Electrochemically activated water as an environmentally safe disinfectant

By

MAPITSI SILVESTER THANTSHA

Submitted in partial fulfilment of the requirements for the degree

Master of Science (Microbiology)

in the

Department of Microbiology and Plant Pathology

Faculty of Natural and Agricultural Sciences

University of Pretoria

Pretoria, South Africa

July 2002

I declare that the thesis, which I hereby submit for the degree M. Sc (Microbiology) at the University of Pretoria, has not been previously submitted by me for a degree at another university

Signed :_____this_____day of_____2002

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude and appreciation to the following people and institutions who contributed to the completion of this study:

- Professor T. E. Cloete, my supervisor and Head of Department:Microbiology and Plant Pathology, of the University of Pretoria, for his support, advice and encouragement.

- The University of Pretoria for Financial Assistance.

- The company Radical Waters for supplying ECA solutions used in the study

- The Department of Microscopy and Microanalysis of the University of Pretoria for helping with all the microscopy work.

- All my laboratory colleagues for creating a good working environment.

- My friends Ivy and Mandla for their support and for proofreading of my thesis.

- My mother, brothers and sisters for always being by my side, always believing in me, praying for me and for their encouragement and love.

- Above all, the almighty God for blessing me and giving me all the strength I needed to make this study a success.

TABLE OF CONTENTS

❖ **Chapter 4: The effect of sodium chloride and sodium bicarbonate derived anolytes, and sodium hypochlorite on biofilms**

❖ **Chapter 5: Handwash trial using anolyte solutions**

❖ **Chapter 6: Disinfection of dairy milking parlour using anolyte**

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ADP: Adenosine diphosphate

AOC: Assimilable organic carbon

ATP: Adenosine triphosphate

BFR: Biofilm formation rate

BIT: Benzisothiazolone

CMIT: 5-chloro-N-methylisothiazolone

CRA: Chlorine releasing agents

DNA: Deoxy ribonucleic acid

ECA: Electro chemical activation

EDIC: Episcopic differential interference contrast

EIS: Electrochemical impedance spectroscopy

EN: Electrochemical noise

EPS: extracellular polymeric substances

ER: Electrical resistance

FeS: Ferrous sulphate

HOCL: Hypochlorite

H_2S: Hydrogen sulphate

IOS: Intelligent optical systems

LPR: Linear polarisation resistance

LPS: Lipopolysaccaharides

LSM: Laser scanning microscopy

MBT: Methylene bis-thiocyanate

MIC: Microbially induced corrosion

MIT:N-menthylisothiazolone

MRSA: Methicilin resistant *Staphylococcus aureus*

NaCl : Sodium chloride

NIAID: National Institute of Allergy and Infectious Diseases

ORP: Oxidation-reduction potential

PAS: Photoaucostic spectroscopy

PHMB: Polyhexamethyne biguanines

PLFA: Phospholipid fatty acid

QCM-D: Quartz crystal microbalance with dissipation

rRNA: r- Ribonucleic acid

SABS: South African Bureau of Standards

SAIMR: South African Institute of Medical Research

SCLM: Scanning Confocal Laser Microscopy

SRB: Sulphate Reducing Bacteria

TEM: Transmission Electron Microscopy

TPC: Total plate count

ELECTROCHEMICALLY ACTIVATED WATER AS AN ENVIRONMENTALLY SAFE DISINFECTANT

By

Thantsha M. S.

Promoter : Prof. T. E. Cloete
Department: Microbiology and Plant Pathology
Degree : M. Sc (Microbiology)

SUMMARY

An increase in the number of bacteria resistant to most of the antibiotics or biocides in common use is a problem faced by industries and the community at large. More bacteria are resistant to moderate levels of biocides, with the bacteria in biofilms being the most difficult to control. High levels of biocides are used and this has detrimental effects on the environment, as biocides are toxic to humans, being carcinogenic, corrosive and producing intolerable odours. Electrochemical activation technology provides an alternative way of controlling microorganisms. ECA water was shown in other countries to have antimicrobial properties. Anolyte, the positively charged solution is benign to fumes and corrosion caused by other biocides. Since anolyte is eco-friendly and present no problems to the environment, it provides a good alternative for controlling microorganisms instead of chemical control. The minimum inhibitory concentration (MIC) of anolyte and its antimicrobial properties against different microorganisms in suspension was evaluated. The 1:10 and neat anolyte gave a 100% kill of all organisms tested while 1:20 dilution gave variable killing percentages ranging from 31% to 100%. Minimum inhibitory concentration was found to be 20% for most Gram positives and 50% for most Gram negatives. Anolyte did have some antimicrobial properties with MIC differing amongst different organisms. Biofilm control using different concentrations of anolyte and sodium hypochlorite was evaluated. Neat and 1:10 anolyte removed biofilm while 1:100 did not have effect on biofilm. The 100 and 300 ppm sodium hypochlorite were effective

in removing the biofilm while 10, 25 and 50 ppm could not remove it. Hand wash trials and hospital disinfection using anolyte resulted in a decrease in the number of cfu/25cm^3 after treatment. Effective disinfection of hands and hospital equipment was achieved. Different surfaces in a milking parlour were treated with anolyte to test its suitability to disinfect a milking parlour. High numbers of microorganisms and spreaders were observed from the plates before the surfaces were cleaned with anolyte. All spreaders were identified as sporeformers. Results were generally better when anolyte was used as a disinfectant, being able to eliminate spreaders as well. Treatment of chicken carcasses with anolyte to evaluate its effect on their shelf life was also tested. The number of colony forming units on chicken carcasses decreased after treatment of the carcasses with anolyte. Anolyte is therefore a naturally safe disinfectant that could be used in most fields including water distribution systems and industries for biofilm control, hospitals for minimising cross infection, food industries for increasing quality and shelf life of food and also in households for washing hands.

ELEKTROCHEMIES-GEAKTIVEERDE WATER AS 'N OMGEWINGS-VRIENDELIKE ONTSMETTINGSMIDDEL

Deur

Thantsha M. S.

Promotor : Prof. T. E. Cloete

Departement: Mikrobiologie en Plantpatologie

Graad : M. Sc (Mikrobiologie)

OPSOMMING

'n Toename in die hoeveelheid bakterieë wat weerstandbiedend is teen meeste van die antibiotika's en biosiede wat algemeen gebruik word, is 'n probleem wat die industrieë en gemeenskap in die gesig staar. Al hoe meer bakterieë word weerstandbiedend teen gematigde hoeveelhede van die biosiede en bakterieë wat in biofilms voorkom is die moeilikste om te beheer. Groot hoeveelhede van die biosiede word gebruik en dit het 'n skadelike invloed op die omgewing. Die biosiede is giftig vir mense omdat hulle karsinogenies en korrosief is en ook onaangename dampe of reuke produseer. Elektrochemiese aktiveringstegnologie (ECA) verskaf 'n alternatiewe metode vir die beheer van mikroörganismes. Die antimikrobiese eienskappe van ECA water is reeds in ander lande bewys. Anolyte een van die produkte van ECA, is 'n positief gelaaide oplossing, is nie korrosief nie en produseer ook nie onaangename dampe nie. Aangesien anolyte omgewings-vriendelik is verskaf dit 'n alternatief vir die beheer van mikroörganismes in plaas van chemiese beheer middels. Die minimum inhibitoriese konsentrasie (MIC) en antimikrobiese eienskappe, teen verskillende mikroörganismes in oplossing, van anolyte is ondersoek. Die 1:10 en gekonsentreerde anolyte het 'n 100% doding van alle organismes wat getoets is gegee, terwyl 'n 1:20 verdunning varieerbare dodings persentasies van 31% tot 100% gegee het. Daar was gevind dat die minimum inhibitoriese konsentrasie vir meeste Gram positiewes was 20% en 50% vir meeste

Gram negatiewes. Anolyte het ant[illegible]gehad met MIC wat verskil tussen verskillende organismes. Die beheer van biofilm deur gebruik te maak van verskillende konsentrasies anolyte en natrium hipochloried is ook ondersoek. Gekonsentreerde en 1:10 anolyte het die biofilm effektief verwyder terwyl 1:100 geen effek op die biofilm gehad het nie. Die 100 en 300 dpm natrium hipochloried was effektief vir die verwydering van die biofilm terwyl, 10, 25 en 50 ppm dit nie kon verwyder nie. Die gebruik van anolyte in handwas-proefnemings en hospitaal ontsmetting het gelei tot 'n afname in die hoeveelheid cfu/25cm^3 na behandeling. Hande en hospitaal apparaat is effektief ontsmet. Verskillende oppervlaktes in 'n melkery is behandel met anolyte om die geskiktheid van anolyte ontsmetting van 'n melkery te toets. Hoë hoeveelhede mikroörganismes en spoorvormers was waargeneem op plate voordat die oppervlaktes met anolyte skoongemaak was. Oor die algemeen was resultate beter wanneer anolyte as ontsmettingsmiddel gebruik was, aangesien dit spoorvormers ook kon elimineer. Hoender karkasse is behandel met anolyte om die effek op hul rakleeftyd te toets. Die hoeveelheid kolonie vormende eenhede op hoender karkasse het afgeneem na behandeling van die karkasse. Anolyte is nie skadelik vir die natuur nie en kan in verskeie toepassings in die samelewing gebruik word insluitend water-verspreidings sisteme en industrieë vir biofilm beheer, hospitale vir minimalisering van kruis-infeksies, voedselindustrie vir verhoging van kwaliteit en verlenging van rakleeftyd van voedsel asook in huishoudings vir die was van hande.

Chapter 1

1.1 INTRODUCTION

A range of bactericidal substances, commonly termed biocides or microbiocides, are available, all of which are claimed by their agents to kill bacteria in aqueous systems quantitatively. However, different bacteria react differently to bactericides, either due to differing cell wall properties (Paulus, 1987), or to other mechanisms of resistance, either inherent or inducible (Heinzel, 1988). Undermining the percentage kill against a standard laboratory pure culture could lead to misleading results (Allsop and Seal, 1986). Bactericides should be evaluated against the organisms which they are expected to kill, i.e. the dominant ones in the system to be treated. The composition of microbial populations in systems varies with the type of water used (Cloete *et al.*, 1989b). A bacterial population structure of South African water-cooling systems was determined by Cloete *et al.* (1989a). Eighteen dominant isolates from this study were used to determine the bactericidal fingerprints of 32 commercially available non-oxidising water treatment bactericides.

Concern over the number of bacteria that are resistant to most antibiotics in common usage has been growing over the years. Not only have strains that are resistant to antibiotics become a problem, but also those that are resistant to moderate levels of chlorine and disinfectants such as cetrimides are on the increase, many of the plasmids coding for resistance in bacteria containing both factors. South African health services have increasingly tight budgets as a result of Provincial Administration cuts. "Stock shrinkage" of disinfectants in the hospital wards and pharmacies exacerbates the problem. Thus staff have to make do with inadequate supplies, especially at the primary health care level. This tends to result in disinfectants being used either as past the in-use expiry date, being overutilized on site (excessive bioloads) and being "watered down" beyond recommended in-use concentrations (Bilgeri *et al.*,1999).

In any developing country, food and water borne diseases pose constant dangers to the population, especially in the more rural areas. The increase in morbidity and mortality resulting from unhygienic food and water leads to a downward spiral of

porvety. Scientists at the Russian Institute for Medical and Scientific Research have developed an alternative method, whereby an electrolytic cell produces a cocktail of powerful radicals, including chlorine dioxide, ozone, hydrogen peroxide, etc., and at the same time effectively stimulating ionic activity increasing the redox potential of the water. These active compounds have a synergistic effect upon each other, providing a more virulent bactericidal effect jointly, than any individual radical on its own. This biocidal activity is one to three hundred times greater than that provided by hypochlorite solutions formed by chlorine and its salts, or in conventional electrolytic sodium hypochloritic cells. Various countries are implementing this new system for sterilization of hospital equipment, floor surfaces, wounds as well as for the disinfection of milking parlours.

Bacterial colonisation of surfaces in an aqueous environment is a basic stratagem for survival in nature as nutrients are more available at the solid - liquid interface (Hoppe, 1984; Lawrence, *et al.*, 1987). The resulting aggregates form microcolonies, which develop into biofilms (McCoy *et al.*, 1981). These biofilms promote corrosion of metals by creating potential differences across surfaces and by harbouring sulphate-reducing bacteria (Iverson, 1987). They also increase fluid frictional resistance (McCoy *et al.*, 1981) and decrease the rate of heat energy transfer. The above phenomena are termed collectively as biofouling (McLeod *et al.*, 1998). As the costs attributable to microbially induced corrosion are high, ca. R400 million in 1988 the South African Industry (Von Holy and Cloete, 1988 ; Bagge *et al.*, 2001), effective control of bacterial numbers in industrial aqueous environments is essential.

Microbial biofilms are problematic in a range of industrial environments where large areas of submerged surfaces are exposed to relatively high nutrient fluxes, providing niches for the formation of copious surface associated growth. Biofilms promote corrosion of ferrous and other metals by the concerted metabollic activity of a number of biofilm associated bacterial types (Cloete *et al.*, 1992; Costerton *et al.*, 1995). MIC is caused by the presence of bacteria in water systems, especially by bacterial biofilms. It is the most obvious conclusion, that such bacterial biofilms must be removed. Five approaches are currently followed: (i) Bacteria are chemically killed by application of bactericidal compounds termed biocides at lethal doses, (ii) biofilms are dispersed by dispersants, (iii) biofilms are removed physically by a variety of

processes, (iv) the biofilm structure is weakened by enzymes or chelants and (v) planktonic bacterial numbers are controlled by ultraviolet light (Cloete and Flemming, 1997).

The objectives of this study were:

- To determine the minimum inhibitory concentration of NaCl derived anolyte and to access the antimicrobial properties of $NaHCO_3$ derived anolyte
- To determine the efficacy of the anolyte and anolyte/catholyte combination for removing biofilms
- To evaluate anolyte as a disinfectant hand washing agent
- To evaluate anolyte as a surface disinfectant for hospital equipment
- To evaluate anolyte as a disinfectant for milking parlours
- To determine the effect of anolyte on the shelf life of chickens

1.2 REFERENCES

Allsop D., and K. J. Seal. 1986. Introduction to biodeterioration. Edward Arnold, London.

Bagge D., M. Hjelm, C. Johansen, and I. Huber. 2001. *Shewanella putrefaciens* adhesion and biofilm formation on food processing surfaces. Appl. Environ. Microbiol. **67:**2319-2325.

Bilgeri Y. R., G. Hinze, and H. J. Koornhof. 1999. Electrochemically activated water as an antimicrobial agent.
Radical/Yvonne/MicrobiologicalAnalysis/publications/publications-Koornhof.doc

Cloete T. E., J. Pressly, and V. S. Brozel. 1989a. A bacterial population structure study of water cooling systems in South Africa. Water SA. **15(1):** 37-42.

Cloete T. E., F. Smith, and P. L. Steyn. 1989b. The use of planktonic bacterial populations in open and closed recirculating water cooling systems for the evaluation of biocides. Int. Biodeterioration. **25:**115-122.

Cloete T. E., V. S. Brözel, and A. Von Holy. 1992. Practical aspects of biofouling control in industrial water systems. Int. Biodeterioration & biodegradation. **29:**299-341.

Cloete T. E., and H-C Flemming. 1997. Monitoring microbial populations in industrial water syatems, p. 20-121. In. Proceedings of Biofouling and its control in Industrial Water Systems-A one -day Management Workshop. University of Pretoria, South Africa.

Costerton J. W., Z. Lewandowski, D. E. Caldwell, D. R. Korber, and H. M. Lappin-Scott. 1995. Microbial biofilms. Annu. Rev. Microbiol. **49:**711-745.

Heinzel M. 1988. The phenomena of resistance to disinfectants and preservatives, p. 52-67. **In: Payne K. R** (ed.) Industrial Biocides. John Wiley and Sons, London.

Hoppe H. G. 1984. Attachment of bacteria:Advantage or disadvantage for survival in the aquatic environment, p. 283-301. **In: Marchall K.C** (ed) Microbial Adhesion and Aggregation. Berlin Springer Verlag.

Iverson W.P. 1987. Microbial corrosion of metals. Adv.Appl.Microbiol. **32:**1-36.

Lawrence J.R., P.J. Delaquis, D.R. Korber, and D.E. Caldwell. 1987. Behaviour of *Pseudomonas fluorescens* within the hydrodynamic boundary layers of surface microenvironments. Microb. Ecol. **14:**1-14.

McCoy W.F., J.D. Bryers, J. Robbins, and J.W. Costerton. 1981. Observation of fouling biofilm formation. Can.J.Microbiol. **27:**910-917.

McLeod E.S., Z. Dawood, R. MacDonald, M. Oosthuizen, J. Graf, P.L. Steyn, and V. S. Brözel. 1998. Isolation and identification of sulphite- and iron reducing, hydrogenase positive facultative anaerobes from cooling water systems. Syst. Appl. Microbiol. **21:**297-305.

Paulos W. 1987. Development of microbiocides for the protection of materials, p. 1-19. **In: Dr Houghton, R N. Smith and HOW Eggins** (eds.)Biodeterioration. London. Elsevier Applied Science.

Von Holy, and T.E. Cloete. 1988. Practical aspects of monitoring biofilms and microbiologically induced corrosion in industrial water systems. S. Afr.J.Science. **84:**17-19.

Chapter 2

LITERATURE REVIEW

2.1 CHEMICAL CONTROL OF MICROORGANISMS

Biocides are toxic chemicals that can affect both microscopic and higher organisms, including humans. They must be carefully selected for appropriate activity and handled with attention to satisfy regulations. The most important parameters to be considered when choosing a biocide for use are: a range of microorganisms to be controlled, the compatibility of the chemical with materials to be protected, the toxicology of the biocide and requirements for its safe disposal and the cost of the of the product (Gaylarde and Morton, 1999).

Biocides are antimicrobial agents employed in various spheres of human activity to prevent, inhibit or eliminate microbial growth. They can be divided into two groups; those occurring naturally and mostly produced by procaryotic organisms (termed antibiotics), and those not occurring readily in nature (termed antiseptics, disinfectants, biocides, bactericides, sanitisers and preservatives). Members of the second group are classified, depending either on their chemical nature, but more often on their specific field of application.

The use of bactericides to control biofouling in water cooling systems is an accepted practice (Bognolo *et al.*, 1992). A recent market survey indicated that the direct prevention costs in terms of bactericide usage in South Africa was R 19.6 million. Although bactericides are employed to reduce bacterial numbers, mere use of the correct bactericides does not necessarily reduce the fouling rate. It is essential to apply the correct dosage at the correct frequency to maintain antibacterial activity in the water. Incorrect use of biocide gives poor results and is expensive. The building blocks of a successful biocides program are ideally considered to be:

- knowledge of the organisms to be killed;
- selection of the correct biocide or combinations and their respective concentrations;

- scientific determination of dosage frequency;
- monitoring the control of microorganisms through analysis and data processing;
- monitoring microbial attachment to surfaces.

The modes of action of a plethora of antibiotics have been investigated in detail. Little has been published on the mechanisms of action of most bactericides and antiseptics. Exceptions are quaternary ammonium compounds and biguanides. Generally, bactericides are not as site-specific as are the antibiotics.

Bactericides attack functional cell components, placing the bacterium under stress (Wainwright, 1988). The type of damage produced by the biocide on the microbial cells determines its final effect, that is, microbiostatic or microbiocidal (Woodcock 1988). Microbiostatic agents react reversibly with the cell components such as nucleic acids and proteins, while microbiocidal compounds cause irreversible damage which leads to lysis of the cells or to coagulation of the cytoplasm (Gaylarde and Morton, 1999). At low concentration bactericides often act bacteriostatically, and are only bacteriocidal at higher concentrations. Targets of bactericide action are components of the cytoplasmic membrane or of the cytoplasm. For bactericides to be effective, they must attain a sufficiently high concentration at the target site in order to exert their antibacterial action. In order to reach their target site(s), they must traverse the outer membrane. Therefore different bacteria react differently to bactericides due to differing cell wall properties. Bacteria with effective penetration barriers to bactericides display a higher inherent resistance than those bacteria which are readily penetrated. The rate of penetration is linked to concentration, so that a sufficiently high bactericide concentration will kill bacteria with enhanced penetration barriers (Gaylarde and Morton, 1999).

It is important to use the correct dose of the biocide because inappropriate doses can lead to failure to reduce cell numbers. In addition, inappropriately low concentrations of the product may lead to the development of biocide resistant population of microorganisms, resulting in the need to change to a different formulation (Gaylarde and Morton, 1999).

From the user's point of view a biocide should be effective against problem causing microorganism, be simple ad safe to use, have no adverse effects on the material to be protected or treated, be inexpensive (low cost/benefit ratio) and lastly be capable of providing protection over the required time scale. Biocide manufacturers have to meet the following additional requirements: (1) long shelf life, (2) low ecotoxicity and (3) biodegradability. The last two have become increasingly important recently, as governments have become more concerned with environmental problems arising from the unregulated disposal of biocides (Gaylarde and Morton, 1999).

Bactericides fall into two categories, oxidising *(e.g.* chlorine and hydrogen peroxide) and non-oxidising. Non-oxidising bactericides can be divided into five groups based on their chemical nature or mode of action, and these will be discussed below.

2.1.1. OXIDISING BIOCIDES

Oxidising biocides are general chemical oxidants. They are not selective for living organisms, but react with any oxidisable matter. However, they are bactericidal because certain bacterial cell components can react readily with them, having a higher oxidation potential than most other chemicals present in water. Three classes of oxidising biocides are available for bactericidal applications; oxidising halogens, peroxides and ozone.

2.1.1.1 Peroxygens

These are unstable oxygen compounds which decompose to form free hydroxyl radicals. These react oxidatively with organic compounds. The peroxides include hydrogen peroxide, peracetic acid, aromatic peroxyacids, persulphates and calcium peroxide.

2.1.1.1.1 Hydrogen Peroxide

Hydrogen peroxide (H_2O_2) is a familiar household antiseptic (Russell *et al.*, 1992). It is an ideal water treatment bactericide as it is stable if stored correctly, non-corrosive and it is totally miscible with water. It has good antimicrobial properties and decomposes to water and oxygen, leaving no toxic waste or by-products (Edstrom, 1995; Cochran *et al.*, 2000). Hydrogen peroxide penetrates cells causing site-directed damage due to metal-dependant OH formation. Its mechanism of action is oxidation

or formation of free radicals which affect enzymes and proteins, DNA. Membranes and lipids resulting in damage of transport systems and receptors, difficulty in maintaining ionic gradients over the cytoplasmic membrane, impairment of replication and (in) activation of enzyme systems (Luppens, 2002). It causes DNA strand breaks and base hydroxylation. Guanine and thymine are the two main targets of peroxide-generated free radical attack. The resulting 7,8-dihydro-8-oxoguanine mispairs with adenine whereas thymine oxidation products stop DNA polymerase, halting replication. Most bacterial mutants cannot survive due to incoherent metabolism, so that peroxide treatment at low concentration leads to slow death. Hydrogen peroxide also inhibits mitochondrial ADP-phosphorylation (Ahern, 1993).

The development of resistance to oxidising bactericides has not been reported in the biofouling control literature. However, a variety of bacteria, mostly fermentative, exhibit oxidising stress response by producing oxidant-degrading and repair enzymes. These include *Escherichia coli Salmonella typhimurium, Bacillus subtilis* and *Pseudomonas fluorescens.* Stress response means that cells become more resistant to a deleterious factor within hours of exposure to sub-inhibitory quantities of the factor. A variety of defense genes have been characterized in *Escherichia coli,* encoding various superoxide dismutases, catalyses, alkyl hydroperoxide reductases and glutathione reductases, as well as DNA repair enzymes. In addition various regulatory genes have been characterized, including *OxyR, Re~ and SoxR.* These regulators determine intracellular redox potential, and activate stress response when cells are exposed to oxidising agents (Farr and Kogoma, 1991).

Hydrogen peroxide is relatively non-toxic, can easily be used in situ, weakens biofilm and supports biofilm detachment (Luppens, 2002). It is environmentally friendly bacause its decomposition products are oxygen and water (Russell *et al.*, 1992).

2.1.1.1.2 Peracetic acid

Peracetic acid (CH_3COOOH) is the best known of the organic peroxides. Like hydrogen peroxide, it forms free hydroxyl radicals which react with various protein structures and DNA. In addition, the dissociation of peracetic acid leads to formation of acetic acid which is mildly antibacterial itself. It finds extensive application in the food industry and for disinfecting sewage sludge (Russell *et al.*, 1992). Application

of peracetic acid to systems does not leave any toxic waste behind. The antibacterial activity of peracetic acid is not affected by water hardness or organic contamination, making it suited for application in cooling water. The great advantage of peracetic acid is that its decomposition products, oxygen and water are innocuous (Russell *et al.*, 1992).

2.1.1.2 Oxidising Halogens

The most important microbiocidal halogens are iodine compounds are chlorine compounds, iodine compounds and bromine (Russell *et al.*, 1992). Hypochloric and hypobromic acids posses excellent antibacterial activity, although within a defined pH range.

2.1.1.2.1 Chlorine Compounds

Hypochloric acid is used in various applications to prevent, control, or decrease bacterial activity. Hypochlorite was first employed as wound disinfectant by Hunter in 1831, and its bactericidal activity was confirmed by Koch in 1881 (Wallhäuser, 1995). Hypochlorite is used among others in industrial water systems to control biofouling.

The antibacterial mechanism of action of hypochlorite is not clear to date although much work on the mechanism of action in eukaryotic cells has been done (Scraufstätter *et al.*, 1990). HOCl does not enter freely into eukaryotic cells but attacks surface and plasma membrane proteins, impairing transport of solutes and the salt balance (Hyslop *et al.*, 1988). It oxidises sulfhydral groups and inhibits plasma membrane ATPases. It appears to halt protein synthesis in cells at low concentrations for ca. 2 hours following exposure. It does, however, not cause any damage to eukaryotic genomic material.

The stability and antimicrobial activity of hypochloric acid is dependent on pH. It dissociates at pH greater than 7, and the undissociated moiety is the antibacterial one. Above pH 7,5 it looses its antibacterial activity. It is excellent for biofouling control as it weakens the extracellular polysaccharide (EPS) structure, leading to sloughing and removal of sections of the biofilm (Wallhäuser, 1995).

2.1.1.2.2 Bromine Compounds

Hypobromous acid works similarly to hypochloric acid. It is, however, stable at a pH up to pH *8.5*. This makes it more suited for application in cooling waters which are often maintained at a slightly alkaline pH. Certain organic compounds release hypobromic and hypochloric acid slowly when in solution. An example is 3-bromo -1-chloro -5,5 -dimethylhydantoin. Such compounds maintain a longer antibacterial level of hypohalous acid in the system treated (Pietersen *et al.*, 1995).

2.1.1.2.3 Iodine compounds

Free iodine was employed in the treatment of wounds, and has been shown to be an efficient microbicidal agent with rapid lethal effects against bacteria and their spores, moulds, yeasts and viruses. It is less reactive chemically than chlorine and is less affected by the presence of organic matter. Its activity is greater at acid than at alkaline pH. Disadvantage of chlorine solutions is that they stain fabric and tend to be toxic (Russell *et al.*, 1992).

Iodophors are compounds formed when iodine is solubilized by certain surface-active agents. They have germicidal action but not have any undesirable properties of iodine. Different concentrations of iodophors are for the antiseptic and disinfectant purposes, but the lower concentrations employed in antisepsis are not claimed to be sporicidal. Povine-iodine was shown to reduce the numbers of viable spores of *Bacillus globigii* on the skin by >99% in one hour, which suggested that it played a role in removing transient sporing organisms from operation sites. Iodophors may be used in the dairy industry and for skin and wound disinfection. Betadine has been widely used in the USA for disinfection of hands for operation sites (Russell *et al.*, 1992).

2.1.1.3 Ozone

Ozone is a strong oxidising material capable of killing bacteria and algae and of inactivating viruses. It is an unstable gas with a pungent odour. It further degrades the EPS holding biofilms together, so that treatment results in loosening of the biofilm. This leads to loosening of scale from the surface. Ozone has a very short half-life and therefore has to be generated on site (Edstrom, 1995). In distilled water its half-life at 20 °C is 25 minutes. Its solubility in water is 13 times that of oxygen. Upon reaction

with organic material it decomposes to oxygen. It does, however, react with several cations and anions such as Fe^{2}+, Mn^{2}+, MnO_4, $N0^{2-}$, and CN. Ozone is toxic to humans, and detectors should be installed together with ozone generators. However treated water is perfectly safe as ozone degenerates to oxygen.

2.1.2 NON-OXIDISING BIOCIDES

These include a variety of organic chemical compounds which have antimicrobial activity. Their modes of action differ vastly, and their only common denominator is that they are non-oxidising organic molecules. They can be grouped into five distinct categories.

2.1.2.1 Detergent-type Biocides

Three groups of surface-active antimicrobial agents have been documented to date; anionic, cationic, and amphoteric (Wallhäuser, 1995). Anionic antimicrobials are only effective at pH < 3.0 and include the aliphatic acids such as sodium dodecyl sulphate. The cationic antimicrobial agents are the quaternary ammonium compounds (QACs) which are well documented and widely used. The best known is benzalkonium chloride.

