experimental period.

Table 4.4b: *E.coli* after treatment with 0.15% $NaHCO_3$ derived anolyte

Time (Hours)	Control	Treated	pH	ORP (mV)	EC(mS),	Temp (ºC)
	Bacterial counts (cfu/ml)					
0,5	$5.70x10^6$	$1.33x10^4$	8.1	362	0.51	23.1
1	$6.00x10^6$	$1.40x10^3$	8.0	353	0.48	23.0
6	$8.60x10^6$	0	8.8	314	0.52	23.1
24	$5.50x10^7$	0	8.8	266	0.50	25.5

cfu = colony forming units

The control count for *E. coli* was $5.70x10^6$cfu/ml, after 30min and increased to $5.50x10^7$cfu/ml after 24h (Table 4.4b). The *E. coli* numbers in the treated sample decreased from $1.33x10^4$cfu/ml after 30min, to $1.4x10^3$cfu/ml after 1h and were completely eliminated after 6h (Table 4.4b).

The pH increased from 8.1 to 8.8 during the 24h experimental period. The ORP remained constant (ca 362mV) between 0 and 6h decreasing to 266mV after 24h.
The temperature fluctuated between 23.1 and 25.5˚C over the 24h experimental period.

Table 4.4c: *P. aeruginosa* treated with 0.15% $NaHCO_3$ derived anolyte

Time (Hours)	Control	Treated	pH	ORP (mV)	EC(mS)	Temp(ºC)
	Bacterial counts (cfu/ml)					
0.5	$8.10x10^6$	$3.80x10^5$	8.4	339	0.47	22.0
1	$3.50x10^7$	$1.00x10^3$	8.5	386	0.53	22.6
6	$4.00x10^7$	0	8.5	253	0.48	23.4
24	$1.27x10^8$	0	9.2	236	0.51	27.1

cfu = colony forming units

The control counts for *P.aeruginosa* was $8.10x10^6$ cfu/ml, after 30min and increased to $1.27x10^8$cfu/ml after 24h (Table 4.4c). The *P.aeruginosa* numbers in the treated sample decreased from $3.8x10^5$cfu/ml after 30min, to $1x10^3$cfu/ml after 1h and were completely eliminated after 6h (Table 4.4c).

The pH increased from 8.4 to 9.2 during the 24h experimental period. The ORP remained constant (ca 339mV) between 0 and 6h decreasing to 236mV after 24h.

The temperature fluctuated between 22.0 and 27.1˚C over the 24h experimental period.

Table 4. 4d: *S. aureus* treated with 0.15% $NaHCO_3$ derived anolyte

Time (Hours)	Control	Treated	pH	ORP (mV)	EC (mS)	Temp (ºC)
	Bacterial counts (cfu/ml)					
0.5	$1.59x10^5$	$6.80x10^4$	8.3	360	0.47	21.5
1	$1.71x10^5$	$5.70x10^3$	8.3	367	0.43	22.4
6	$6.20x10^6$	0	8.8	243	0.50	23.3
24	$5.50x10^6$	0	9.1	222	0.53	27.8

cfu = colony forming units

The control count for *S. aureus* was $1.59x10^5$cfu/ml, after 30min and increased to $5.50x10^6$cfu/ml after 24h (Table 4.4d). The *S.aureus* numbers in the treated sample

decreased from $6.8x10^4$cfu/ml after 30min, to $5.7x10^3$cfu/ml after 1h and were completely eliminated after 6h (Table 4. 4d).

The pH increased from 8.3 to 9.1 during the 24h experimental period. The ORP remained constant (ca 360mV) between 0 and 6h decreasing to 222mV after 24h.
The temperature fluctuated between 21.5 and 27.8˚C over the 24h experimental period.

The use of biocides to control biofilm growth is a common practice (Cloete *et al*., 1998). In this study, the effect of NaCl anolyte on the biofilm was monitored using the Rotoscope. The addition of NaCl anolyte as a biocide, caused some detachment of the microbial cells (Fig. 4.4), indicated by a slight increase in light reflectance (Fig. 4.2). During disinfection, dead biomass will however stay in place and may provide nutrients for the new cells and the incoming cells leading to rapid regrowth of biofilm (Schulte *et al*., 2004). In this study, the decrease in light reflectance, indicating an increase in biofilm formation was attributed to microbial regrowth.

All anolyte samples used at a 10% dosage resulted in a 100% kill after 6 hours against all the microorganisms used as a challenge.

It was interesting to note that the ORP for the $NaHCO_3$ anolyte did not decrease to the same low levels observed with NaCl anolyte after 24 hours. Recent research in commercial and model postharvest water systems has shown that, if necessary, ORP limits can be relied on to determine microbial kill potential across a broad range of water quality (http://vric.ucdavis.edu).

4.4 CONCLUSIONS

The Rotoscope proved to be sensitive to slight changes in biofilm thickness offering an on-line, real-time, non-destructive method for monitoring biofilms. With respect to biofilm research, the Rotoscope offers a significant advantage in that the biofilm can be investigated in an undisturbed state.

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CHAPTER 5

THE ANTIMICROBIAL ACTIVITY OF SODIUM NITRITE ON AEROBIC BACTERIA COMMONLY ENCOUNTERED IN BIOFILMS

Abstract

Low concentrations of sodium nitrite are used in food safety. The combination of sodium nitrite with sodium chloride or potassium nitrate has been used as preservatives in hot-smoked fish products and cold smoked rainbow trout. The antimicrobial effect of sodium nitrite on aerobic bacteria was evaluated. Four bacterial species used were *B. subtilis*, *P. aeruginosa*, *S. aureus* and *E. coli*. These cells were exposed to different concentrations of $NaNO_2$ (ranging between 1ppm and 2000ppm) at 37˚C for 24h. Exposure of the cells to all the $NaNO_2$ concentrations tested for 24h did not have any effect on all the bacterial cells treated. Nitrite oxidation was probably taking place within the cells. It can therefore be concluded that nitrites have a very limited or no effect on these bacteria.

Keywords: Sodium nitrite, antimicrobial effect, nitrite oxidation, nitrifying bacteria

5.1 INTRODUCTION

Nitrite ions (NO_2) are intermediates in the biological oxidation of ammonia to nitrate (NO_3) and also in the biological reduction of nitrate to nitrogen gas. Nitrite ions in the environment are unstable. They are slowly oxidized to nitrate by atmospheric air or dissolved oxygen in the water, and they may be susceptible to react with certain types of organic pollutants that may be present in wastewaters (Patnaik and Khoury, 2004).

They are found at trace concentrations in many industrial wastewaters, and are sometimes produced by microbial reduction of more commonly occurring nitrate ions, (NO_3). Under anaerobic conditions, nitrate is reduced to nitrite by bacteria, and it can accumulate in solution in significant concentrations depending on temperature, pH, nitrate concentration and salinity (Weon *et al.*, 2002).

Sodium nitrite plays an important role in cheese and fish processing and in meat for the development of desirable colour, flavour and texture and for protection against oxidative rancidity and pathogenic microorganisms, especially *Clostridium botulinum* (Ahn *et al*., 2003). High levels of nitrite in cured meat cause the formation of carcinogenic N-nitrosamines (this is the reaction with amino compounds in foodstuffs, which might occur during processing, preservation and preparations). Therefore the addition must be in low concentrations (0.5-5ppm) acceptable for food safety (Cammack *et al*., 1999). Combinations of sodium chloride (NaCl) and sodium nitrite ($NaNO_2$), or potassium nitrate (KNO_3) have been used as preservatives in hot-smoked fish products, and cold-smoked rainbow trout (Lyhs *et al*., 1998).

Nitrite is already used together with other inorganic compounds (e.g. molybdate, chromate, etc.) or organic compounds (e.g. organic phosphates, phosphates etc.) to inhibit the corrosion of mild steel, especially in industrial cooling processes (Kielemos *et al*., 2000). Nitrite and other nitrogen oxides have been documented to have toxic effects on various bacteria such as methanogenic bacteria (Clarens *et al*., 1998; Karlik *et al.,* 1995; Klüber and Conrad, 1998). This suggests that $NaNO_2$ may be used to control the growth of microorganisms in industrial water systems.

The present investigation was carried out to determine the effect of sodium nitrite on aerobic bacteria. The bacteria used were *Pseudomonas aeruginosa*, *Staphylococcus aureus, Escherichia coli* and *Bacillus subtilis.*

5.2 MATERIALS AND METHODS

5.2.1 Cultures

Microorganisms used were *B. subtilis* (spore-forming bacteria), *P. aeruginosa*, *E. coli* (Gram negative bacteria) and *S. aureus* (Gram positive bacteria). These cultures were obtained from the culture collection, Department of Microbiology and Plant Pathology, University of Pretoria, South Africa. The stock cultures were kept on Nutrient agar plates.

5.2.2 Chemicals

All the chemicals used were obtained from MERCK Chemicals (PTY) LTD (SA). Sodium nitrite stock solution (1000ppm) was prepared by dissolving 1.0g of sodium nitrite in distilled water and brought to 1L.

5.2.3 Sensitivity test

Bacteria were subcultured on Nutrient agar plates and checked for purity by Gram staining before use. Sensitivity was determined by the agar diffusion method where 100µl of colony suspension in sterile distilled water was evenly spread on Nutrient agar. Filter paper discs containing different concentrations of sodium nitrite were then placed on the agar surface of this Nutrient agar plate. The concentration of sodium nitrite on each of the disc was specified. Plates were incubated at 37°C for 24h, 48h and 72h. After incubation, the presence or absence of inhibition zones around the discs were noted.