Benzalkonium chloride adsorbs to the cell surface of negatively charged cells (pH > 7.0) in an irreversible way (Russell and Chopra, 1990). The pH minimum for antimicrobial activity is 3.0. It is membrane active and induces leakage of cytoplasmic constituents (Wallhäuser, 1995). Upon exposure to benzalkonium chloride, membranes of *P. cepacia* appeared irregular, indicating membrane damage (Richards and Cavill, 1980). At 37 °C it is twice as active as at 20 °C. It is active against Gram positive and also against Gram negative cells, but not against spores. Cations such as Ca^{2}+ and Fe^{3}+ decrease its activity, as does NaCI (Wallhäuser, 1995).

Benzalkonium chloride has been employed for the preoperative disinfection of broken skin, for application to mucous membrane and for bladder and urethra irrigation. Creams are used in treating napkin (diaper) rash caused by ammonia producing organisms, and lozenges for the treatment of superficial mouth and throat infections. Other uses of benzalkonium chloride are hard contact-lens soaking solutions and it is

also applied to wounds as an aqeous solution (0.1%) and as a solution (0.2%) in alcohol and acetone for pre-operative skin disinfection and for controlling algal growth in swimming pools (Russell *et al.*, 1992).

Cetrimide is used for cleaning and disinfecting burns and wounds and for pre-operative cleansing of the skin. A mixture of cetrimide and chlorhexidine (savlon) is employed for general disinfection purposes. Solutions containing 1-3% of cetrimide are employed as hair shampoos for seborrhea capitis and seborrhoeic dermatitis. Cetylpyridinium chloride is employed pharmaceutically for skin disinfection and for antiseptic treatment of wounds surfaces, as an oral and pharyngeal antiseptic and as a preservative in emulsions. Cosmetically it is used at concentrations between 0.1 and 0.5% in hair preparations and in deodorants. Lower concentrations are incorporated into face and shaving lotions (Russell *et al.*, 1992).

QACs in the veterinary context have been used for the disinfection of automatic calf feeders and have been incorporated into sheep dips for controlling microbial growth in fleece and wool. QACs are generally very useful disinfectants and pharmaceutical and cosmetic preservatives (Russell *et al.*, 1992).

2.1.2.2 Biguanides

Biguanides are polymer derivatives of a general guanidine structure. Two biguanides are currently used as industrial bactericides. These are polyhexamethylene biguanide (PHMB) and 1,6-di-(4-chlorophenyldiguanido)-hexane, better known as chlorhexidine (Wallhäuser, 1995). Both are not corrosive and all are well suited for application in cooling water (Woodcock, 1988). Biguanides are bacteriostatic at low concentrations and bactericidal at higher concentrations, and have a wide spectrum of activity, especially against gram negative bacteria (Wallhäuser, 1995). They are membrane active agents and attach rapidly to negatively charged cell surfaces (pH neutral or alkaline). By making use of ^{14}C-radiolabelled PHMB, it has been shown that PHMB 15 absorbed into cells of *E. coli* within 20 S after exposure (Fitzgerald *et al.*, 1992). Bactericidal action, however, requires a few minutes. Biguanides compete with divalent cations for negative sites at LPS, displacing these. PHMB then interacts by elecrostatic interactions with the charged headgroups of phosphatidylglycerol and diphosphatidylglycerol (negative), but not with the neutral phosphatidylethanolamine

(Broxton *et al.*, 1984). By binding to phospholipids of the inner leaflet of the outer membrane and of the outer leaflet of the inner membrane, the two membranes attain net positive charges and are repelled from each other, causing membrane damage by distortion. This is supported by TEM studies on *P. cepacia* where both membranes acquired a distinct irregular appearance after treatment with chlorhexidine (Richards and Cavill, 1980). Cytoplasmic constituents start leaking out of the cell due to rupturing of the membranes, and the cell loses its viability.

Chlorhexidine is widely used combined with cetyltrimethylamonium bromide as a topical antiseptic. It has wide spectrum of antibacterial activity against both Gram positive and Gram negative bacteria. It is not sporicidal, and is not lethal to acid-fast organisms or generally viruses. Its main use is in medical and veterinary antisepsis. It is used in catheterization procedures, bladder irrigation and in obsterics and gynaecology. It is also a recommended bacteriocide for inclusion in eye drops. In the veterinary context, chlorhexidine fulfils major functions for the application of a disinfectant to a cow's teat after milking and can also be used as an antiseptic wound application (Russell *et al.*, 1992).

2.1.2.3 Aldehyde-type Biocides

Two aldehydes are commonly used as antimicrobial agents, i.*e.* formaldehyde and gluteraldehyde. Further there is a range of bactericides such as the hydroxyethyl- and ethyltriazine- bactericides available which all release formaldehyde. Formaldehyde has a high polarity and high nucleophilic reactivity, so that it reacts primarily with free primary amino groups, but also with amines, amides, suffides, purines and pyrimidines (Rossmoore and Sondossi, 1988). In water it hydrates to methylene glycol. Reaction with primary amino groups leads to the formation of methyloamines which react further with cellular components. 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 (Wallhäuser, 1995). Formaldehyde is employed both in liquid and gaseous states. Vapour phase formaldehyde is used in the disinfection of sealed rooms, for the disinfection/sterilization of heat sensitive medical materials, hospital bedding and blankets and fumigation of poultry houses, of considerable importance in hatchery hygiene. Formaldehyde in liquid form has been used as a virucidal agent in the

production of viral vaccines. It has also been employed for the treatment of warts, as an antiseptic mouthwash, for the disinfection of membranes in dialysis equipment and as a preservative in hair shampoos (Russell *et al.*, 1992).

Gluteraldehyde also reacts with amino and sulfhydryl groups (Russell and Chopra, 1990). It is stable in acid solution but is 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 (Wallhäuser, 1995). Its reactivity is related to temperature; a 2 % solution kills spores of *Bacillus anthracis* in 15 min at 20 °C, whereas it requires only 2 min at 40 °C. In Gram positive bacteria it reacts with, and binds to, peptidoglycan and teichoic acid, and is also sporicidal. In Gram negative bacteria it reacts primarily with lipoproteins of the outer membrane, preventing the release of membrane-bound enzymes (Russell and Chopra, 1990). Gluteraldehyde has been recommended for the disinfection of medical equipment notably cytoscopes and anaesthetic equipment. In the veterinary field it is used for disinfection of utensils and of premises. It has potential mutagenic and carcinogenic effects which makes it harzadous to personnel. Its advantages are it has broad spectrum of activity with a rapid microbicidal action and it is non-corrosive to metals, rubber and lenses (Russell *et al.*, 1992).

2.1.2.4 Phenol Derivatives

Phenol was the antimicrobial agent which revolutionized invasive surgery, and was pioneered by Lister in 1870 (Franklin and Snow, 1982). It enters the cell by dissolving in the membrane, and upon entry into the cytoplasm, precipitates proteins. It is, however, harmful to humans, and its antibacterial activity is not very high. A range of halogenated phenols, cresols, diphenyls and bisphenols have been developed from phenol, and have excellent antimicrobial activity, many being applied in the preservation of pharmaceutical products. Halogenation increases the antimicrobial activity of phenol, as does the addition of aliphatic and aromatic groups (Wallhäuser, 1995). Bisphenols have the highest antimicrobial activity of the phenol derivatives, especially halogen substituted ones. Hexachlorophen and 2,2'-methylenebis(4-chlorophenol) (dichlorophen) fall into this group (Brözel and Cloete, 1993).

Phenol derivatives are membrane active agents. They penetrate into the lipid phase of the cytoplasmic membrane, inducing leakage of cytoplasmic constituents. 3- and 4-chlorophenol uncouple oxidative phosphorylation from respiration by increasing the permeability of the cytoplasmic membrane to protons (Gillbert and Brown, 1978).

2.1.2.5 Organic acids and inorganic acids: esters and their salts

Organic acids. Both aromatic and aliphatic, and one or two inorganic acids, have found application as preservatives more especially in the food industry. Others, foe example benzoic acid, are also used in the preservation of pharmaceutical products while salicylic acid is used for topical treatment of fungal infections of the skin. Vinegar, containing acetic acid is used as wound dressing, hydrochoric and sulphuric acids are used in veterinary disinfection, HCL being sporicidal at high concentrations. It is also used for disinfecting hides and skin contaminated with anthrax spores. Sulphuric acid is used in combination with phenol for decontamination of floors, feed boxes and troughs. Citric acid is an approved disinfectant against foot-and-mouth virus. Acid preservatives act by preventing the uptake of substrates which depend on a proton motive force for their entry into the cell, thuis acting as uncoupling agents (Russell *et al.*, 1992).

2.1.2.6 Alcohols

Several alcohols have been shown to posses antimicrobial properties but only a few will be discussed here. They generally have rapid bactericidal activity including acid-fast bacilli, but are not sporicidal and have poor activity against many viruses. Ethanol (CH_3CH_2OH) is rapidly lethal to non-sporulating bacteria, destroys mycobacteria but is totally ineffective at all concentrations against bacterial spores. It is the lethal alcohol of choice in cosmetic products because of its relative lack of odour and irritation. Methyl alcohol (methanol) has poor antibacterial activity and is not sporicidal. Freshly prepared mixtures of methanol and sodium chloride are however highly sporicidal. Isopropanol is more effective than ethanol for inhibiting bacterial growth. It is used in various cosmetics as an alternative to ethanol, either as a solvent or as a preservative. Chlorbutol is an antibacterial and antifungal agent used at concentrations of 0.5% w/v as a bactericide in injections (Russell *et al.*, 1992).

2.1.2.7 Thiol-oxidising biocides

Thiols on amino acids such as cysteine are important groups which influence the tertiary structure of proteins by forming disulphide bridges. Three groups of antimicrobial agents, isothiazolones, Bronopol (2-bromo-2-nitropropane-1,3-diol), mercury and other heavy-metal compounds, react with accessible thiols, altering the three dimensional structure of enzymes and structural proteins (Collier *et al.*, 1991). Mercury interacts with sulfhydryl groups by complexing with sulphur. Bronopol oxidises thiols to disulphides, reacting especially with the active center of hydrogenase enzymes (Wallhäuser, 1995).

Three isothiazolones possess antibacterial activity; 5-chloro-N-methylisothiazolone (CMIT), N-methylisothiazolone (MIT) and benzisothiazolone (BIT). Isothiazolones react oxidatively with accessible thiols such as cysteine and glutathione. These thiols are oxidised to their disulphide adjuncts which, in the case of cysteine, leads to an alteration of protein conformation and functionality. Isothiazolone is hereby reduced to mercaptoacrylamide, which in the case of CMIT tautomerises to thioacyl chloride, the latter reacting with amines such as histidine and valine. Isothiazolones are primarily bacteriostatic, and are only bactericidal at high concentrations (Collier *et al.*, 1990).

2.1.2.8 Miscellaneous Biocides

The mechanisms of action of various antimicrobial agents, employed to control bacterial growth in cooling water systems, have not been formally published to date. These include phosphonium chloride (tetra-alkyl phosphonium chloride), thiocarbamates (Na diethyldithiocarbamate) and MBT (methylene bis-thiocyanate) (Igarashi and Watanabe, 1992). Phosphonium chloride probably has surfactant properties, damaging the bacterial cell envelope. The mechanism of antimicrobial action of MBT is not known to date. Thiocarbamates are used as agents for the extraction of trace metals such as Fe, Cd, Co, Cu, Mn, Ni, Pb and Zn (Lo *et al.*, 1982). This would imply that it chelates iron, a vital trace element of most bacteria. The nucleophilic sulphur atom indicates potential reactivity with accessible thiols. Thiocarbamates do react with accessible thiols and amines. Therefore their antibacterial mechanism of action would rest partially on denaturation of surface proteins. We have found that the antibacterial mechanism of action depends on the

alkyl chain length of the thiocarbamate. Sodium diethyldithiocarbamate is inactivated by free ferrous iron, indicating that it removes iron (a growth factor) from the cell. Sodium dimethyl dithiocarbamate is not inactivated to the same degree, showing that its antibacterial activity does not rest as much on iron removal (Igarashi and Watanabe, 1992).

2.1.3 Factors influencing the efficacy of antimicrobial agents

The activity of disinfectants against microorganisms depends on the external physical environment, the nature, structure and composition and condition of the organism itself and the ability of the organism to degrade or inactivate the particular substance converting it to an inactive form. There are pre-treatment, in-use and post treatment factors influencing activity of biocides. Only those parameters influencing the in-use activity or activity during treatment will be discussed here. These factors include among others, the concentration of the antimicrobial agent, the number, type and location of the microorganisms, the temperature and pH of treatment and the presence of interfering substances such as organic matter and metal ions.

2.1.3.1 Concentration of biocide

Kinetics studies involving the effect of concentration on the lethal activity of microbial substances employ the concentration exponent, also termed the dilution coefficient. Concentration exponent is a measure of the effect on changes in concentration on cell death rate. A decrease in concentration of substances with high concentration exponent values result in a marked increase in the time necessary to achieve a compatible kill, other conditions remaining constant. In contrast, compounds with low concentration exponent values are much less influenced. A knowledge of the effect of concentration on antimicrobial activity is essential in the evaluation of biocidal activity; testing sterility of pharmaceutical and medical products; in ensuring adequate preservative levels in pharmaceutical products and in deciding what dilution instructions are reasonable in practice (Russell *et al.*, 1992).

2.1.3.2 Numbers and location of microorganisms

An antimicrobial agent is effective when there are few microorganisms against which it has to act. Location of microorganisms must also be considered in assessing activity. Difficulties may arise in the penetration of a disinfectant to all parts of the

equipment especially in the cleaning of equipment used in large-scale production of creams. Affectivity of the agent is high when all microorganisms on equipment are accessible to the antimicrobial agent (Russell *et al.*, 1992).

2.1.3.3 Temperature

The activity of a disinfectant or preservative is usually increased when the temperature at which it acts is increased. The activity of ascorbic acid increases markedly at elevated temperatures. Gluteraldehyde shows a very marked temperature dependent activity with its alkalinized or 'potentiated' form being more powerful at 20°Cthan the more stable acid formulation. At temperatures of about 40°C and above however, there is a little, if any, difference in the activity, although the alkaline formulation is less stable at higher temperatures (Russell *et al.*, 1992). Formaldehyde polymerizes when exposed to polar compound or high temperatures and oxidizes to formic acid when exposed to air. Isothiazolones are unstable at temperatures above 40°C and chlorhexidine is unstable above 70°C (Cloete and Flemming, 1997).

2.1.3.4 Environmental pH

pH can influence biocidal activity in many ways. Firstly, it may cause changes in the molecule. For example, phenol and benzoic acid are effective only or mainly in the unionized form. As the pH rises there is an increase in their degree of dissociation. Gluteraldehyde is more stable at acid pH but more potent at alkaline pH. Secondly, the change in pH may result in 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. Thirdly, partitioning of a compound between a product in which it is present and the microbial cell may be influenced by pH (Russell *et al.*, 1992).

2.1.3.5 Interfering substances

2.1.3.5.1 Organic matter

Organic matter may interfere with the microbicidal activity of disinfectants and other antimicrobial compounds. The reaction between the biocide and organic matter leaves a reduced concentration of antimicrobial agent for attacking microorganisms. There is a possibility that organic material protects microorganisms from attack. Organic matter decreases the effect of hypochlorite against bacteria, viruses and

fungi. Phenol may also show reduced activity in the presence of organic matter. Disinfectants use in the cosmetic, pharmaceutical food and dairy industries are influences by the reduction of activity that may occur in the presence of organic soil. This problem may be overcome by pre-cleaning before employment of antimicrobial agent. In the food industry invisible milkstone may protect microorganisms against disinfection (Russell *et al.*, 1992).

2.1.3.5.2 Metal ions

The presence of metal cations may reduce, enhance or have no effect on the activity of antimicrobial agents. Manganese ions (Mn^{2+}) reduce the anti-pseudomonal activity of salicylaldehyde, zinc ions (Zn^{2+}) enhance the activity while calsium (Ca^{2+}) and magnesium (Mg^{2+}) ions have no effect. The anti-staphylococcal potency of anionic surfactants is increased in the presence of low concentrations of divalent cations, whereas the bactericidal activity of long chain fatty acids is diminished greatly in the presence of Mg^{2+}, Ca^{2+} and Ba^{2+} ions (Russell *et al.*, 1992).

2.1.3.6 Humidity

Relative humidity influences the activity of vapour-phase disinfectants. The gaseous disinfectants influenced are ethylene oxide, β-propiolactone and formaldehyde. With bacterial spores dried on cotton patches as test pieces, ethylene oxide is most active at relative humidity of 28 to 32%, β-propiolactone at 70% and formaldehyde at relative humidity about 60% (Russell *et al.*, 1992).

2.1.3.7 Type of organism

2.1.3.7.1 Gram positive bacteria

Gram positive bacteria are generally more sensitive to biocides than are Gram negative bacteria. Their sensitivity is caused by the composition of the cell wall envelope. The cell wall of gram positive bacteria is basically made up of peptodoglycan layer, with other molecules such as teichoic and teichuronic acids and lipids (though to a smaller extent) whereas Gram negative organisms have lipid rich envelopes. The cell walls of gram positives may not form a barrier to entry of antibacterial substances as does lipid rich envelope of gram negative bacteria. Gram positives are more sensitive to QACs and salicylanilides than Gram negatives (Russell *et al.*, 1992).

2.1.3.7.2 Gram negative bacteria

Resistance of Gram negative bacteria to biocides is linked to the lipid content of their cell envelopes. Resistance is also liked to R-plasmid mediated enzymatic inactivation or to intrinsic resistance. R+ strains of Gram negative bacteria may destroy mercury compounds. Some R-plasmids may be associated with sensitivity and resistance to sodium deoxycholate (Russell *et al.*, 1992).

2.1.3.7.3 Mycobacteria

Resistance of mycobacteria to many disinfectants is linked to the composition of their cell walls. The cell walls of mycobacteria contain alanine, glutamic acid and meso-diaminopimelic acid, monosaccharides galactose and arabinose. The muramic acid found in mycobacteria is N-acetylated whereas that in other bacteria is N-glycosylated. They possess an unusually high wall lipid content whose hydrophobic nature contributes to their high resistance. QACs and dyes inhibit M tuberculosis growth, but are not tuberculocidal. M tuberculosis is resistant to chlorhexidine, acids and alkalis. Alcohols, formaldehyde, formaldehyde-alcohol, ethylene and iodine-alcohol are tuberculocidal. Gluteraldehyde had been confirmed to be mycobactericidal (Russell *et al.*, 1992).

2.1.3.7.4 Moulds and yeasts

Many compounds have both antibacterial and antifungal activity. They include QACs, oxine, diamine and esters of ρ-hydroxybenzoic acids, the parabens (Russell *et al.*, 1992).

2.1.3.7.5 Viruses

Several bactericidal agents possess virucidal properties. Morphological changes in viruses sometimes occur following exposure to biocides. Chlorine disinfectants are considered to be effective in killing all types of viruses and to be useful in preventing the spread of foot and mouth disease. Mercury compounds are inactive against viruses. Some agents are much less active in destroying non-lipid enveloped viruses, eg. polio, than lipid enveloped ones. Formaldehyde is used in preparation of viral vaccines but require an extensive period in order to be virucidal. Gluteraldehyde is a virucidal agent with the activity against many types of viruses. Hepatitis B virus

(HBV) and human immunodeficiency virus (HIV) can be readily inactivated by gluteraldehyde and chlorine releasing agents (Russell *et al.*, 1992).

2.1.4 Environmental problems associated with biocides

Enhanced public perception of environmental matters and increasingly restrictive legislation has led to changes in the development and introduction of new biocides. The need to conform to the new legislation has substantially increased the cost of bringing new products to the market so that few companies consider that the development of new products is worthwhile as long as older, cheaper products are still allowed to be sold. The European Biocidal Products Directive, to be used in the control of biocides in the countries of the European Union, obliges biocide manufacturers to carry out extensive toxicological and ecotoxicity testing on their products. It requires manufactures to provide data on human exposure risks, environmental destiny and the efficiency of the biocide, in addition to the overall risk of use. Risk is defined as the multiple of exposure and hazard, where exposure is equal to the release rate into the environment multiplied by the persistence of the biocide in that environment, and hazard is the toxic effect on the environment. Their ecotoxicity has led to some of the old, established products being discontinued and the modern generation of biocidal products is largely composed of mixtures of less toxic compounds (Gaylarde and Morton, 1999).

2.1.5 Safety precautions for handling biocides

All commercial formulations of biocides are harzadous chemicals that must be handled and dispensed with great care to avoid injury. Regardless of the chemical being handled, eyes must be protected with approved chemical goggles, safety spectacles are not sufficient. Wearing clean clothing that covers the arms and legs completely. Hands must be protected with rubber gloves and there should be adequate ventilation in the work area. Clothing contaminated by splashes or spills of biocide must be removed immediately, and not worn again unless thoroughly washed with soap and water. Empty biocide barrels should be thoroughly washed before being discarded. Barrels should not be reused.

Other personal safety equipment should include rubber gloves, rubber boots, aprons and face shields. If chlorine is used, a chemical cartridge respirator or a self-

contained breathing apparatus should be available for respiratory protection in the vent of leaks or other equipment failure. Most industrial water treatment biocides are classified as primary irritants and sensitizers. Others like methylene bisthiocyanate and sodium dimethyldithiocarbamate depress the central nervous system. Symptoms of poisoning include drowsiness, fatigue, headache, unconsciousness, narcosis and convulsions. Pentachlorophenol if inhaled, ingested or absorbed through the skin, increases the basal metabolism rate, and it interferes with oxidative phosphorylation. Prompt first aid is essential, particularly when the eyes are affected. Eyes should be irrigated with clean water for at least fifteen minutes, rolling back eyelid to facilitate removal of chemical, after which the accident victim should be examined by a medical doctor. Spills on the skin should be washed off immediately and thoroughly with soap and water. if the skin is reddened, blistered or ulcerated, or if any chemicals are ingested, a medical doctor should be consulted immediately. If the victim accidentally swallowed dodecylguanidine hydrochloride or methylene bisthiocyanate, milk, olive oil or egg white should be given if available, otherwise large volumes of water will dilute the toxicant. Vomiting should not be induced, as it is likely to cause mucosal damage (Cloete and Flemming, 1997).

2.2 ELECTROCHEMICAL ACTIVATION (ECA) TECHNOLOGY

Water is the essence of life and as a chemical compound, its composition and characteristics have no analogue in nature. The molecular composition of water comprises two hydrogen and one oxygen atom, and the opposite polarity of the charges between the molecules, results in a process of "hydrogen bonding". It is the very nature of this polarity of charge that imparts the capacity of water to be activated into metastable states with distinctive and unique attributes.

ECA technology is based upon the generation, by means of specialised electrochemical systems, of metastable or activated solutions being derivatives of basic water molecules, which display abnormal physico-chemical and catalytic activity. The source components of these activated water solutions are simply potable water and a small quantity (0.2-5g/l) of salt.

During the period of increased activity, these meta-stable solutions have been shown to have both real and potential applications in a diverse array of technological processes, often as a substitute for traditional chemical agents.

Water has been described as the universal solvent, and hence the variety of applications for the use of the activated water solutions is potentially beyond constraint. Irrespective of the characteristics of the specific solution, where activation status may extend from hours to days, the resultant product following decay of the state of activation remains benign water.

The ability to consistently produce a solution of a specific quality, possessed of unique and proven attributes, on a demand driven basis, with no adverse environmental consequences, significantly differentiates ECA technology from current customary interventions in water treatment and its allied applications. Water of varying mineralisation is passed through an electrochemical cell, the specific design of which, permits the harnessing of two distinct and electrically opposite streams of activated water.

Aside from its distinctive attributes, the negatively charged anti-oxidant solution (Catholyte) can also be channeled back into the anode chamber, thereby modulating the quality of the positively charged oxidant solution (Anolyte) that is produced. Depending on the specifications of the required application, variations in the design of the hydraulic systems can be effected to meet the requisite objectives.
The design of the cell is such, as to ensure a uniformly high voltage electrical field through which each micro-volume of water must pass. This unipolar electrochemical activation created by potential gradients of millions of volts per cm^2 between the anode and cathode terminals, results in the creation of solutions whose pH, Oxidation Reduction Potentials (ORP) and other physico-chemical properties, lie outside of the range which can be achieved by conventional chemical means.

2.2.1 Concept of electrochemical activation

ECA of water involves the exposure of water and the natural salts therein or salts added to it, to a substantial electrical potential difference. If one places anode (+) and a cathode (-) in pure water and applies a direct current, electrolysis of water occurs at

poles, leading to the breakdown of water into its constituent elements, producing gaseous oxygen and hydrogen. Electroplating is a similar process, where one adds chromium salts to water and applies a potential difference, the chromium plating out onto the material attached to the cathode. 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 (http://www.camwell.net/electrochemical_activation.htm).

The key innovation of the ECA process is the interposition of an ion permeable membrane between the positive and negative electrodes, as well as the design and materials used for the electrodes. The Russian electrochemical reactor separated by a patented zirconium oxide diaphragm (certain new designs use polymer membranes). The base solution used in this reactor is a 5% solution of NaCl, which is split into two channels, one running through the anode chamber and the other through the cathode chamber. Salt, which is a solution in its ionised form (Na^+ and Cl^-), is exposed to a controlled electrical potential difference between the cathode and the anode. This potential difference causes the Na^+ and Cl^- ions to migrate towards the pole of the opposite charge. The specially designed membrane, which separate the two chambers, allows ions to pass unimpeded, but is largely impermeable to unionised water as well as organic molecules. The net result is an enrichment of chlorine ions in the anode chamber and sodium ions in the cathode chamber. Similarly, water is also ionised extensively and will also migrate to opposite pole ($H_2O \rightarrow H^+ + OH^-$). As high concentrations of Cl^- and OH^- without compensation of Na^+ and H^+ build on either side of the membrane, this unstable chemical state result in complex reactions which produces a metastable solution containing a variety of very reactive ions and free radicals. Some of the reactive ions and free radicals formed in the anolyte chambers are given in Table 2.1.

Table 2.1: Reactive ions and free radicals formed in the anolyte and catholyte solutions by electrochemical activation

	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

It is the formation of these complex chemical species which leads to describing the solutions formed as electrochemically activated water. Some of the more important reactive constituents formed include, hypochlorite (HClO), hydrogen peroxide (H_2O_2), ozone (O_3), chlorine and $HClO_3$. Most of the compounds are formed in the anolyte chamber, they are acidic in nature and are very strong oxidising compounds. The 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. Analogously, the anolyte and catholyte solutions develop opposing potentials, the anolyte having a redox potential of +1200mV, while the catholyte reaches a value of –1000mV, relative to a potential of +300-400mV for the starting NaCl solution (http://www.camwell.net/electrochemical_activation.htm).

2.2.2 Properties of Activated water

The properties of the activated solutions are dependent upon a number of factors. These comprise the solution flow rate through the reactor cell, the current being applied, temperature, and degree of feedback of catholyte into the anolyte chamber and the degree of mineralisation of the water.

During electrochemical activation, three categories of products within the solution are generated. They comprise:

Stable products which include acids and bases which influence the pH of the solutions,

Highly active unstable products including free radicals and electrolytic gases in the form of micro-bubbles which influence the ORP of the solutions, and

Quasi-stable products comprising complexes of hydrated membranes which form clusters of water molecules which impart the catalytic activity of the solutions.

Without maintenance of the activated state, these diverse products degrade to the relaxed state of benign water and the anomalous attributes of the activated solutions such as altered conductivity and surface tension similarly revert to pre-activation status.

It is important to note that the level of mineralisation of input water required to generate optimally metastable solutions is insignificantly different from the composition of benign potable water. However, the heightened electrical activity and altered physico-chemical attributes of the solutions differ significantly from the benign state, but yet remain non-toxic to mammalian tissue and the environment.

2.2.3 Biocidal properties of Anolyte

Earlier technologies that have employed electrochemical activation to generate biocidal solutions have not been capable of separating the output the anolyte and catholyte solutions. In these cases the two opposing solutions have neutralised each other with regard to potential electrical activity.

The advantages of the current ECA technology has been confirmed, wherein the biocidal activity of hypochlorous acid generated by the current ECA technology is 300 times more active than the sodium hypochlorite generated by earlier systems. Additionally comparison of neutral anolyte (pH=7), with alkaline Gluteraldehyde (pH=8.5), showed that the latter required a concentration of 2% versus 0.05% of the former, in order to achieve the same biocidal efficacy. Similarly, it has been shown that a 5% solution of sodium hypochlorite (Jik) can only be used for purposes of disinfection whilst a 0.03% solution of neutral anolyte, has both disinfectant and sterilising properties. In general, the biocidal activity of non-activated neutral anolyte (only stable products and no electrical charge) is 80 times the potential activity of the hypochlorite solution, but still exhibits only one third of the full biocidal potential of the optimally activated ECA solution.

The anolyte solution has more oxidative microbial destruction power than chlorine based solutions. As a result, the anolyte has superior sterilizing and disinfectant properties, since the reactive species present in the solution are more effective in destroying microorganisms and organic molecules than chlorine alone. Organic molecules such as pesticides, tannins and phenols, which are of concern in terms of toxicity, colour and off-flavours are effectively oxidised. Chlorine, which is a key component of the anolyte solution, effectively chlorinates the water, however, because of the low redox potential of the added anolyte, the formation of potentially toxic chlorinated hydrocarbons is minimised because the low redox potential does not favour their formation. As such, the anolyte is an effective means of eliminating organisms of public health concern from water, while simultaneously destroying organic constituents, which are commonly associated with off flavour and colour.

Thus, activated solutions have been conclusively shown to exceed chemically derived equivalents both in low dosage effectiveness as well as physico-chemical purity. This heightened biocidal capacity relative to traditional chemical solutions permits the incorporation of ECA solutions at substantially lower dose rates, therein obviating the risk of intoxication, adverse environmental impact, while providing cost effective resolutions.

ECA sterilizing solutions have a well proven track record in killing microorganisms as diverse as spores like *subtilis ver niger* to methicilin resistant *Staphyococcus aureus* (MRSA) the super bug found in hospitals. It has strong bactericidal effects on pathogenic bacteria including *S. typhimurium* (http://www.baeg.engr.uark.edu/FACULTY/yanbinli/projects/project1.htm).

These solutions can be administered as neat or diluted blends. The exact mechanism by which the biocidal action operates is not yet fully understood. The fact is that the biocidal action can be quantified, is proven technology and looks impressive, giving log 6 reductions in spores like *Bacillus subtilis var niger* in 30s. The breakdown of the cell wall of microorganisms is swift, violent ensuring that no immunity can be achieved.

The solutions produced, particularly the anolytes are benign in terms of fume, corrosion or effect on the skin of humans or animals. The solutions are oxidative but

only mildly having quite a low chemical load. They are in this respect eco-friendly and present no problems to the environment. Other biocides based on chlorine are highly oxidative in their action and rely on this property to carry out the kill on bacteria and viruses. Neutral anolyte has a different way of killing. It is proven to be far more effective (even on spores) without the need for aggressive (and some would say corrosive) oxidation reactions to achieve a kill efficiency. This is in essence what makes this remarkable biocidal solution a valuable tool in the fight against unhealthy microorganisms (http://www.camwell.net/electrochemical_activation.htm).

The antimicrobial activity of anolyte was analysed regarding the following microorganisms: *Mycobacterium tuberculosis*, *Mycobacterium avium-intracellulare*, *Mycobacterium chelonae*, *Escherichia coli* (including 0157 type), *Enterococcus faecalis*, *Pseudomonas aeruginosa*, methycillin resistant spores of *Bacillus subtilis var niger*, *Staphylococcus aureus*, *Candida albicans*, polio virus of type 2 and human-immunodeficienc virus HIV-1. It has been established that the absence of organic load freshly made anolyte has a high biocidal activity towards all of the above listed microorganisms and provides five log 10 reduction in contamination levels in 2 minutes or less. Using membrane filters British scientists explored anolyte biocidal effect on spores of *Clostridium difficile*, *Helicobacter pylori*, vancomycin resistant varieties of *Enterococcus*, *Candida albicans* and certain varieties of *Mycobacterium*. In all experiments there was either 1 and 5 vol. % equine serum added or no serum at all. Distilled water, 0.35% peracetic acid (Nu-Cidex) and 2% gluteraldehyde were used as reference substances. The ratio of anolyte to spore-containing suspension being 9:1, 5% equine serum was added, and the content of microorganisms were used: spores of vancomycin resistant *H. pylori*, varieties of *Enterococcus*, *Candida albicans* and four atypical varieties of *Mycobacterium- M. avium*, *M. chelonei*, *M. xenopi* and *M. smegmatis*. The sporicidal effect of anolyte on *Clostridium difficile* was notably decreased when 5% equine serum was added (Leonov, 1999).

2.2.4 Antibacterial assessment of anolyte

Various researchers had conducted a number of experiments, testing the ability of activated water produced by Radical Waters to kill or inhibit growth of different microorganisms. The conducted experiments are described briefly:

Bilgeri *et al.* 1999 conducted an experiment in which anolyte was used as a biocidal agent on a number of Gram positive and Gram negative organisms. The Gram positive organisms tested were *Ent. faecalis* (vanomycin-resistant ward isolate), *L. monocytogens*, *Clostridium perfringes*, *S. aureus* (ATCC strain, fully sensitive as well as methicilin resistant), *B. subtilis* and *B. cereus*. The Gram negative organisms included *L. pneumophila*, *E. coli* (SATCC, fully sensitive strain, obtained from SABS) as well as *E. coli* O157: H7 (obtained from SAIMR), *K. pneumoniae* (ward isolate, highly mucoid) and *P. aeruginosa*. Bacteria with excessive capsular material were chosen because it was thought that anolyte might act by transferring its positive electrical charge to the bacterial membrane and that the presence of capsular material would be a possible factor that will decrease antibacterial action. The organisms were exposed to anolyte for 5 min. A 100% kill of all the organisms tested was obtained except for *C. albicans* where a >99.99% kill was achieved after 5 min and a 100% kill by 30 min. This experiment showed that anolyte had antibacterial properties and that capsular material does not decrease the antibacterial action of anolyte.

Kruger and Buys (1998) examined if there was a reduction of beef carcass contamination after treatment with anolyte. Twenty-four beef carcasses were used; twelve were treated with anolyte while the remaining twelve were used as control. The carcasses were all microbiologically monitored using Rodac plates, which were incubated at 25°C for 3d. Total counts per 24 cm^3 showed that anolyte treated group of beef carcasses had significantly lower total counts than the control group of carcasses.

Division of Hospital epidemiology and infection control of the SAIMR also evaluated the antimicrobial properties of anolyte on three organisms known to be difficult to eradicate from the hospital environment. Various dilutions (1:1, 1:50, 1:100 and 1:150) were used for the exposure times of 5 and 30 min. The organisms tested in the study were Staphylococci, both coagulase- positive and coagulase negative found on the skin, methicilin resistannt *S. aureus* (cross resistance of MRSA to chlorhexidine has also been reported). The second organism was *P. aeruginosa* which is well documented not only as resistant to a large number of antibiotics, but also able to replicate in savlon and chlorhexidine when the concentration of ready-to-use ward disinfectant stock solution is sufficiently low. The third organism was *L.*

pneumophila isolated from hospital air-conditioning systems. *L. pneumophila* is notoriously difficult to eradicate despite attempts to raise the temperature of the water pipes to 60°C in the water supply. Ringer's solution, the diluent used, and sodium thiosulphate were used as control. In the organisms treated with Ringer's solution and sodium thiosulphate no significant drop in the cfu/ml of the organisms was observed, indicating that any drop in cfus obtained was purely due to the effect of anolyte. With the organisms that had an initial concentration of 10^5 cfu/ml showed no growth (became non-viable) after being exposed to any of dilutions of anolyte (1:1, 1:50, 1:100 and 1:150) for both exposure times. A 100% kill was obtained for all the organisms with initial concentration of 10^6 cfu/ml after 30-min exposure. The slightly lessened activity against *S. aureus*, which is Gram positive, necessitated a comparative study using Gram positive and Gram negative bacteria.

Swift Micro laboratories (1997) determined the efficacy of disinfection of stainless steel cutting utensils with anolyte at various dilutions rates and exposure times at ambient temperatures, compared to water at 20°C and 82°C. The neat anolyte reduced the total plate counts (TPC) from 2200 cfu/ml to 10 cfu/ml after 2 min and zero after 5 min. The Coli counts were reduced to zero after 2-min. Anolyte (1:4) sterilized equipment after 5-min exposure while 1:9 anolyte sterilized equipment only after 30 min. Water at 20°C did not eliminate coli counts even after 30-min exposure though the TPC was zero. Water at 82°C eliminated TPC both after 5 min and 30 min, but Coli counts were still 20 cfu/ml and 10 cfu/ml after 5 and 30 min, respectively. Anolyte was a better disinfectant for stainless steel cutting utensils than water at 82°C.

2.2.5 Safety assessment of activated water

Biocon research (1999a, b and c) undergone a study to determine the acute oral toxicity to rats of Radical Waters activated water (anolyte, catholyte and anolyte-catholyte combination), to provide information on health hazards likely to arise from short term exposure to the test item by oral route. Five male and five female rats were exposed by oral gavage at a single limit dose of 20mg/kg body weight. Rats were observed daily and examined for any clinical sign of an adverse effect. No mortality occurred and no abnormal clinical signs were observed for all the three products

tested. The results obtained up to day 13 after acute oral dosing of the animals suggested that anolyte, catholyte and their combination are non-toxic to rats.

Acute dermal irritation/corrosion of anolyte, catholyte and their combination in rabbits was assessed by Biocon research. Two patches, each moistened with 0.5 ml of the test item were applied to skin of three male New Zealand white rabbits. Exposure time was 4 h, after which bandages were removed and skin rinsed with purified water. The test sites were examined for dermal irritation at points of time 1, 24, 48 and 72 h after the patches were removed. In cases were anolyte was tested, erythema of the skin was observed in only treated animal. No oedema was observed in any of the animals. The reaction of skin to anolyte (in one animal) was reversible and has disappeared by 48 h. No other clinical signs of adverse reactions were observed in any of the animals. For catholyte and anolyte-catholyte combination, no erythema or oedema of skin was observed in any of the treated animals, or any abnormal clinical sign of adverse reactions. The potentials of the test items to irritate the skin by all the test items were considered negligible in terms of response categories given in ISO 10993-10: 1995. It was concluded from the results that the potential of activated water to cause irritation to the skin is negligible (Biocon research, 1999d, e and f). The activated water was also shown by Biocon research to have negligible potential to irritate or corrode the eyes. Thus there is no much of risk to people using anolyte as disinfectant, thus anolyte is a very user-friendly product (Biocon research, 1999g, h and i).

2.2.6 ECA technology advantages

The system is interesting for specialists in applied fields, since it allows solving their industrial problems in the easiest way. As a sure rule, they do not need complicated mathematical or economic calculations to recognize its efficiency. Economic efficiency of ECA is associated with reduced quantities of chemical reagents which were previously used in technological processes, or their replacement with most ordinary reagents, for instance, cooking salt; with augmentation of industrial processes; with achieving new optimal results which were unattainable when traditional technologies were used; with better quality of end products, and with sewage decontamination (Leonov, 1999). ECA solutions may come to play useful role in surface and instrument disinfection, since gluteraldehyde, peracetic acid and

other disinfectants in common use are toxic and unpleasant to inhale (Bilgeri *et al.*, 1999)

2.3 BIOFILMS

Biofilms have been defined in number of ways by various researchers. Such definitions are usually constructed to be inclusive of the many environments in which biofilms are found and disciplines that the subject covers. Characklis and Marshall (1990) defined a biofilm as consisting of "cells immobilized at a substratum and frequently embedded in an organic polymer matrix of microbial origin". Costerton *et al.*, (1995) supplied a broader definition of biofilms as "matrix enclosed bacterial populations adherent to each other and/or to surfaces or interfaces". Palmer and White (1997) tried to be more comprehensive still and defined biofilms as "a collection of microorganisms (including cells in culture) and their associated extracellular products at an interface and generally attached to a biological (other cells or tissues, including matrix polymers) or abiological (mineral or synhetic) substratum (Stoodley *et al.*, 1999).

Examples of places where biofilms can be found are as adverse as teeth, ship hulls, prosthetic implants, (drinking) water systems and food contact surfaces. In short, biofilms can be found in all places where the following requirements are met: presence of a surface, nutrients, moisture, a suitable temperature and microorganisms (Luppens, 2002). Biofilms are characterized by a high population density and by structural organization. Factors influencing their growth include the chemical nature of the substratum, the nature of the carbon source, carbon source concentration, osmolarity, shear stress and population composition (Heydorn *et al.*, 2000). Temperature amongst others influences which microorganisms will form a biofilm. Above mentioned examples are all places where biofilm formation is detrimental. In those places biofilms can cause energy losses and deterioration or create health risks. However, biofilms can also be useful, for instance in the human gut and when they are used for wastewater treatment, bioremediation or for the production of vinegar and ethanol (Luppens, 2002).

Although biofilms have long been known and used, only about two decades ago they started to become of increasing interest to both research and industry, and their importance in aquatic, soil, clinical and industrial environments was established. The perception of biofilms evolved from a "homogenous matrix" towards complex heterogeneous multispecies biocoenoses. These insights were largely influenced by the availability of suitable analytical monitoring equipment and representative sampling techniques. At early stages, biofilm monitoring was often carried out off-line and ex-situ, for example by microscopical methods, which usually required biofilm destruction and necessitated specimen dehydration in the case of electron microscopy. This led to distortion of the natural biofilm structure and to a biased interpretation of the images, considering biofilm structure essentially homogenous. This model was later challenged by nondestructive, in-situ techniques such as confocal laser scanning microscopy, combined with microelectrode and nuclear magnetic resonance imaging. This showed that biofilms grow in microcolonies embedded in an exopolysaccharide matrix structure, which are interspersed with less dense regions containing highly permeable water channels. From these results, the importance of in-situ and non-destructive techniques which are able to monitor biofilms in their natural environments becomes apparent. Several microscopic, spectrochemical, electrochemical and piezoelectrical techniques have been developed for noninvasive, in-situ biofilm monitoring during the past decade, focusing on a wide range of biofilm characteristics. Thereby, on-line techniques are of special importance, as they allow for real-time monitoring (Wolf *et al.*, 2002).

Conventional monitoring systems are mostly based on test surfaces, so-called "coupons" of materials exposed and removed after a given period of time and subsequentlt investigated by microbilogical and biochemical methods. This approach provides results only after a relatively long time and usually requires sophisticated laboratory equipment and skills. What is needed is early warning systems which provide rapid information on line, in situ, in real time, non-destructively and suitable for computer aided automization- without requiring sample removal, staining or other secondary procedures. Such systems will be mainly based on physical principles. To be considered 'continuous" and "non-destructive", a technique must i) function in an aqueous system, ii) not require sample removal, and iii) minimize signal from organisms or contaminants in the bulk phase, and iv) provide real time data.

Interestingly, many of these sensors are either locally immobilized, delivering information an integrated signal, such as provided by measurement of friction or heat transfer resistance. However, sensors which allow for scanning of surfaces on demand would be very feasible for assessment of cleanliness of, e.g. food surfaces, but there is no information available about the successful development of such sensors.

In technical systems, pure biofilms are rare in most cases, deposits develop which consist of biological material, adsorbed particulate matter and adsorbed dissolved substances. Thus mixed deposits have to be expected which require combined countermeasures. Three levels of information can be clearly distinguished, in which current monitoring devices can be divided:

Level 1 systems, which detect the kinetics of deposition of material and changes of thickness of deposits layer but cannot differentiate between microorganisms and abiotic deposit components. Some examples for this category are:

- Fiber optical device (FOS) consisting of optical fibers, intergrated into the walls of pipes, using the intensity of backscattered light for assessing the thickness of the deposit
- Differential turbidity measurement device (DTM), consisting of two turbidity measurement devices, one of them being continuosly cleaned. The difference between the cleaned and non-cleaned device is caused by the deposit
- Quartz crystal microbalance device (QMB), exploiting the decrease of vibration caused by material deposited on the crystal surface
- Surface accoustic waves (SAW), determining the difference of the speed of surface waves on surfaces with and without deposits
- Friction resistance measurement, exploiting the pressure drop which is caused by increasing thickness and roughness of a given deposit
- Heat transfer exchange resistance device, based on the decrease of the heat transfer rate by fouling layers
- Electrochemical measurement devices which are based on the change in electric conductivity of a surface caused by a deposit or on cathodic depolarization which is indirectly attributed to microorganisms (Flemming, 2002).

The signal as provided by these methods can be attributed to microorganisms and biofilms if it has been shown that it is caused by an independent method.
Level 2 systems, which can distinguish between biotic and abiotic components of a given deposit. A suitable way to accomplish this is the specific detection of signals of biomolecules. Examples are:

- FTIR-spectroscopy specific for amid bands. This approach is suitable for system which usually do not contain biological molecules, e.g. cooling or process water systems
- Use of auto-fluorescence of amino acids such as tryptophane or other biomolecules
- Microscopical observation of biofilm formation in a bypass flow chamber and morphological identification of microorganisms (Flemming, 2002).

Level 3 systems, which provide detailed information about the chemical composition of the deposit or directly address microorganisms. Some examples:

- FTIR-ATR-spectroscopy in a flow-through cell.
- Optical fiber sensors using intensity modulation and image analysis. A fourth level would be the discrimnination of living from dead microorganisms on a surface (Flemming, 2002).

We can only monitor physical quantities in biofilm systems, such as biofilm thickness, biofilm activity, biofilm intensity, etc., and use the measurements to generate opinions about specific functions of these biofilms. For example how thick, active, dense, etc., should a biofilm be to accelerate corrosion or significantly decrease heat transfer resistance. To successfully implement biofilm monitoring, it is imperative to quantify biofilm functions that are relevant to the system being monitored from the monitored physical quantities in a biofilm, and there are no clear notions of ho this should be done. Monitoring biofilms can provide insight into fundamental biofilm process. However. One needs to be fully aware of what is actually measured, and how the measured quantities correlate with fundamental features describing biofilm systems, like rates of biofilm accumulation and microbial activity (Lewandowski and Beyenal, 2002).

There is increasing recognition that microbes such as bacteria play an even larger role in all forms of corrosion than previously thought. The development of biofilm can be described in four basic steps:

1) Conditioning film deposition

Surfaces in natural environments are never totally clean from small particles and molecules adhering to it, neither are the surfaces in paper machines. Bacteria approaching a surface do not sense the underlying material, but rather this covering conditioning film with its own characteristics and components (Green, 2001).

2) The initial microbial approach and adhesion

Following the establishment of a conditioning film, the attachment process of microbes in initiated. There have been presented several theories about the complex interactions occurring during this process and of them the Derjaguin Landau Verwey and Overbeck {DLVO} and the theory of thermodynamics will be mentioned here:

The DLVO theory predicts two distances of separation between the particles and the surface, as a result of physico-chemical interactions. The outer distance is where the bacterium is weakly held by van der Waals attraction forces, and is called the secondary minimal, {10-20 nm} [2]. From this position the bacterium can easily be removed by shear forces. As the bacterium advances towards the surface, repulsive electrostatic forces act as a barrier. Within a boundary area between the secondary and the primary minimas there is an interplay between repulsive and attractive electrostatic forces, if the bacterium comes even closer on to the surface the short range (< 1.5 nm) attraction of hydrogen bonding, hydrophobic effects and ion pair formation form irreversible binding of the bacterium to the surface. The DLVO theory can not alone account for all direct contact between the bacterium and the surface.

The thermodynamic interaction theory considers the requirement of minimal surface free energy, which is an important factor for the adhesion to take place. According to this theory, the attachment is a spontaneous change to an energetically more favorable state. Between the bacterium, the surface, and the fluid environment, three different interactions can occur. These interactions and the energy balance between them is described by a thermodynamic equation:

$\Delta F^{adh} = \gamma BS - \gamma BL - \gamma SL$

ΔF^{adh} is the free energy of adhesion, γBS is the bacterium-substratum interfacial tension, γBL is the bacterium-liquid interface interfacial, and γSL is the substratum-liquid interfacial tension. If $\Delta F^{adh} < 0$, indicating a decrease in surface-free energy, a thermodynamically favorable situation exists for microbial adhesion (Green, 2001).

3) Growth and colonization

When bacteria once have attached to the surface, they begin to grow and colonize on it, to establish themselves firmly. Among the first steps following is the production of polysaccharides, which anchor the organisms to the surface. Exopolysaccharides (EPS) production by biofilm bacteria serves many functions, including the stabilizing effect during attachment of bacteria to surfaces, and the formation and maintenance of microcolony and biofilm structure by keeping microorganisms in their 3D arrangement. The EPS (slime) also enhance biofilm resistance to environmental stress and antimicrobial agents, and give protection of the biofilm from protozoan grazing. Further more, it is assumed that EPS in a biofilm bind nutrients essential for growth, thereby creating a nutrient rich micro-environment in an otherwise nutrient-poor macroenvironment. Studies have shown that bacterial polysaccharides have the ability to bind cations. When the supply of nutrients are too low, the bacteria starts to digest EPS itself, according to the study of *P. fluorescens*. In this bacterium, production of an exopolysaccharide lyase has been observed under starvation conditions. The enzyme degrades the biofilm-associated EPS for consumption and frees cells from the biofilm scaffold to seek more favorable environments (Green, 2001).

There have been identified different stages of EPS production in biofilms: in a study of the bacterium *Pseudomonas sp.* S9 the EPS was first closely associated with the cell surface during growth, but when the cells were starved a second loosely associated polymer was produced, and bacteria detached. Essentially, little is known about the chemical structure or physical properties of biofilm-associated EPS, it contains though a number of distinct monosaccharides and non-carbohydrate substituents. Polysaccharide chains vary in size from 10^3-10^8 kDa. Depending upon the components of the repeat units, polysaccharides are usually negatively charged, sometimes neutral or rarely positively charged. Although

polysaccharides are significant subsequent to attachment and in the cell accumulation in biofilms, there is little evidence that they are involved in initial adhesion (Green, 2001).

(4) Mature biofilm formation

In the final stage of microbial colonization the cells spread over the surface and form a mature biofilm structure. Recent studies show living biofilms to consist of a variable distribution of cells and cellular aggregates, their extracellular polymers, and void spaces or water channels, which may or may not be continuous with the bulk liquid phase. The aggregates form at different horizontal and vertical sites, and not necessarily seek the direct contact with the surface. In a study it has been shown that *Vibrio parahaemolyticus* biofilms exhibited an inverted pyramid structure, with most biofilm biomass located nearest the biofilm liquid interface. Many reports confirm that living biofilms are highly hydrated, with 50-90% of the total area at each certain sectioning depth consisting either of polymer and/or void space. Therefore, biofilms can be considered as hydrogels. Normally, the depth of biofilm lies between μm and up to a few 100 μm (Green, 2001).

Evidence exists that different genes are transcribed in the planktonic and biofilm associated phases of the bacteria; life cycle. In *E. coli* it has been found that flagellin synthesis is decreased in biofilm-associated cells, while the production of EPS is increased. Flagellin is the building stone in the motility-generating organelle flagellum that accelerates the initial interactions with the surface. A reason for the decrease in flagellin synthesis could be that it destabilizes the biofilm, or is not needed anymore. In another study of *P. aeruginosa* it was found that the sigma factor σ22 positively regulates the production of the exopolysaccharide alginate, while it at the same time negatively regulates the synthesis of flagellum (Green, 2001).

When bacteria in the laboratory use are removed from where they are stored, e.g. the plastic agar encasements, they show to be viable and cultivable, even though they did not divide before. This state of zero population growth may be the norm for biofilm-associated cells, because the spatial constraints are such that surrounding exopolysaccharides impedes cell division. Instead of funneling excess energy from consumed nutrients direct into procreation, as planktonic bacteria do, the biofilm cells may use it to make exopolysaccharides (Green, 2001).

The formation of biofilms is caused by attachment of microorganisms to surfaces followed by growth (van der Kooij *et al.*, 2002). Biofilms are not formed randomly but are structured as a response to the surrounding environment, nutrient conditions and metabolic processes (Jayaraman *et al.*, 1998). The development of biofilm is promoted by microbial utilization of biodegradable compounds, either originating from treated water or from the exposed material. Biofilms play an important role in water treatment and distribution. They contribute to the removal of organic and inorganic biodegradable compound and also play a role in the removal of pathogens. Under certain conditions however, biofilm formation seriously hampers treatment process, e.g. biofouling of membranes and clogging of filter beds. Also abstraction wells or recharge wells may become clogged due to the biological process. In water distribution, excessive biofilm formation leads to a deterioration of the microbiological quality of treated water. Major disadvantages include regrowth of coliforms, multiplication of opportunistic pathogens such as *Mycobaterium spp.*, *Legionella spp.*, *Aeromonas spp.* and *Pseudomonas spp.* *Legionella* especially multiply at elevated temperatures in plumbing systems. Other problems include increased heterotrophic plate counts, complaints about invertebrates, color, taste and odor and microbially induced corrosion (van der Kooij *et al.*, 2002).

The need for increased research on biofilms is based on many factors. A better understanding of biofilm behavior is particularly important because of the many problems associated with biofilm colonization, ranging from medical infections, to the fouling of industrial components (Dawood and Brözel, 1998; Stoodley *et al.*, 1999). Physical and chemical eradication measures are typically partially effective at partial biofilm removal, with regrowth occurring over a time scale of hours to days (Saxena *et al.*, 2002). Biofilms are remarkably difficult to treat with antimicrobials (Russell *et al.*, 1997; Percival and Walker, 1999; Stoodley *et al.*, 1999). Antimicrobials may be readily inactivated or fail to penetrate into the biofilm. In addition, bacteria within biofilms have increase (up to 1000-fold higher) resistance to antimicrobial compounds, even though these same bacteria are sensitive to these agents if grown under planktonic conditions (NIH, 1998).

There are several causes for the increased resistance of biofilm cells. (i) the extracellular material of the biofilm excludes or influences the access of the disinfectant, (ii) the outer layers of the biofilm may react with and quench the

disinfectant, (iii) attachment changes the physiology of the bacteria, (iv) limited availability of key nutrients within the biofilm results in alteration of the physiology of the bacteria and induction of stress response, (v) the high cell density in the biofilm results in expression of different phenotypes (Cloete, 2002). All of these parameters have been shown to be applicable to biofilms but not all for the same biofilm or for a specific antimicrobial. It has been suggested that the structure of a biofilm can moderate the delivery of an antimicrobial agent to cells within the biofilm which may give cells time to adopt a physiologically protective set of changes against the antimicrobial agent (Luppens, 2002).

Consequently, biofilm control is costly, time consuming and frequently ineffective (Stoodley *et al.*, 1999). Biofilms increase the opportunity for gene transfer between or among bacteria. This is important since bacteria resistant to antimicrobials or chemical biocide can transfer the genes for resistance to neighboring susceptible bacteria. Gene transfer can convert a previous avirulent commensal organism into a highly virulent pathogen. Certain species of bacteria communicate with each other within the biofilm. As their density increases, the organisms secrete low molecular weight molecules that signal when the population has reached a critical threshold. This process, called quorum sensing, is responsible for the expression of virulence factors. For example, *Pseudomonas aeruginosa* produces destructive proteinases when the number of these bacteria reaches a high enough density in the airway biofilms of cystic fibrosis patients (NIH, 1998).

Bacteria express new and sometimes more virulent phenotypes when growing within a biofilm. Such phenotypes may have not been detected in the past because the organisms were grown on rich nutrient media under planktonic conditions. The growth conditions are quite different particularly in the depths of biofilms, were nutrients and oxygen are usually limited, and waste products from neighbors can be toxic. In short, bacteria found at the bottom of the biofilm look and act different than species located at the surface. Bacteria embedded within biofilms are resistant to both immunological and specific defense mechanisms of the body. Contact with a solid surface triggers the expression of a panel of bacterial enzymes which catalyse the formation of sticky polysaccharides that promote colonisation and protection (NIH, 1998; Marais and Brözel, 1998; O'Toole *et al.*, 2000). The structure of biofilms is

such that immune responses may be directed only at those antigens found on the outer surface of the biofilm, and antibodies and other serum or salivary proteins often fail to penetrate into the biofilm. In addition, phagocytes are unable to effectively engulf a bacterium growing within a complex polysaccharide matrix attached to a solid surface. This causes the phagocyte to release large amounts of pro-inflammatory enzymes and cytokines, leading to inflammation and destruction of nearby tissues (NIH, 1998).

The commonly used method to monitor hygienic water quality used by the water industry is the heterotropic plate count. However, in order to fully detect changes in the microbial community, information of the size of the population (biomass quantification), the activity of the present microorganisms and the distribution among various (active) groups of organisms (diversity) is needed (Boe-Hansen *et al.*, 2002). Controlling biofilm formation is a precondition in all stages of water supply, enabling optimal use on one side and effective limitation on the other (van der Kooij *et al.*, 2002). Until now, the dominant element of antifouling measures is still the more or less blind use of biocides in which improvement of process parameters or product quality are used for assessing the efficacy of the action (Flemming, 2002). There is therefore a need for real-time on-line methods of biofilm detection and quantification in critical applications such as dental unit water lines, dialysis machines, as well as other medical or industrial applications (Saxena *et al.*, 2002).

2.3.1 EFFECTS OF BIOFOULING

The attachment, anchoring and growth of organisms such as bacteria, algae, barnacles and blue mussels or in fresh water, zebra mussels, can significantly degrade the performance of water based facilities and vessels. Biofouling has degraded the performance of hydroelectric power plants by clogging pipes involved in cooling water intake system and compromising the performance of heat exchangers and condensers. For vessels, biofouling creates unnecessary drag and, therefore increases the fuel consumption and decreases the speed of large vessels (http://www.sri.com/psd/technologies/biocidylcoatings.html).