5.2.4 Exposure to nitrite

Sterile tubes were set up containing 800µl sterile tap water and 100µl of bacterial suspension (*E. coli, P aeruginosa, B. subtilis and S. aureus*) and 100µl of nitrite solution at concentrations of 1ppm, 10ppm, 100ppm 1000ppm and 2000ppm. The test was replicated for each concentration of nitrite. The negative control was sterile tap water with 100µl of bacterial suspension without sodium nitrite. The tubes were closed and incubated at 37˚C for 6h and 24h.

5.2.5 Viability testing

After incubation with and without sodium nitrite, 100µl samples were taken at 0h, 6h and 24h. 100µl of the culture was serially diluted (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5}) in sterile tap water for viability testing. 100µl of each dilution was spread plated on Nutrient agar plates in duplicates and incubated at 37˚C for 24h for calculation of colony forming units per ml (cfu/ml) and the death rate.

5.3 RESULTS AND DISCUSSION

5.3.1 Sensitivity test

The four bacteria tested were resistant to nitrite. No clear zones were observed around the discs. The highest nitrite concentration (2000ppm) for an incubation time of 72h did not have any effect on the bacteria.

5.3.2 Viable bacterial counts

The number of viable colonies for all the bacterial species tested remained the same after exposure to different concentrations of $NaNO_2$ (Table 5.1). All the bacteria tested were therefore resistant to sodium nitrite at all concentrations tested.

Table 5.1: Growth of different bacterial species after exposure to varying concentrations of nitrite

Biocide concentrations (ppm)	Bacterial species			
	B. subtilis	*E. coli*	*P. aeruginosa*	*S. aureus*
	Total counts (cfu/ml)			
0	>3.00 x 10^7	>3.00 x 10^7	>3.00 x 10^7	>3.00 x 10^7
1	>3.00 x 10^7	>3.00 x 10^7	>3.00 x 10^7	>3.00 x 10^7
10	>3.00 x 10^7	>3.00 x 10^7	>3.00 x 10^7	>3.00 x 10^7
100	>3.00 x 10^7	>3.00 x 10^7	>3.00 x 10^7	>3.00 x 10^7
1000	>3.00 x 10^7	>3.00 x 10^7	>3.00 x 10^7	>3.00 x 10^7
2000	>3.00 x 10^7	>3.00 x 10^7	>3.00 x 10^7	>3.00 x 10^7

ppm = parts per million, cfu = colony forming units

The four bacteria used in this experiment are capable of denitrification (Brock and Madigan 1991; Sakai *et al*., 1996) and can reduce nitrite to nitrate. This implies that nitrite oxidation was probably taking place within the cells. Addition of nitrite to the media therefore served as a nutrient source rather than a biocide.

Nitrite within the cells is reduced to ammonia or other gaseous compounds and this usually happens when not all of the nitrite is converted to nitrate (Brock and Madigan, 1991). Some denitrification strains reduce and accumulate nitrate from nitrite whereas others only reduce nitrite and don't accumulate it (Sakai *et al*., 1997).

5.4 CONCLUSIONS

- Nitrite will therefore have no or a very limited effect on nitrifying organisms and hence a limited effect in biofouling control, since *Pseudomonas* spp. are predominant organisms in biofilm formation and biofouling.

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CHAPTER 6

READY TO USE CHLORINE DIOXIDE AS A MEANS OF CONTROLLING BIOFILM

Abstract

Biofilm control is important in industrial systems because biofilms cause biofouling problems. Chlorine dioxide (ClO_2) has been successfully used to eliminate chlorine's taste and odours in potable water treatment. The interest of ClO_2 as a biofilm control disinfectant has increased. In this study, we examined the effect of ready-to-use ClO_2 on planktonic bacteria and biofilms. Planktonic bacteria were exposed to different concentrations of ClO_2 for 6h at room temperature. The results indicated that *P. aeruginosa, S. aureus and E.coli* were immediately killed by ClO_2 at concentrations of 80ppm. *B. subtilis* was eliminated after 1h of exposure. The addition of ClO_2 to established biofilm, caused detachment and death of bacterial cells, indicated by an increase in light reflectance. SEM analysis indicated a decreased cell deposit confirming biofilm detachment. It can be concluded that ClO_2 was effective in killing planktonic and in removing biofilm. Therefore it is a broad disinfectant.

Keywords: biofouling, chlorine dioxide, chlorine's taste and odours, broad disinfectant

6.1 INTRODUCTION

In water systems, water is passed over large surface areas in pipelines, providing favourable conditions for bacterial attachments. These surfaces offer bacteria a nutritional advantage by attracting amphipathic molecules such as proteins. Free floating or planktonic bacteria attach readily to most surfaces upon reaching these surfaces and this attachment and subsequent growth lead to the formation of biofilm (Brözel and Cloete, 1993). The presence of biofilm in water systems cause significant problems such as heat transfer resistance, filter plugging, product contamination and spoilage and metal corrosion. Detachment of cells from biofilm in food production facilities and drinking water systems may result in the potential transmission of pathogens via contaminated

food, drinking water or aerosols (Stoodley *et al*., 2002). They cause not only economical losses but health diseases as well. Therefore their control is a real challenge within engineered systems (De Saravia and De mele, 2003).

The use of biocide is an accepted and commonly used practice. They are employed to prevent, inhibit or eliminate bacterial growth in water systems. Biocides place bacterial cells under stress by attacking targets of cell functions. These targets are essentially components of the cytoplasmic membrane or the cytoplasm contents. In order to reach their target sites, biocides must transverse the outer membrane and attain a minimum active concentration at that site. Biocides are used in a wide variety of processes and matrices, cosmetics, food, industrial process waters, marine antifouling paints, plastics, wood and swimming pools, etc. (Chapman, 2003).

Chlorination of drinking water has for many decades played a leading role in reducing mortality and morbidity rates associated with waterborne pathogens (Monarca *et al*., 2004). However, during treatment, chlorine reacts with naturally occurring organic material to produce numerous disinfectants-by-products (DBPs), some of which are suspected carcinogens (Korn *et al.,* 2002). Again chlorine affects taste, odour and appearance of drinking water. Although it is a reliable disinfectant, chlorine is affected by pH. The control of pH is an important precaution; low pH values minimize the formation of Trihalomethanes (THMs) and promote the formation of haloacetic acids and other DBPs (Meyer, 2003). There is strong evidence that reduced efficacy results from limited penetration of chlorine into the biofilm matrix and reduced activity at high concentrations of organic matter (Elvers *et al*., 2002).

Chlorine dioxide (ClO_2), a greenish yellow solution in water with a distinctive odour, similar to chlorine, has been successfully used to eliminate chlorine's tastes and odours in potable water treatment (White, 1992). When compared with ozone and chlorine, chlorine dioxide is less reactive, and will therefore not react with natural organic compounds. Although it reacts with reduced sulphur compounds, secondary and tertiary amines, the chances of producing by-products are zero. It does not promote THMs

formation, and is at the same time effective in reducing THM precursors. Unlike chlorine, ClO_2 is unaffected by pH. Other advantages of ClO_2 are its ability to remove ammonia and manganese, control musty tastes, fishy tastes and odours (White, 1992), and destruction of phenols, which cause taste and odour problems in potable water supplies. It kills microorganisms by disruption of the transport of nutrients across the cell wall. ClO_2 has the ability to kill spores, viruses and fungi at low concentrations. It is also used in cooling towers, in vegetable washing, in hot and cold water systems (http://www.lenntech.com/chlorine_dioxide.htm). In the United States and Europe, ClO_2 has been used to treat municipal drinking water for more than 50 years and it is recognized as a superior disinfectant alternative to chlorine (http://www.pristine.ca/chlorine.html).

At the moment, available information on ClO_2 behaviour mainly comes from data acquired in batch scale (Vischetti *et al*., 2004). The small number of real applications indicates the necessity to go deep into some essential aspects like ClO_2 dosages and contact time. The objective of this study was to investigate the effect of the recent ready-to-use (RTU) chlorine dioxide on four bacterial cultures i.e. *P. aeruginosa*, *B. subtilis*, *S. aureus* and *E.coli*. These cultures were previously isolated from water systems. The effect of chlorine dioxide on already established biofilm was also tested.

6.2 MATERIALS AND METHODS

6.2.1 Cultures and media

Four cultures: *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Escherichia coli* and *Staphylococcus aureus*; obtained from the culture collection in the Department of Microbiology and Plant Pathology, University of Pretoria, SA, were used. These were maintained on Nutrient Agar (NA) plates, and subcultured after every second week. The cultures were checked for purity by Gram staining technique before use.

6.2.2 Ready to use ClO_2 biocide

Ready-to-use (RTU) chlorine dioxide (ClO_2) was obtained from BTC Products and

Services Company, South Africa. The concentration of ClO_2 was 2025ppm with pH 6.4, a claimed shelf life of 4weeks when stored at room temperature in the dark.

6.2.3 Determination of the minimum inhibitory concentration (MIC)

One colony of a 24h pure culture was suspended in test tubes containing 10ml of sterile quarter strength Ringer's solution (Merck). The biocidal effect of different concentrations of ClO_2 (10ppm, 20ppm, 30ppm, 40ppm, 50ppm, 60ppm, 70ppm, 80ppm, 90ppm, 100ppm, 200ppm and 500ppm) was tested. ClO_2 was used after 1week of production. The control tube contained 10ml of bacterial suspension without ClO_2. From each tube, the control and the one containing the relevant bacteria and ClO_2 concentration, 100µl were taken out at time 0h, after 6h and after 24h of incubation for serial dilutions (from 10^{-1} to 10^{-6}) and spread on Nutrient Agar (NA) (Biolab) plates. The plates were incubated at 37ºC for 24h. The lowest concentration of bactericide showing absence of growth was taken to be the minimum inhibitory concentration.