Multiplication of microorganisms such as *Actinomycetes* or fungi in biofilms along the drinking water distribution systems results in the deterioration of the bacteriological quality of drinking water, the development of odour or colour, taste problems and the acceleration of the phenomenon of corrosion within the pipework (Camper *et al.*, 1999; Momba *et al.*, 2000). Opportunistic pathogens that colonize pipe surfaces results in cases of gastroenteritis or other nosocomial infections. Iron bacteria may grow on ferrous metal surfaces and result in the presence of iron particulates in finished water. There is potential for coliform and heterotropic regrowth, where biofilm organisms are shed into water and place a utility in violation of regulations (Camper *et al.*, 1999). Reduced industrial performance occurs as a result of energy losses, increased heat transfer resistance and increased frictional resistance caused by presence of biofilms (McCoy *et al.*, 1981; Herbert-Guillou *et al.*, 2000).

Anaerobic bacteria can propagate in the deeper layers of the biofilm where little oxygen reaches. Some of these anaerobes are capable of metabolizing carbon from stainless steel, and some produce nitric, sulfuric or other organic acids that further accelerate corrosion. The depletion of oxygen in the wetted surfaces under bacterial colonies can result in the formation of "differential aeration cells" that can lead to galvanic corrosion. During the past several years, MIC has become recognized as a major cause of tuberculation, pitting and subsequent pipe failure in fire sprinkler systems. In steel, polymers and concrete marine construction, biofouling can be detrimental, resulting in unwanted excess drag structures and marine craft in seawater or causing blockages in pipe systems. Expensive removal by mechanical means is often required (http://marine.copper.org/1-biofouling.html). Fouling of the pipework reduce the flow channel and leads to pumping costs. The colonizing biofilm also impairs the efficiency of heat transfer (Mott *et al.*, 1998).

2.3.2 MICROBIALLY INDUCED CORROSION

The physical presence of microbial cells on the surfaces, as well as their metabolic activities can cause biocorrosion or microbially induced corrosion (Edstrom, 1995). It is defined as the deterioration of a metal by corrosion process which occur either directly as a result of metabolic activity of microorganisms (Von Holy and Cloete, 1988). The biofilms grow on the surfaces and adheres firmly and form a gel like mass

around bacterial deposits, which promotes the formation of differential oxygen cell corrosion (Bauer, 1999). The forms of corrosion caused by bacteria are not unique. Biocorrosion result in pitting, crevice corrosion, selective dealloying, stress corrosion crackling and under deposit corrosion (Edstrom, 1995).

Five factors believed to contribute to MIC are: (1) formation of oxygen concentration cell, (2) formation of ion concentration cells, (3) activities of ion and manganese oxidizing bacteria, (4) microbiological acid production and (5) creation of anaerobic conditions promoting growth of sulphate reducing microorganisms (Von Holy and Cloete, 1988).

2.3.2.1 Microorganisms involved in MIC

Various microorganisms including bacteria, fungi and algae cause MIC. Sulphur oxidizing bacteria, mainly of the genus *Thiobacillus*, form sulphuric acid which is a strongly corrosive agent to metals and concrete structures. The aerobic bacteria in the genera *Gallionella*, *Pedomicrobium* and *Siderococcus* are able to oxidize ferrous iron to ferric iron, catalyzing the deposition of tubercles on, for example, stainless steel weld-seams. Members of the filamentous bacterial genus *Leptothrix* deposit ferric oxides in their sheath. *Metallogenium*, *Pedomicrobium* and *Leptothrix* species oxidize manganese (Mn^{2+}) and deposit Mn^{4+} salts outside the cell wall (Cloete and Flemming, 1997).

The moulds *Hormonis resinae* and *Aspergillus fumigatus* are able to cause MIC in fuel tanks by production of organic acids, e.g. lactic and acetic acid. Various algae growing on metal surfaces promote corrosion by forming concentration cells, producing organic acids and by supplying nutrients to other corrosion causing microorganisms (Cloete and Flemming, 1997).

2.3.2.1.1 SRB in biocorrosion

SRB have been cited as the most important causes of MIC under anaerobic conditions. In their presence, steel and other alloys in anaerobic aqueous surroundings corrode up to four times faster than by ordinary oxygen promoted corrosion. SRB are associated with corosion where metal structures are covered by biofilms, and on metal structures on seabed since these structures give rise to anaerobic conditions. MIC caused under these conditions is accompanied by the

formation of sulphide deposits, chiefly ferrous sulphide (FeS). Hydrogen sulphide (H_2S) and some iron sulphides produced as a result of microbiological sulphate reduction are also corrosive and thus compound the severity of the problem (Cloete and Flemming, 1997).

SRB act as cathodic depolarizers and produce corrosive hydrogen sulphide. The cathode depolarization is thought of as a biological catalyst, involving the enzyme hydrogenase present in the sulphate reducing bacterial cell. Bacterial action reduces the sulphate present in the water to sulphides, using the hydrogen to form hydrogen sulphide. The hydrogen reacts with the dissolved ferrous iron, which is released at the anode to form an iron sulphide precipitate (black). Predisposing factors to MIC by SRB have been identified as high levels of sulphate, anaerobic conditions, areas of low or no flow and the presence of biological slime or other deposits (Von Holy and Cloete, 1988).

2.3.2.1.2 Acid producing bacteria

Bacteria can produce aggressive metabolites, such as organic or inorganic acids. For example *Thiobacillus thiooxidans* produces sulfuric acid and *Clostridium aceticum* produces acetic acid. Acids produced by bacteria accelerate corrosion by dissolving oxides and accelerating the cathodization reaction rate (Edstrom, 1995).

2.3.2.1.3 Hydrogen producing bacteria

Many microorganisms produce hydrogen gas as a product of carbohydrate fermentation. Hydrogen gas can diffuse to metals and cause hydrogen embrittlement (Edstrom, 1995).

2.3.2.1.4 Iron bacteria

Iron bacteria such as *Gallionella*, *Sphaerotilus*, *Leptothrix* and *Crenothrix*, are aerobic and filamentous bacteria which oxidise iron from a soluble ferrous form to an insoluble ferric form (Edstrom, 1995). These organisms thus have the ability to convert ferrous iron to ferric hydroxide. The ferric hydroxide forms a sheath on the organism that deposits on the walls of the pipes, thereby restricting flow. If a difference in oxygen concentration develops between the metal and solution, a localized corrosion well will be produced, causing tuberculation and a high corrosion

rate. The tuberculation caused by bacteria can grow until it almost closes off a pipe, and the corrosion products' sloughing off process causes blockage in sprinklers. Tuberculation can cause a severe head loss within a few months due to MIC and other types of corrosion, thereby reducing flow below safety requirement levels (Bauer, 1999).

There are mainly two strategies for handling biofilm formation: either to stop the production at regular intervals for cleaning, or instead to add biocides at constant level that continuously kill microorganisms in the process water. Along with increasing demands on environmentally friendly production and economic profit, the need increases for an effective way to control and eliminate biofilm formation. In actual practice, biofilm development is often detected by a decrease of plant performance or product quality. This is the most expensive way of monitoring. Even though there exists some techniques (e.g. based on sampling methodology) today, the ideal monitoring system is left to be invented: a nondestructive, simple, cheap, robust system that works in situ and on-line (Green, 2001).

2.4 ON-LINE TECHNIQUES FOR BIOFILM MONITORING

2.4.1 Biofilm monitoring using a fiber-optic probe

An optical fiber for the detection and quantification of biofilm has been developed by Intelligent Optical Systems (IOS). This probe detects biochemical markers only present in living cells. The probe was tested with suspended cultures and biofilms comprised of *Pseudomonas aeruginosa*. Results indicated that the probe is sensitive to the presence and concentration of bacterial cells in suspension (Saxena *et al.*, 2002).

2.4.2 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 about biofilms as present in water treatment, in distribution systems, in biofilm monitoring devices and in material testing gives a framework for evaluation of the observed concentrations. This framework has been designated as the Unified Biofilm Approach. This Unified

Biofilm approach is used in the Nederlands in combination with assimilable organic carbon (AOC) and biofilm formation rate (BFR) measurements to elucidate microbiological processes in water treatment and distribution. The objectives are to control biofilm formation in water treatment and to achieve biological stability in the distribution system (van der Kooij *et al.*, 2002).

2.4.3 Biofilm monitoring by photoaucostic spectroscopy (PAS)

Photoaucostic spectroscopy is based on the adsorption of electromagnetic radiation inside a sample where non-radiative relaxation processes convert the absorbed energy into heat. Due to the thermal expansion of the medium, a pressure wave is generated which can be detected by microphones or piezoelectric transducers. If a short laser pulse is used for excitation, he time resoled recording of the photoaucostic signal allows the depth-resolved investigation of the light absorption inside radiated part of the sample. Thus changes in the optical adsorption properties of a sample can be investigated, if the sound velocity inside the sample is known (Schimid *et al.*, 2002).

2.4.4 Biofilm assessment using 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 glass wall itself to 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 (Tinham and Bott, 2002).

2.4.5 Measuring biofilm formation using Quartz crystal microbalance with dissipation monitoring (QCM-D).

The core of the QCM-D technique is a thin quartz crystal disc. When an alternating current electric field is applied to the electrodes, a sensor crystal disc starts to oscillate

along is thickness shear-mode (along the surface). The frequency of the oscillation depends on the attached mass. The frequency (mass) is the first parameter measured by the QCM-D. A deposited film, which is visco-elastic, does not follow the crystal oscillation to the full extent and frictional energy losses occur in the film (dissipation). Consequently, the crystal oscillation is damped, causing a faster decay of the amplitude. By measuring the extent of the damping, a measure of the visco-elasticity of the layer is obtained. This is the second parameter measured by QCM-D. The quartz disc can be coated with a wide range of materials. The QCM-D can also be used in liquids, however, due to viscous damping of the vibration of the crystal film detection is considerably more involved. Density ρ and viscosity of the contacting liquid also generate a distinct frequency shift (Rudh *et al.*, 2002).

This dependence has been exploited as a liquid viscosity monitor. A visco-elastic behavior of the biofilm may appear as one reasonable drain. A non-uniform film growth will also increase the equivalent resistance. Although a detailed interpretation of such experimental results is not expected to be easy, the resistance has already been proven to be a very sensitive tool for thin biofilm investigation (Rudh *et al.*, 2002).
One of the advantages of QCM-D technique is the real time mode of measurement, allowing kinetic studies. Another advantage of the technique is that the two parameters (D-factor and f-factor) can be plotted against each other to give specific "fingerprints" of the different reactions measured (Green, 2001).

2.4.6 Detection of biofilms and pathogens using scanning confocal laser microscopy and episcopic differential interference contrast microscopy

Knowledge of biofilm structure and function has changed significantly in the last few years due to advances in light microscopy. One pertinent example is the use of scanning confocal laser microscopy (SCLM) to visualize corrosion pits caused by the biofilm mosaic footprint on corroding metal surfaces. Nevertheless, SCLM has some limitations, including cost, inability to observe motile bacteria and eukaryotic grazers within biofilms, and difficulty to scan a curved surface. By contrast, episcopic differential interference contrast (EDIC) microscopy has provided a rapid, real time analysis of biofilms on opaque, curved, natural or manmade surfaces without the need for cover slips and oil. EDIC, coupled with epi-fluorescence (EDIC/EF), microscopy has been used successfully to visualize the 3-D biofilm structure, physiological

niches, protozoal grazing and iron biomineralization, and the location of specific pathogens such as *Legionella pneumophila*, *Campylobacter jejuni* and *Cryptosporidium parvum*.

These species were identified using gold nanoparticles or fluorophores coupled to monoclonal antibodies or 16S rRNA probes, respectively. Among its many potential uses, the EDIC technique will provide a rapid procedure to facilitate the calibration of the modern generation of biofilm-sensing electrodes (Keevil, 2002).

2.4.7 Biofilm monitoring by electrochemical techniques

2.4.7.1 Linear Polarisation Resistance (LPR)

Polarisation resistance is commonly used in commercial instrument corrosimeters to measure uniform corrosion. LPR gives direct measurement of current vs. potential within 10 mV from the free corrosion potential, assuming that in this range, an applied current density is approximately linear with potential. In this range, the corrosion rate is inversely proportional to polarisation resistance at potential close to the free corrosion potential. The polarisation resistance measurement obtained in the LPR measurement represents the sum of the polarization resistance and the solution resistance. When the electrolyte conductivity is lower than 0.5-1 mS cm^{-1} (dependent geometry of probes), it is necessary to measure the ohmic drop in the electrochemical probe in order to calculate the correct corrosion current. The analysis of the LPR technique is valid if there are not other electrochemical reactions contributing to the response of the interface which would result in a very complicated system to interpret. It has been reported that problems could occur with use in MIC testing because the biofilm layer itself behaves as a resistance with a magnitude inversely proportional to the corrosion current, and the interference could act as a combination of parallel resistance-capacitance because of intermediate reactions. Nevertheless, because simplicity of measurement and instrumentation, the technique is widely used. LPR has given good result in association with other determinations (as weight loss and chemical analyses) to quantify MIC in aerobic conditions (Cristiani *et al.*, 2002).

2.4.7.2 Electrochemical Impedance Spectroscopy (EIS)

EIS is an alternate current polarisation technique where e.g., sinusoidal potential perturbation is applied to the electrode at a high frequency range. The use of this

sophisticated method is especially favourable for nonconducting and semiconducting films or in media with low conductivity. EIS has been applied for MIC studies and monitoring with differing success. Recent applications for MIC were performed, but results suggested that the technique is not sensitive enough for detecting biofilm growth thickness less than about 200 micron. However, for a rough estimation of corrosion rate the technique has proved to be useful with the same limitations given for carbon steel in anaerobic sulphide media as given for LPR. High capacitances can occur for steel in sulphide environment leading to very long measurement time. This technique is mainly applied in laboratory experiments and provides better information than LPR, for mechanistic studies in particular. A general limitation of this technique is that the data interpretation is not simple and requires specialist knowledge (Cristiani *et al.*, 2002).

2.4.7.3 Electrochemical Noise (EN)

EN is non-destructive measure of potential or current fluctuations and can be conducted to measure open circuit potential without applying an external signal. This 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. Good results have been obtained by combining this technique with corrosion rate measurements in laboratory applications. One critical aspect of this technique is the analyses and interpretation of data, e.g. simple statistical evaluation (root mean square, variance and standard deviation) (Cristiani *et al.*, 2002).

2.4.7.4 Redox potential

The oxidation-reduction (redox) 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. This technique can be useful in combination with other electrochemical measurements, such as LPR and corrosion potential, to monitor MIC. The processing of paper in paper mills was selected as a model industrial process for the monitoring if biofilms. The pulp and paper industry is an optimal environment for microorganisms on account of the abundant supply of nutrients coupled with favourable pH values and temperatures. The formation of biofilms in a waste paper medium was studied in a pilot plant by analysing the redox potential in the biofilm.

Miniaturised redox electrodes were applied at the reactor wall/biofilm phase boundary. With this measurement set-up it was possible to demonstrate the effectiveness of biocides and thus to avoid under- and over-doses of biocides. The redox signals measured were correlated with reference methods, such as colony-forming units and dehydrogenase activity. The signal of the redox sensors can, however, be measured on-line and in situ, thus permitting automation. This means that the essential demands made of a monitoring concept have been fulfilled (Cristiani *et al.*, 2002).

2.4.7.5 Electrical Resistance (ER)

ER is a traditional direct corrosion rate measuring technique, which can be applied like weight loss. The response is usually fairly long and only accumulated corrosion rate can be measured. A new, improved and patented instrument (VN instrument, Denmark, International Patent) and probe construction has increased sensitivity and reduced response time to the levels of e.g. electrochemical techniques like LPR. Since ER can work in all media without limitations regarding e.g. conductivity, this technique has the potential to be very useful for MIC. Tests have been with MIC in laboratory studies in soil and sea media with promising results (Cristiani *et al.*, 2002).

2.4.8 Microbial community structure in biofilms and water distribution network determined by lipid biomarkers

The aim of the work was to study the development of microbial communities in biofilms in a drinking water distribution system, and to compare the microbial communities in biofilms and water. The microbial communities were studied by phospholipid fatty acid (PLFA) profiles. In drinking water samples the most common PLFAs, with proportion of 58.9% to 62.9%, were monoenoic fatty acids indicating gram-negative bacteria. Instead, in biofilm samples saturated fatty acids, accounted for 54.9% to 78.9% of the total PLFAs. The proportions of monosaturated straight-chain fatty acids in water increased from 11.4% to 31.2% with water age from 22h to 62h, respectively (Keinanen *et al.*, 2002).

2.4.9 Off-line, multiparameter monitoring of major biofilm features

One of the most versatile tools to investigate biofilms in situ is laser scanning microscopy (LSM). LSM allows optical sectioning of fully hydrated living microbial

biofilms. By this technique employing 1-photon and 2-photon excitation, several important biofilm parameters can be assessed. Confocal laser scanning microscopy (single-photon excitation) has become a routine approach to investigate biofilms, whereas two-photon excitation is an emerging technique for examining microbial communities. These techniques can be used to determine biofilm thickness, reflective material, autofluorescence, cell distribution, EPS distribution, viability cell identity, enzyme activity and various parameters of the environment (Neu *et al.*, 2002).

2.4.10 Monitoring of biofilm reactors using natural fluorescence fingerprints

The concept of employing fluorescence for bioprocess monitoring, using intracellular fluorophors as indicators of the physiological state of the bacterial culture has long been known. However, it was mostly limited to monoculture fermentations and to suspended biomass systems, and typically focused on single fluorophor monitoring. However, fluorometry is prone to influences by environmental factors (such as temperature, pH and aeration) and to cascade and inner filter effects which may distort the spectra and result in shifts in the fluorophor peak location and intensity. Therefore, multiple fluorophor monitoring and scanning (bidimensional) fluorometry were introduced. The latter monitors a range of excitation and emmision wavelengths simultaneously, resulting in a three-dimensional fluorescence map (emission-vs-excitation wavelengths-vs-intensity), which has the advantage that changes outside the optical fluorophor positions are also assessable. Since changes in environmental conditions are common in bioreactors, and may even be part of process control strategies, bidimensional fluorometry is a more adequate technique than single fluorophor monitoring. Moreover, it is especially indicated for complex bioprocess employing bacterial consortia, where peak overlapping may occur due to different metabolic patterns (Wolf *et al.*, 2002).

Bidimensional fluorometry was used for monitoring a multispecies membrane-attached biofilm system. Each fluorescence map can be considered a fingerprint of the physiological state of the mixed culture, and differences in the fluorescence response can be used for assessing process status. However, due to the complexity of the multispecies system and the effects mentioned earlier, the fluorometric maps are bound to be convoluted. This recesatates deconvolution technique where the utilization of all the fluorometric information obtained is guaranteed, including

possible shifts in the maps. It is the basis for developing a comprehensive handle for assessing and predicting process status, without necessitating off-line measurements (Wolf *et al.*, 2002).

2.4.11 On-line biofilm monitoring by electrochemical probe "BIOX"

A new version of an electrochemical biofilm sensor called BIOX (CESI international patent, 1996) based on the effect of "cathodic depolarisation" was recently set by CESI in collaboration in CNR-ICMM of Genova in order to optimize the antifouling treatments based on oxidant biocide (mainly chlorination). This new system measure the coupling current between stainless steel and a sacrificial anode connected by a resistance of appropriate value, in order to decrease the coupling current and detect both cathodic process of biofilm growth and the cathodic chlorine discharge. An electrical voltage in the range 400-1400 mV represents the probe output, as a function flowing through the probe. In the case of addition of chlorine to the solution, the probe response is immediate in the case of biofilm in the case of biofilm growth onto electrodes the time response is quite longer, as the complete biofilm covering of a clean probe may require several days. For this reason, analysing the electric signal trend under regular operating conditions, it is possible to distinguish the contributions of the two phenomena to the probe output. Because of the very low current circulating, the BIOX probe could, in principle, be applied in water with relative low conductivity also (Mollica and Cristiani, 2002).

2.4.12 The use of the "Biowatch" system for biofilm monitoring

The "biowatch" system is available through ONDEO-Nalco and is based on a rotating transparent disc with a diameter of approximately 30 cm, where one half of the disc is exposed to the system water, in a chamber which houses the disc. Measurement of biofouling is done by rotating the disc out of the water and taking a photometer reading by transmitting a light beam through the transparent disc. 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.

2.4 REFERENCES

Ahern H. 1993. How bacteria cope with oxidatively damaged DNA. ASM News **59:** 119-112.

Bauer R.O. 1999. Microbially influenced corrosion (MIC) in fire sprinkler systems. Info@sprinklersolutions.com.

Bilgeri Y. R., G. Hinze, and H. J. Koornhof. 1999. Electrochemically activated water as an antimicrobial agent. Radical/Yvonne/MicrobiologicalAnalysis/publications/publications-Koornhof.doc.

Biocon Reasearch (Pty) Ltd. 1999a. Activated water: Anolyte solution (Radical waters). Acute oral toxicity (LD_{50}) study in rats. Study number 01746/01 (Interim report, unaudited).

Biocon Reasearch (Pty) Ltd. 1999b. Activated water: Catholyte solution (Radical waters). Acute oral toxicity (LD_{50}) study in rats. Study number 01747/01 (Interim report, unaudited).

Biocon Reasearch (Pty) Ltd. 1999c. Activated water: Anolyte-catholyte combination (Radical waters). Acute oral toxicity (LD_{50}) study in rats. Study number 01748/01 (Interim report, unaudited).

Biocon Reasearch (Pty) Ltd. 1999d. Activated water: anolyte solution (Radical waters). Acute dermal irritation/corrosion study in rabbits. Study number 01749/01 (Interim report, unaudited).

Biocon Reasearch (Pty) Ltd. 1999e. Activated water: catholyte solution (Radical waters). Acute dermal irritation/corrosion study in rabbits. Study number 01750/01 (Interim report, unaudited).

Biocon Reasearch (Pty) Ltd. 1999f. Activated water: anolyte-catholyte combination (Radical waters). Acute dermal irritation/corrosion study in rabbits. Study number 01751/01 (Interim report, unaudited).

Biocon Reasearch (Pty) Ltd. 1999g. Activated water: anolyte solution (Radical waters). Acute eye irritation/corrosion study in rabbits. Study number 01752/01 (Interim report, unaudited).

Biocon Reasearch (Pty) Ltd. 1999h. Activated water: catholyte solution (Radical waters). Acute eye irritation/corrosion study in rabbits. Study number 01753/01 (Interim report, unaudited).

Biocon Reasearch (Pty) Ltd. 1999i. Activated water: anolyte-catholyte combination (Radical waters). Acute eye irritation/corrosion study in rabbits. Study number 01754/01 (Interim report, unaudited).

Boe-Hansen R., A. C. Martiny, E. Arvin, and H-J Albrechtsen. 2002. Monitoring of biofilm formation and activity in drinking water distribution networks under oligotrophic conditions, p. 23-26. In: Proceedings of the International Specialised Conference on Biofilm Monitoring, Porto, Portugal.

Bognolo G., G. R. John, and J. G Evans. 1992. Surfactants and the environment-some recent developments, p. 23-28. **In: Karsa, D. R** (ed.), Industrial applications of surfactants, Cambridge. The royal Society of Chemistry.

Broxton P., P. M. Woodwock, F. Heatley, and P. Gilbert. 1984. Interaction of some polyhexametylene-biguanides and membrane phospholipids in *Escherichia coli*. J.Appl.Bacteriol. **57:**115-124.

Brözel V. S., and Cloete T.E. 1993. Bacterial resistance to conventional water treatment biocides. CAB Biodeterioration Abstracts. **7:**387-395.

Camper A. M., M. Burr, B. Ellis, P. Butterfield, and C. Albernathy. 1999. Development and structure of drinking water biofilms and techniques for their study. J. Appl. Microbiol. Symposium Supplement. **85:**1S-12S.

Characklis W.G., and K.C. Marshall. 1990. Biofilms:a basis for an interdisciplinary approach, p. 3-15. **In:Characklis W.G, Marshall K.C**. (eds). Biofilms:Wiley, New York.

Cloete T. E. 2002. Resistance mechanisms of microorganisms to antimicrobial compounds(In Press: Int. J. Biodeterioration and Biodegradation).

Cloete T. E., and H-C Flemming. 1997. Monitoring microbial populations in industrial water systems, p. 20-121. In. Proceedings of Biofouling and its control in Industrial Water Systems-A one -day Management Workshop. University of Pretoria, South Africa.

Cochran W. L., G. A. McFeters, and P. S. Steward. 2000. Reduced susceptility of thin *Pseudomonas* biofilms to hydrogen peroxide and monochloramine. J.Appl.Micrbiol. **88:**22-30.

Collier P. J., P. Austin, and P. Gilbert. 1991. Isothionolone biocides:enzyme-inhibiting prodrugs. Int.J.Pharm. **74:**195-206.

Collier P. J., A. Ramsey, R.D. Waigh, K. T. Douglas, P. Austin, and P. Gilbert. 1990. Chemical reactivity of some isothiazolone biocides. J.Appl.Bacteriol. **69:**578-584.

Costerton J.W., Z. Lewandowski, D.E. Caldwell, D. R. Korber, and H.M. Lappin-Scott. 1995. Microbial biofilms Annu.Rev.Microbiol. **49:**711-745.

Cristiani P., G. Perboni, L. Hillbert, A. Mollica, and R. Gubner. 2002. Experiences on MIC monitoring by electrochemical techniques, p. 197-200. In: Proceedings of the International Specialised Conference on Biofilm Monitoring, Porto, Portugal.

Dawood Z., and V. S. Brözel. 1998. Corrosion-enhancing potential of *Shewanella putrefaciens* isolated from industrial cooling towers. J.Appl.Microbiol. **84:**929-936.

Edstrom industries inc. 1995. http:www.edstrom.com/Lab/WaterQualityBulletins/biofilm/biofilm13.htm.

Farr S. B., and T. Kogoma. 1991. Oxidative stress response in *Escherichia coli* and *Salmonella typhimurium*. Microbiol. Rev. **55:**1309-1316.

Fitzgerald K. A., A. Davies, and A. D. Russell. 1992. Bacterial uptake of ^{14}C-chlorhexidinediacetate and ^{14}C-benzyl alcohol and the influence of phenoxyethanol and azolectin:studies with gram-negative bacteria. Microbios. **70:**77-91.

Flemming H-C. 2002. Role and levels of real time monitoring for sucessful antifouling strategies- an overview, p. 8-10. In: Proceedings of the International Specialised Conference on Biofilm Monitoring, Porto, Portugal.

Franklin T. J., and G. A Snow. 1982. Biochemistry of Antimicrobial action. 3rd edn. Chapman and Hall. London.

Gaylarde C. C., and L. H. G. Morton. 1999. Deteriogenic biofilms on buildings and their control: a review. Biofouling. **14(1):**59-74.

Gillbert P., and M. R. W. Brown. 1978. Influence of growth rate and nutrient limitation on the gross cellular composition of *Pseudomonas aeruginosa* and its resistance to 3- and 4-chlorophenol. J. Bacteriol. **133:**1066-1072.

Green H. 2001. Detection of biofilm formation using the quartz crystal microbalance with dissipation monitoring, p. 6-10. Master's thesis. Linkopings Universiteit, Department of Physics and Measurement Technology, SE-581 83 Linkoping, Sweden.

Herbert-Guillou D., B. Tribollet, and D. Festy. 2000. Influence of the hydrodynamics on the biofilm formation by mass transport analysis. Bioelectrochemistry. **53:**119-125.

Heydorn A., B. K. Ersbøll, M. Hentzer, M. R. Parsek, M. Givskov, and S. Molin. 2000. Experimental reproducibility in flow-chamber biofilms. Microbiol. **146:**2409-2415.

http://marine.copper.org/1-biofouling.html

(http://www.baeg.engr.uark.edu/FACULTY/yanbinli/projects/project1.htm).

http://www.camwell.net/electrochemical_activation.htm

http://.sri.com/psd/technologies/biocidylcoatings.html

Hyslop P.A., D.B. Hinshaw, W.A. Hasley, I.U. Schraufstätter, R. D. Sauerheber, R.G. Spragg, J.H. Jackson, and C.G. Cochrane. 1988. Mechanisms of oxidant-mediated cell injury. J.Biol.Chem. **263:**1665-1675.

Igarashi Y., and S. Watanabe. 1992. Antimicrobial activities of 2-arylthio-N-alkylmaleimides. J.Ind.Microbiol. **9:**91-96.

Jayaraman A., A. K. Sun, and T. K. Wood. 1998. Characterization of axenic *Pseudomonas fragi* and *Escherichia coli* biofilms that inhibit corrosion of SAE 1018 steel. J. Appl. Microbiol. **84:**485-492.

Keevil C. W. 2002. Rapid detection of biofilms and pathogens using scanning confocal laser microscopy and episcopic differential interference contrast microscopy, p. 169-178. In: Proceedings of the International Specialised Conference on Biofilm Monitoring, Porto, Portugal.