6.2.4 Biofilm control using ClO_2

A Rotoscope, which was designed and described in the chapter 4, was used to grow biofilm. Water was collected from LC de Villiers dam of the University of Pretoria (SA). Biofilm was allowed to grow on the rotating disc and on the removable glass slides, which were placed inside the Modified Peterson Device attached to the Rotoscope. Slides were removed daily and fixed in 10ml fixing solution (2.5% Gluteraldehyde in 0.0075M Phosphate buffer) for SEM. For SEM, slides were washed 3x with 0.0075M Na_2PO_4 buffer (diluted in 1:1 with dH2O) to remove all the gluteraldehyde. This was followed by the dehydration steps, which were done by washing with a series of increasing concentrations of ethanol as follows: 1 x 15min 50%, 1 x 15min 70%, 1 x15min 90% and lastly 3 x 15min 100% ethanol. The slides were dried overnight using a critical point dryer, and then coated with gold.

Light reflectance measurements were taken daily. On the 3rd day of biofilm growth, ready to use (RTU) chlorine dioxide (ClO_2), which had been stored for two weeks, was added

at a concentration of 80ppm. Samples for SEM were removed and prepared as previously.

6.2.5 Enumeration of viable populations of biofilm and planktonic bacteria

Microscopic slides were removed from the Modified Peterson device and the biofilm was removed from the slides by shaking the slide in 10ml of ¼ strength Ringer's solution containing 10 sterile glass beads. 100µl of this suspension were plated on NA plates. The plates were incubated at 37ºC for 24h.

6.3 RESULTS AND DISCUSSION

6.3.1 Exposure of bacteria to ClO_2

The control count of *Bacillus subtilis* was $>3.00x10^7$ cfu/ml. Exposure of *B. subtilis* cells to ClO_2 concentrations lower than 80ppm did not eliminate/kill the cells but reduced the cell numbers. Increasing the exposure time reduced the cell numbers very slowly (Table 6.1. Additions of ClO_2 in the concentrations of 80ppm killed all the cells after 1h of exposure. Higher concentrations of ClO_2 (100, 200 and 500ppm) killed all the cells immediately upon exposure. The MIC of ClO_2 for *B. subtilis* was therefore found to be 80ppm (Table 6.1).

Table 6.1: The effect of Ready To Use (RTU) chlorine dioxide on *Bacillus subtilis*

Time (Hours)	ClO_2 concentrations (ppm)							
	Control	20	40	60	80	100	200	500
	Bacterial counts (cfu/ml)							
0	$>3.00x10^7$	$>3.00x10^7$	$3.33x10^6$	$8.80x10^2$	$3.00x10^1$	0	0	0
1	$>3.00x10^7$	$>3.00x10^7$	$7.05x10^5$	$5.08x10^2$	0	0	0	0
6	$>3.00x10^7$	$>3.00x10^7$	$6.08x10^5$	$3.10x10^1$	0	0	0	0
24	$>3.00x10^7$	$>3.00x10^7$	$4.20x10^4$	$2.09x10^1$	0	0	0	0

cfu = colony forming units, ppm = parts per million

Table 6.2 The effect of Ready To Use (RTU) chlorine dioxide on *Pseudomonas aeruginosa*

Time (Hours)	ClO_2 Concentrations (ppm)							
	Control	20	40	60	80	100	200	500
	Bacterial counts (cfu/ml							
0	$>3.00x10^7$	$>3.00x10^7$	$7.80x10^3$	$9.89x10^1$	0	0	0	0
1	$>3.00x10^7$	$>3.00x10^7$	$9.70x10^3$	$7.91x10^1$	0	0	0	0
6	$>3.00x10^7$	$>3.00x10^7$	$7.30x10^2$	$4.05x10^1$	0	0	0	0
24	$>3.00x10^7$	$>3.00x10^7$	$5.50x10^2$	$2.25x10^1$	0	0	0	0

cfu = colony forming units, ppm = parts per million

The control count for *Pseudomonas aeruginosa* was $>3{,}00x10^7$ cfu/ml before incubation and after 24h of incubation. The number was not reduced by the addition of 20ppm, but reduced by addition of 40 and 60ppm of ClO_2. Increase in exposure time for these concentrations had an effect on the bacteria although it was not much. The addition of 80, 100, 200 and 500ppm ClO_2 resulted in a 100% kill immediately after exposure (Table 6.2). The MIC of ClO_2 for *P. aeruginosa* was also 80ppm.

Table 6.3 The effect of Ready To Use (RTU) chlorine dioxide on *Staphylococcus aureus*

Time (Hours)	ClO_2 Concentrations (ppm)							
	Control	20	40	60	80	100	200	500
	Bacterial counts (cfu/ml							
0	$>3.00x10^7$	$>3.00x10^7$	$8.91x10^4$	$3.78x10^2$	0	0	0	0
1	$>3.00x10^7$	$>3.00x10^7$	$8.08x10^3$	$1.81x10^2$	0	0	0	0
6	$>3.00x10^7$	$>3.00x10^7$	$5.03x10^3$	$3.01x10^1$	0	0	0	0
24	$>3.00x10^7$	$>3.00x10^7$	$3.00x10^2$	$1.07x10^1$	0	0	0	0

cfu = colony forming units, ppm = parts per million

The control count for *Staphylococcus aureus* was >3.00x10^7 cfu/ml before incubation and after 24h of incubation. This number was not reduced by the addition of 20ppm, but reduced by addition of 40 and 60ppm of ClO_2. The addition of 80ppm resulted in a 100% kill immediately after exposure (Table 6.3) and the MIC was 80ppm.

Table 6.4 The effect of Ready To Use (RTU) chlorine dioxide on *Escherichia coli*

Time (Hours)	ClO_2 Concentrations (ppm)							
	Control	20	40	60	80	100	200	500
	Bacterial counts (cfu/ml							
0	>3.00x10^7	>3.00x10^7	7.05x10^5	3.20x10^2	0	0	0	0
1	>3.00x10^7	>3.00x10^7	6.03x10^3	2.22x10^2	0	0	0	0
6	>3.00x10^7	>3.00x10^7	2.01x10^3	5.03x10^1	0	0	0	0
24	>3.00x10^7	>3.00x10^7	1.24x10^3	1.11x10^1	0	0	0	0

cfu = colony forming units, ppm = parts per million

The control count for *Escherichia coli* was >3.00x10^7 cfu/ml before incubation and after 24h of incubation. This number was not reduced by the addition of 20ppm, but reduced by the addition of 40 and 60ppm of ClO_2. The addition of 80ppm resulted in a 100% kill immediately after exposure (Table 6.4), and the MIC was 80ppm.

The MIC of a 1-week-old ClO_2 solution investigated in this study was 80ppm. This was higher than indicated in other studies. Han *et al*. (2000) indicated that the MIC of ClO_2 on *B. subtilis, E. coli* and *S. aureus* was as low as 1.5mg/l at 20˚C. Junli *et al*. (1997) indicated that bacteria inoculated on injured surfaces (of green pepper), were completely inactivated by the addition of 1.2mg/l of ClO_2 after 20min, ClO_2 in 1mg/l with a contact time of 18h, was sufficient to reduce the viable counts of planktonic bacteria in a continuous culture model of a drinking water system (Walker and Morales, 1997). In all the previous experiments, gaseous ClO_2 was used whereas in our experiment, liquid ClO_2 was used. Gaseous ClO_2 seemed to be more effective than liquid.

6.3.2 Control of biofilm using ClO_2

Table 6.5 The effect of RTU ClO_2 on viable populations in biofilm samples

Time (min)	Bacterial counts (cfu/ml)
0	$1.81x10^5$
2	$1.30x10^3$
30	0
60	0
120	0

cfu = colony forming units

The control count for viable populations was $1.81x10^5$ cfu/ml, which decreased to $1.30x10^3$ cfu/ml after 2min of bacteriocide exposure. After 30min of exposure, these cells were completely eliminated, indicating that ClO_2 killed all bacterial biofilm (Table 6.5). The MIC for all the biofilm organisms was 80 ppm.

6.3.3 Biofilm control with ClO_2 as measured with the Rotoscope

Light reflectance started at 68.5mV and decreased slowly to 60mV indicating biofilm formation. The addition of ClO_2 caused the reflectance to increase to 70mV indicating biofilm removal. The light reflectance reading again decreased to 55.5 mV after the third day of exposure indicating biofilm regrowth (Fig 6.1).

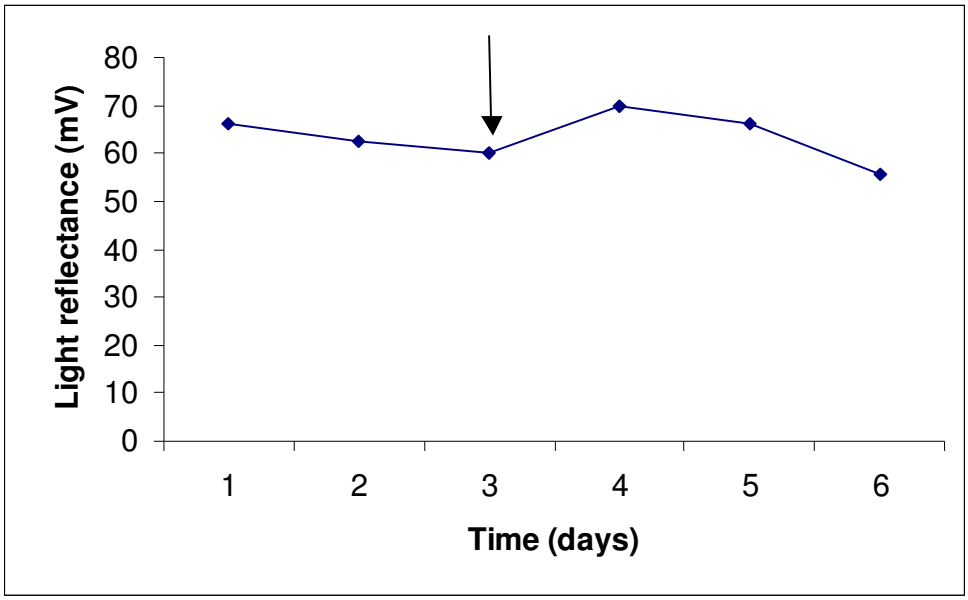


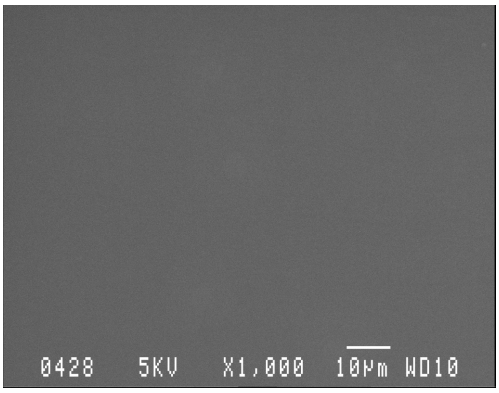
Figure 6.1 The average reflectance readings as affected by biofilm growth on the rotating disc. The arrow indicates the time ClO_2 was added.

The decrease in light reflectance was due to the attachment of bacteria on the rotating disc. The addition of ClO_2 caused a decrease in bacterial attachment, which caused an increase in light reflectance. Decrease of light reflectance during the second and the third days of exposure, might also be due to dead biomass that remained attached on the disc. ClO_2 killed the bacterial cells and removed biofilm when used at a concentration of 80ppm. Total viable counts of bacteria in the biofilm were completely eliminated after 30min of biocide addition.

6.3.4 Scanning electron microscopy (SEM)

Bacterial attachment on surfaces was very fast (Fig 6.2). The number of bacterial cells attached increased with time. Bacteria attached and moved towards each other to produce extracellular polymeric substances (EPS). Cells continued to attach until they covered almost the whole surface indicated on the slide taken after 48h (Fig 6.2).

Addition of ClO_2 at a concentration of 80ppm caused detachment of the biofilm over time (Fig 6.3). Within 2min of exposure, cells were already removed from the slide as



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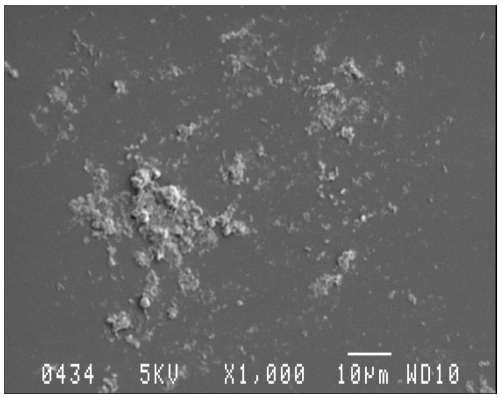
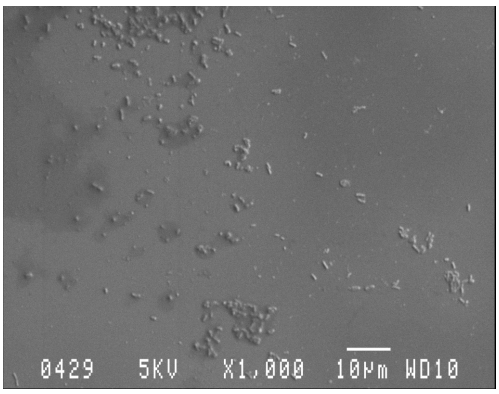
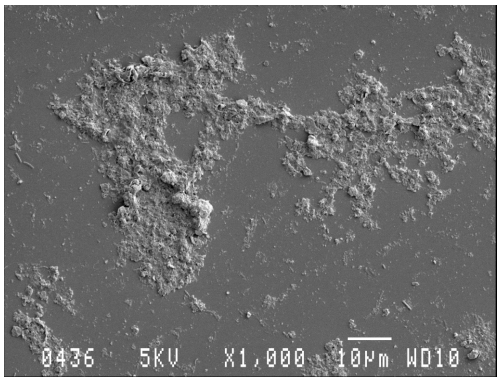


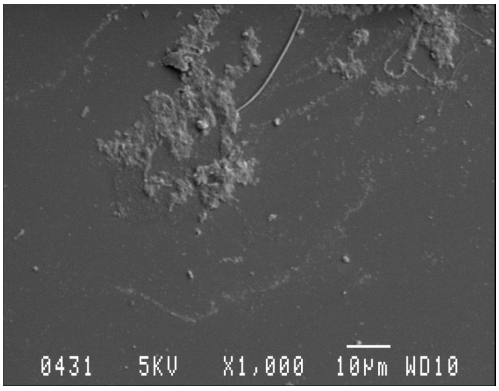
after 24h



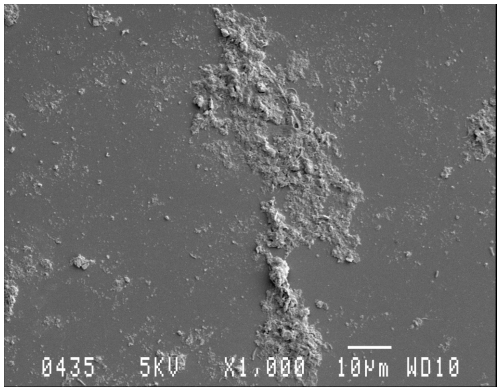
after 3h



after 48h

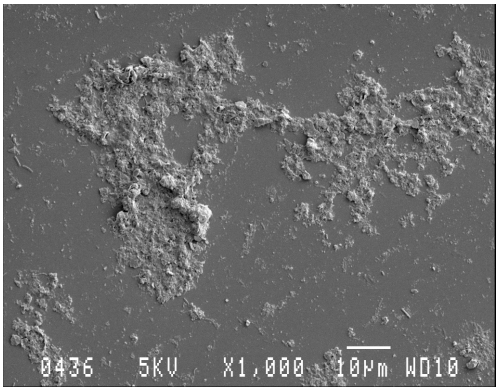


after 6h

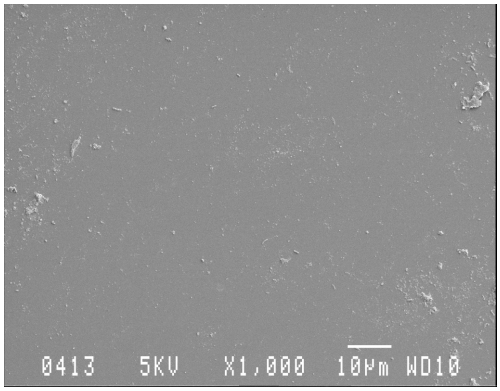


after 48h

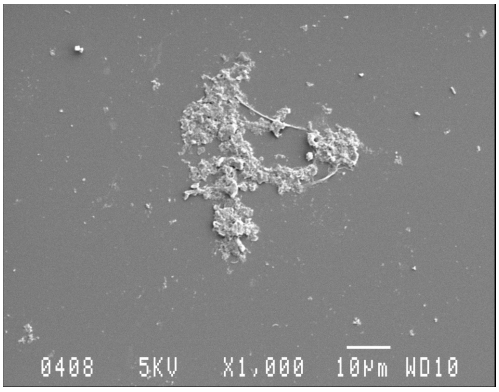
Figure 6.2 SEM micrographs of biofilm growth over 3days



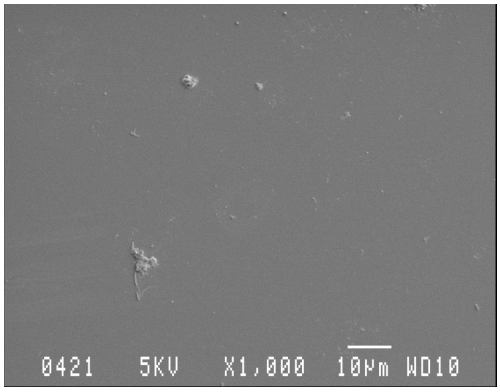
control



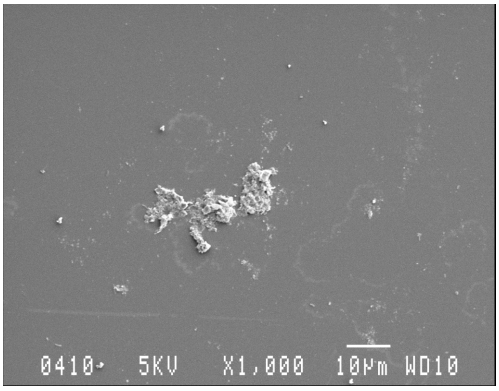
after 30min



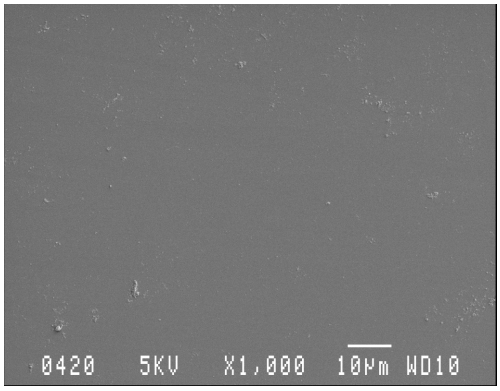
after 2min



after 6h



after 30min



after 24h

Figure 6.3 SEM micrographs of biofilm after addition of chlorine dioxide

compared to the control. After 24h of exposure, most of the bacterial cells were removed (Fig 6.3). The treatment effectively removed the established biofilm and inhibited the formation of new biofilm on the slide. Exposure to ClO_2 changed the surface structure of the biofilm.