Keinänen M.M., L.K. Korhonen, P.J. Martikainen, and M.H Suurtari. 2002. Microbial community structure in biofilms and water of a drinking water distribution

network determined by lipid biomarkers, p. 304-306. In: Proceedings of the International Specialised Conference on Biofilm Monitoring, Porto, Portugal.

Kruger J., and E. M. Buys 1998. Reduction of beef carcass contamination with anolyte. ARC-Animal Nutrition and Animal Products Institute, Irene. Meat industry Centre.

Leonov B. I. 1999. Electrochemical systems for man and mankind, p. 15-21. In:Proceedings of the Second International Symposium on Electyrochemical Activation in Medicine, agriculture and Industry, summaries of papers and brief reports. Moscow.

Lewandowski Z., and H. Beyenal. 2002. Biofilm monitoring:A perfect solution in search for a problem, p. 34-37. In: Proceedings of the International Specialised Conference on Biofilm Monitoring, Porto, Portugal.

Lo J.M., J. C. Yu, F. I. Hutchison, and C. M Wai. 1982. Solvent extraction of dithiocarbamate complexes and back extraction with mercury (II) for determination of trace metals in seawater by atomic absorption spectroscopy. Anal. Chem. **54:**2536-2539.

Luppens S. B. I. 2002. Suspensions or biofilms and other factors that affet didinfectant testing on pathogens, p. 1-2. PhD. Thesis. Wageningen University, Wageningen, The Netherlands.

Marais J.T., and V.S. Brözel. 1999. Electrochemically activated water in dental unit lines. Br.Dent.J. **187:**154-158.

McCoy W.F., J.D. Bryers, J. Robbins, and J.W. Costerton. 1981. Observation of fouling biofilm formation. Can.J.Microbiol. **27:**910-917.

Mollica A., and P. Cristiani. 2002. On-line biofilm monitoring by electrochemical probe "BIOX", p. 201-203. In: Proceedings of the International Specialised Conference on Biofilm Monitoring, Porto, Portugal.

Momba M. N. B., R. Kfir, S. N. Venter, and T. E. Cloete. 2000. An overview of biofilm formation in distribution systems and its impact on the deterioration of water quality. Water SA **26(1):**59-66.

Mott I. E. C., D. J. Stickler, W. T. Coakley, and T. R. Bott. 1998. The removal of bacterial biofilm from water-filled tubes using axially propagated ultrasound. J. Appl. Microbiol. **84:**509-514.

Neu T. R., A. Eitner, C. Staudt, and J. R. Lawrence. 2002. Off-line, multi-parameter monitoring of major biofilm features, p. 314-316. In: Proceedings of the International Specialised Conference on Biofilm Monitoring, Porto, Portugal.

NIH (National Institude of Health). 1998. Research on microbial biofilms. http://grants.nih.gov/grants/guide/pa-files/PA-98-070.html

O'Toole G., H. B. Kaplan, and R. Kolter. 2000. Biofilm formation as microbial development. Annu. Rev. Microbiol. **54:**49-79.

Palmer R.J., and D.C. White. 1997. Developmental biology of biofilms: implications for treatment and control. Trends Microbiol. **5:**435-439.

Percival S.L., and J. T. Walker. 1999. Potable water and biofilms:A review of the public health implications. Biofouling. **14(2):**99-115.

Pietersen B., V.S. Brözel, and T.E. Cloete. 1995. The reaction of bacterial cultures to oxidizing water treatment bactericides. Water SA. **21:**173-176.

Richards R. M. E., and R. H. Cavill. 1980. Electron microscope study of the effect of benzalkonium chlorhexidine and polymyxin on *Pseudomonas cepacia*. Microbios. **29:**21-23.

Rossmoore H.W., and M. Sondossi. 1988. Application and mode of action of formaldehyde condensate biocides. Adv. Appl. Microbiol. **33:**223-275.

Rudh M., H. Green, E. Lie, and L. Sjöström. 2002. Measuring biofilm formation in real-time by Quartz Crystal Microbalance with Dissipation monitoring (QCM-D), p. 57-60. In: Proceedings of the International Specialised Conference on Biofilm Monitoring, Porto, Portugal.

Russell A. D., and I. Chopra. 1990. Understanding antibacterial action and resistance. Ellis Horwood, New York.

Russell A. D., W. B. Hugo, and G. A. J. Ayliffe. 1992. Principles and Practice of Disinfection, Preservation and Sterilization. Second edition, p. 9-106. Blackwell scientific, London.

Russell A.D., J.A. Furr, and J.Y. Mailard. 1997. Microbial susceptibility and resistance to biocides. ASM News. **63:**481-487.

Saxena I., P.J. Sturman, and J.W. Costerton. 2002. Development and testing of a fiber-optic probe for biofilm detection and quantification, p. 15-17. In: Proceedings of the International Specialised Conference on Biofilm Monitoring, Porto, Portugal.

Schimid T., U. Panne, C. Haisch, and R. Niessner. 2002. Biofilm monitoring by photoaucostic spectroscopy (PAS), p. 49-52. In: Proceedings of the International Specialised Conference on Biofilm Monitoring, Porto, Portugal.

Schraufstätter I.U., K. Browne, A. Harris, P.A. Hyslop, J.H. Jackson, O. Quehenberger, and C.G. Cochrane. 1990. Mechanisms of hypochlorite injury in target cells. J.Clin.Invest. **85:**554-562.

Stoodley P., J.D. Boyle, D. DeBeer, and H.M. Lappin-Scott. 1999. Evolving perspectives of biofilm structure. Biofouling **14(1):**75-90.

Swift Micro Laboratories. 1997. Disinfection of stainless steel cutting utensils with anolyte. Johannesburg. South Africa.

Tinham P., and T.R. Bott. 2002. Biofouling assessment using an infrared monitor, p. 53-56. In: Proceedings of the International Specialised Conference on Biofilm Monitoring, Porto, Portugal.

Van der Kooij D., J.S. Vrouwenvelder, and H. R. Veenendal. 2002. Elucidation and control of biofilm formation processes in water treatment and distribution using the unified biofilm approach, p. 18-22. In: Proceedings of the International Specialised Conference on Biofilm Monitoring, Porto, Portugal.

Von Holy, and T.E. Cloete. 1988. Practical aspects of monitoring biofilms and microbiologically induced corrosion in industrial water systems. S. Afr.J.Science. **84:**17-19.

Wainwright M. 1988. Structure and biology of bacteria relevant to the action of disinfectants, p. 1-18. **In: Payne, K. R** (ed.), Industrial biocides. John Wiley and Sons, Chichester.

Wallhäuser K. H. 1995. Praxis der Sterilisation:Desinfektion-Konservierung, Keimidentizierung-Betriebshygiene. Georg Thieme Verlag, Stuttgart.

Wolf G., J.S. Almeida, J. G. Crespo, and M.A.M. Reis. 2002. Monitoring of biofilm reactors using natural fluorescence fingerprints, p. 329-332. In: Proceedings of the International Specialised Conference on Biofilm Monitoring, Porto, Portugal.

Woodcock P. M. 1988. Bioguanines as industrial biocides, p. 19-36. **In; Payne, K. R** (ed.), Industrial Biocides. John Wiley and sons, Chichester.

Chapter 3

ANTIMICROBIAL EFFECTS OF ECA WATER USING NaCl AND $NaHCO_3$ AS REAGENTS

3.1 INTRODUCTION

A range of bactericidal substances, commonly termed biocides or microbiocides, are available, all of which are claimed by their agents to kill bacteria in aqueous systems quantitatively. However, different bacteria react differently to bactericides, either due to differing cell wall properties (Hoppe, 1984; Paulos, 1987), or to other mechanisms of resistance, either inherent or inducible (Heinzel, 1988; Von Holy and Cloete, 1988; Cloete *et al.*, 1998).

The bacterial cell membrane provides the osmotic barrier for the cell and catalyses the active transport of substances into that cell. Alternations in transmembrane potential caused by the action of electro donor or electron acceptor factors are associated with powerful electro-osmotic processes accompanied by water diffusion against ORP gradients with resultant rupture of the membranes and outflow of the bacterial cell contents. The bacterial membrane itself has an electrical charge. The anions present in anolyte act on this membrane. Anolyte can disrupt other functions of the cell. Unlike higher organisms, single celled organisms such as bacteria obtain their energy sources from the environment immediately outside the cell. Small molecules are transported across the cell membrane via an electrochemical gradient. Thus, any significant change in the ORP of the immediate environment has drastic consequences for the cell. Even if instantaneous death of the cell does not occur, all enzymatic functions in the membrane are affected and this will result in the loss of cell viability.
A novel way of the electro chemical activation of water was recently introduced in South Africa. During Electro Chemical Activation (ECA) of water, a dilute saline solution is "activated" by passing through a cylindrical electrolytic cell in which the anodic and cathodic chambers are separated by a permeable membrane. Two separate streams of activated water are produced: Anolyte with a pH range of 2-9 and an oxidation- reduction potential (ORP) of +400 mV to +1200 mV. Anolyte is an oxidizing agent due to a mixture of free radicals and has an antimicrobial effect.

Catholyte with pH of 12 to 13 and an ORP of about –900mV, it has reducing and surfactant properties and is an antioxidant. During the process of electrochemical activation three broad classes of product are produced:

- Stable products – these are acids (in the anolyte) and bases (in the catholyte) which influence the pH of the solution in question, as well as other active species.
- Highly active unstable products – these include free radicals and other active ion species with a typical lifetime of less than 48 hours. Included here would be electrically and chemically active micro bubbles of electrolytic gas 0,2 – 0,5 micrometer in diameter and with concentrations up to 10^7 ml^{-1}, distributed uniformly through the solution. All these species serve to enhance the oxidation – reduction potentials (ORP) of the anolyte.
- Quasi-stable structures – these are structures formed at or near the electrode surface as a consequence of the very high voltage drop (10^7 V cm^{-1}) in those regions. These are free structural complexes of hydrated membranes around ions, molecules, radicals and atoms. The size of these water clusters is reduced to approximately 5-6 molecules per cluster. All these features enhance the diffusion, catalytic and biocatalytic properties of the water.

The chemical composition of ECA solutions may be altered by utilizing various hydraulic arrangements linking electrolytic cell modules, together with other supplementary devices, in order to optimally address the requirements of specific areas of application. Some other variables are flow rate; hydraulic pressure; current density and voltage on the electrodes.

One of the most important problems for researchers into ECA processes' mechanism is that of the nature of the state metastable water and diluted water solutions find themselves in after unipolar electrochemical exposure. Until now this problem has not been satisfactorily solved, nevertheless it is not an obstacle to wide practical application of electrochemically activated liquids. The problem lies in the fact that it is extremely difficult to assess activation contribution of purely chemical and purely physical components of electrochemical effect on para-electrode environment.

During anode electrochemical treatment, water acidity grows. ORP increases due to the formation of stable and unstable acids (Sulfuric, hydrochloric, hypochlorous, persulfuric), as well as hydrogen peroxide, peroxo-sulfates, peroxo-carbonates, oxygen-containing chlorine compounds and different intermediate compounds arising in the process of spontaneous decomposition and interaction of the indicated substances. Also, as a result of anode electrochemical treatment surface tension somewhat decreases, electric conductivity rises, as does the content of dissolved chlorine and oxygen, concentration of hydrogen and nitrogen decreases, and water structure changes.

Electrochemically synthesized reagents are rapidly finding application in a variety of manmade environments. Not only does this technology offer a cost-effective alternative to existing technologies, but is also contributing to the protection of the environment through the implementation of electrochemical-based effluent treatment and waste minimization processes. Electrochemically synthesized reagents (with reference to the anolyte) have antimicrobial properties and can potentially be used as surface disinfectants (http://www.camwell.net/electr.ochemical_activation.htm).

The advantages of electrochemically synthesized reagents can be listed as follows:

- These reagents are synthesized from diluted solutions of inorganic salts. This is in contrast to traditional chemical reagents, which are prepared by dissolving corrosive acids and alkalis.
- Important parameters such as pH and oxidation-reduction potential (ORP) are manipulated by variation of the inorganic salt mixture. To achieve a similar level of manipulation for chemical reagents, additional compounds have to be added.
- Reaction capacity and electrochemical reagent parameters can be varied according to the customer's requirements.
- Since electrochemical synthesis of reagents occur on-site, transportation costs are eliminated. This is in contrast to the use of traditional chemicals, which require timely planning, purchasing and proper storage infrastructure.
- Since electrochemically synthesized reagents are produced by an environmentally safe technology, the need for neutralization or purification prior to use is absent.

The objective of this study was to determine the minimum inhibitory concentration of NaCl derived anolyte using 15 reference bacterial strains, and to assess the antimicrobial properties of $NaHCO_3$ derived anolyte.

3.2 MATERIALS AND METHODS

3.2.1 Minimum inhibitory concentration determination

Test isolates were obtained from the culture collection of the Department of Microbiology and Plant Pathology, University of Pretoria. Bacterial cultures were grown on nutrient agar for 24h at 30°C. Suspensions were diluted to ca. 3 x 10^8 cfu/ml using the McFarland scale. Initial counts of suspensions were determined by spreading 0.1 ml quantities of a serial dilution series onto nutrient plates. Anolyte produced using a 3% NaCl solution was initially added to the culture suspensions to a final concentration of 1: 10 (anolyte: water), 1: 20 and 100% undiluted anolyte. Suspensions were incubated at 25°C for 6h. Viable counts were determined as described above. The kill percentage was calculated for each individual culture-anolyte combination using the following formula:

$$\% \text{ Kill} = 100 - (\text{survivor count/initial count}) \times 100$$

3.2.2 Preparation of cell suspension

The test organisms (*Escherichia coli*, *Bacillus subtilis*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*) were grown in Nutrient broth (Biolab) for 24h at 37°C. 2 ml of this cell suspension was centrifuged for 15 min at 2200 rpm. The supernatant was discarded and the pellet resuspended in sterile quarter strength Ringer's solution (Merck). This cell suspension was used to prepare the appropriate test dilutions. The experiment was designed to construct a matrix evaluating the effect of decreasing anolyte concentration versus decreasing cell concentration of each organism. The $NaHCO_3$ anolyte was distributed in 9.9 ml quantities in sterile borosilicate test tubes. 0.1 ml of the cell suspension (at the predetermined concentration) was added to the disinfectant solution, and 0.1 ml was added to sterile quarter strength Ringer's solution as a control. After exposure period of 8min, 0.1 ml was plated on Nutrient agar plates. The plates were inspected for bacterial growth after 24-48h incubation at 37°C. 100% kill was assumed when no growth was visible on the agar plates.

3.3 RESULTS AND DISCUSSION

3.3.1 Minimum inhibitory concentration

NaCl derived anolyte gave a 100% kill of all test isolates at a concentration of 100% and 10% (Table 3.1). Anolyte at a 1:20 dilution gave a 100% kill of *A. calcoaceticus*, *L. brevis*, *M. luteus*, *S. aureus* and *M. roseus* (Table 3.1). The kill percentages of other test organisms using a 1:20 anolyte dilution varied, ranging from 31%-100% (Table 3.1). This indicated variable susceptibility of different bacteria to anolyte. This is not an uncommon phenomenon. Many organisms are intrinsically more tolerant of antimicrobial substances than others (Brözel and Cloete, 1991).

Table 3.1: Percentage kill of bacterial strains at different anolyte (produced using 3% NaCl) concentrations.

		Anolyte concentration		
Bacterial strain	**Gram stain**	**100%**	**1:10**	**1:20**
Bacillus subtilis	+	100	100	78
Pseudomonas aeruginosa	-	100	100	87
Acinetobacter calcoaceticus	-	100	100	100
Lactobacillus brevis	+	100	100	100
Micrococcus luteus	+	100	100	100
Streptococcus faecalis	+	100	100	31
Pseudomonas fluorescens	-	100	100	66
Staphylococcus aureus	+	100	100	100
Pseudomonas alcalgenes	-	100	100	52
Pseudomonas medocina	-	100	100	88
Pseudomonas putida	-	100	100	90
Bacillus cereus	+	100	100	92
Micrococcus roseus	+	100	100	100
Pseudomonas stutzeri	-	100	100	57
Pseudomonas syringae	-	100	100	87

Anolyte was more effective against Gram positive bacterial strains at 1:20 dilution. A 100% kill against all Gram positive strains was obtained except *S. faecalis, Bacillus cereus* and *B. subtilis* (Table 3.1). A 100% kill of *A calcoaceticus* (Gram negative) was also obtained at a 1:20 anolyte dilution (Table 3.1).

3.3.2. Antibacterial properties of sodium bicarbonate anolyte

Table 3.2: Antimicrobial effect of $NaHCO_3$ anolyte on test microorganisms

Test organism: *Escherichia coli*					
Anolyte concentration	**cfu/ml**				
	10^6	10^5	10^4	10^3	10^2
Neat	G	NG	NG	NG	NG
1:10	G	G	G	G	G
1:50	G	G	G	G	G
1:100	G	G	G	G	G
1:1000	G	G	G	G	G
1:10 000	G	G	G	G	G
Test organism: *Pseudomonas aeruginosa*					
Anolyte concentration	**cfu/ml**				
	10^6	10^5	10^4	10^3	10^2
Neat	NG	NG	NG	NG	NG
1:10	NG	NG	NG	NG	NG
1:50	NG	NG	NG	NG	NG
1:100	G	G	NG	NG	NG
1:1000	G	G	G	G	NG
1:10 000	G	G	G	G	G
Test organism: *Bacillus subtilis*					
Anolyte concentration	**cfu/ml**				
	10^6	10^5	10^4	10^3	10^2
Neat	G	NG	NG	NG	NG
1:10	G	G	NG	NG	NG
1:50	G	G	G	NG	NG
1:100	G	G	G	NG	NG
1:1000	G	G	G	NG	NG
1:10 000	G	G	G	NG	NG
Test organism: *Staphylococcus aureus*					
Anolyte concentration	**cfu/ml**				
	10^6	10^5	10^4	10^3	10^2
Neat	G	NG	NG	NG	NG
1:10	G	G	G	G	G
1:50	G	G	G	G	G
1:100	G	G	G	G	G
1:1000	G	G	G	G	G
1:10 000	G	G	G	G	G

cfu= Colony forming units,G= Growth,NG= No growth

The sodium bicarbonate derived anolyte showed to have some antimicrobial properties (Table 3.2). Neat anolyte was effective against *E. coli* cells at a concentration of 10^5 cfu/ml but not at 10^6 cfu/ml. All other concentrations of anolyte tested (1:10-1:10000) were ineffective against *E. coli* at all concentrations tested (Table 3.2). The neat, 1:10 and 1:50 anolyte killed *P. aeruginosa* at cell concentration of 10^6 cfu/ml. The 1:100 anolyte was effective against *P. aeruginosa* at a concentration of 10^4 cfu/ml. The 1:1000 killed the same cells at 10^2 cfu/ml while 1:10000 was ineffective even at the lowest cell concentration tested (Table 3.2). *Bacillus subtilis* at cell concentration of 10^5 cfu/ml was killed by neat anolyte while 1:10 anolyte was effective against *B. subtilis* at 10^4 cfu/ml concentration (Table 3.2). The 1:50, 1:100, 1:1000 and 1:10000 were effective against *B. subtilis* cell concentration of 10^3 cfu/ml (Table 3.2). The sodium bicarbonate anolyte was moderately effective against *B. subtilis* at high concentrations (10^4-10^6) but very effective at lower concentrations. The response of *S.aureus* to anolyte treatment was the same as that of *E. coli*. Neat anolyte was effective against 10^5 cfu/ml of *S. aureus* while the other anolyte concentrations tested were ineffective to *S aureus* at all cell concentrations tested (Table 3.2).

3.4 CONCLUSIONS

- The MIC of sodium bicarbonate anolyte was lower for *P. aeruginosa* than any other bacterial strain tested. The highest MIC was against *E. coli* and *S. aureus*
- Neat and 1:10 NaCl anolyte were effective against all organisms tested. Efficacy of 1:20 anolyte among organisms ranged from 31%-100%
- NaCl anolyte was more effective against Gram positive bacteria than Gram negatives
- The neat anolyte was more effective than the dilute anolyte

3.5 REFERENCES

Brözel V. S., and T. E. Cloete. 1991. Fingerprinting of commercially available water treatment bacteriocides in South Africa. Water SA. **17:**57-66.

Heinzel M. 1988. The phenomena of resistance to disinfectants and preservatives, p. 52-67. **In: Payne K. R** (ed.). Industrial biocides. John Wiley and Sons.

http://www.camwell.net/electr.ochemical_activation.htm.

Hoppe H. G. 1984. Attachment of bacteria: advantages and disadvantages for survival in the aquatic environment, p. 283-301. **In: Marshall K. C** (ed.). Microbial adhesion and aggregation. Berlin Springer Verlag.

Paulos W. 1987. Development of microbiocides for the protection of materials, p. 1-19. **In: Houghton, R. N. Smith and H. Eggins** (eds.). Biodeterioration. Elsevier Applied Sciences, London.

Von Holy, and T. E. Cloete. 1988. Practical aspects of monitoring biofilms and microbiologically induced corrosion in industrial water systems. S. Afr. J. Science. **84:**17-19.

Chapter 4

THE EFFECT OF SODIUM CHLORIDE AND SODIUM BICARBONATE DERIVED ANOLYTES, AND SODIUM HYPOCHLORITE ON BIOFILMS

4.1 INTRODUCTION

Adhesion to surfaces is a common and well known behaviour of microorganisms in oligotrophic habitats. This adhesion and subsequent metabolism lead to the formation of biofilms (McCoy *et al.*, 1981). Bacterial biofilms promote increased biomass deposition (Whitekettle, 1991), resulting in fluid flow resistance, loss of heat exchange and microbial induced corrosion in industrial water cooling systems (Cloete *et al.*, 1992).

Industries control unwanted biofilms, with varying degrees of success, by using biocides (Cloete *et al.*, 1992). The use of biocides, especially chlorine, in water reticulation and heat exchange systems is effective only if the biofilm is removed manually. Chlorination of a mature biofilm is usually unsuccessful because the biocide only reacts with the outer portion of the biofilm, leaving a healthy and substantial bacterial community on the surface that rapidly regrows (Brözel and Cloete, 1992). Bacteria within biofilms develop increasing resistance to the biocide on repeated dosing (Cloete *et al.*, 1992).

Microbial biofilms are problematic in a range of industrial environments where large areas of submerged surfaces are exposed to relatively high nutrient fluxes, providing niches for the formation of copious surface-associated growth (Cloete *et al.*, 1992; Costerton *et al.*, 1994). Bacterial colonisation of surfaces in an aqueous environment is a basic strategy for survival in nature as nutrients are more available at the solid - liquid interface (Hoppe, 1984; Lawrence *et al.*, 1987). The resulting aggregates form microcolonies which develop into biofilms (McCoy *et al.*, 1981). Biofilms increase fluid frictional resistance and decrease the rate of heat energy transfer, collectively termed biofouling. These biofilms also promote corrosion of ferrous and other metals by the concerted metabolic activity of a number of biofilm-associated bacterial types

(McLeod *et al.*, 1998), a process collectively termed microbially influenced corrosion (MIC). MIC encompasses a number of specific mechanisms relating either directly or indirectly to the metabolic activity of a variety of microorganisms, notably the action of sulphidogenic bacteria (Lee *et al.*, 1995; Dawood and Brözel, 1998). The food industry has also realized that adhesion and colonisation of bacteria may cause problems. Bacteria colonizing the processing equipment may be important source of bacterial contamination, and studies have shown that both spoilage bacteria like *Pseudomonas* spp. and pathogenic bacteria like *Listeria monocytogens* may contaminate products directly from the processing environment (Bagge *et al.*, 2001). As the costs attributable to MIC and biofouling are high, effective control of bacterial numbers in industrial aqueous environments is essential.

A range of bactericidal substances, commonly termed biocides or microbiocides, are available, all of which are claimed by their producers to kill bacteria occurring in aqueous systems quantitatively. Biocides target a range of cellular loci, from the cytoplasmic membrane to respiratory functions, enzymes and the genetic material (Russel and Chopra, 1990). However, different bacteria react differently to bactericides, either due to inherent differences such as unique cell envelope composition and non-susceptible proteins (Brözel and Cloete, 1991), or to the development of resistance, either by adaptation or by genetic exchange (Brözel and Cloete, 1993). Bactericides should therefore be evaluated against the organisms which they are chosen to control, i.e. the dominant ones in the system to be treated. The composition of microbial populations in systems varies with the type of water used, and changes considerably following treatment with various biocides by selection for resistant strains. Bacteria growing as biofilms are also significantly more resistant to most antimicrobial agents known currently, so that methods for their control pose an ongoing challenge (Costerton *et al.*, 1994; Cochran *et al.*, 2000).

Successful biofouling control depends on rationally developed treatment strategies which are based on information of the specific system. The primary target should always be the biofilm-associated flora as it is the catalyst for MIC and impacts negatively on system operation. Five approaches are currently available and may be used in combination:

(i) bacteria are chemically killed by application of biocides;

(ii) cells are dislodged from surfaces by dispersants;

(iii) the biofilm structure is weakened by enzymes or chelants of divalent cations;

(iv) biofilms are removed physically by a variety of processes, and

(v) biocide efficacy is enhanced by applying an alternating current or ultrasonic sound across the biofilm.

Chlorine is commonly used for treating industrial water systems as an antifouling strategy. The most important types of chlorine-releasing agents (CRAs) are sodium hypochlorite, chlorine dioxode, and the N-chloro compounds such as dichloroisocyanurate (NaDCC), with chloramine-T being used to some extent. Sodium hypochlorite solutions are used for hard surface disinfection (household bleach). In water, sodium hypochlorite ionizes to produce Na^+ and the hypochlorite ion, OCl^-, which establishes an equilibrium with hypochlorous acid (HOCl). Between pH 4 and 7, chlorine exists predominantly as HClO, the active moiety, whereas above pH 9, OCl^- predominates. Although CRAs have been predominantly used as hard surface disinfectants, novel acidified sodium chlorite (a two component system of sodium chlorite and mandelic acid) has been described as an effective antiseptic (Norwood and Gilmour, 2000).

Surprisingly, despite being widely studied, the actual mechanism of action of CRAs is not fully known. CRAs are highly active as oxidizing agents and thereby destroy the cellular activity of proteins; potentiation of oxidation may occur at low pH, where activity of CRAs is maximal, although increased penetration of outer cell layers may be achieved with CRAs in the unionized state. Hypochlorous acid has long been considered the active moiety responsible for bacterial inactivation by CRAs, the OCl^- ion having a minute effect compared to undissolved HOCl. CRAs at higher concentrations are sporicidal; this depends on the pH and concentration of available chlorine. CRAs also possess virucidal activity (Norwood and Gilmour, 2000).

Research has indicated the problem of microbial resistance to biocides. Very little information is further more available on the biodegradability of these compounds in natural water systems. This makes these compounds harzadous from an environmental point of view. Chlorine is the most widely used oxidising biocide, with its own limitations. An environmentally sensible alternative to chlorine and

other commonly used biocides is needed. Electrochemically activated water may provide such an alternative. Water of varying mineralization is passed through an electrochemical cell, the specific design of which permits the harnessing of two distinct and electrically opposite streams of activated water. Aside from its distinctive attributes, the negatively charged antioxidant solution (catholyte) can also be channelled back into the anode chamber, thereby modulating the quality of the positively charged oxidant solution (anolyte) that is produced.

Without maintenance of the activated stage these diverse products degrade to the relaxed state of benign water and the anomalous attributes of the activated solutions such as altered conductivity and surface tension similarly revert to pre-activation status. However, the heightened electrical activity and altered physico-chemical attributes of solutions differ significantly from benign state, but yet remain non-toxic to mammalian tissue and the environment. ECA water is less toxic, less volatile, easier to handle, compatible with other water treatment chemicals, effective against biofilms and generates no by-products compared to currently used biocides. The objectives of this study were to use microscopy to:

- Evaluate the biofilm removal efficiency of Sodium Hypochlorite.
- Evaluate biofilm removal efficiency of sodium chloride and sodium bicarbonate and NaCl derived anolytes.
- Evaluate the biofilm removal efficiency of a combination of anolyte and catholyte

4.2 MATERIALS AND METHODS

4.2.1 Experimental procedures

A continuous flow - through system Pedersen device was used to determine the biofilm removal of *Pseudomonas aeruginosa* on a stainless steel surface and on glass.

4.2.2 pH and oxidation reduction potential (ORP) measurements

Serial dilutions of the Sodium hypochlorite solution (10,25,50,100 and 300 ppm) were prepared and the pH and ORP values were measured. The pH and ORP were measured using probes from Radical waters.