6.4 CONCLUSIONS

- The biofilm was effectively killed and removed using RTU ClO_2 at 80ppm.
- ClO_2, whether gaseous or liquid, is a broad-spectrum disinfectant.

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CHAPTER 7

SDS PAGE ANALYSIS OF TOTAL CELL PROTEINS OF BACTERIA TREATED WITH ELECTROCHEMICALLY ACTIVATED WATER

Abstract

Bacteria have varying degrees of susceptibility to disinfectants. There are two major mechanisms of resistance, namely intrinsic and acquired. Bacterial resistance has serious implications in many applications such as drinking water systems. In the present work the possibility of bacterial resistance to electrochemically-activated water (ECA) was examined, and the protein profiles of the treated and the untreated cells were compared using SDS-PAGE. Bacterial cells were exposed to different concentrations of NaCl anolyte and non-halide anolyte, at different time intervals at room temperature. Cells exposed to 10% NaCl anolyte were immediately killed, whereas cell numbers exposed to the non-halide anolyte at this concentration were reduced after 6h. The 1:100 anolyte dilutions did not completely eliminate any of the bacteria used in this study. The protein bands of bacteria treated with 1:10 dilution of NaCl derived anolyte were fewer and faint when compared with those of untreated bacteria. Protein bands from bacteria treated with 1:100 NaCl anolyte dilutions were less than those from the untreated but more than those from bacteria treated with 1:10 dilution. The protein profile of bacteria treated with 1:100 dilution of non-halide derived anolyte indicated more bands in these bacteria than in their untreated counterparts. The 1:10 anolyte dilution destroyed vital proteins for bacterial survival, causing cell death while 1:100 dilution modified protein structures, causing reduction of cell numbers.

Keywords: disinfectants, intrinsic and acquired resistance, electrochemically activated water, anolyte, protein profiles, SDS-PAGE

7.1 INTRODUCTION

Resistance can be defined as the temporary or permanent ability of microorganisms to grow and multiply or to remain viable under conditions that would usually destroy or inhibit other members of the strain (Brözel and Cloete, 1993). The mechanism of resistance can be due to several reasons: Gram-negative bacteria are protected from

biocides by negatively charged lipopolysaccharides in their outer membrane that limit the entry of hydrophobic biocides in the cell. Although Gram-positive bacteria are not well protected, they can modify their cell wall structures and decrease the permeability of the biocides. Bacteria can also acquire resistance through mutation of bacterial genomes and gaining of new genes through horizontal gene transfer to protect them. Exposure to sub-inhibitory concentrations of biocides leads to phenotypic adaptation, resulting in a resistant cell population (Russell, 2004; Russell, 1998).

Resistance has serious economic and environmental implications in many applications like cooling water, papermaking, medical implants, drinking-water distribution, secondary oil recovery, metal working and food processing (Cloete, 2003).

Electrochemical activation (ECA) is based on the generation of activated solutions featuring extra-ordinary physico-chemical and catalytic activity, using special electrochemical systems. The main material used is ordinary, mineral, natural, tap or potable water to which a small amount of various salts, sodium chloride or sodium bicarbonate is added. Water is passed through a special electrochemical cell or cells, consisting of positive electrode (anode) and a negative electrode (cathode). The molecules of water in the anolyte and the catholyte acquire special properties that cannot be reproduced by other means. This electrochemical treatment results in the creation of anolyte and catholyte solutions whose pH, oxidation-reduction potential (ORP) and other physico-chemical properties lie outside of the range which can be achieved by conventional chemical means. ECA solutions (anolyte and catholyte) are clear and colourless aqueous solutions with a faint clean smell of sterilants and disinfectants. The ECA devices are mostly used in potable water systems. Anolytes are used for disinfection and sterilization, while catholytes are used for life support and enhancement and to modify viscosity and surface activity. ECA is less toxic, less volatile, easier to handle, compatible with other water treatment chemicals and effective against biofilms and generates no by-products (http://www.cdtwater.co/mobile.php).

Bacteria can be exposed to a wide range of stresses. Among the stress conditions to which these organisms may be subjected are starvation, excessive heat or cold, high

levels of metal ions, oxidative compounds or other potentially lethal chemicals or extreme pH (Rowbury and Goodson, 1999). Oxidative stress can come from both endogenous and exogenous sources, and is ubiquitous to all aerobic organisms (Shackelford *et al*., 2000). The oxidative attack on proteins may lead to amino acid modification, fragmentation, and loss of secondary structure. Therefore the affected proteins expose hydrophobic residues, favouring aggregation due to hydrophobic interactions and cross-linking reactions (Janig *et al.,* 2005). Stress factors may affect several biochemical pathways and their coordinated behaviour differently. Reactive oxygen species (ROS) are continuously produced during stress, due to various metabolic activities and the production of these increases along with the activation of various defence genes that may include ROS scavenging and stress proteins (Aertsen and Michiels, 2004; Kochhar and Kochhar, 2005).

Proteins can be denatured by a variety of agents such as urea and guanidinium hydrochloride as well as strong positive or negative ionic detergents. Sodium dodecyl sulphates are the negatively charged detergents that are usually used in connection with polyacrylamide gels to denature and separate proteins. SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis) is a powerful tool to dissociate proteins into individual chains and separate them according to their molecular weight. Samples to be run are first boiled for 5min, in the sample buffer containing β-mercaptoethanol and SDS. The β-mercaptoethanol reduces any disulfide bridges present that hold together the protein tertiary structure. SDS binds strongly to and denatures the protein. Each protein in the mixture is therefore fully denatured by this treatment and opens up into a rod-shaped structure with a series of negatively charged SDS molecules along the polypeptide (Walker, 2002).

The advantages of SDS-PAGE can be summarized as follows:

- It separates proteins strictly according to a single molecular parameter and molecular size.
- The technique is inexpensive and easy to set up and perform.
- The resolution is generally very high.
- The separation is fast.

- The method is applicable to most separation problems, as SDS solubilizes most existing proteins (Jason and Rydén, 1998).

SDS-PAGE is therefore an ideal technique to use for demonstrating antimicrobial affectivity and has previously been used to study resistance mechanisms in bacteria (Brözel and Cloete, 1993).

The objectives of this study were:

- To determine the disinfectant capability of the anolyte against selected bacterial cultures.
- To compare protein profiles of the treated and untreated bacterial cells using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) in order to determine the effect of anolyte on cell proteins.

7.2 MATERIALS AND METHODS

7.2.1 Cultures

Pseudomonas aeruginosa, *Staphylococcus aureus, Bacillus subtilis* (spore-former) and *Escherichia coli* (the most pathogenic bacterium) were used in this study. Radical Waters Ltd, SA, kindly donated these cultures. Cultures were maintained on Nutrient Agar plates and sub-cultured after every two weeks. Gram staining was used to check purity. Sodium chloride (NaCl) labelled (AS1) and non-halide (labelled AS2) derived anolytes were supplied by Radical Waters Ltd. These were stored at 4˚C and used within 24h of production.

7.2.2 Properties of anolyte solutions

The ORP, EC, temperature and pH were measured before the solutions were used. A Waterproof ORPScan (Double Junction) with replaceable Double Juction Electrode and 1mV Resolution was used to measure ORP of the anolyte solutions. Electrical conductivity and temperature were measured using Waterproof ECScan with Replaceable Electrode, Temperature Display and 0.01mS resolution. In order to measure pH, a Waterproof pHScan 2 tester was used.

7.2.3 Determination of minimum inhibitory concentration (MIC)

Cultures were grown on Nutrient agar (NA) plates and incubated at 37ºC for 24 h. An overnight culture on NA plate was suspended with freshly prepared ¼ strength Ringer's solution (Merck). 1ml of each bacterial suspension was added to four different tubes, each containing a different concentration of anolyte (neat, 1:10, 1:100 and 1:1 000 (anolyte: Ringer's solution)). The fifth tube was prepared as control containing 1ml of the bacterial suspension and 9ml of ¼ strength Ringer's solution instead of the anolyte. All the dilutions were prepared in fresh Ringer's solution. The tubes were vortexed after addition of the culture and then 100µl was taken from each tube as 0h sample. All the test tubes were incubated at room temperature for 6h. At the end of 6h another 100µl sample was taken. The 100µl aliquots were serially diluted (from 10^{-1} to 10^{-6}) in ¼ strength Ringer's solution and plated out on NA plates in duplicates. The plates were incubated at 37ºC for 24h. The lowest concentration of bacteriocide showing the absence of growth was taken to be the MIC.

7.2.4 Determination of minimum exposure time

A 24h culture on NA plate was suspended in freshly prepared ¼ strength Ringer's solution. 1ml of the bacterial suspension was added to 9ml of the MIC of the anolyte determined above. For the control, 1ml of the bacterial suspension was added to 9ml of ¼ strength Ringer's solution. Tubes were incubated at room temperature for a 6h. 100µl samples were taken from each tube immediately after mixing (0min) and after 5min, 10min, 15min, 20min, 25min, 30min and 60min. Samples were serially diluted. The appropriate dilutions were plated out on NA plates in duplicates by spread plate technique. The plates were incubated at 37°C for 24 h. The plates with the lowest cfu/ml were taken to be the minimum exposure time.

7.2.5 SDS-PAGE

7.2.5.1 Sample preparation

Cells were grown on NA plates for 24h at 37ºC and suspended in 20ml of ¼ strength Ringer's solution. The suspensions were transferred into 50ml Falcon tubes and centrifuged for 10min at 10 000rpm using Eppendorf Centrifuge 5804R. The supernatant was discarded, and the pellet was washed four times with 20ml phosphate buffer of pH 6.8 (112.5ml of 0.2M $NaH_2PO_4.2H_2O$ + 137.5ml of 0.2M $Na_2HPO_4.12H_2O$). The pellet was resuspended in 1ml of phosphate buffer and then

transferred to a preweighed Eppendorf tube. Cells were centrifuged for 11min at 10 000rpm and the supernatants were discarded. The mass of the pellet was determined by weighing the tubes. 100µl of 20% SDS were mixed with 900µl of STB (sample treatment buffer) for use with proteins. 100µl of SDS-STB solution was added to the pellet and heated for 3min at 96˚C. Cells were lysed by sonification for about 5s using 4710 Series Ultrasonic Cole Palmer Homogenizer with an output (40 watt) using 15 pulses. Another 100µl of SDS-STB mixture was added and cells were centrifuged for 11min at 10 000rpm. The supernatant was transferred to the sterile Eppendorf tube and stored according to Ehlers (1997).

7.2.5.2 Protein analysis

SDS-PAGE was performed by the method described by Hames and Rickwood, (1990), modified according to Janson and Rydén (1998). Proteins were separated on gels (1.5mm thick and 125 mm long), which were run in a Hoefer SE600 dual cooled vertical slab unit. The separation gel (12%, 1.5M Tris-HCl pH 8.66 with conductivity of 17.5mS) and the stacking gel (5% 0.5M Tris-HCl pH 6.68 with conductivity 28-33.5mS) were prepared from monomer solution containing 29.2% (m/V) acrylamide (BDH Electran) and 0.8% (m/V) N^1-N^1-bismethyleneacrylamide (BDH Electran). After pouring the separation gel, it was overlayed with Butan-1-ol. The separation gel was allowed to polymerize for 30min. After polymerization, Butan-1-ol was removed, and the gel was washed several times with distilled H_2O before addition of stacking gel. The gel was then covered with plastic and the stacking gel was allowed to polymerize overnight. Two microlitres (2µl) of each sample was boiled at 100˚C for 10min before loaded on the gel. Electrophoresis was performed at a constant current of 22mA through the stacking gel (for 1.45h), and at 32mA through the separation gel (for 3.15h) at 20˚C. After electrophoresis, gels were stained in solution containing Coomassie Blue stock solution, methanol and acetic acid in the ratio 1:4:2 for 1h at room temperature. Gels were destained overnight in a solution containing methanol, acetic acid and double distilled H_2O (dd H_2O) in the ratio 5:2:20 at room temperature. Gels with the proteins were scanned using Amersham Pharmacia biotech ImageScanner.

7.3 RESULTS AND DISCUSSION

7.3.1 Properties of anolyte solutions.

The oxidation-reduction potential (ORP), pH, electrical conductivity (EC) and temperature of the anolyte solutions were found to be as tabulated (Table 7.1 and 7.2).

Table 7.1 The ORP, pH, EC and temperature of the halide (NaCl) anolyte solutions.

Solution	ORP (mV)	pH	EC (mS)	Temp (ºC)
Ringers	234	6.9	5.38	25.4
NaCl anolyte (neat)	902	6.8	3.04	24.0
1:10	700	6.4	4.58	24.4
1:100	593	6.4	4.44	29.5
1:1 000	365	6.6	2.35	25.1

Table 7.2 ORP, pH, EC and temperature of the non-halide anolyte (NHA) solutions.

Solution	ORP (mV)	pH	EC (mS)	Temp (˚C)
Ringer	234	6.9	5.38	25.4
NHA (neat)	785	6.7	1.62	27.3
1:10	548	6.4	4.08	27.0
1:100	535	6.3	5.53	27.0
1:1 000	450	6.2	5.89	24.2

The ORP value of neat halide anolyte was higher than that of neat NHA (Tables 7.1 and 7.2). Dilution of both anolytes caused a decrease in ORP reflecting the decrease in biocidal effect of the solution. Only the neat solution produced an ORP value of more than 700mV. The pH of both anolytes remained very constant at 6.8 to 6.2. EC values of the halide anolyte fluctuated, while that of the nonhalide increased (Tables 7.2 and 7.3). The temperatures of both halides were fluctuating between 29.5˚C and 24˚C.

7.3.2 Minimum inhibitory concentration (MIC) determination

Halide anolyte

All the bacteria exposed to neat NaCl derived anolyte were killed immediately on exposure (Table 7. 3). The 1:10 anolyte dilution also killed cells exposed immediately

except for *B. subtilis* where bacterial cell numbers were reduced from $1.67x10^7$ to $1.42x10^5$ immediately after exposure. However, all *B. subtilis* cells were killed by a 1:10 dilution after 6h. The neat anolyte and 1:10 dilution killed all the bacteria. The 1: 100 dilution reduced the *B. subtilis* cell numbers from $1.67x10^7$ to $1.88x10^4$ after 6h, while exposure to 1:1 000 did not have any biocidal effect on these cells. Exposure of *P. aeruginosa*, *E. coli* and *S. aureus* to 1:100 and 1:1 000 did not show any biocidal effect on the cell numbers or in some cases reduction of numbers was insignificant (Table 7.3).

Non-halide anolyte (NHA)

Exposure to neat NHA resulted in a 100% kill of all the bacteria (Table 7.4). Exposure of *B. subtilis* and *E.coli* cells to 1:10 dilutions of NHA did not have any biocidal effect. Cells of *P. aeruginosa* and *S. aureus* were reduced from $>3.00x10^7$ to $2.04x10^6$ and $7.70x10^6$cfu/ml, respectively, after 6h exposure to 1:10 NHA anolyte. (Table 7.4). The neat NHA was effective as a biocide, however a 1:10 dilution of NHA was not considered biocidal. (Table 7.4). The 1:100 dilution did not have any biocidal effect on all the bacterial cells used in this experiment (Table 7.4).

Table 7.3 Effect of various dilutions of NaCl derived anolyte on bacterial cultures

Species	Control (ORP=234mV)		Neat (ORP=902mV)		1:10 (ORP=700mV)		1:100 (ORP=593mV)		1:1 000 (ORP=365mV)	
	0h	6h	0h	6h	0h	6h	0h	6h	0h	6h
	Bacterial counts (cfu/ml)									
B. subtilis	$1.67x10^7$	$2.14x10^7$	0	0	$1.42x10^5$	0	$2.40x10^6$	$1.88x10^4$	$2.50x10^6$	$>3.00x10^8$
P.aeruginosa	$>3.00x10^8$	$1.68x10^8$	0	0	0	0	$1.85x10^8$	$8.40x10^8$	$8.40x10^8$	$9.51x10^8$
S. aureus	$>3.00x10^8$	$>3.00x10^8$	0	0	0	0	$6.40x10^6$	$2.14x10^8$	$6.40x10^8$	$6.60x10^8$
E. coli	$>3.00x10^8$	$>3.00x10^8$	0	0	0	0	$2.00x10^8$	$1.80x10^8$	$1.80x10^8$	$>3.00x10^8$

cfu = colony forming units, h = hours

NaCl derived anolyte with a redox potential of above 700mV resulted in a 100% kill of all the test organisms after 6h (Table 7.3); as the ORP decreased (upon dilution) the biocidal effect decreased (Table7.3). This suggests that the biocidal effect of the anolyte was directly related to the ORP value.

Table 7.4 Effect of various dilutions of non-halide (NHA) on bacterial cultures

Species	Control (ORP=234mV)		Neat (ORP=785mV)		1:10 (ORP=548mV)		1:100 (ORP=535mV)	
	0h	6h	0h	6h	0h	6h	0h	6h
	Bacterial counts (cfu/ml)							
B. subtilis	$>3.00x10^7$	$>3.00x10^7$	0	0	$>3.00x10^7$	$>3.00x10^7$	$>3.00x10^7$	$>3.00x10^7$
P.aeruginosa	$>3.00x10^7$	$>3.00x10^7$	0	0	$>3.00x10^7$	$2.04x10^6$	$>3.00x10^7$	$>3.00x10^7$
S. aureus	$>3.00x10^7$	$>3.00x10^7$	0	0	$>3.00x10^7$	$7.70x10^6$	$>3.00x10^7$	$>3.00x10^7$
E. coli	$>3.00x10^7$	$>3.00x10^7$	0	0	$>3.00x10^7$	$>3.00x10^7$	$>3.00x10^7$	$>3.00x10^7$

cfu = colony forming units, h = hours

7.3.3 Determination of minimum exposure time

P. aeruginosa, *E. coli* and *S. aureus*, samples taken immediately after addition of the MIC indicated that the anolyte was very effective for killing the bacteria with no cells growing on NA plates (Table 7.5). The cells of *B. subtilis* were reduced from $>3.00x10^8$ to $6.00x10^2$ after 30min of exposure and then to zero after 60min exposure. Thus *B. subtilis* cells, the most resistant cells, were only eliminated after an extended exposure time of 60min when compared with the other bacteria tested (Table 7.5).