4.2.3 Biofilm removal

Pseudomonas aeruginosa bacterial cells were allowed to adhere to glass slides and stainless steel coupons in Nutrient broth for four weeks for sodium hypochlorite treated biofilm and two weeks for anolyte treated biofilm. A continuous flow through system was used to determine biofilm removal on the glass and stainless steel surfaces. Samples were removed before and after treatment with Sodium hypochlorite, NaCl or $NaHCO_3$ anolyte. Tap water was allowed to flow through the reactor for 30 min before treatment with Sodium hypochlorite, and for 20 min before treatment with $NaHCO_3$ anolyte. Samples removed after treatment with tap water were used as the control. The samples were exposed to different concentrations of Sodium hypochlorite, exposure time being 30 min for each solution. Exposure time for anolyte was 20 min. The sodium hypochlorite concentrations used were 10 ppm, 25 ppm, 100 ppm and 300 ppm. The anolyte dilutions used were 1:10, 1:100 and neat (undiluted) for $NaHCO_3$ anolyte and only 1:10 for NaCl anolyte. The experiment was performed starting from the less concentrated to the more concentrated biocide solution. The glass slides were viewed under the light microscope while the stainless steel coupons were prepared for scanning electron microscopy.

For biofilm treatment using NaCl anolyte and anolyte/catholyte combination, bacteria were allowed to adhere to stainless steel coupons for 168h in R2A broth. The experiment was allowed to proceed for 78h. Samples were removed before treatment and hourly for 6h following treatment. The anolyte/catholyte combination at a ratio of (2:1) and the anolyte were used at a concentration of 1:10. Some of the coupons were used for Epifluorescence microscopy while others were prepared for SEM. For both the NaCl anolyte and anolyte/catholyte experiments a control system, using dam water with no biocide added, was included. All ECA solutions were supplied by Radical waters.

4.2.4 Light Microscopy

Attached bacteria were observed under oil immersion using light microscopy.

4.2.5 Scanning Electron Microscopy (SEM)

Coupons (25 x 27 x 1mm) were removed form the modified Pederson device after 30 min exposure to Sodium hypochlorite solutions. After removal the coupons were

rinsed with sterile distilled water for 30 s to remove any unattached cells and then fixed for SEM by the following series of treatments: 2.5 % gluteraldehyde (30 min); 0.15M Phosphate- buffer (3 x 15min); 50% ethanol (1 x 15min); 70% ethanol (1 x 15min); 90% ethanol (1 x 15 min) and 100% ethanol (3 x 15min). The coupons were thereafter dried in a critical point dryer, mounted on studs and coated with gold plasma and examined using the JEOL 840 scanning electron microscope.

4.2.6 DAPI staining

Quantification of attached bacteria using 4,6-diamino-z-phenylindole. Attached bacteria were observed under oil immersion using epifluorescence microscopy. Ten randomly chosen microscope fields were counted under the 800x magnification

4.3 RESULTS AND DISCUSSION

Table 4.1: pH and ORP values for the different sodium hypochlorite solutions

Solution concentration	**pH**	**ORP** (mV)
10 ppm	9.82	468
25 ppm	10.43	415
50 ppm	10.95	461
100 ppm	11.26	527
300 ppm	11.64	730

A matured biofilm had formed on both the glass and stainless steel surfaces within four weeks (Fig. 4.1 A and Fig. 4.2A). The 10 and 25 ppm sodium hypochlorite did not have an effect on the biofilms. The biofilm was still intact as though it had not undergone any treatment (Fig.4.1 B,C and Fig. 4.2 B,C). The 50 ppm sodium hypochlorite disrupted some areas of the biofilm (Fig. 4.2 D). The layer attaching the cells was ruptured at 50 ppm of sodium hypochlorite (Fig. 4.2 D), but the biofilm was not removed, leaving some areas of the biofilm intact. The biofilm was mostly disrupted by the 100 and 300 ppm solutions. Most of the cells on the glass slides treated with 100 ppm solution appeared dispersed all over the glass surface, which is an indication that the clusters were completely disrupted (Fig.4.1 D). The 300 ppm solution managed to remove the cells from the glass as only a few cells were remaining on the surface (Fig.4.1 E). The SEM for both the 100 and 300 ppm treated samples showed improved results. A large surface area of the stainless steel was clean, though there were still a number of cells remaining (Fig. 4.2 E, F). These results are in agreement with that of Norwood and Gilmour (2000), who indicated that when steady state biofilms in the constant-depth film fermenter were exposed to increasing strengths of hypochlorite; 200, 500 and 1000 ppm free chlorine, a substantial two log cycle drop in bacterial numbers was only achieved at 1000 ppm free chlorine. In planktonic culture the organisms were completely eliminated when exposed to 10 ppm free chlorine for 30s period.

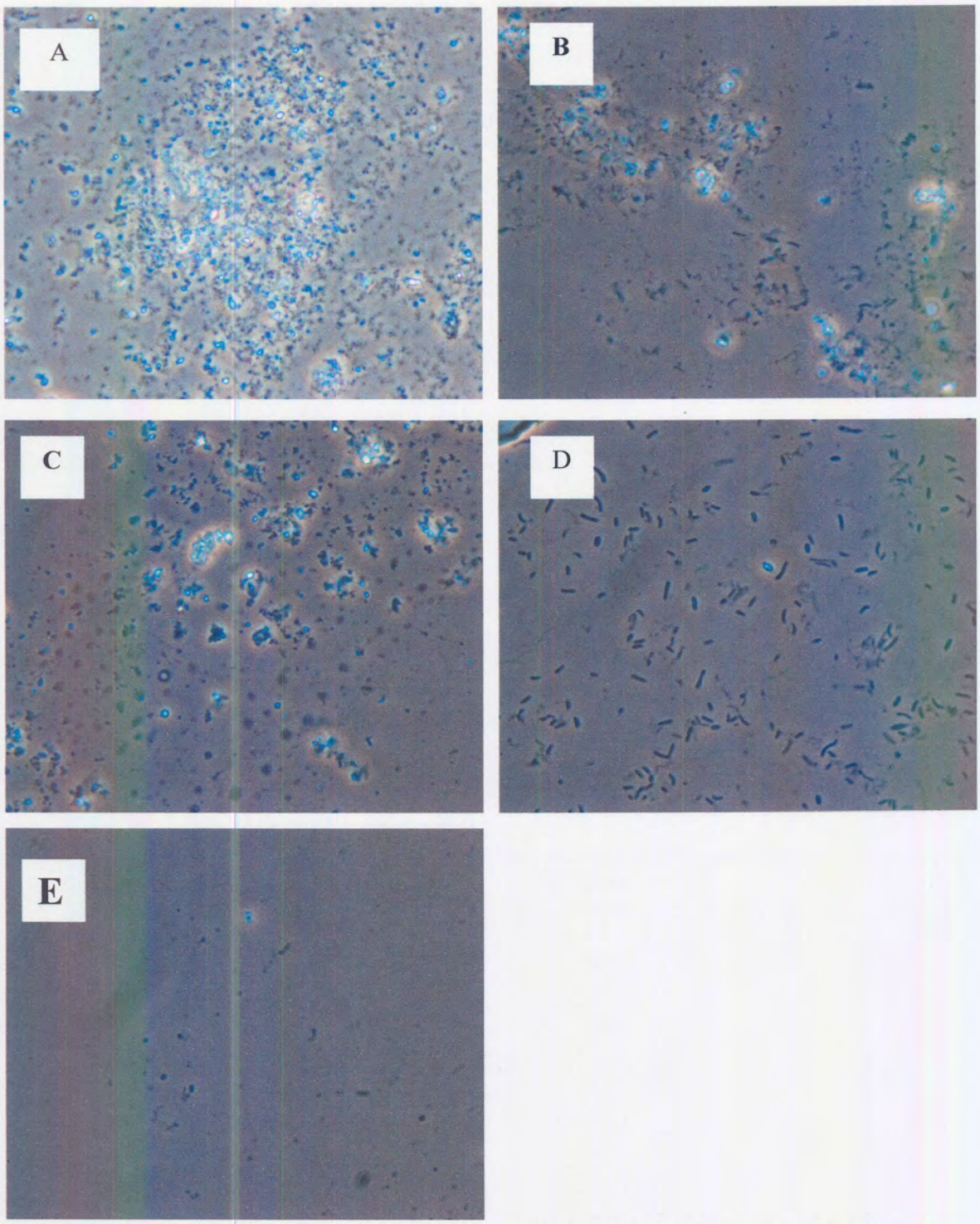


Fig. 4.1: Light microscope biofilm pictures (A) control and after treatment with (B) 10ppm, (C) 25 ppm, (D) 100 ppm and (E) 300 ppm sodium hypochlorite

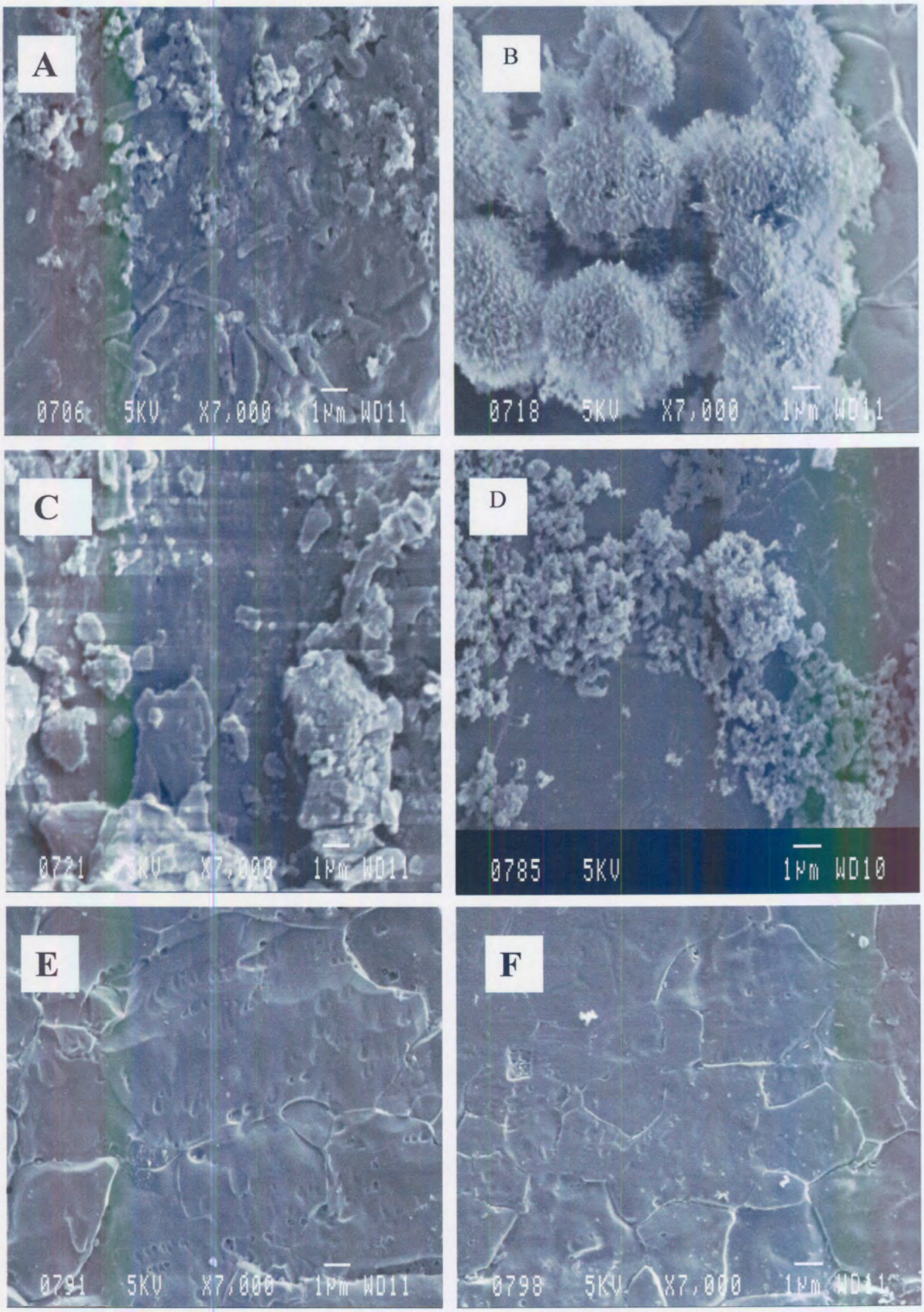


Fig. 4.2: SEM biofilm pictures (A) control and after treatment with (B) 10 ppm, (C) 25 ppm, (D) 50 ppm, (E) 100 ppm and (F) 300 ppm sodium hypochlorite

Light microscopy results indicated that a mature biofilm had formed on the glass slides after two weeks (Fig 4.3 (a)). Exposure of the biofilm to a 1:100 dilution of anolyte did not yield any noticeable removal of biofilm (Fig. 4.3 (b)). A 1:10 and a neat (undiluted) solution of anolyte disrupted and removed the biofilm that had formed on the surface of the glass slide after a 20 min exposure (Fig. 4.3 (c and d)).

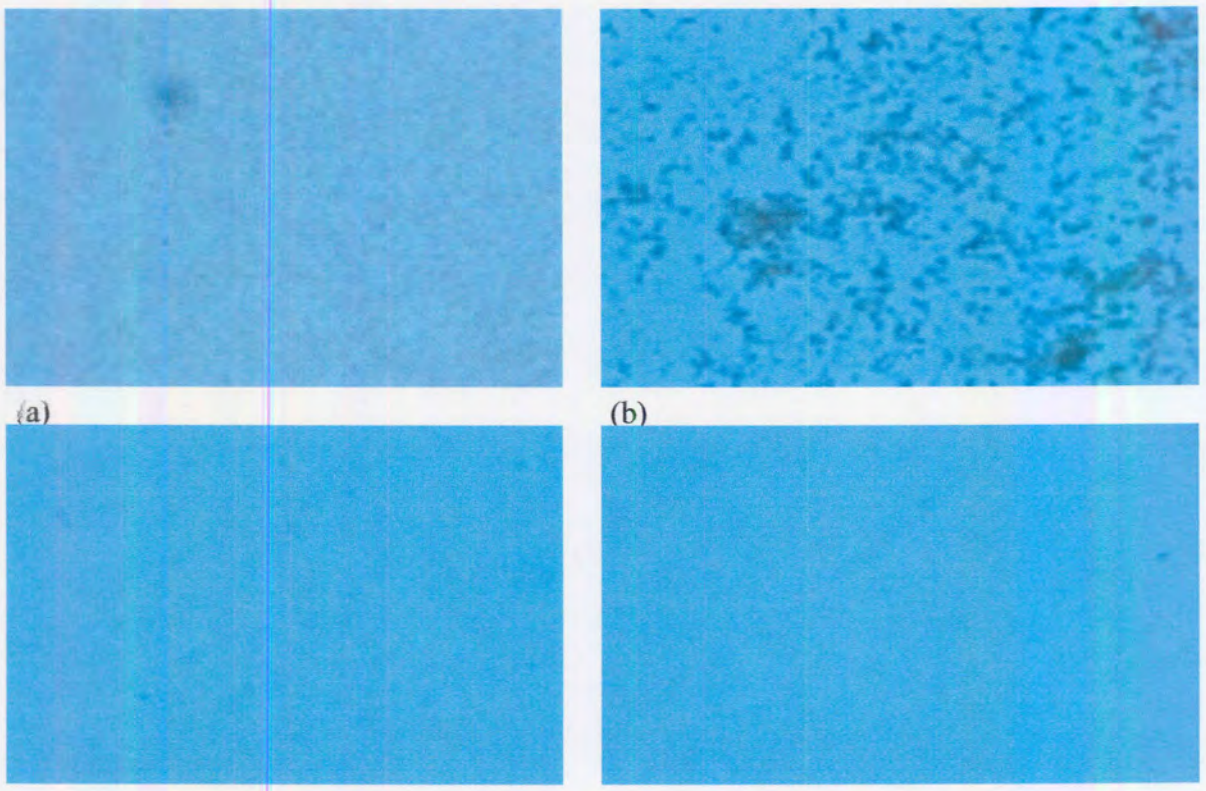


Fig 4.3: Light microscope pictures of biofilm (a) control and after treatment with (b) 1: 100, (c) 1:10 and (d) neat sodium bicarbonate derived anolyte

The same observations as those of light microscopy were found on the SEM pictures. The control (Fig. 4.4(control) showed that a mature biofilm had also formed on the stainless steel coupons after two weeks. Exposure of the biofilm to a 1:100 dilution of anolyte did not have any noticeable removal of biofilm (Fig 4.4(1:100)). The biofilm structure remained intact as though the biofilm was not subjected to any treatment with a disinfectant (Fig. 4.4(1:100)). A 1:10 dilution and neat solutions of anolyte resulted in dispersion and removal of the biofilm after a 20 min exposure (Fig. 4.4(1:10 and neat)). That is, effective removal of biofilm was achieved when these two concentrations of anolyte were used.

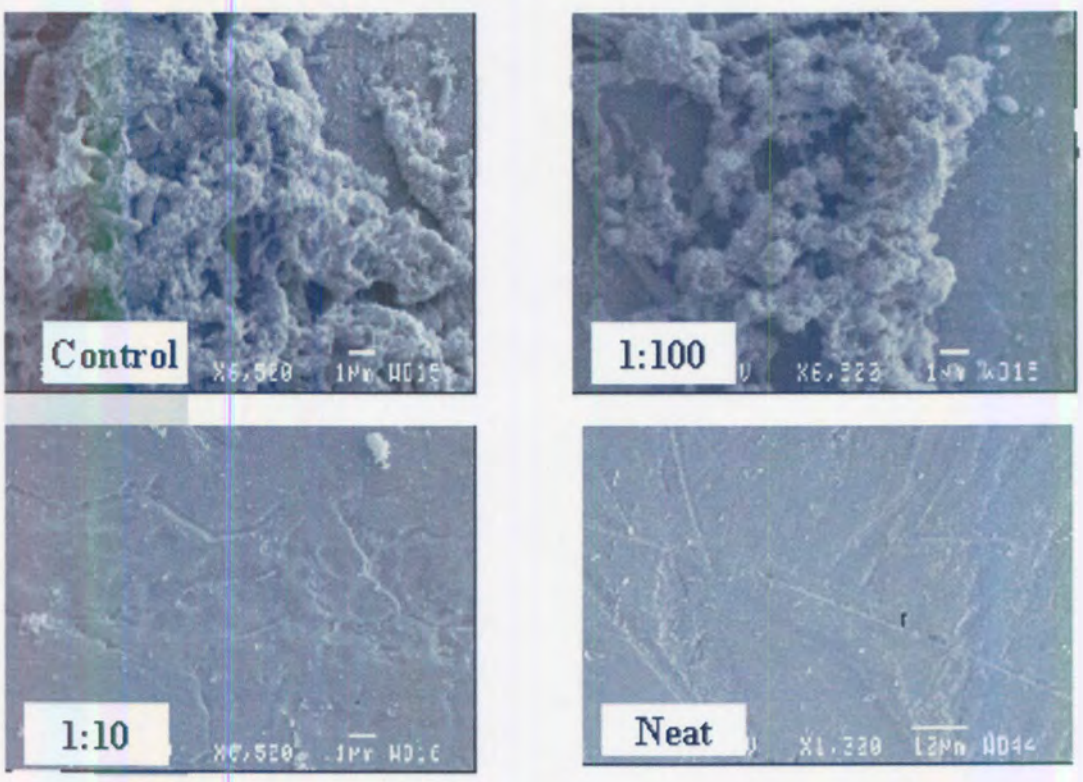


Fig. 4.4. SEM pictures of biofilm after treatment with sodium bicarbonate derived anolyte.

Biofilm treatment with NaCl anolyte and combination of anolyte and catholyte

The anolyte solution (1:10 dilution) effectively removed a mature *P.aeruginosa* biofilm within 6h (Fig 4.5). The anolyte also reduced the planktonic bacteria numbers from 2,41 x 10^7 cfu ml^{-1} to <10 cfu ml^{-1} during the same period (Table 4.2). The anolyte killed the bacteria in the biofilm within 1h indicated by the fading of the DAPI stain (Fig 4.5). The system was operated for a further 72h to determine whether biofilm regrowth would occur. Regrowth of the biofilm was observed 24h after treatment (Fig 4.6). Regrowth of the planktonic bacteria also occurred as reflected by the increase in cfu to 1,33 x 10^6 cfu ml^{-1} after 72 h (Table 4.2). These results are in agreement with Brözel and Cloete (1992) who indicated that regrowth normally occurs within 48 h after biocide treatment. Regrowth can be attributed to mainly two factors: firstly, in some instances, a microbial population shift may occur to organisms resistant to the biocide, or secondly, the biocide is "consumed" by organic matter allowing the regrowth of the surviving bacteria.

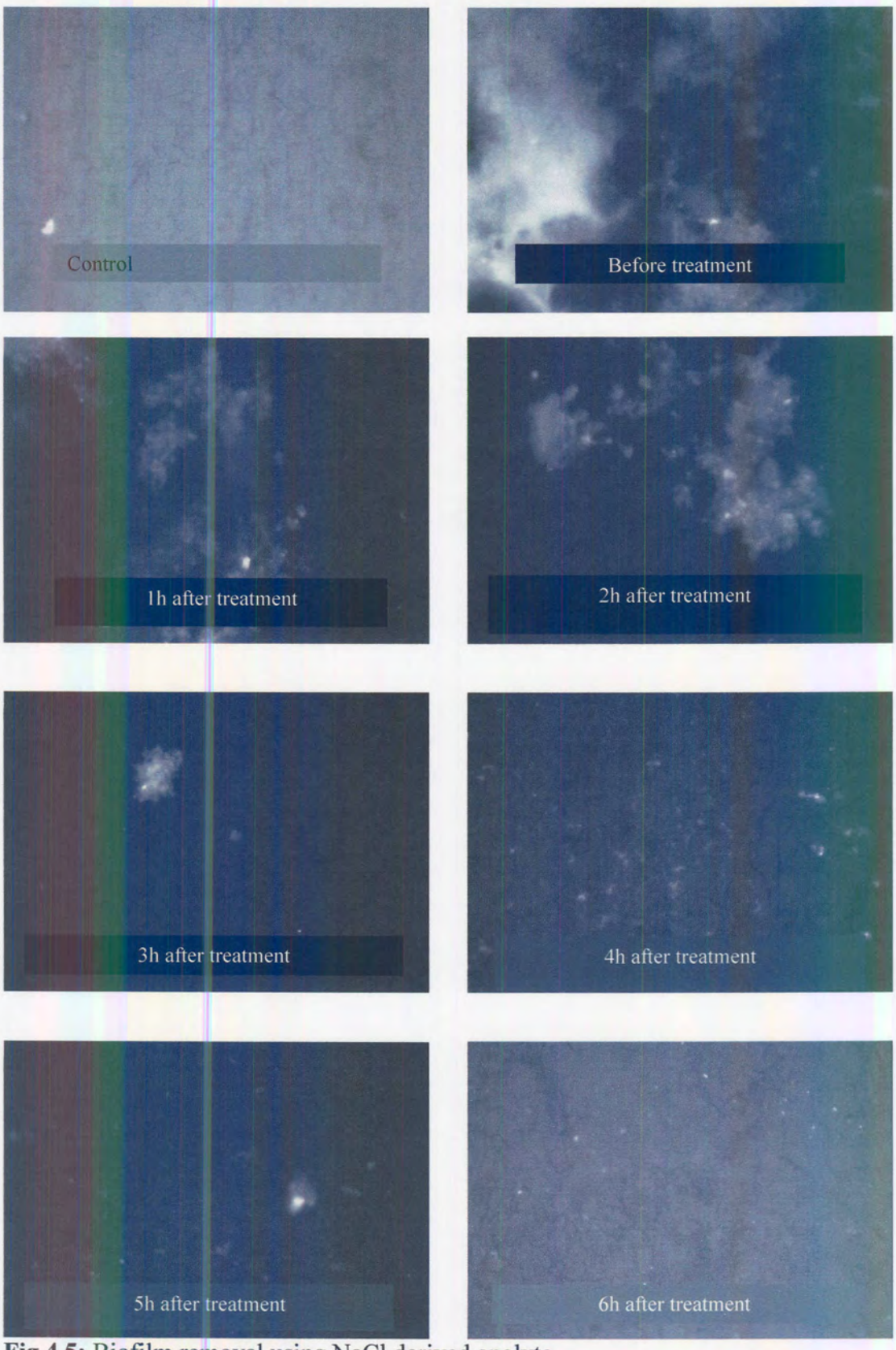


Fig 4.5: Biofilm removal using NaCl derived anolyte

Table 4.2: Planktonic bacterial numbers before and after treatment with NaCl anolyte

Time	cfu/ml
Before treatment	2.41×10^7
6h after treatment	<10
24h after treatment	<10
72h after treatment	1.33×10^6

cfu=colony forming units

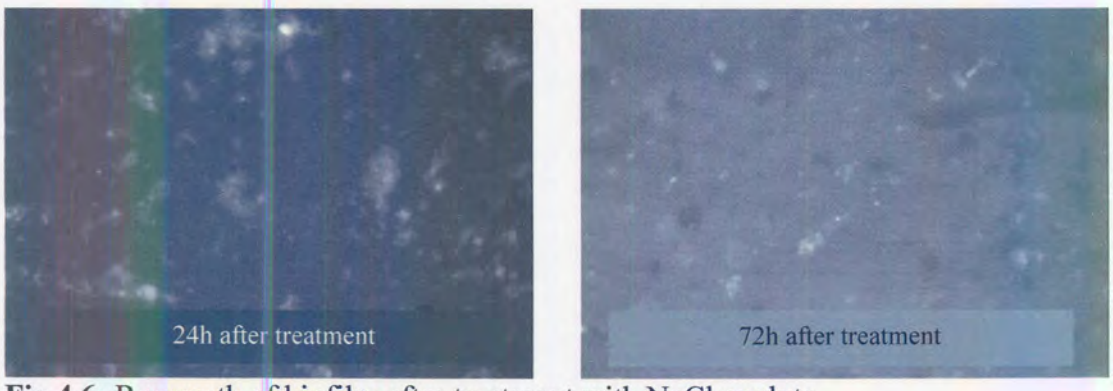
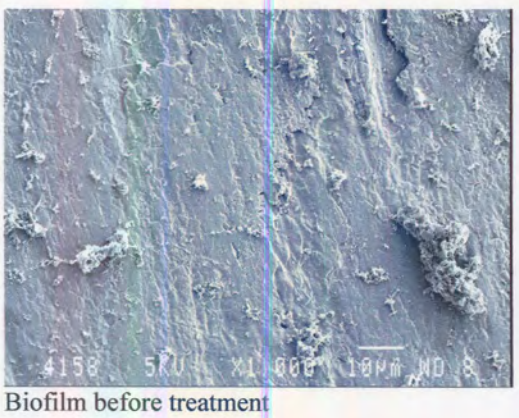
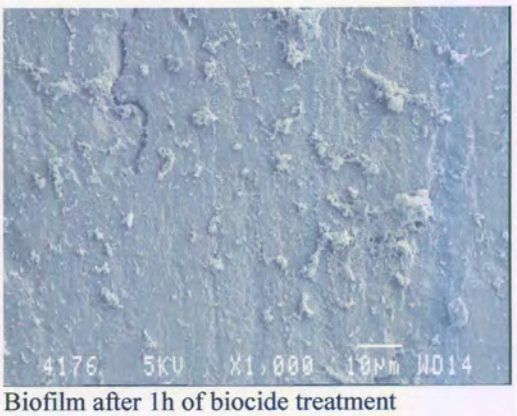


Fig 4.6: Regrowth of biofilm after treatment with NaCl anolyte

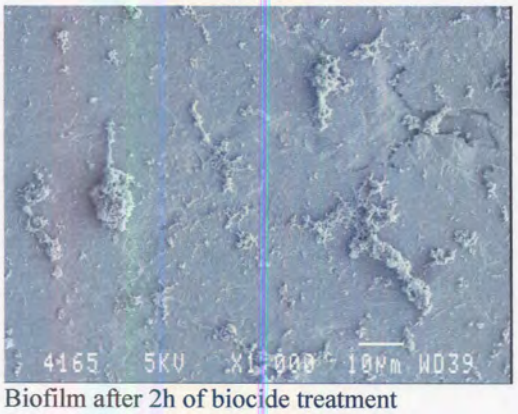
Figure 4.7 shows scanning electron micrographs of the biofilm behaviour before and after biocide treatment. Surface colonization can clearly be seen by numerous microcolonies. Also noticeable is the dehydrated biofilm structure (Fig. 4.7). These micrcolonies are still visible after 2h and 3h of treatment (Fig. 4.7). The microcolonies seen after 2h and 3h of treatment are however fewer in number and smaller in size than those at 0h and 1h (Fig. 4.7). After 4h of treatment very few microcolonies were observed and the biofilm was no longer noticeable (Fig 4.7). After 24h of treatment the situation remained unchanged (Fig 4.7). Neverthless, DAPI staining indicated regrowth of the biofilm (Fig 4.6). This difference was attributed to the difference in the method of preparation for DAPI and SEM, where the preparation of slides for DAPI is less harsh than for SEM.