Table 7.5 Bacterial counts after varying exposure times to 1:10 NaCl derived anolyte solution (ORP 700mV)

Organism	Time of Exposure (min)							
	0	5	10	15	20	25	30	60
	Bacterial counts (cfu/ml)							
B. subtilis	$>3.00x10^8$	$>3.00x10^6$	$3.20x10^5$	$1.90x10^5$	$2.25x10^4$	$2.75x10^3$	$6.00x10^2$	0
P. aeruginosa	$>3.00x10^8$	0	0	0	0	0	0	0
S. aureus	$>3.00x10^8$	0	0	0	0	0	0	0
E. coli	$>3.00x10^8$	0	0	0	0	0	0	0

cfu = colony forming units

The above results support previous work indicating that the change of the molecular state of water from a stable to a metastable state was important in the activation process leading to the biocidal effect of the solution

(http://www.wcp.net/column.cfm? T=W&ID=1823). The ORP results reflect the activation process as demonstrated by the biocidal effect wherever the ORP exceeded 700mV. Dilution of the anolyte resulted in a decreased biocidal effect and ORP.

7.3.4 Protein analysis of bacterial cells after anolyte treatment

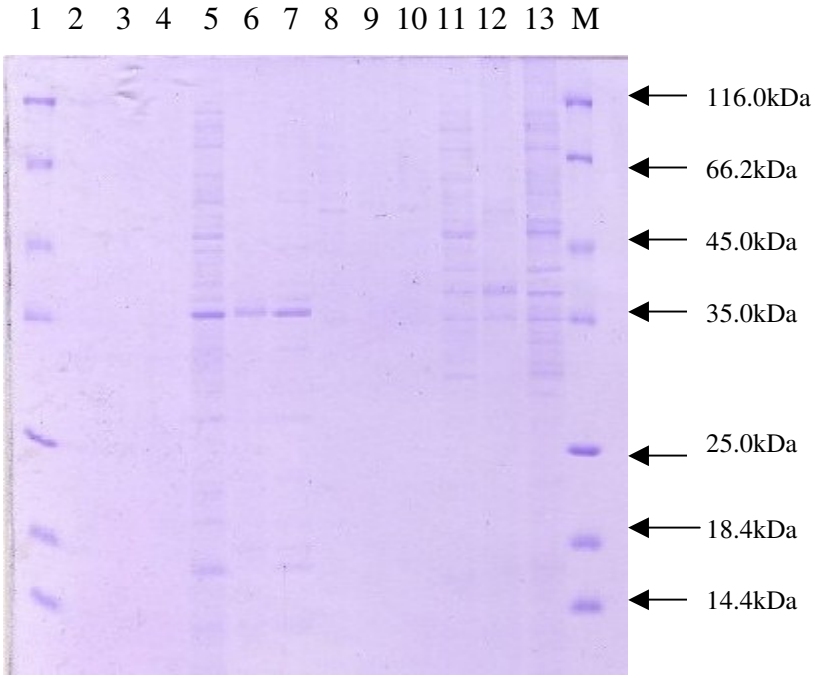

Figure 7.1 SDS-PAGE analysis of whole protein extracts from bacterial isolates treated and untreated with NaCl derived anolyte (AS1). Lanes 2, 5, 8 and 11 are untreated cells of *B. subtilis*, *P. aeruginosa*, *S. aureus* and *E. coli*, respectively. Lanes 3, 6, 9 and 12 are bacterial proteins from *B. subtilis*, *P. aeruginosa*, *S. aureus* and *E. coli,* respectively, treated with 1:10 dilution of NaCl derived anolyte whereas lanes 4, 7, 10 and 13 are bacterial proteins of *B. subtilis*, *P. aeruginosa*, *S. aureus* and *E. coli,* respectively treated with 1:100 dilutions. Lanes 1 and M contain molecular weight marker of the sizes indicated.

The proteins of treated and untreated *B. subtilis* were not detected by SDS-PAGE (Fig 7.1, lanes 2, 3 and 4). It has previously been found that the proteins with a high concentration of carbohydrate or proline content are notorious for migration in SDS with unpredictable relative mobility, and fail to stain with standard methods (htt://www.ruf.rice.edu/~bioslabs/studies/sds-page/gellab3.html). If the protein concentration is too low, they are also not visible on SDS-PAGE, and therefore an

alternative extraction method is necessary (http://www.mirador.ca/corporate 2/pdf/TroubleshootingWeb_05Mar2004.pdf). These were probably the reasons why *B. subtilis* (Fig 7.1 lanes 2, 3 and 4) did not show any protein bands.

The SDS-PAGE protein profile for untreated *P. aeruginosa* cells showed presence of several/many proteins in this cell evidenced by many protein bands on the gel (Fig 7.1, lane 5) as compared to cells treated with 1:10 and 1:100 NaCl derived anolyte (Fig, 7.1, lanes 6 and 7 respectively). Only one protein band from cells treated with 1:10 anolyte dilution was visible on the gel (Fig 7.1, lane 6) whereas there were several protein bands in untreated, but fewer than those from cells treated with 1:100 anolyte dilution, with band intensities lower than bands from untreated cells (Fig 7.1, lanes 6 and 7). The observations made indicated that 1:10 anolyte dilution destroyed all the vital proteins for bacterial cell survival, as there was no cell growth. 1:100 dilution destroyed some of the proteins and only reduced the number of the vital proteins (protein bands with lower intensities).

For untreated *S. aureus cells* only one faint protein band was visible on the gel (Fig 7.1, lane 8). No proteins were detected from *S.aureus* treated with 1:10 and 1:100 NaCl anolyte dilutions (Fig 7.1 1anes 9 and 10, respectively). It has been observed that the 1:10 NaCl derived anolyte killed the cells of *S. aureus,* as no cell growth was present when cells treated with this concentration of anolyte were plated out (Table 7.3). The 1:100 dilution reduced the number of cells possibly by fragmenting their proteins into small peptides or by unfolding tertiary structures. The absence of protein bands from bacteria treated with 1:100 dilution might be due to presence of a high carbohydrates or proline concentration (htt://www.ruf.rice.edu/~bioslabs/studies/sds-page/gellab3.html) in the remaining protein or the protein concentrations were too low, requiring an alternative extraction method (http://www.mirador.ca/corporate2/pdf/TroubleshootingWeb_05Mar2004.pdf).

Observations made from *E. coli*, were similar to those of *S. aureus*, with no growth for cells treated with 1:10 and a reduced number of cells treated with 1:100 NaCl derived anolyte. The protein bands from untreated *E. coli* cells were fewer than those from cells treated with 1:100 (Fig.7.1, lane 11 untreated and 13 treated with 1:100). Protein bands from *E. coli* cells treated with 1:10 dilution were fewer than those of

untreated cells. Observations made indicated that 1:10 dilution destroyed all the vital proteins for bacterial survival with no cell growth (Table 7.3). The 1:100 dilution reduced number of cells. The protein of the *E. coli* cells exposed to a 1:100 dilution of anolyte were fragmented into small peptides that were detected in the treated cells (Fig 7.1 lane 13). This resulted in a longer exposure time required to kill the cells.

Unfavourable environmental conditions induce a stress response in bacteria. The level of stress response varies with the type of organism and the type of environment (Kochhar and Kochhar, 2005). Exposure of bacterial cells to NaCl derived anolyte resulted in a decrease in the number of proteins of *P. aeruginosa* and *S. aureus*. The decreases in protein numbers were brought about by oxidative stress. The oxidative stress (caused by the high ORP of the anolyte) on proteins caused amino acid modification and the loss of secondary structures, where some of the proteins were destroyed causing bacterial death. However, the exposure of *E. coli* to 1:10 and 1:100 NaCl derived anolyte, caused the fragmentation of the native protein to small peptides, resulting in a longer exposure time to cause death of the bacteria.

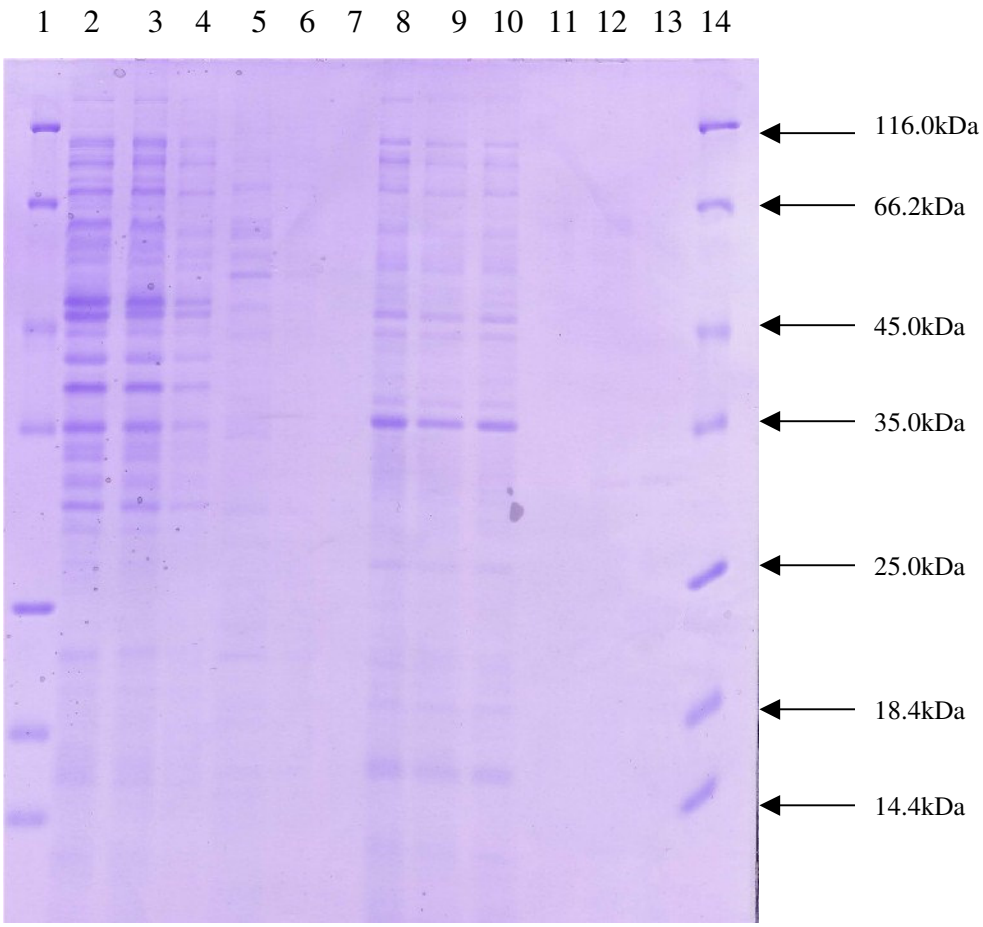

Figure 7.2 SDS-PAGE analysis of whole protein extracts from bacterial isolates treated and untreated with non-halide anolyte (NHA). Lanes 4, 7, 10 and 13 are untreated proteins of *E. coli*, *S. aureus*, *P. aeruginosa* and *B. subtilis* respectively. Lanes 3, 6, 9 and 12 are proteins *E. coli*, *S. aureus*, *P. aeruginosa* and *B. subtilis* treated with 1:10 dilution of NHA respectively. Lanes 2, 5, 8 and 11 are proteins of *E. coli*, *S. aureus*, *P. aeruginosa* and *B. subtilis* treated with 1:100 dilution of NHA respectively. Lanes 1 and 14 contain molecular weight marker of the sizes indicated.

Fewer proteins were detected in untreated cells of *E.coli* (Fig 7.2, lane 4) as compared to the treated cells (Fig.7.2, lanes 2 and 3). The non-halide anolyte resulted in more protein bands detected in *E.coli* cells treated with both 1:100 and 1:10 dilutions (Fig 7.2, lanes 2 and 3 respectively). Extra protein bands that were present in treated bacteria resulted from fragmentation of the original protein into small peptides by the anolyte.

Proteins from the untreated *S. aureus* and the one treated with 1:10 dilutions were not detected (Fig 7.2, lanes 7 and 6 respectively). Treatment of *S. aureus* with 1:100 dilution of non-halide derived anolyte resulted in many faint fragments (Fig 7.2, lane 5). The observation was similar with that of the cells treated with NaCl derived anolyte (Fig 7.1).

The protein profiles of the *P. aeruginosa* were the same in both the untreated and the treated bacteria (Fig 7.2 lanes 8, 9 and 10). Though the profiles were similar, the band intensities of cells treated and untreated differed, with the treated samples showing fainter bands, indicating potential elimination and/or fragmentation of the proteins.

There were no protein bands visible on the gel for *B. subtilis* both treated and untreated with non-halide derived anolyte (Fig 7.2, lanes 11, 12 and 13) by using SDS-PAGE. This result was similar to that observed when *B.subtilis* cells were treated with NaCl derived anolyte (Fig 7.1, lanes 2, 3 and 4).

Analysis of extracted proteins on SDS-PAGE indicated that the dilute NHA anolyte did not destroy the bacteria. However, it was observed that NHA caused oxidative stress to bacterial proteins. This stress caused amino acid modification, fragmentation and loss of secondary structures. In our study, this was indicated by the increase in number of proteins detected in treated cells as compared to untreated cells. The affected proteins exposed hydrophobic residues, favouring aggregation due to hydrophobic interactions and cross-linking reactions. Under stress conditions, native proteins unfold to adopt a misfolded intermediate state. The misfolded proteins intermediates in the cytoplasm can be refolded, and the ubiquitinated proteins can be degraded to small peptides and amino acids. (Janig *et al*., 2005; Li *et al*., 2005). In this experiment, the results obtained indicated that the native proteins in untreated cells were misfolded during treatment and degraded to small peptides. Oxidative stress modified proteins may contain two or three oxidized amino acids that probably indicate that most of the amino acids from an oxidized and degraded protein were reutilized for protein synthesis. Therefore during oxidative stress, many stress proteins synthesised as damage replacements, are likely to contain a high percentage of recycled amino acids, giving rise to altered pattern from the native protein (Davies, 2001). These were clearly indicated by the proteins of *P. aeruginosa*, *S. aureus* and *E.*

coli after treatment with both (1:10 and 1:100) dilutions of non-halide anolyte. Zinkevich *et al.* (2000) discovered that when *E. coli* was exposed to neat Sterilox for 5min, its DNA, nucleic acids and proteins were completely destroyed.

7.4 CONCLUSIONS

- Generally, anolyte treatment affected bacterial proteins causing cell death.
- Anolyte caused oxidative stress in bacteria leading to amino acid modification, loss of secondary structures and fragmentation to small peptides. In the case of halide the damage was more serious and resulted in bacterial cell death.
- The non-halide anolyte was only effective when undiluted.
- The neat and the 1:10 dilution of NaCl derived anolyte was effective in killing isolated bacteria immediately upon exposure.

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CHAPTER 8

General conclusions

- Decrease in light reflectance was an indication of an increase in biofilm thickness. Light reflectance changed with biofilm thickness and the thicker the biofilm, the lesser the light reflectance.

- Dilute anolyte (1:100) did not have any biocidal effect on the bacterial tested. Less dilute (1:10) and concentrated (neat) anolyte removed biofilm. Anolyte derived from NaCl was effectively at 1:10 dilution within 6h of bacterial exposure. The aged anolyte stored at ambient temperatures was biocidal on bacterial cells and effective for biofilm removal.

- The developed Rotoscope was very sensitive to a slightest change in biofilm characteristics. In addition, the Rotoscope met the requirements for an on-line, real-time and non-destructive biofilm monitoring. Further improvement of this system (Rotoscope) will include the addition of a data logger that will allow the operator to link the data between the monitor and a computer that will automate biofouling control programmes.

- Sodium nitrite had a very limited or no effect on aerobic bacteria mostly encountered in biofilms.

- Liquid ready-to-use chlorine dioxide was effective in killing bacteria and removing biofilm. The MIC for liquid ClO_2 was found to be 80ppm, which was higher than values reported for gaseous ClO_2.

- SDS-PAGE studies indicated that exposure of bacterial cells to anolyte caused oxidative stress to bacterial proteins. During the oxidative stress, the original proteins were fragmented into small peptides and amino acids, causing death of the bacteria at high concentrations of anolyte.

Appendix

Protein concentration determination

5µl of each protein extract was blotted on filterpaper by using a micropipette
The filterpaper was stained with Coomassie Brilliant Blue R solution for 10min
The filter was destained with destaining solution for 2min and left to dry
The intensity of the colour of each blot was used as an indication of the protein concentration – the darker the blot, the higher the concentration.

Preparation of a SDS-PAGE gel for a vertical gel apparatus

Preparation of 5% Stacking gel

H_2O (double distilled)	11.4ml
Stacking gel buffer	5ml
Acrylamide-Bis Monomer solution	3.4ml
10% SDS	200µl
10% $(NH_4)_2S_2O_8$	100µl

Preparation of separation gel at 12%
After assembling the glass plates in the casting stand, the following was mixed in a clean flask with a magnetic stirrer

H_2O (double distilled)	26.8ml
Separation gel buffer	20ml
Acrylamide-Bis Monomer solution	32.0 ml
10% SDS	0.8ml
10% $(NH_4)_2S_2O_8$	0.28ml
TEMED	40µl

Sample Treatment Buffer (STB)

- Weigh 3.75g Tris in a beaker
- Add 200 – 250ml ddH_2O and stir until Tris is completely dissolved
- Add 25ml mercapto-ethanol and 50ml glycerol, stir until mixed
- Measure 17.25ml of 1.75 N HCl into a beaker
- Add HCl to the solution whilst measuring pH and conductivity
- Continue adding HCL until pH = 6.8

Conductivity = 3.87mS/cm

Store in the freezer (-20ºC)

Upper Buffer (prepared fresh)

Tris	1.5g
Glycine (UniVar)	7.2g
SDS 10%	5ml
dH_2O	500ml

Tris-glycine buffer (Tank/Running buffer)

Tris	12g
Glycine (UniVar)	57.5g
SDS (BDH)	4g
H_2O (double distilled)	4 000ml

Monomer solution

Acrylamide (BDH)	29.2g
NN'-Methylenebisacrylamide (BDH)	0.8g
H_2O (double distilled)	100ml

Store at 4ºC

Stain stock solution

Coomassie blue (UniLab)	10g
ddH_2O	500ml

Staining solution

Stain stock solution	125ml
Methanol (AR)	500ml
Acetic acid (	100ml
dH_2O up to	1 000ml

Store at room temperature

Destaining solution

Methanol (AR)	500ml

Acetic acid	100ml
dH_2O up to	2 000ml

Store at room temperature

10% Ammonium peroxodisulphate $(NH_4)_2S_2O_8$

Ammonium peroxodisulphate $[(NH_4)_2S_2O_8]$	0.103g
H_2O (double distilled)	1ml

Cover with foil and store at 4ºC