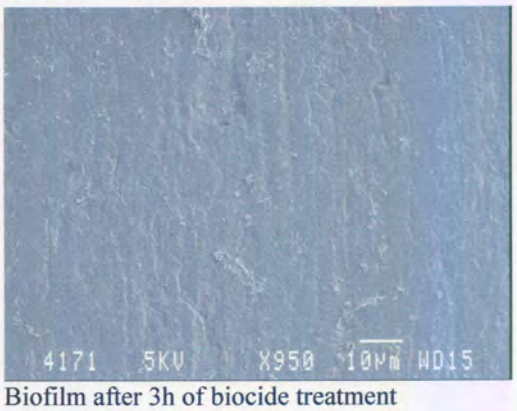
Biofilm before treatment



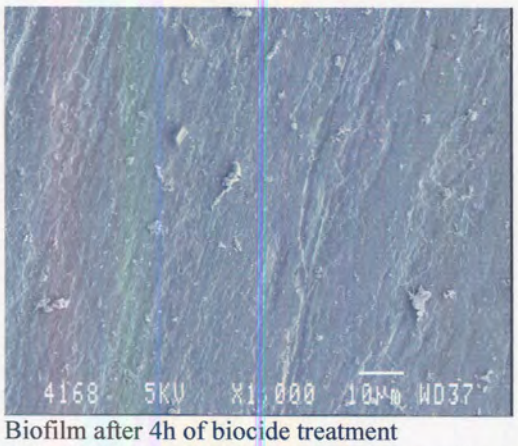
Biofilm after 1h of biocide treatment



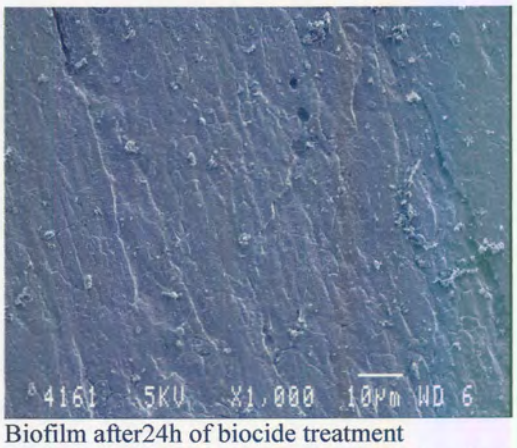
Biofilm after 2h of biocide treatment



Biofilm after 3h of biocide treatment



Biofilm after 4h of biocide treatment



Biofilm after24h of biocide treatment

Fig 4.7: SEM pictures of biofilm after treatment with anolyte/catholyte combination

The anolyte/catholyte (2:1 ratio) solution added at a 1:10 ratio also effectively removed the mature *P.aeruginosa* biofilm. The anolyte/catholyte solution effectively removed the biofilm within 3-4h (Fig 4.8). There was no removal of biofilm 1h after treatment with anolyte/catholyte combination though there was a noticeable dispersion of the biofilm structure (Fig 4.8). Regrowth of the biofilm started taking place 24h after treatment (Fig 4.8). Regrowth of the planktonic bacteria occurred after 72h of treatment with anolyte catholyte combination (Table 4.3).

Table 4.3: Planktonic bacterial numbers before and after treatment with anolyte/catholyte combination

Time	**cfu/ml**
Before treatment	1.14×10^7
6h after treatment	<10
24h after treatment	<10
72h after treatment	1.50×10^6

cfu=colony forming units

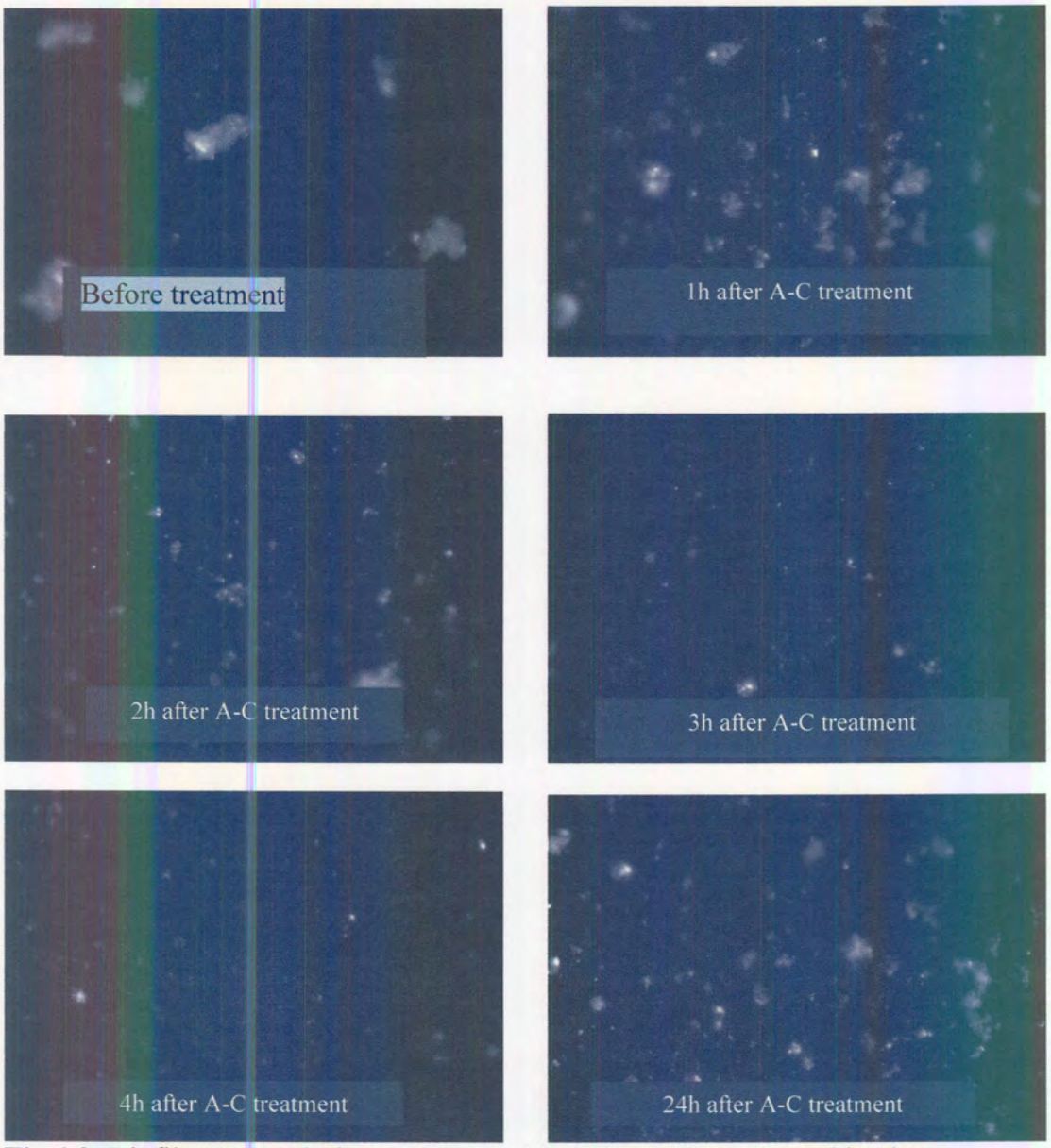


Fig 4.8: Biofilm removal after treatment with anolyte/catholyte combination

4.4 CONCLUSIONS

- The 10, 25, 50 ppm sodium hypochlorite and 1:100 sodium bicarbonate anolyte did not remove the biofilm.
- The 100 and 300 ppm sodium hypochlorite solutions were effective for removing the biofilm.
- The neat and 1:10 sodium bicarbonate anolyte effectively removed the mature *P. aeruginosa* biofilm after 20 min exposure.
- Both the NaCl anolyte and the anolyte/catholyte combination effectively removed a mature biofilm and reduced the bacterial numbers from $>10^7$cfu/ml to less than 10 cfu/ml within 6h.
- Regrowth of both the biofilm and planktonic bacteria occurred after 24h of treatment with both biocides

4.5 REFERENCES

Bagge D., M. Hjelm, C. Johansen, and I. Huber. 2001. *Shewanella putrefaciens* adhesion and biofilm formation on food processing surfaces. Appl. Environ. Microbiol. **67:**2319-2325.

Brözel V. S., and T. E. Cloete. 1991. Fingerprinting of commercially available water treatment biocides in South Africa. Water SA. **17:**57-66.

Brözel V. S., and T. E. Cloete. 1992. The effect of bactericide treatment on planktonic bacterial communities in water cooling systems. Water SA. **18:**87-92.

Brözel V. S., and T. E. Cloete. 1993. Bacterial resistance to conventional water treatment biocides. CAB Biodeterioration Abstracts. **7:**387-395.

Cloete T.E., V. S. Brözel, and A. Von Holy. 1992. Practical aspects of biofouling control in industrial water systems. Int. Biodeterioration and Biodegradation. **29:**299-341.

Cochran W. L., G. A. McFeters, and P. S. Stewart. 2000. Reduced susceptibility of thin *Pseudomonas aeruginosa* biofilms to hydrogen peroxide and monochloramine. J. Appl. Microbiol. **88:**22-30.

Costerton J. W., B. Ellis, K. Lam, F. Johnson, and A. E. Khoury. 1994. Mechanism of electrical enhancement of efficacy of antibiotics in killing biofilm bacteria. Antimicrob. Agents Chemother. **38:**2803-2809.

Dawood Z., and V. S. Brözel. 1998. Corrosion enhancing potential of *Shewanella putrefaciens* isolated from industrial cooling waters. J. Appl. Microbiol. **84:**929-936.

Hoppe H. G. 1984. Attachment of bacteria:Advantage or disadvantage for survival in the aquatic environment, p. 283-301. **In: Marshall K. C** (ed.) Microbial adhesion and aggregation. Berlin Springe Verlag.

Lawrence J. R., P. J. Delaquis, D. R. Korber, and D. E. Caldwell. 1987. Behaviour of *Pseudomonas fluorescens* within the hydrodynamic boundary layers of surface microenvironments. Microb. Ecol. **14:**1-14.

Lee W., Z. L. Lewandowski, P. H. Nielsen, and W. A. Hamilton. 1995. Role of sulphate reducing bacteria in corrosion of mild steel: A Review. Biofouling. **8:**165-194.

McLeod E. S., Z. Dawood, R. MacDonald, M. Oosthuizen, J, Graf, P. L. Steyn, and V. S. Brözel. 1998. Isolation and identification of sulphite- and iron reducing, hydrogenase positive facultative anaerobes from cooling water systems. Syst. Appl. Microbiol. **21:**297-305.

McCoy W. F., J. D. Bryers, J. Robbins, and J. W. Costerton. 1981. Observations of fouling biofilm formation. Can. J. Microbiol. **27:**910-917.

Norwood D.E. and A. Gilmour. 2000. The growth and resistance to sodium hypochlorite of *Listeria monocytogens* in a steady-state multispecies biofilm. J. Appl. Microbiol. **88**:512-520.

Russel A. D., and I. Chopra. 1990. Understanding antibacterial action and resistance. Ellis Horwood, New York.

Whitekettle W. K. 1991. Effects of surface-active chemicals on microbial adhesion. J. Ind. Microbiol. **7:**105-116.

Chapter 5

ANOLYTE AS A DISINFECTANT (ANTISEPTIC) HANDWASHING SOLUTION

5.1 INTRODUCTION

Normal human skin is a complex organ and the bacterial populations associated with it are complex in kind and number. The skin supports the growth of both aerobic and anaerobic bacteria (Evans, 1950; Nester *et al.*, 1995). Microorganisms on the skin can be classified into two categories: resident flora and transient flora. The resident organisms are those that multiply and persist on the skin. They are mainly non-pathogenic organisms or potential 'opportunistic' pathogens. Examples are micrococci, coryneform bacilli and *Propionibacterium acnes*. Transient organisms are those that are deposited but do not multiply on the skin. In hospitals transient organisms include bacteria transmitted by various channels from other patients, including such wound pathogens as *Staphylococcus aureus* and *Pseudomonas aeruginosa* (Russell *et al.*, 1992; Alcamo, 2001). Microscopic studies indicated that the ducts of the sebaceous glands were the major sites of growth. Sweat gland ducts contained little or no bacteria. Organism on the surface of the skin may have grown on or in the skin itself or may be casual contaminants picked up from external sources.

Microorganisms on the skin can be reduced in numbers by removing them physically with soap or some other detergent and water (skin cleansing) or by killing them on the skin, through the application of bactericidal agents (skin disinfection). The skin cannot be sterilized (rendered free from all microbial flora, including bacterial spores) (Russell *et al.*, 1992).

Quantitation of the bacteria of the skin may be greatly influenced by the method of sampling (Ulrich, 1964). The techniques most commonly employed are the contact plate, swabbing and streaking, scrubbing and flushing (Price, 1938) and Tape stripping (Updegraff *et al.*, 1963). Each of these methods determines only a portion of the total population present on or in the skin. The contact plate method is the

simplest of the techniques and gives good reproducibility, but only those microorganisms on the surface will be determined. The scrubbing and flushing is a more involved procedure and removes surface bacteria as well as those deeper lying organisms that are more easily brought to the surface.

The use of swabs to quantify bacteria on the skin gives variable results unless the method is rigidly controlled and strict attention is given to such details as the pressure applied while swabbing, whether dry or moist swabs are employed, the length of time that an area is swabbed. No one method can give the complete picture of the bacterial population on and in the skin, but a combination of the above procedures can be used to produce a better idea of the bacterial distribution and changes in the population.

While the pattern of distribution is constant, the level of the bacterial population of the skin appears to vary greatly among individuals. Spore formers are not common in adults (Ulrich, 1967). Variation in bacterial level among subjects is expected but a single individual tends to maintain a relatively stable level of microbial populations over long periods of time. Although individuals tend to maintain certain microbial populations over long periods of time, it is possible to change the level by a variety of methods. Peculiarly enough, the numbers of bacteria on the skin are significantly increased by ordinary bathing or showering. Scrubbing with germicidal detergents as is employed in a surgical scrub can reduce the numbers of bacteria on the skin (Ulrich, 1967). The objective of this study was to evaluate Anolyte products as disinfectant hand washing agents.

5.2 MATERIALS AND METHODS

5.2.1 Solutions used in the trial

Sterile water was used as a control.

The physicochemical attributes of the disinfectant solutions used in the Hand washing trial were as follows:

- NaC1 derived Anolyte – salt concentration in catalyst solution: 5gm/litre ORP: 840mV, Conductivity – 7400microsiemens, pH-7.2
- $NaHCO_3$ derived Anolyte – Bicarb concentration in Catalyst solution: 3gm/litre ORP: 780mV, Conductivity – 2400microsiemens, pH – 7.1

5.2.2 Methodology

a. Contact Plate method

The RODAC plate technique was used to sample the palm of the hands of subjects. Samples were taken on unwashed hands and again after washing with the solutions.

b. Wash and rinse method.

Subjects washed their hands in 400 ml of the solutions for approximately 60 s. The wash water was collected and microbiological analysis conducted.

c. Microbiological analysis

-The total bacterial number was determined using Nutrient agar and incubation at 37°C for 48h

- The numbers of coliforms bacteria were determined using McConkey agar and incubation at 37°C or 24h.

- The numbers of Salmonella bacteria were determined using Salmonella agar and incubation at 37°C for 24h

Table 5.1: Microbiological analysis of the water collected after washing of hands.

Sample	**Number of total bacteria cfu/ml**	**Number of coliforms/ml**	**Number of *Salmonella*/ml**	**TPC/ 25 cm^2**
Pre water wash	ND	ND	ND	3.52×10^2
Pre water wash	ND	ND	ND	>300
Pre water wash	ND	ND	ND	>300
Pre water wash	ND	ND	ND	3.60×10^2
Sterile H_2O (1)	2.4×10^2	45	0	1.49×10^2
Sterile H_2O (2)	1.17×10^4	<100	0	6.24×10^2
Sterile H_2O (3)	1.96×10^4	78	0	3.80×10^2
Sterile H_2O (4)	2.26×10^2	48	0	2.25×10^2
Bicarb anolyte (1)	5×10^2	0	0	6.48×10^2
Bicarb anolyte (2)	1.4×10^1	0	0	3.48×10^2
Bicarb anolyte (3)	8.2×10^3	<100	0	>300
Bicarb anolyte (4)	7.1×10^3	1.34×10^2	0	2.30×10^2
NaCl anolyte (1)	<10	0	0	2.30×10^2
NaCl anolyte (2)	<10	0	0	0
NaCl anolyte (3)	0	0	0	1.48×10^2
NaCl anolyte (4)	0	0	0	0
NaCl anolyte (5)	0	0	0	79
Container washed with alcohol	ND	ND	ND	0

ND: Not determined

TPC: Total plate count using the RODAC contact plate method

cfu : colony forming unit

The TPC on the pre wash, as determined using the contact plate method, indicated a high level of contamination, with most of the RODAC plates completely covered with bacterial colonies (Table 5.1). After washing hands with sterile water, the results on the wash water indicated the removal of bacteria, indicated in the bacterial numbers (Table 5.1). Coliforms were also present, indicating that the workers hands were

contaminated (Table 5.1). The contact plate results indicated that the hands washed with sterile water were not sanitized completely, with between 200 and 700 bacteria/25 cm^2 remaining on the hands (Table 5.1). The results obtained with the sodium bicarbonate anolyte were very similar to those for sterile water, when comparing the TPC determined by the contact plate method, with bacterial numbers ranging between 200 and 600 bacteria/25 cm^2 (Table 5.1). However, when comparing the average number of total bacteria/ml in the hand wash solution, the sodium bicarbonate anolyte resulted in a significant 1 log lower bacteria number, than sterile water (3,9 X 10^3 cfu/ml compared to 7,94 x 10^4 cfu/ml for sterile water). This indicated that the sodium bicarbonate anolyte did have some disinfectant properties. The NaCl anolyte virtually sterilized the hands after washing. The total number of bacteria as determined by the contact plate method was 230, 148 and 79 cfu/25 cm^2 in three cases and 0 cfu/25 cm^2 in two cases (Table 5.1). The efficacy of NaCl anolyte as a disinfectant was confirmed by the sterility of the hand wash water sampled (Table 5.1).

5.4 CONCLUSIONS

- The bicarbonate anolyte was more effective as disinfectant than sterile water
- NaCl Anolyte was very effective as a disinfectant and hand-washing agent

5.5 REFERENCES

Alcamo, I.E. 2001. Fundamentals of Microbiology, Jones and Bartlett Publishers, London.

Evans, C.A., W.M. Smith., E.A. Johnston, and E.R. Gibbett. 1950. Bacterial Flora of the Normal Human Skin. J. Inv. Dermatol. **15:**305-324.

Nester, E.W., C. E. Roberts, and T. M. Nester. 1995. Microbiology. A human perspective. Wm. C. Brown Publishers, Oxford.

Price P. B. 1938. Bacteriology of the normal skin; A new quantitative test to a study of the bacterial flora and the disinfection action of mechanical cleaning. J. Disinf. Diseases. **63:**301.

Russell A. D., W. B. Hugo, and G. A. J. Ayliffe. 1992. Principles and Practice of Disinfection, Preservation and Sterilization, p. 312. Second edition. Blackwell Scientific, London.

Ulrich, J.A. 1964. Technics of Skin Sampling for Microbial Contaminants. Health Laboratory Science. **1:**135-136.

Ulrich J. A. 1967. Skin sanitation. Presentation at E. L. technical Symposium. Research and Developmental Department. Economics Laboratory, Inc. Mendota Heights, Minnesota.

Updegraff, D.M., M.H. Halbert., K..M. Keenan., H.P. Putman., O.R. Rushmeyer, and D. Vesely. 1963. Development of a Method for Microbial Sampling of Surfaces with Special Reference to Reliability. Contract RH- 86-62-182. Division of Hospital and Medical Facilities, HEW.

Chapter 6

DISINFECTION OF A DAIRY MILKING PARLOUR USING ANOLYTE

6.1 INTRODUCTION

Dairy products such as milk, butter, cream and cheese are all susceptible to microbial spoilage because of their chemical composition. Milk is an excellent growth medium for all of the common spoilage organisms, including moulds and yeasts. Fresh, non-pasteurized milk generally contains varying numbers of microorganisms, depending on the care employed in milking, cleaning, and handling of milk utensils. Raw milk held at refrigerator temperatures for several days invariably shows the presence of several or all bacteria of the following genera: *Enterococcus, Lactococcus, Streptococcus, Leuconostoc, Lactobacillus, Microbacterium, Propionibacterium, Micrococcus,* coliforms, *Proteus, Pseudomonas, Bacillus,* and others. Those unable to grow at the usual low temperature of holding tend to be present in very low numbers. The pasteurization process eliminates all but thermoduric strains, primarily streptococci and lactobacilli, and spore formers of the genus *Bacillus* (and clostridia if present in raw milk). The spoilage of pasteurized milk is caused by the growth of heat-resistant streptococci utilizing lactose to produce lactic acid, which depresses the pH to a point (about pH 4.5) where curdling takes place. If present, lactobacilli are able to grow at pH values below that required by *Lactococcus lactis*. These organisms continue the fermentative activities and may bring the pH to 4.0 or below. If mould spores are present, these organisms begin to grow at the surface of the sour milk and raise the pH towards neutrality, thus allowing the more proteolytic bacteria such as *Pseudomonas* spp. to grow and bring about the liquefaction of the milk curd. The use of a well-balanced cleaning and sanitizing program will aid in the production of raw milk of exceptionally high microbiological quality. Farms with well disciplined and carefully organized sanitation programs which extend from cow preparation to bulk tank cleaning, are easily achieving Standard Plate Counts of <10 000/ml and coliforms counts of <10/ml for raw milk – standards usually not reached for fluid pasteurized milk by most processing plants in South Africa (Jay, 1992).

Chemical sanitizers are normally used and iodophors (25 ppm iodine) and chlorine (100 ppm chlorine) are the most widely used. Allow a minimum contact time of two minutes for best results.

The bacterial cell membrane provides the osmotic barrier for the cell and catalyses the active transport of substances into that cell. Alternations in transmembrane potential caused by the action of electron donor or electron acceptor factors are associated with powerful electro-osmotic processes accompanied by water diffusion against ORP gradients, with resultant rupture of the membranes and outflow of the bacterial cell contents. The bacterial membrane itself has an electrical charge. The anions present in Anolyte act on this membrane. Anolyte can also disrupt other functions of the cell. Unlike "higher" organisms, single celled organisms such as bacteria obtain their energy sources form the environment immediately outside the cell. Small molecules are transported across the cell membrane via an electro-chemical gradient. Thus, any significant change in the ORP of the immediate environment has drastic consequences for the cell. Even if instantaneous death of the cell does not occur, all enzymatic functions in the membrane are affected and this will also result in loss of cell viability. The objective of this study was to evaluate anolyte as surface disinfectant for milking parlours and to determine whether anolyte possessed sporicidal properties.

6.2 MATERIALS AND METHODS

Three milking stations (same everyday) were analysed on a daily basis after disinfection. Four different surfaces on each of the three stations were sampled each day. The four different surfaces sampled were (1) the inside of the teat cluster (2) teat cluster top (mouth), (3) float control flow sensor inside and (4) float control flow sensor lid. A sterile swab was used to sample each surface and streaked out on Nutrient agar plates. The plates were incubated at 37°C for 48h and the number of colonies formed on each plate was counted.

Sporeformer analysis

Cell suspensions were prepared from the plates that gave spreaders in the dairy disinfection experiment. The colonies were suspended by adding 5 ml of sterile distilled water to the plate and suspending the colony using a sterile loop. The

suspensions were transferred to test tubes and the test tubes were incubated at 80°C for 10 min. 1 ml of each of the cell suspensions was plated out on Nutrient agar plate. The plates were incubated at 37°C for 48h and then they were checked for growth.

6.3 RESULTS AND DISCUSSION

Table 6.1: Microbiological analysis (cfu) of different surfaces in a milking parlour after disinfection using the standard disinfection procedure.

	1	2	3	4	5	6	7	8	9	10	11
Station 1											
FSL	Spr	>300	0	>300	14	Spr	Spr	Spr	>300	Spr	>300
FSI	Spr	119	154	115	0	>300	>300	Spr	>300	Spr	246
TCI	0	3	0	7	0	2	0	>300	105	0	0
TCT	>300	Spr	>300	>300	Spr	>300	>300	>300	>300	>300	>300
Station 2											
FSL	Spr	>300	Spr	>300	Spr	Spr	Spr	Spr	>300	Spr	Spr
FSI	279	>300	>300	>300	Spr	>300	149	Spr	98	Spr	0
TCI	0	51	0	6	61	38	1	2	0	Spr	90
TCT	>300	Spr	>300	>300	6	Spr	>300	>300	>300	Spr	>300
Station 3											
FSL	3	>300	Spr	Spr	Spr	Spr	Spr	Spr	>300	Spr	Spr
FSI	>300	>300	>300	273	9	Spr	292	>300	>300	82	>300
TCI	0	6	186	6	0	0	1	Spr	1	3	182
TCT	>300	>300	>300	>300	Spr	>300	>300	Spr	>300	Spr	>300

Spr= spreader

cfu= colony forming units

FSL=float control flow sensor lid

FSI =float control flow sensor inside

TCI=teat cluster inside

TCT=teat cluster top

The results in Table 6.1 and Table 6.2 are qualitative rather than quantitative, since it was impossible to sample exactly the same surface area, due to the nature of the sampled surface. The normally used method of disinfection (indicated as control in the results) did have some degree of microorganisms removal as (Table 6.1). In most cases, the teat cluster inside had the lowest level of contamination. However, most of the surfaces were not satisfactorily disinfected, indicated by spreaders (Table 6.1), and some surfaces were number of microorganisms exceeded the maximum number that could be counted on the plates. The relatively higher numbers of microorganisms in the float control flow sensor lid and teat cluster top, was attributed to these surfaces not being exposed to the disinfectant solutions (Table 6.1 and Table 6.2). The anolyte however eliminated the spreaders on these sampling sites. When anolyte was used as a disinfectant, the results were generally better. This is particularly evident when comparing the results of the FSI sampling point (Table 6.1 and Table 6.2).

Table 6.2: Microbiological analysis (cfu) of different surfaces in a milking parlour after disinfection with anolyte

	1	2	3	4	5	6
Station 1						
FSL	246	94	>300	0	69	0
FSI	1	0	0	1	0	0
TCI	0	0	0	1	0	4
TCT	>300	>300	0	>300	>300	>300
Station 2						
FSL	110	5	>300	>300	185	>300
FSI	0	0	1	0	1	0
TCI	0	0	2	0	0	9
TCT	>300	>300	44	78	>300	>300
Station 3						
FSL	69	>300	Spr	>300	6	0
FSI	3	0	Spr	0	0	0
TCI	0	13	0	0	4	9
TCT	>300	164	109	>300	>300	>300

Spr= spreader

cfu= colony forming units

FSL=float control flow sensor lid

FSI =float control flow sensor inside

TCI=teat cluster inside

TCT=teat cluster top

Table 6.3 : Growth of cells after exposure to a high temperature

Plate number	Results
1-17	G

G=Growth

All the spreaders were resistant to heating at 80°C for 10 min (Table 6.3). This indicated that all the spreaders were a result of growth of sporeforming organisms that contaminated the milking parlour.

6.4 CONCLUSIONS

- The contact between the disinfectant and the surface to be disinfected is essential for removing the number of microorganisms
- All spreaders were identified as sporeforming microorganisms
- Anolyte eliminated the spore forming bacteria
- Overall, the anolyte gave better disinfection than the control disinfectant
- Where anolyte made contact with a surface, disinfection was at an acceptable level, with most surfaces being sterilized

6.5 REFERENCES

Jay J. M. 1992. Modern Food Mocrobiology. 4th edition. Chapman & Hall, New York.

Chapter 7

HOSPITAL DISINFECTION USING ANOLYTE SOLUTION

7.1 INTRODUCTION

The hospital may not only be a place where sick people get well, it may also be a place where sick people get sicker. The fact is that cross infection from patient to patient or from hospital personnel to patients presents a constant hazard. Hospital infections are partly due to the prevalence of diseased patients, but are largely due to the presence of pathogenic microorganisms, which are selected by the hospital environment (Brock *et al.*, 1994). These hospital acquired infections are generally referred to as nosocomial infections (Volk and Wheeler, 1984; Brock *et al.*, 1994). The prevalence of nosocomial infections is due to the concentration of highly susceptible persons in the hospital, the ubiquity of opportunistic pathogens, medical procedures that encourage infection, and breaches in sound aseptic practices (McKane and Kandel, 1996).

Hospitals are especially harzadous for the following reasons: many patients have weakened resistance to infectious diseases because of their illness. Hospitals must of necessity treat patients suffering from infectious disease and these patients may be reservoirs of highly virulent pathogens. The crowding of patients in rooms and wards increases the chance of cross infections. There is much movement of hospital personnel from patient to patient, increasing the probability of transfer of pathogens. Many hospital procedures, such as catheterization, hypodermic injection, spinal puncture and the removal of tissue samples or fluids, carry with them the risk of introducing pathogens to patients. Surgical procedures are a major hazard, since not only are highly susceptible parts of the body exposed to sources of contamination but stress of surgery often diminishes the resistance of patients to infection. Many drugs used for immunosuppression increase susceptibility to infection (Brock *et al.*, 1994).

The majority of nosocomial infections are caused by microorganisms that make up part of the patient's normal flora. Such microorganisms are termed opportunists because they normally produce infections under the following conditions: when they

are in a host whose immune system has been impaired, when they can bypass anatomical barriers following burns or surgery or are implanted by contaminated catheters, syringes or respirators (Volk and Wheeler, 1984).

Although a variety of pathogens are encountered in the hospital environment, a relatively limited number cause the majority of hospital infections. Opportunistic pathogens such as *Escherichia coli*, *Enterococcus faecalis* and *Pseudomonas aeruginosa* are common residents of the human colon and skin surrounding anus (McKane and Kandel, 1996). *E. coli*, introduced from the normal flora, is the most common cause of urinary tract infections. *Staphylococcus aureus* is the most important and widespread hospital pathogen (Brock *et al.*, 1994).

The tendency of pathogens to form biofilms complicates the prevention and treatment of disease. Hospital acquired infections also pose a potential hazard to the general community since symptoms often do not develop until after the patient is discharged, and many of these patients harbor virulent pathogens. Infectious disease introduced into the community may be difficult to control as individuals freely and continuously enter and exit (McKane and Kandel, 1996). The objective of this study was to use the anolyte as disinfectant for hospital equipment.

7.2 MATERIALS AND METHODS

Hospital disinfection trials were done at the Union hospital in Alberton. Rodac plates with Nutrient agar were used to take samples from the different surfaces and equipment. The samples were taken before and after treatment with anolyte. The plates were incubated at 37°C for 48h.

7.3 RESULTS AND DISCUSSION

Table 7.1: Number of colonies counted from the plates (cfu/25 cm^2) before and after treatment with anolyte

Sample	Before treatment	After treatment
Pre(1) swab	>300	0
Pre (2) swab	137	0
Pre (2)swab	61	ND
Pre (3) swab	>300	ND
Basin surface	0	0
Basin surface	>300	0
Basin surface	>300	0
Basin surface	214	70
Basin surface	86	0
Basin surface	11	2
Basin surface	9	7
Basin surface	207	117
Basin surface	>300	114
Bed pan	4	0
Soap handle	72	9
Ward table	31	Not treated
Basin ward	0	0
Cold tap	43	0
Soap handle	>300	0
K wall	14	ND
K wall	>300	ND

cfu = colony forming units.

ND = not determined

The numbers of organisms obtained from the surfaces before treatment with the anolyte were higher than the numbers obtained after treatment with the anolyte (Table 7.1). According to the results the heavily contaminated surfaces were the drains in the basins. However, even these highly contaminated areas were effectively disinfected

(Table 7.1). That is, treatment with anolyte gave a decrease in the number of live organisms, in some cases the surfaces did not show any growth on the plates. Effective disinfection was achieved.

7.4 CONCLUSIONS

- Overall effective disinfection of hospital equipment was achieved using the anolyte.

7.5 REFERENCES

Brock T. D., M. T. Madigan, J. M Martinko, and J Parker. 1994. Biology of Microorganisms, p. 517-519. 7th edition. Simon and Schuster Asia Pte. Ltd., Singapore.

McKane L., and J. Kandel. 1996. Microbiology, Essentials and Applications, p. 681-700. 2nd edition. Mcgraw-Hill inc., USA.

Volk W. A., and M F. Wheeler. 1984. Basic Microbiology, p. 224-225. 5th edition. Harper and Row publishers, New York.

Chapter 8

SHELF LIFE STUDY OF CHICKENS TREATED WITH ANOLYTE AND AN ALTERNATIVE DISINFECTANT

8.1 INTRODUCTION

Poultry is an important part of the animal food market and production is increasing to satisfy public demand world-wide (Bryan, 1980; Anand *et al.*, 1989). Poultry and its products are also a major dietary item for the South African population (Bok *et al.*, 1986). According to the Directorate of Veterinary Public Health, 4.4 billion rands worth of poultry meat products were purchased by South Africans in 1994 (AFMA, 1996; SAPA, 1996). Therefore, it becomes necessary to maintain absolute hygiene and strict control at different stages of processing to produce a safe and wholesome chicken product.

Healthy chickens ready for processing harbour a tremendous amount and variety of bacteria. These bacteria are present on the surfaces of feet, feathers, skin and also in the intestines. During processing, a high proportion of these organisms will be removed, but further contamination can occur at any stage of the processing operation. The procedure for converting a live, healthy bird into a safe and wholesome poultry product provides many opportunities for microorganisms to colonise on the surface of the carcass. During the various processing operations, opportunities exist for the contamination of the carcasses from the environment, the process in the plant itself, contamination via knives, equipment, the hands of workers and also by cross-contamination from carcass to carcass. Some processing operations encourage an increase of contamination or even multiplication of contaminating organisms. As a result, the microbial population changes from mainly Gram-positive rods and micrococci on the outside of the live chicken to Gram-negative microorganisms on the finished product (Bryan, 1980; Thomas *et al.*, 1980; Eustace, 1981; Roberts, 1982; Grau, 1986; Bailey *et al.*, 1987; Connor *et al.*, 1987; Banwart, 1989; Mead, 1989).

Poultry processing has a number of unique features which make control of microbial contamination more difficult than the processing of any other conventional meat animal. Among them is the rapid rate of processing in some processing plants, a condition which favours the spread of microorganisms. The carcasses must be kept whole throughout the process and the viscera have to be removed rapidly through a small opening in the abdomen without breakage, to minimise contamination of the carcasses with intestinal organisms. After defeathering, the skin provides a complex surface with many holes which are capable of trapping bacteria (Mead, 1982; Grau, 1986; Mead, 1989).

The microorganisms are widely distributed over the carcasses under normal circumstances and are spread over the skin during scalding and defeathering and on the inner and outer surfaces during evisceration and further processing (Bailey *et al.*, 1987). Efforts should be made to prevent the build-up of contamination peaks during processing. Rinsing of the carcasses, especially during defeathering and evisceration is therefore of great importance (McMeekin and Thomas, 1979; Brown *et al.*, 1982; Mead, 1982; Anand *et al.*, 1989; Mead, 1989). Spoilage bacteria grow mainly on the skin surfaces, in the feather follicles and on cut muscle surfaces under the skin. The nature and rate of attachment of the microorganisms depends upon several factors including the bacteria involved and their concentration and also the conditions under which attachment occurs, namely, pH, temperature and contact-time. It was also found that *Pseudomonas* strains attach to meat surfaces more rapidly than any other bacteria (Firstenberg-Eden, 1981).

The structure of the skin also has a crucial influence on attachment of bacteria. The organisms adhere by way of flagella and fimbrae and cannot easily be removed by rinsing, especially after a delay. There is still some disagreement on the role and importance of flagella in the attachment process of bacteria to meat. Research also shows that mesophilic bacteria are more heat-resistant when attached to skin than are the same bacteria not attached. (Barnes *et al.*, 1973; Green, 1974; Notermans *et al.*, 1974; Notermans *et al.*, 1975; Harrigan, 1976; Firstenberg-Eden, 1981;

Thomas *et al.*, 1981; Faber *et al.*, 1984; Lillard, 1985). The skin serves as a barrier to microorganisms that might otherwise contaminate the underlying muscle and therefore the deep muscles are normally free of bacteria (Bryan, 1980; Mead, 1982). The few bacteria found in the deep muscle are of types that can only multiply slowly or not at all at low temperatures. The important microbiological changes take place on the surfaces of the carcasses. It appears that some parts of the carcass are more favourable than others for bacterial growth, depending on the type of muscle and pH. Studies conducted over the last few years show that the sites most heavily contaminated are the neck skin and less frequently on the back and the area around the vent. Fewer organisms are found around the breast, legs and under the wings. *Acinetobacter* and *Alteromonas* grow better in leg muscle where pH is 6.4 to 6.7 than in breast muscle where pH is 5.7 to 5.9. *Pseudomonas* spp. can grow well at both pH ranges (Patterson, 1972; Barnes *et al.*, 1973; Green, 1974; McMeekin and Thomas, 1979; Bryan, 1980; Thomas *et al.*, 1981; Mead, 1982; Gill, 1983; Grau, 1986; Anand *et al.*, 1989). The presumable reason for the neck skin being the most heavily contaminated is that the washings from the rest of the carcass run down the neck while the carcass hangs on the conveyor (Patterson, 1972; Connor *et al.*, 1987). The objective of this study was to determine the effect of anolyte on the shelf life of chickens.

8.2 MATERIALS AND METHODS

8.2.1 Sampling

One chicken with red and blue packaging respectively was sampled at 0 d, then daily from 5 d to 12 d. The red packaged chicken was treated with anolyte while the blue was treated with the standard disinfectant used for poultry treatment. A composite skin sample was prepared, by taking samples from the neck, back between the wings and under the wing. 10g of sample were added to 90 ml of sterile Ringers solution to give a 1:10 dilution. From this a dilution series (10^{-1} - 10^{-6}) was prepared for plating. This composite sample was placed into quarter strength Ringers solution in a 1+9 mass/volume ratio based on exact mass. Each composite sample was homogenised for 20 min (Seward Medical 400 Stomacher Lab Blender). Tenfold serial dilution's in Ringers solution were plated out in duplicates, using the spread-plate technique and

incubated aerobically at 37°C for 48 h. Plates showing between 30 and 300 colony forming units (cfu) (or the highest number if below 30) were counted.

8.2.2 Microbiological analyses

The following microbiological analyses were performed on every sampling period, except on day zero, when only total aerobic bacteria were enumerated:

Table 8.1: Microorganisms enumerated and microbiological media (Biolab) used in the study.

Microorganisms enumerated	Microbiological media used
Total aerobic bacteria	Nutrient Agar
Salmonella-Shigella spp.	SS Agar
Enterobacteriaceae	MacConkey Agar
Staphylococcus aureus	Baird-Parker Agar Base
Pseudomonads	Cetrimide Agar Base

8.3 RESULTS AND DISCUSSION

Total bacteria Count (TBC)

The initial TBC was 1 log lower (10^4 cfu/g) in the anolyte treated samples compared to those treated with standard disinfectant (10^5 cfu/g) (Table 8.2 and Table 8.3). After 5 d the TBC in the anolyte samples were 2 log units lower than in the standard disinfectant treated samples, and remained 2 log units lower up to day 7 (Fig 8.1, Fig. 8.2, Table 8.2 and Table 8.3). On days 8, 9 and 10 TBC was 1 log unit lower in anolyte treated samples than in the standard disinfectant treated samples (Table 8.2 and Table 8.3). On day 11 and 12 the TBC in the standard disinfectant treated samples were 1 log lower than in the anolyte treated samples (Fig 8.1, Fig. 8.2, Table 8.2 and Table 8.3). This was attributed to the bacteria reaching the stationary phase due to the depletion of nutrients. This stage had not yet been reached in the anolyte treated samples at 12 days. These results indicate a significant difference between the two treatments, with the anolyte treated samples having the best result and longest shelf life.

Table 8.2: Microbiological analysis (cfu/g) of chicken using anolyte

Time (d)	Total bacteria	*Salmonella-Shigella*	*S. aureus*	*Enterobacteriaceae*	Pseudomonads
0	2.9×10^4	ND	ND	ND	ND
5	2.9×10^5	1.5×10^5	3.9×10^3	1.5×10^5	0
6	3.0×10^5	1.2×10^5	3.0×10^3	1.6×10^5	1.6×10^2
7	3.8×10^6	1.8×10^5	2.3×10^3	2.2×10^5	1.7×10^2
8	3.0×10^7	3.1×10^5	1.4×10^4	2.0×10^5	8.7×10^3
9	4.1×10^7	3.3×10^4	2.1×10^3	2.7×10^5	1.7×10^3
10	8.3×10^7	2.2×10^5	1.3×10^4	1.2×10^6	2.7×10^3
11	2.3×10^8	9.4×10^5	1.7×10^4	9.3×10^5	9.0×10^4
12	1.4×10^8	9.0×10^4	0	3.1×10^5	3.5×10^3

cfu= colony forming units

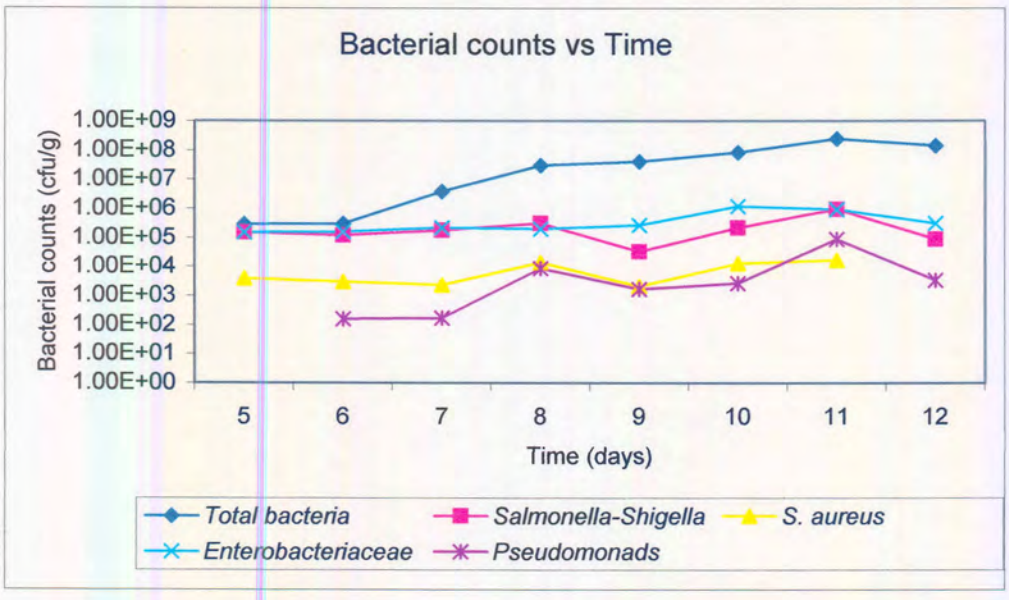


Fig 8.1: The microbiological analysis of anolyte treated chickens over the experimental period

Table 8.3: Microbiological analysis (cfu/g) of chicken using standard disinfectant

Time (d)	Total bacteria	*Salmonella-Shigella*	*S. aureus*	*Enterobacteriaceae*	Pseudomonads
0	9.5×10^5	ND	ND	ND	ND
5	3.1×10^7	2.4×10^6	2.5×10^4	2.3×10^6	3.8×10^4
6	2.0×10^7	9.0×10^6	2.3×10^4	1.2×10^7	9.9×10^4
7	1.7×10^8	2.4×10^7	2.4×10^5	6.6×10^7	3.6×10^5
8	2.3×10^8	2.3×10^6	3.3×10^5	1.5×10^7	1.8×10^5
9	1.2×10^8	2.3×10^6	1.9×10^4	4.9×10^6	2.5×10^4
10	2.4×10^8	9.6×10^5	7.5×10^4	1.1×10^7	7.0×10^4
11	8.1×10^7	1.5×10^6	2.6×10^5	2.3×10^6	1.6×10^4
12	5.0×10^7	1.7×10^5	0	2.0×10^6	2.9×10^3

cfu = colony forming units

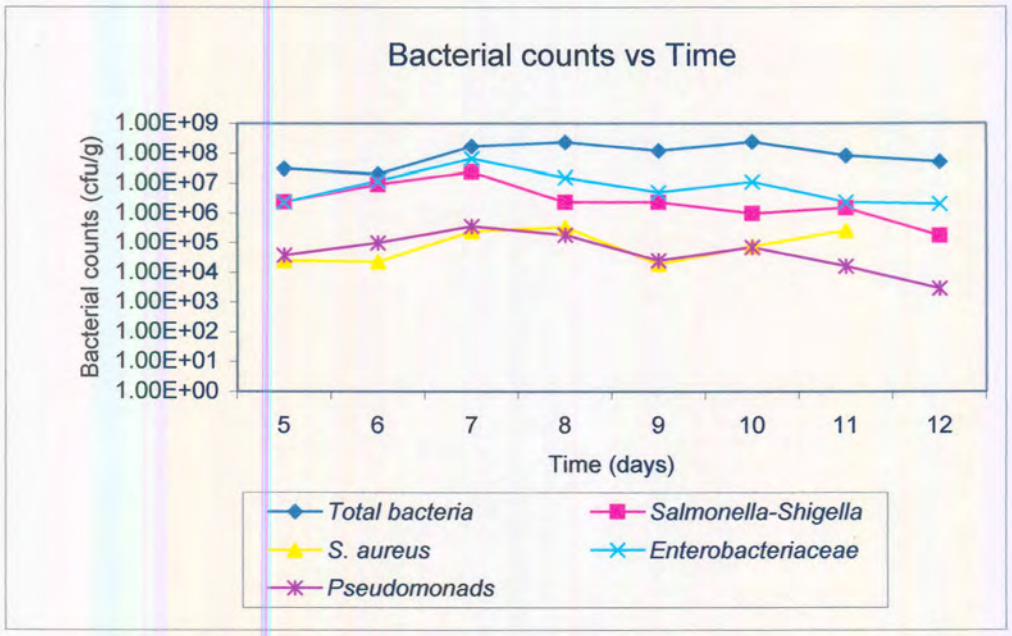


Fig 8.2: The microbiological analysis of standard disinfectant treated chickens over experimental period

***Salmonella-Shigella* count (SSC)**

The SSC was high in both treatments. Nevertheless, the SSC was significantly lower in the anolyte treated samples.

***Staphylococcus aureus* (SAC)**

The SAC followed the same trend as the TBC and SSC, being consistently lower in the anolyte treated samples.

***Enterobacteriaceae* (EC)**

The *Enterobacteriaceae* count was lower in the anolyte treated samples than in the standard disinfectant treated samples and did not increase significantly over the study period.

Pseudomonads

The *Pseudomonas* count was at least two log units lower in the anolyte treated samples compared to the standard disinfectanttreated samples up to day 8.

The Red samples (anolyte treated) overall, consistently had a significantly lower bacteria number than the Blue samples (standard disinfectant treated). The TBC in the Red samples after 8 days was equivalent to the TBC in the Blue samples after 6 days and after 11 days in the Red samples had the same TBC as the Blue samples after 8 days. From this it was concluded, that the anolyte treated samples, on average, would have an increase in shelf life of 2 days over the samples treated with a standard disinfectant. The TBC was used to compare shelf life, since none of the other bacterial numbers increased over the study period in any of the two treatments.

8.4 CONCLUSIONS

- Both treatments reduced the number of microorganisms on the chicken carcasses
- The anolyte was superior compared to the standard disinfectant with regards to extending the shelf life of fresh chickens.

8.5 REFERENCES

AFMA. 1996. Braaikuikenbedryf in Suid-Afrika. *AFMA MATRIX* **5(3):**27.

Anand S.K., C. M. Mahapatra, N. K. Pandey., and S. S. Verma. 1989. Microbial changes on chicken carcasses during processing. Indian J. Poult. Sci. **24(3):**203-209.

Bailey J.S., J. E. Thomson., and N.A. Cox. 1987. Contamination of poultry during processing, p. 193-211. **In: Cunningham F.E. and N.A. Cox** (eds.). The Microbiology of Poultry Meat Products. Academic Press, Orlando.

Banwart G. J. 1989. Basic Food Microbiology. 2nd edition. Van Nostrand Reinhold.

Barnes E. M., C.S. Impey, and R. T. Parry. 1973. The Sampling of Chickens, Turkeys, Ducks and Game Birds, p. 63-73. **In: Board R.G. and D.W. Lovelock** (eds.). Sampling - Microbiological Monitoring of Environments, Society of Applied Bacteriology Technical Series No.7, Academic Press, London.

Bok H.E., W. H. Holzapfel, E. S. Odendaal, and H. J. van der Linde. 1986. Incidence of foodborne pathogens on retail broilers. Int. J. Food Microbiol. **3:**273-285.

Brown M.H., and A. C. Baird-Parker. 1982. The microbiological examination of meat, p. 423-520. **In: Brown M.H** (ed.). Meat Microbiology. Applied Science Publishers, London.

Bryan F.L. 1980. Poultry and meat products, p. 410-458. **In: Silliker J.H., R. P. Elliot, A. C. Baird-Parker, F. L. Bryan, J. H. B. Christian, D. S. Clark, J. C. Olson, and T.A. Roberts** (eds.). Microbial Ecology of Foods, Vol.2: Food Commodities, (ICMSF).

Connor J.T., T. A. McMeekin, and J. T. Patterson. 1987. Prevention of Microbial Contamination in the Poultry Processing Plant, p. 163-175. **In: Smulders F.J.M** (ed.). Elimination of Pathogenic Organisms from Meat and Poultry. Proceedings of the International Symposium: Prevention of Contamination, and Decontamination in the Meat Industry. Zeist, The Netherlands, 2 - 4 June 1986. Elsevier Science Publishers.

Eustace I.. J. 1981. Control of bacterial contamination of meat during processing. Food Technology in Australia **33(1):**28–32.

Faber J.M., and E. S. Idziak. 1984. Attachment of psychrotrophic meat spoilage bacteria to muscle surfaces. J. Food Prot. **47(2):**92-95.

Firstenberg-Eden R. 1981. Attachment of bacteria to meat surfaces: A review. J. Food Prot. **44(8):**602-607.

Gill C.O. 1983. Meat spoilage and evaluation of the potential storage life of fresh meat. J. Food Prot. **46(5):**444-452.

Grau F.H. 1986. Microbial Ecology of Meat and Poultry, p. 1-36. **In: Pearson A.M. and T.R. Dutson** (eds.). Advances in Meat Research, Vol.2: Meat and Poultry Microbiology. Avi Publishing Company, Inc.

Green S. 1974. Microbiology in the poultry processing industry. Process Biochem. **9(1):**27-28.

Harrigan W.F., and M. E. McCane. 1976. Laboratory Methods in Food and Dairy Microbiology. Academic Press Inc., London.

Lillard H.S. 1985. Bacterial cell characteristics and conditions influencing their adhesion to poultry skin. J. Food Prot. **48(9):**803-807.

McMeekin T.A., and C. J. Thomas. 1979. Aspects of the microbial ecology of poultry processing and storage: A Review. Food Technology in Australia **31:**35-43.

Mead G.C. 1989. Hygiene Problems and Control of Process Contamination, p. 183-220. **In: Mead G.C.** (ed.). Processing of Poultry. Elsevier Science Publishers Ltd.

Mead G.C. 1982. Microbiology of poultry and game birds, p. 67-101. **In: Brown M.H** (ed.). Meat Microbiology. Applied Science Publishers Ltd.

Notermans S., and E. H. Kampelmacher. 1974. Attachment of some bacterial strains to the skin of broiler chickens. Br. Poult. Sci. **15:**573-585.

Notermans S., and E. H. Kampelmacher. 1975. Further studies on the attachment of bacteria to skin. Br. Poult. Sci. **16:**487-496.

Patterson J.T. 1972. Microbial sampling of poultry carcasses. J. Appl. Bacteriol. **35:** 569-575.

Roberts D. 1982. Bacteria of public health significance, p. 319-386. **In: Brown M.H** (ed.). Meat Microbiology. Applied Science Publishers Ltd.

South African Poultry Association (SAPA). 1996. What's happening in the poultry and egg industry? Food Ind. S. A. **9:**9-15.

South African Poultry Association (SAPA). 1993. Code of Practice for Broiler Processing Plant (Abattoirs) Annexure G.

Thomas C.J., and T. A. McMeekin. 1981. Attachment of *Salmonella* spp. to chicken muscle surfaces. Appl. Environ. Microbiol. **42(1):**130–134.

Thomas C.J., and T. A. McMeekin. 1980. Contamination of broiler carcass skin during commercial processing procedures: an Electron microscopy Study. Appl. Environ. Microbiol. **40(1):**133–144.

Chapter 9

GENERAL DISCUSSION

The degree of resistance of bacteria to biocides differs from organism to organism. The increasing resistance of bacteria to moderate amounts of commonly used biocides is a major concern. The response of bacteria to anolyte disinfection is not different from that of other biocides. Various organisms responded differently to the treatment with anolyte. Some were very sensitive even at low concentrations of anolyte while others were resistant. This is in agreement with literature that different bacteria react differently to bacteriocides due to differing cell wall properties (Gaylarde and Morton, 1999). However, anolyte inhibited growth of most of the microorganisms tested resulting in complete sterilisation of others or just a reduction in number of colony forming units. Therefore anolyte showed some antibacterial properties, with MIC varying among microorganisms.

Bacteria in biofilms are of importance because of the deleterious effects they have. Biofilm bacteria in drinking water distribution pipeworks can be detached from the pipes resulting in deterioration of the bacteriological quality of water and development of colour and odour. In industrial systems biofilms lead to the phenomenon of microbially induced corrosion which result in reduction of plant performance and product quality. Along with increasing demands on environmentally friendly production and economic profit, the need increases for an effective way to control and eliminate biofilm formation (Green, 2001). Evaluation of the effect of anolyte and sodium hypochlorite showed there is a possibility of the two reagents being used for biofilm control. Dilute anolyte (1:100) did not have any effect on the mature *P. aeruginosa* biofilm as the structure remained intact as if it was not subjected to any treatment. Less dilute (1:10) and concentrated (neat) anolyte dispersed and removed the biofilm. This was evident from both the light and scanning electron microscopy pictures. Control of biofilm using sodium hypochlorite require concentrated solutions of 100 and 300 ppm as the diluted had less or no effect on the biofilm.

The human skin supports growth of both aerobic and anaerobic bacteria, some of which are opportunistic pathogens. Thorough disinfection of hands before handling of food is important for minimisation of contamination that can result in infections. Washing hands with ordinary water does not necessarily sterilise the hands. The number of colony forming units were higher when subjects washed hands with ordinary water. There was a reduction in the number of bacterial counts when anolyte was used as disinfectant washing agent. Hospital equipment is highly contaminated with different types of pathogens. These surface microorganisms are the main cause of cross infections as patients use equipment like basins, beds, etc. Anolyte was tested for ability to reduce numbers of microorganisms on hospital equipment. The numbers of microorganisms from surfaces were also higher in this instance before than after treatment with anolyte. Not only the numbers of live organisms were reduced, but also some surfaces were completely sterilised showing no growth on the plates. The use of anolyte in hospitals may decrease the level of nosocomial infections.

Milk is an excellent growth media for all common spoilage organisms, including yeasts and moulds. In order to produce raw milk of exceptionally high microbiological quality, farms need the use of a well balanced cleaning and sanitation programme. Testing effects of anolyte on the surfaces in the milking parlour showed some interesting results. Anolyte gave better disinfection than the control disinfectant especially on surfaces that had maximum contact with disinfectant. Spreaders, which were identified as sporeformers because of their resistance to a high temperature, were observed on the plates after the use of the control disinfectant, but were eliminated when anolyte was used. Anolyte eliminated the sporeformers. Thus, when anolyte made contact with a surface, effective disinfection was obtained with more surfaces being sterilised. Anolyte was a better disinfectant for milking parlours than the standard disinfectant used.

Chicken carcasses are exposed to high numbers of microorganisms in the poultry processing plants, some of which are spoilage microorganisms. They cause food poisoning and therefore are detrimental to public health. Failure to remove these microorganisms on chicken carcasses affects shelf life negatively, especially for people in rural areas not having refrigerators. The numbers of microorganisms on

chicken carcasses decreased when the carcasses were treated with anolyte. These results were in agreement with those of Kruger and Buys (1998), who conducted a trial on the microbiological quality of beef carcasses after fogging with anolyte. They found that anolyte treated group of carcasses had significantly lower total counts than the control group of beef carcasses.

REFERENCES

Gaylarde C. C. and L. H. G. Morton. (1999). Deteriogenic biofilms on buildings and their control: a review. Biofouling. **14(1):**59-74.

Green H. (2001). Detection of biofilm formation using the quartz crystal microbalance with dissipation monitoring, p. 6-10. Master's thesis. Linkopings Universiteit, Department of Physics and Measurement Technology, SE-581 83 Linkoping, Sweden.

Kruger J. and E. M. Buys (1998). Reduction of beef carcass contamination with anolyte. ARC-Animal Nutrition and Animal Products Institute, Irene. Meat industry Centre.

Chapter 10

CONCLUSIONS

- Both the sodium chlorite and sodium bicarbonate derived anolytes had antimicrobial properties against a number of microorganisms with the killing efficacy increasing with the decrease in cell concentrations

- Response of different microorganisms to anolyte is not different from the response to other biocides. The degree of resistance of different microorganisms to sodium chloride derived anolyte differs among microorganisms. Therefore the minimum inhibitory concentration of anolyte to microorganisms differs among different bacteria, as is the case with other commonly used biocides.

- Anolyte had the potential to remove biofilms. Concentrated anolyte was more effective for removal of biofilms than the diluted anolyte solutions.

- The bicarbonate anolyte was effective as a hand disinfectant.

- Overall effective disinfection of hospital equipment was achieved using anolyte with some of the surfaces being sterilised. Anolyte could therefore be used for disinfection of hospital equipment and this may decrease the spread infections in the hospital environment.

- Maximum contact between the milking parlour surface and anolyte is important for effective removal of microorganisms present on that surface. Anolyte effectively removed microorganisms, including sporeformers that contaminated milking parlour surfaces.

- The numbers of microorganisms on chicken carcasses were reduced by the use of anolyte. The use of anolyte for treatment of chicken carcasses increased their shelf life.